Invited and Short Oral Talks

1. **Re-conceiving the Pill: From Revolutionary Therapeutic to Lifestyle Drug.**
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   This paper traces the shift in the conceptualization of the pill from life-changing to life-enhancing, from revolutionary to commonplace, and the implications for the trajectories of women, birth control, and pharmaceutical consumerism. In the 1960s, the birth control pill extended the reach of pharmacy beyond the treatment or prevention of disease or illness and effected a contraceptive revolution in the United States and in many other countries by changing the ways people thought about, discussed, and used birth control. Although much ink was spilled either crediting or blaming the pill for fomenting sexual revolution, it is clear from the historical record that the pill played only a supporting role as one of many factors contributing to the liberalization and democratization of sexual behaviors and attitudes. It played a similarly auxiliary part in the revolutionary appeal of second wave feminist activism that swept the United States in the late 1960s and 1970s. In concert with a host of other social, cultural, and political forces, the pill helped to make women’s lives in the 1980s look very different from those of their mothers in the 1950s. By the 1990s, the pill had become part of the birth control establishment, prescribed and used more often than any other method of reversible contraception. Well past its revolutionary heyday, it still served as the standard to which newer methods were compared. Manufacturers of oral contraceptives shifted the focus of their marketing strategies away from the primary indication of family planning to emphasize instead the secondary effects of relieving discomforts resulting from the menstrual cycle, such as pimples, irritability, and monthly bleeding. The transition in the pill’s social status – from a radically innovative drug that upended therapeutic and social conventions to an time-honored member of the pharmacopeia, considered so basic that it is marketed for its secondary effects – offers an interesting perspective for delineating the contours of this particular therapeutic revolution.

2. **Germ Line Modification to Create Large Animal Models of Human Disease.**
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   Genetically-modified domestic animal models are of increasing significance in biomedical research. Large animal models are especially valuable where rodent models do not recapitulate the phenotype observed in human patients as exemplified by animal models for cystic fibrosis. In addition, outbred large animal models are closer in size, physiology and longevity to humans than rodent models, thereby providing a more suitable platform for the development of treatment strategies. With robust ES cells from domestic animals largely lacking, the currently established approaches for engineering genetic modifications in large animals are pronuclear microinjection and somatic cell nuclear transfer (SCNT or cloning). Both pronuclear microinjection and SCNT are relatively inefficient, costly, and time-consuming. In animals produced by pronuclear microinjection, the transgene is usually inserted randomly into the genome, which results in highly variable expression patterns and levels in different founders. Therefore, significant efforts are required to generate and screen multiple founders to obtain animals with optimal transgene expression. For SCNT, specific genetic modifications (both gain-of-function and loss-of-function) can be engineered and selected in the somatic cell nucleus before nuclear transfer. SCNT has been used to generate a variety of genetically modified animals such as goats, pigs, sheep and cattle; however, animals resulting from SCNT frequently suffer from developmental abnormalities associated with incomplete nuclear reprogramming. Other strategies to generate genetically-modified animals proposed the use of the spermatozoon as a natural vector to introduce genetic material into the female gamete. This sperm mediated DNA transfer (SMGT) combined with intracytoplasmatic sperm injection (ICSI) has reportedly relatively high efficiency but has not found widespread application. An approach developed to complement SCNT for producing genetically modified animals is germ cell
transplantation using genetically modified male germ line stem cells (GSCs). This approach relies on the ability of GSCs that are genetically modified in vitro to colonize the recipient testis and produce donor derived sperm upon transplantation. As the genetic change is introduced into the male germ line just before the onset of spermatogenesis, the time required for the production of genetically modified sperm is significantly shorter using germ cell transplantation compared to cloning or embryonic stem (ES) cell based technology. The GSC-mediated germ line modification also circumvents problems associated with embryo manipulation and nuclear reprogramming. Currently, engineering targeted mutations in domestic animals using GSCs remains a challenge as GSCs from those animals are difficult to maintain in vitro for an extended period of time. Recent advances in genome editing techniques such as Zinc-Finger Nucleases (ZFNs), Transcription Activator-like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) greatly enhance the efficiency of engineering targeted genetic change in domestic animals as demonstrated by the generation of several gene knock-out pig and cattle models using those techniques. The potential of GSC-mediated germ line modification in making targeted genetic modifications in domestic animal models will be maximized if those genome editing techniques can be applied in GSCs.

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We study the molecular pathways that regulate eukaryotic gene expression by affecting the stability or translation of mRNAs. Much of our work is on microRNAs (miRNAs), which are ~22 nt RNAs that pair to sites within mRNAs to direct the posttranscriptional down-regulation of these messages. Because most of the mammalian miRNAs are conserved regulatory targets of miRNAs, it will be hard to find a developmental or physiological process that is not influenced by miRNAs. For example, when the miRNAs of Zeb transcription factors are altered such that the miR-200 family of miRNAs can no longer repress them, female mice are sterile because they do not ovulate. We also study miRNAs, with particular interest in the untranslated regions and poly(A) tails, and how these regions recruit and mediate regulatory phenomena. We have developed a high-throughput method to measure the lengths of miRNA poly(A) tails. When we used this method to measure tail lengths of millions of individual mRNAs isolated from early zebrafish and frog embryos, we found a very strong correlation between tail length and translational efficiency, as would be expected if mRNAs with longer tails were more efficiently translated. However, this strong coupling diminished at gastrulation and was absent in nonembryonic samples, which indicated a previously unrecognized developmental switch in the nature of translational control.

4. Maternal Vitamin D Deficiency Programs Hypothalamic-Pituitary Dysfunction and Subfertility in Affected Female Offspring.
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Vitamin D (VD) deficiency affects over one billion people worldwide. In the United States, as much as 40% of pregnant women and 20% of children are VD deficient. The vitamin D receptor (VDR) is expressed throughout the hypothalamic-pituitary-gonadal (HPG) axis raising the possibility that VDR signaling is an important regulator of HPG function and reproduction. We previously reported female mice exposed to peripubertal VD deficiency have delayed puberty and develop estrous cycle dysfunction in adulthood that is rescued by dietary VD supplements. To determine the critical developmental periods during which VD deficiency might affect reproductive function in adult female mice and program subfertility in affected offspring, we exposed female mice to dietary VD deficiency at three specific developmental stages: 1) in utero, 2) lactation or 3) in utero and lactation (early life). We hypothesize that in utero VDR signaling is necessary for normal female reproductive function and fertility. We used vaginal opening and cytology to assess puberty and estrous cyclicity in females and we monitor litter sizes and placental site nodules to assess fertility across three generations of female offspring. Developmental VD deficiency did not affect puberty in any exposure group. Interestingly, regardless of when in development female mice were exposed to VD deficiency they developed prolonged irregular estrous cycles, characterized by oligo-ovulation suggesting developmental VD signaling is important for programming normal HPG axis function in females. To determine if early life VD deficiency disrupts HP- axis function, we collected serum samples on estrus, diestrus and proestrus and quantified serum LH. Females exposed to early life VD deficiency released 40% less LH release on the day of proestrus. To determine if early life vitamin D deficiency affects fertility we mated females exposed to early life VD deficiency with proven fertile males and found females exposed to early life VD deficiency and their female offspring have significantly fewer placental site nodules and give birth to fewer pups than control females. Our studies suggest that maternal VD deficiency may program multiple generations of reproductive dysfunction in female offspring.

5. The Intersection of Epigenetics, Genetics, and Reproduction.
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Epigenetics classically involves the study of heritable changes in gene function that cannot be explained by changes in the primary DNA sequence. Epigenetic programming is unique in each cell type and undergoes extensive dynamic changes during development, particularly in the pre-implantation embryo and germ line in mammals. Because of the plasticity of the epigenome, it is not surprising that disruptive environmental effects can alter normal epigenetic programming resulting in epimutations. When such disruptions directly impact the epigenome, they are referred to as primary epimutations. Some primary epimutations, e.g. those induced in imprinted genes by assisted reproductive technologies, have been shown to be corrected by germline-specific epigenetic reprogramming. However, other epimutations, e.g. those induced by exposure to endocrine disruptors in utero, have been shown to be transmitted transgenerationally, thus appearing to escape correction by germline reprogramming. Secondary epimutations are those caused by an initial genetic mutation that leads to altered epigenetic programming, and can be transmitted transgenerationally on the basis of genetic inheritance of the causative mutation. To determine if in utero exposure to vinclozolin induces primary or secondary epimutations, we exposed pregnant female rats carrying the lacI mutation-reporter transgene to vinclozolin and compared the frequency of mutations detected in the transgene recovered from kidney tissue and spermatozoa of F1 and F3 generation descendants of vinclozolin-treated or control (vehicle-treated) dams. Our results indicate that vinclozolin is not directly mutagenic and thus does not appear to have the capacity to induce secondary epimutations. Therefore, it appears the initial defects induced by in utero exposure to vinclozolin are primary epimutations. However, our results also reveal the surprising finding that a subset of F3 generation descendants of vinclozolin-treated dams showed a significantly higher frequency of mutations than that detected in F3 descendants of control dams. We therefore propose the existence of a third type of epimutations—“tertiary epimutations”—which we define as initial primary epimutations that disrupt epigenetic programming in a manner that facilitates a subsequent accelerated accumulation of genetic mutations. Our results suggest a functional interaction between mechanisms regulating epigenetic and genetic integrity in cells, and provide insight into a novel paradigm potentially contributing to transgenerational transmission of epimutations.

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Methylation reprogramming in vivo occurs in the pre-implantation embryo, and during primordial germ cell (PGC) development post-implantation. Combined these two events are hypothesized to remove methylated alleles acquired in gametogenesis, and to establish the epigenetic ground state for embryo and germline differentiation respectively. In order to understand in vivo DNA methylation reprogramming in a human genome, my laboratory evaluated DNA methylation in human post-implantation PGCs between 57 to 137 days of prenatal development. To characterize the germline during this period we first used RNA-Seq of germ cells isolated by fluorescence Activated Cell Sorting to evaluate changes in gene expression with time and with sex. Surprisingly, we show that the major determinant in transcriptional identity in the cKIT positive human germ cell lineage is age post fertilization rather than sex of the embryo. Therefore, based on this unsupervised RNA-Seq classification, we categorized the human prenatal germline cell as either PGCs or advanced germ cells (AGCs), with the PGC period in females and males corresponding to 67 and 74 days of development and younger respectively. Notably, this transcriptional shift between PGCs to AGCs correlates with loss of H3K27me3 from the genome in both sexes. Using whole genome bisulfite sequencing (WGBS) of human prenatal germline cells in the PGC and AGC period, we show that human AGCs have the most globally hypomethylated genome described to date (<20% average CpG methylation). However, DNA methylation is not completely erased in PGCs or AGCs with persistent methylation found at intragenic CG islands (CGi) in promoters, exons and 3’UTRs as well as certain transposable elements of the endogenous retrovirus and human-specific LINE1 classes. We also discovered that similar to the mouse, the human germline erases DNA methylation in a stage-specific manner, with both gains and losses in intragenic DNA methylation between the PGC and AGC period. Taken together, we have generated the first whole genome DNA methylation map of prenatal human germline cells that will be critical for future studies aimed at understanding DNA methylation inheritance through the germline, as well as the emergence of in vitro demethylated cell types from a global DNA demethylation reprogramming event.

7. DNA methylation dynamics during mammalian preimplantation development.
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In mammals, cytosine methylation is predominantly restricted to CpG dinucleotides and stably distributed across the genome, with local, cell type-specific regulation directed by DNA binding factors. This comparatively static landscape dramatically contrasts the events following fertilization, where the paternal genome is globally reprogrammed. Paternal genome demethylation includes the majority of CpGs, though methylation is maintained at several notable features. To date, these dynamics have been extensively characterized in the mouse, with limited observations from other mammals, and the extent to which early embryonic landscapes are conserved cannot be understood without direct measurement. We present genome-scale DNA methylation maps of human preimplantation development and embryonic stem cell (ESC) derivation, confirming a transient state of global hypomethylation that includes most CpGs, while sites of persistent maintenance are primarily restricted to gene bodies. While most features share similar dynamics to those in mouse, maternally contributed methylation is divergently targeted to discrete sets of species-specific sets of CpG island (CGI) promoters that extend beyond known Imprint Control Regions (ICRs). Retrotransposon regulation is also highly diverse and transitions from maternally to embryonically expressed, species-specific elements. Together, our data confirm paternal genome demethylation as a general attribute of early mammalian development that is characterized by specific modes of epigenetic regulation.

8. Developmentally Essential DNA Methylation is Regulated by the DNMT1 Intrinsically Disordered Domain.
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The DNMT1 cytosine methyltransferase contains a large ~300-amino acid intrinsically disordered domain (IDD) in its amino-terminal regulatory region. To determine IDD’s role in controlling genomic methylation, we generated seven mouse lines with different missense mutations in the IDD. Homozygous mutant mice of five lines developed normally with no alterations in genomic methylation. Homozygous mutants of a line with missense mutations in 14 contiguous codons (IDel13 allele) died in early postimplantation development with associated profound loss of genomic methylation, including the loss of methylation associated with imprinted differentially methylated domains (DMDs). In this case, we attributed the embryonic lethality to loss of the inherited methylation on DMD sequences. Interestingly, IDel13/IDel13 embryonic stem (ES) cells were normally methylated, indicating a specific role of the IDD in regulating genomic methylation during fetal development. Homozygous mice of another mutant mouse line (P allele), in which a 6-aa motif was substituted by the orthologous rat sequence, survived to adulthood. Although these mice exhibited significant fetal and postnatal growth restriction (~half the size of P+/+ and wild-type (WT) littersmates), no gross phenotypic abnormalities were observed. Whole-genome bisulfite sequencing (WGBS) and reduced representation bisulfite sequencing (RRBS) of whole E15.5 homozygous WT and P/P embryos and brains showed ~50% loss of genomic methylation in P/P tissues. Mean CpG methylation of WT embryos 66.7%; P/P embryo 34.7%; WT brain 75.2 %, and; P/P brain 33.2%. There was a similar reduction of methylation on X-chromosome sequences as there was on autosomal sequences in a female E15.5 P/P embryo. Interestingly, DMDs of imprinted genes were retained in the mutant mice. This retained imprinting presumably contributed to the survival of P/P mice. In addition, a number of other CpG-rich regions retained their methylation in P/P embryos and brain samples (>400bp, minimum 15 CpGs, >50% methylated, no less than 20% difference between WT and P/P). We infer from these in vivo studies that there are at least two DNMT1-dependent methylation processes during fetal development. One process maintains the bulk of genomic methylation on non-imprinted sequences, and this is largely dispensable for fetal development. The other process maintains methylation on a much smaller class of developmentally essential sequences that includes but may not be limited to imprinted DMDs. These findings also indicate that mammalian genomic methylation can be simplified to a smaller essential fraction whose identification should lead to an improved understanding of the role of DNA methylation in mammalian development. This work was supported by NIH HD044133 (JRC), NIH069316 (JRC) and CIHR MOP110987 (JT).

9. Ancient regulators of animal development are epigenetically poised in male germ cells of five mammalian species.
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In mouse, germ cells maintain an epigenetically poised state, defined by the simultaneous presence of the opposing histone modifications H3K4me3 and H3K27me3, at the promoters of many somatic developmental regulatory genes. This state is retained at some promoters in mature sperm, implying that it may play a role in regulating gene expression in the next generation. To establish the relevance of germline poising outside the mouse model, and to identify a core set of germline-poised genes, we compared genome-wide chromatin and transcriptional states in sorted male meiotic and postmeiotic germ cells of five mammalian and one avian species. We identified a set of 40 genes whose promoters are robustly poised in germ cells across the mammalian lineage, from marsupial to man. These genes share a cohesive molecular and biological function: the majority encode homeodomain transcription factors that control gene regulatory circuits governing early body part and tissue pattern and signaling in the embryo. They also share a common evolutionary origin: with few exceptions, they arose at the base of the metazoan tree and are associated with developmental patterning throughout the animal kingdom. We propose that this set of genes comprises an ancient metazoan developmental toolkit critical for guiding basic patterning during animal embryogenesis. As the metazoan body plan gained complexity, these genes may have acquired a tightly regulated, epigenetically privileged state in germ cells and embryos, manifested by H3K27me3/H3K4me3 bivalency in mammals.

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10. Transcription and DNA Methylation Dynamics in Developing Postnatal Male Germline Stem Cells.
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Postnatal spermatogonial stem cells (SSCs) progress through proliferative and developmental stages to populate the testicular niche prior to productive spermatogenesis. To better understand germ cell developmental process, we conducted extensive genomic profiling involving DNA methylation, chromatin and transcription at multiple postnatal stages. Our profiles reveal dynamic changes in transcription and signaling networks correlated with stage-specific SSC adhesion/migration, proliferation, self-renewal, and development - and reveal novel candidate receptor-ligand networks involving SSCs and the niche. Surprisingly, we find that the establishment of methylation patterns at a subset of imprinted and random monoallelic genes to be developmentally regulated and largely completed before puberty (P12/P14). Taken together, we reveal stage-specific transcription/signaling profiles of developing SSCs, and identify a new phase of extensive DNA/chromatin reprogramming during SSC development.

11. Uniting Major Constituents of the Genome: The Role of piRNAs in the Germline.
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The eukaryotic genome has vast intergenic regions containing transposons, pseudogenes, repetitive sequences, and noncoding genes that produce numerous long non-coding RNAs (lncRNAs) and PIWI- interacting RNAs (piRNAs). Yet the functions of the intergenic regions remain largely unknown. In mammals, a unique set of piRNAs, pachytene piRNAs, is abundantly expressed in late spermatocytes and early spermatids. Recently, we showed that piRNAs derived from transposons and pseudogenes mediate the degradation of a large number of mRNAs and lncRNAs in mouse late spermatocytes. In particular, they have a large impact on the lncRNA
transcriptome, as a quarter of IncRNAs expressed in late spermatocytes are upregulated in mice deficient in piRNA pathway. Furthermore, our genomic and in vivo functional analyses revealed that retrotransposon sequences are frequently found in the 3' UTR of mRNAs that are targeted by piRNAs for degradation. Similarly, the degradation of spermatogenic cell-specific IncRNAs by piRNAs are mediated by retrotransposon sequences. Moreover, we have shown that pseudogenes regulate mRNA stability via the piRNA pathway. The degradation of mRNAs and IncRNAs by piRNAs requires Miwi1 and, at least in part, depends on its slicer activity. Together, these findings reveal a highly complex and global RNA regulatory network through which transposons and pseudogenes regulate target mRNA and IncRNA stability via the piRNA pathway to promote meiosis-spermiogenesis transition.

12. Retromer Vesicles Interact With RNA Granules In Haploid Male Germ Cells.
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Spermatogenesis is a complex process that leads to the production of mature spermatooza starting from spermatogonial stem cells. The first characteristic step in this process is the half of the genome brought about during meiosis. After meiosis the cells undergo major morphological changes: compaction of the chromatin in the sperm head, development of an acrosome, production of a long flagellum and disposal of most of the cytoplasm. All these processes must be thoroughly controlled in order to produce working mature sperm. During spermatogenesis, many RNA regulation pathways, including PIWI-interacting small RNAs, are particularly concentrated in a specific cytoplasmic RNA-protein complex in haploid round spermatids called chromatoid body (CB). Besides controlling different RNA-dependent pathways, the CB protein composition suggests that it actively interacts with other cellular compartments. In this study, we identified a novel interaction between the CB and VPS26A/VPS35 positive vesicles. In somatic cells these proteins form the Retromer, a protein complex involved in recycling cargo-receptor proteins from endosomes to the Golgi. We demonstrated that in haploid spermatids, the Retromer vesicles are associated with the acrosome formation and their function is dependent on the endosome-to-lysosome pathway. In the light of our findings, we suggest that in addition to its known function in retrograde transport from endosomes to the Golgi complex, the Retromer may be involved in vesicle trafficking between the acrosome and the Golgi/endosomal compartments in haploid male germ cells. Furthermore, while the exact role of the Retromer vesicles in the CB function remains unclear, our results suggest a direct functional link between the vesicle transport and the CB-mediated RNA regulation.

13. Sex-specific micro-RNA response of primate fetal kidney to maternal caloric restriction (CR) and high-fat diet (HFD) in late gestation.
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Maternal caloric restriction (CR) and maternal high-fat diet (HFD) have been implicated in predisposition of the offspring to metabolic syndrome. We reported (Cox LA et al. J Physiol, 2006) that maternal CR induced significant changes in gene expression in fetal kidney leading to accelerated differentiation in the mid gestation (0.5G); however, the impact of maternal CR at late gestation (0.9G) has not been determined. Further, the knowledge on the impact of maternal HFD on primate kidney development in 0.9G is still lacking. Micro-RNAs (miRNAs) are small, non-coding RNA molecules and play important roles in many cellular processes via epigenetic regulation of gene expression. Here we report for the first time the changes in transcriptome and miRNAome of the primate fetal kidney in vivo functional analyses revealed that retrotransposon sequences are frequently found in the 3' UTR of mRNAs that are targeted by piRNAs for degradation. Similarly, the degradation of spermatogenic cell-specific IncRNAs by piRNAs are mediated by retrotransposon sequences. Moreover, we have shown that pseudogenes regulate mRNA stability via the piRNA pathway. The degradation of mRNAs and IncRNAs by piRNAs requires Miwi1 and, at least in part, depends on its slicer activity. Together, these findings reveal a highly complex and global RNA regulatory network through which transposons and pseudogenes regulate target mRNA and IncRNA stability via the piRNA pathway to promote meiosis-spermiogenesis transition.

14. SLY modulates the expression of multiple chromatin regulators and affects chromatin structure during sperm differentiation.
FOCUS SESSION 2: Impact of the Environment on Development and Function of the Mammalian Ovary.

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Cyclophosphamide is a pro-drug requiring hepatic biotransformation for conversion to phosphoramide mustard (PM), which destroys rapidly dividing cells, and is thus an effective anti-cancer chemotherapeutic. PM is also an ovotoxicant and depletes ovarian oocyte-containing follicles at all developmental stages. To determine direct ovarian mechanisms involved in PM-induced ovotoxicity, we have employed an ex vivo rat whole ovarian culture system. Postnatal day 4 (PND4) rat ovaries were trimmed of excess tissue and placed onto a membrane floating upon culture media in the presence or absence of PM (60 µM) for 1, 2, 3 or 4 days. At each time point, follicle classification and enumeration was performed (n = 5/treatment). In addition, PM-induced alterations to abundance of mRNA and protein of cellular components involved in DNA repair, chemical biotransformation, and cell viability over the temporal pattern of exposure were evaluated (n = 3; 10 ovaries per pool). Furthermore, electron microscopy was performed to identify histological changes to the oocyte and granulosa cells during PM-induced ovotoxicity (n = 5/treatment). An unexpected finding was that PM is transformed to a volatile metabolite, chloroethylaziridine (CEZ), thus for all experiments, vehicle-control treated ovaries were maintained in a separate incubator from the PM-exposed ovaries, and all PM exposures are considered to receive CEZ exposure. CEZ exposure depleted (P < 0.05) primordial follicles from 4 days of exposure onwards, and did not affect any other specific follicle type across the timecourse examined. In contrast, PM destroyed (P < 0.05) all follicle stages from 4 days onwards. Cell vacuolization, appearance of double-membranated structures indicating induction of autophagy and abnormal golgi apparatus were observed by electron microscopy 1 day after PM exposure onwards. After 24h of exposure onwards, mRNA encoding members of DNA repair response were increased (P < 0.05) in abundance in response to PM exposure. mRNA and protein encoding chemical biotransformation enzymes were also increased prior to PM-induced follicle loss, demonstrating the ovarian response to assault by PM. A key sensor of DNA damage, ataxia telangiectasia mutated (ATM), was consistently induced during PM exposure, thus we utilized a chemical ATM inhibitor (KU55933; 10 nM) and evaluated impacts on PM-induced ovarian follicle demise after 4 days in culture. In the absence of ATM, PM-induced primordial, large primary and secondary follicle depletion was prevented. In addition, microsomal epoxide hydrolase (Ephx1), was found to increase at the mRNA and protein level in response to PM. Using a competitive EPHX1 inhibitor (cyclohexene oxide; 2mM), the biotransformation role of EPHX1 during PM exposure was investigated and greater PM-induced follicle loss was observed in the absence of EPHX1 after 4 days of treatment. These data suggest that the DNA repair response pathway may shunt damaged follicles towards destruction, and that ATM inhibition may retain potentially poor quality oocytes. Further, these data support that the action of ovarian EPHX1 biotransforms PM to a lesser ovotoxic metabolite. These data identify both pathways as being involved in the ovarian response to PM, as well as being potential candidates for manipulating the ovarian outcomes of PM exposure. (Supported by ES016818 to AFK).
16. Environmental Exposures and Gametogenesis: Are We Messing with Mendel?
Patricia A. Hunt

Mammalian gametogenesis is complex and strikingly sexually dimorphic. Although the global features are shared between the sexes (e.g., the production of haploid gametes from diploid germ cell precursors and extended periods of gamete maturation), oogenesis and spermatogenesis differ strikingly in the timing of events, their order, and in the propensity for and response to errors during the process. Our interest is in the factors – both endogenous (e.g., age) and exogenous (e.g., environmental exposures) - that influence the genetic quality of the resultant gametes. Increasing evidence suggests that both male and female reproduction are susceptible to the effects of endocrine disrupting chemicals, and our focus has been on understanding sex-specific effects of these chemicals on gametogenesis. Data from our studies in mouse suggest that exposure to BPA in the female, and to either BPA or other exogenous estrogens in the male, induces subtle changes that profoundly affect levels of meiotic recombination. Temporal differences in the timing of events, however, result in different developmental windows of vulnerability in the two sexes. Further, our findings suggest that EDCs induce meiotic effects by altering the germline epigenome and, importantly, demonstrate strikingly different effects and consequences in males and females.

17. Bisphenol A exposure disrupts germ cell nest breakdown in cultured neonatal mouse ovaries.
Changqing Zhou, Wei Wang, Jackye Peretz, Jodi Flaws

Bisphenol A (BPA) is a known endocrine disrupting chemical and reproductive toxicant in animal models. In females, studies suggest that BPA exposure during development has the potential to affect germ cell nest breakdown and follicle formation in the ovary. Previous studies indicate that low-dose in utero BPA exposure increases the number of germ cells in nests and decreases the number of primordial follicles compared to controls. However, the mechanism by which BPA affects germ cell nest breakdown is unknown. Thus, we tested the hypothesis that BPA inhibits germ cell nest breakdown by interfering with oxidative stress and apoptosis pathways. To measure oxidative stress, we focused on the expression of major antioxidant genes [superoxide dismutase 1 (Sod1), catalase (Cat), glutathione peroxidase (Gpx), and glutathione reductase (Gsr)], as well as the level of reactive oxygen species (ROS). To measure apoptosis, we focused on the expression of several key anti-apoptotic and pro-apoptotic genes [B cell leukemia/lymphoma 2 (Bcl2), Bcl2-like 1 (Bclxl), Bcl2-associated X protein (Bax), Bcl2-related ovarian killer protein (Bok), Bcl2-associated agonist of cell death (Bad), Fas cell surface death receptor (Fas), and caspase 8 (Casp8)]. To test our hypothesis, ovaries from newborn mice [postnatal day (PND) 0] were collected and cultured with vehicle (dimethylsulfoxide, DMSO) or low doses of BPA (0.1, 1, 5, and 10 µg/mL) for 1, 2, 4, and 8 days. After culture, the ovaries were collected for histological evaluation of germ cell nest breakdown and for biochemical analyses of oxidative stress and apoptosis pathways. Our results indicate that BPA treatment did not alter Sod1 expression at any doses, but it significantly increased Cat, Gpx, and Gsr expression levels on PND 1, 2, and 4 compared to control (5 and 10 µg/mL, n=3-6, p<0.05). On PND 8, BPA (1, 5, and 10 µg/mL) significantly decreased the percentage of primary follicles compared to control (n=3, p<0.05). Our results indicate that BPA treatment did not alter Sod1 expression at any doses, but it significantly increased Cat, Gpx, and Gsr expression levels on PND 1, 2, and 4 compared to control (5 and 10 µg/mL, n=3-4, p<0.05). Additionally, BPA treatment did not affect ROS levels compared to control on PND 2 and 4 (n=3, p>0.05), but BPA (5 µg/mL) significantly increased ROS levels at PND 8 (n=3, p<0.05). Our results from analyses of the apoptosis pathway indicate that BPA significantly increased expression of anti-apoptotic genes (Bcl2, Bclxl), and reduced expression of some pro-apoptotic genes (Fas, Casp8) (n=3-4, p<0.05). Collectively, these data suggest that low doses of BPA exposure significantly inhibit germ cell nest breakdown by inhibiting the expression of key ovarian apoptotic genes, but not by interfering with the oxidative stress pathway. Supported by NIH PO1 ES022848 (JAF), EPA RD-83459301 (JAF), and an Environmental Toxicology Fellowship (CZ).

18. Space irradiation causes premature ovarian failure in mice.
Birendra Mishra, Birendra Mishra, Laura Ortiz, Ulrike Luderer

Background: About 15% of astronauts are women. While traveling in space they are exposed to galactic cosmic rays and solar particle events, which are characterized by charged particles, including iron (56Fe). Treatment for cancer with ionizing radiation to the pelvis is known to destroy ovarian follicles and cause premature ovarian failure. However, most studies have focused on gamma or x-radiation, and the risks of space radiation to the ovary remain poorly understood. To protect the health of women astronauts, it is important to understand whether space radiation has similar effects on the ovary as the types of radiation exposure that are common on earth. We hypothesized that irradiation of adult mice with 56Fe charged particles induces apoptosis, resulting in the destruction of ovarian follicles and causing premature ovarian failure. Methods: Three month old female C57BL/6J mice were divided into three groups: 1) sham-irradiated and fed with normal rodent chow (0 cGy group); 2) irradiated with a single dose of 50 cGy 56Fe at an energy of 600 MeV (LET=179 keV/µm) and fed normal chow, and 3) irradiated with 50 cGy 56Fe and fed rodent chow supplemented with 150 mg/kg diet alpha lipoic acid (N=8/group). Feeding with supplemented chow began 1wk before irradiation and continued until euthanasia. Ovaries were collected 6h, 1wk, and 8wk after irradiation. Follicles were counted in serial ovarian sections stained with hematoxylin and eosin. DNA double strand breaks and apoptosis were assessed by gamma H2AX and cleaved caspase 3 immunostaining, respectively. Serum follicle stimulating hormone (FSH) and luteinizing hormone (LH) were measured at 8wk. Results: At 6h after 50 cGy 56Fe, the percentages of primordial, primary and secondary follicles with gamma H2AX-positive oocytes and the percentages of secondary and antral follicles with gamma H2AX- positive granulosa cells were significantly higher than in the 0 cGy group. At 6h, the percentages of primordial and secondary follicles with cleaved caspase-3-positive granulosa cells were significantly elevated in the 50 cGy 56Fe group compared to the 0 cGy group. At 1wk, the percentages of gamma H2AX-positive and caspase-3-positive secondary follicles remained elevated in 50 cGy 56Fe exposed mice. Mice that received the lipoic acid supplemented diet along with 50 cGy 56Fe had decreased gamma H2AX and caspase-3 immunostaining compared to 50 cGy 56Fe alone. At 1wk after irradiation, the numbers of primordial and primary follicles were significantly reduced in the ovaries of mice treated with 50 cGy 56Fe compared to control. The lipoic acid supplemented diet did not
significantly alter the effects of 50 cGy $^{56}$Fe on ovarian follicle numbers at 1 wk. The serum FSH level was significantly higher in the mice treated with 50 cGy $^{56}$Fe compared to 0 cGy at 8 wk.

Conclusions: Our results show that exposure to 50 cGy $^{56}$Fe irradiation induces ovarian follicular DNA double strand breaks and apoptosis, and depletes the ovarian follicle pool. In addition, the increased serum FSH level is consistent with loss of negative feedback due to follicle depletion. The results further suggest that dietary supplementation with alpha lipoic acid is partially protective against these effects of $^{56}$Fe exposure. These results raise the concern that exposure to space radiation may increase the risk of early ovarian senescence and its attendant adverse consequences in female astronauts. (Supported by NASA NNX14AC50G to UL).

19. EFFECTS OF IN UTERO AND LACTATIONAL EXPOSURE TO NEW GENERATION “GREEN” PLASTICIZERS: A COMPARATIVE STUDY WITH DEHP.

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Phthalates, widely used plasticizers, leach from their innumerable supports into the environment and have been well-established to exert anti-androgenic action on male reproductive functions. This has raised concerns in the general population and public health authorities. Still, the use of phthalates in consumer products is poorly regulated, while the need for innocuous replacement compounds is stressed increasingly. The objective of this study is to identify novel “green” plasticizers that will meet the stringent demands of both industry and regulatory agencies. Among the 20 candidate molecules developed by McGill University's Department of Chemical Engineering to mimic the properties of diethylhexyl phthalate (DEHP), the most commonly used plasticizer, and following numerous in vitro tests on several immortalized cell lines, two compounds displayed the most promising innocuous profiles. We have tested the hypothesis that these compounds, namely dibenzooate and succinate plasticizers, exert fewer endocrine disrupting effects than DEHP on rat reproductive function after in utero and lactational exposure. Nine groups of Sprague Dawley dams (n=15/group) were dosed daily with either corn oil (vehicle), or 30 or 300 mg/kg of DEHP (positive control), 1,2-cyclohexane dicarboxylic acid diisononyl ester (also known as DINCH, and currently used as a replacement for DEHP despite a gap in the peer reviewed literature on its potential health effects), 1,4-butanediol dibenzoate (1,4-BDD) or dioctyl succinate (DOS), from gestational day 8 (GD8) to postnatal day 21 (PND21). One pup of each sex per litter was sacrificed at PND 3, 8, 21, 46 and 90 and the dams were sacrificed at weaning; multiple developmental endpoints were monitored throughout the study. Analysis of these endpoints and organ weights lead us to conclude that: (i) our model responds to the positive control: in accordance with the literature, in F1 males exposure to the higher dose of DEHP caused a significant decrease in anogenital index (AGI) on PND3, providing evidence of a decreased exposure to androgens during fetal life, and an increased incidence of hemorrhagic testes at PND8. In F1 females exposure to a low dose of DEHP caused a significant decrease in weight at vaginal opening; a similar effect has been reported after exposure to low doses of diethylstilbestrol or bisphenol A; (ii) DINCH exerts endocrine disruptive properties in the F1 females: exposure to a low dose of DINCH caused a significant decrease in AGI in F1 females at PND21, suggesting a disruption of the hormonal balance during fetal life and potential consequences on follicular recruitment; (iii) neither of the new generation “green” plasticizers showed any significant effect on the weight of vital and reproductive organs in the dams or the pups, age or weight at puberty in the offspring or cycling capabilities of the F1 females. This lack of significant effects suggests that they are safer alternatives to DEHP and DINCH. While further experiments are needed to fully assess the safety profile of our candidate compounds, the evidence obtained to date indicates that the endocrine disruptive properties of existing phthalates such as DEHP and DINCH can be circumvented without producing any untoward effects. These studies were supported by the CIHR Institute of Human Development, Child and Youth Health, the CIHR Training Program in Reproduction, Early Development, and the Impact on Health, and the Fonds de la Recherche du Québec en Santé.

20. Environmental Toxicant Exposure Induced Dysregulation of Ovarian Function.

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Widespread human exposure to environmental contaminants and residue concentrations in human tissues and reproductive fluids has been revealed mainly through advances in analytical chemistry. Although exposure to environmental toxicants has been widely documented, the concentrations measured are frequently low relative to concentrations shown to produce reproductive hazards to ovarian function (steroidogenesis and follicle development) in animal models. Even in the case of lifestyle behaviors such as cigarette smoking with well-established adverse effects on reproductive health the underlying mechanisms remain poorly defined. Through a series of animal experiments we discovered that cigarette smoke exposure induces ovarian follicle loss is driven by accelerated follicle recruitment as opposed to direct toxicity to primordial follicles. Subsequent experiments revealed up-regulation of AMP-κ α1+α2, a known initiator of autophagic signaling, in cigarette smoke exposed mice compared to controls. In contrast, two pro-survival factors AKT and mTOR were decreased in expression, an outcome that favors induction of the autophagy pathway. Moreover, cigarette smoke exposure induced down-regulation of CDKN1B, suggestive of cell cycle dysregulation. Taken together, we suggest that cigarette smoke exposure induces oxidative stress in the ovary leading to activation of reparative autophagy leading to attenuated follicle growth and ultimately follicle loss. We speculate that chronic cellular stress induced by environmental toxicants triggers the reparative autophagy pathway potentially leading to sub fertility.

21. Finding Hazards to the Ovary.

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Methods for identifying ovarian toxicants in humans are limited. The aim of this presentation is to stimulate research to investigate the potential use of antimullerian hormone (AMH) as a biomarker for detection of ovarian hazards.
AMH is produced by granulosa cells during follicular development starting in primary follicles and continuing as they grow during secondary, pre-antral and early antral follicle stages. Circulating AMH concentration has been correlated with the number of primordial follicles (ovarian reserve), and has been used most for assessment of infertility patients. Circulating concentrations decline with age after a peak in the early reproductive years. Several characteristics of AMH make it a potentially valuable tool in epidemiologic studies. Importantly it is relatively stable over the menstrual cycle, so blood draws do not need to be timed for specific days of the cycle. Also, its sensitivity to hormonal contraception and early pregnancy appears to be minor, so studies of premenopausal women could be done without the potential biases created by excluding these major groups. Toxicant exposures that damage growing follicles are expected to show decreases in AMH during the exposure. Toxicants that result in damage to primordial follicles will reduce ovarian reserve and show decreased in age-specific AMH levels compared to unexposed women, even after exposures ends. Both short and long-term effects are seen with chemotherapy treatments, the exposure that has been studied most regarding effects on AMH. Laboratory animal studies of the drugs lend further support to the distinct short- and long-term effects. Other exposures (e.g., other drugs or medical treatments, pesticides, solvents, and lifestyle factors) have been associated with alterations in AMH in either humans or laboratory animals, though few have been studied in both. Prospective studies to follow exposure effects over time are generally lacking. Critical next steps in a research agenda to investigate the feasibility of using AMH as a biomarker for detecting ovarian hazards include: 1) longitudinal studies of AMH in healthy, population-based samples, including infants and pre-pubertal children to better document life course changes, 2) coordination of research designs for studies in humans and laboratory animals to examine AMH changes in response to model toxicants with exposure timing at different life stages (prenatal, infancy, prepubertal childhood, puberty, early reproductive years, and late reproductive years) with laboratory animal exposures designed to mimic human exposure.


Cigarette smoking (CS) has been linked with decreased circulating estradiol concentrations, shorter menstrual cycle length, decreased fertility, and premature menopause in women who smoke compared to non-smokers. We previously demonstrated that CS exposure induces autophagy in ovarian granulosa cells in preference to apoptosis and is a potential novel alternative cell death pathway. However, the mechanisms regulating autophagy in ovarian granulosa cells are poorly defined. Therefore, adult female mice were exposed to mainstream CS twice daily for a total of 8 weeks; equivalent to a pack of cigarettes a day, using a whole body exposure system. One ovary/mouse was harvested for gene and protein analysis. Data were checked for normality and treatment effects were measured using t-test. Using a gene array, we found that CS induced a greater than 2-fold significant increase in the expression of pro-autophagy genes Cdkn1b, Map1lc3a, Bad and Systm1/p62. Expression of Cis7 and Pikh3c part of the PI3K/PIK signalling pathway, were attenuated by more than half the expression of the control group. Q-PCR analysis revealed a significant decrease in Akt-t (p=0.0041) expression only. Since mRNA changes are not always complemented with comparable protein expression changes and it is ultimately the post-translational changes that are the most notable indicators of cell signalling, we next investigated for changes in protein expression. Western blot analysis revealed significant CS-induced up-regulation of AMP-k α1+α2 (p=0.0119) and ATG7 (p=0.0006) and down-regulation of AKT-1 (p=0.0021), mTOR (p=0.0005), CDKN1B/p27 (p<0.0001), and CXCR4 (p=0.005) proteins. Up-regulation of AMP-k α1+α2, a known inhibitor of autophagic signalling, and ATG7 further suggests activation of the autophagy cascade. CS-exposure induced down-regulation of AKT and mTOR, two pro-survival factors, an outcome that favours induction of the autophagy pathway whereas down-regulation of CDKN1B is suggestive of cell cycle dysregulation. In summary, our data suggest that CS exposure induces ovarian follicle loss through induction of the autophagic cascade via the AMPK pathway together with inhibition of the anti-autophagic AKT and mTOR pathways. We further postulate that toxicant-induced dysregulation of reparative autophagy is a novel pathway central to impaired follicle development and subfertility. Funding support from Canadian Institute of Health Research (CIHR), Training Program in Reproduction, Early Development, and the Impact on Health (REDIH).

23. Wild caught Norway rats’ reproduction was reduced by 95% after taking liquid fertility management bait containing the active ingredients 4-vinylcyclohexene diepoxide and triptolide. Brandy Pyzyna, Stefanie Whish, Gary Witmer, Rachel Moulton, Cheryl A. Dyer, Loretta P. Mayer.

Norway rats pests cause extensive crop loss, infrastructure damage, and are vectors for several zoonotic diseases. The most common method used to reduce rat pest populations is the use of acute and anticoagulant poisons. However, poisons are non-specific and may kill non-target animals, such as companion animals, that consume them or may kill predators that eat rats killed by poisons such as hawks and owls. The worst possible scenario is the accidental poisoning of children, sometimes leading to their death. Due to these problems US EPA has restricted the retail sale of rodenticides for residential use. Finally, poisons do not cause a long-term reduction in rat populations due to the rapid reproductive rebound by the survivors and immigration of nearby rats. A more sustainable approach to reduce and manage rat populations is suppression of rat gonadal function. We have developed an oral, liquid palatable bait with two active ingredients, 4-vinylcyclohexene diepoxide (VCD) at 0.09% and triptolide at 0.001%. VCD targets primordial follicles causing their accelerated depletion leading to ovarian failure. Triptolide targets growing follicles stopping their ovulation. In addition, triptolide targets the epididymis and testes reducing sperm number making male rats infertile. In a proof of principle of experiment, we fed female and male Norway rats the bait over 15 nights and as a result they did not produce a single pup, while rats that consumed bait without active ingredients were fertile, producing litters with an average of 11 pups. Given our results with research rats we next tested if the bait formulation would cause infertility in wild caught Norway rats. Rats were caught at a sheep farm and brought into the animal research facility to acclimate, and, if pregnant, to give birth before being used in the study. For a total of 50 nights, the bait was given to wild caught female and male Norway rats with unlimited rodent chow and water available. There were 2 study groups, control rats that ate bait without active ingredients and treated rats that ate bait with active ingredients. After the first 15 nights of baiting, females (n=10) and males (n=10)

2015 Abstracts – Page 9
Heat Stress Induces Autophagy in Pig Ovaries during Follicular Development.
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Heat stress (HS) occurs when heat dissipation mechanisms are overwhelmed by heat accumulation processes and body temperature increases. HS is associated with seasonal infertility and therefore is a major source of production losses in the swine industry. Increased body temperature as a result of HS compromises swine reproduction through many mechanisms and potentially contributes to compromised oocyte integrity and reduced developmental competence of ensuing embryos. We have previously shown that HS can interfere with molecular pathways contributing to follicle recruitment and development in pigs. Autophagy is the process by which somatic cells recycle energy through the reutilization of cellular components and is activated by a variety of stressors. During autophagy the cytoplasm is sequestered into a double-membrane cytosolic vesicle, the autophagosome, which fuses with a lysosome to form an autolysosome enabling degradation by lysosomal hydrolases. In this process beclin 1 (BECN1) regulates membrane isolation after the initial induction of autophagy. Autophagosomal membrane extension is regulated by two ubiquitin-like conjugation pathways; one involving autophagy related 12 (ATG12) and one involving microtubule-associated protein 1 light chain 3 beta (LC3B). In autophagosomal membrane extension ATG12 forms a complex with ATG5 and LC3B is cleaved to form LC3B-II. The conversion of LC3B-I to LC3B-II is indicative of autophagic activity, and the amount of LC3B-II directly correlates to the abundance of autophagosomes. The objective of this study was to characterize changes in protein abundance of BECN1, ATG12 and LC3B-II, which correspond to autophagy induction, following cyclical HS experienced during the follicular phase preceding ovulation. To accomplish this, twelve gilts were synchronized using Matrix administered orally for 14 days and subjected to cyclical HS (n = 6) or thermal neutral (TN; n = 6) conditions for 5 days immediately following Matrix withdrawal during the synchronized follicular phase for all gilts. During HS the average room temperature for each day was 20.3°C ± 0.5°C for TN conditions and 31.1°C ± 1.4°C for HS conditions. During the maximal HS load for each day, the HS pigs had increased (P = 0.001) average rectal temperatures (39.8°C ± 0.2°C) compared to the TN pigs (38.8°C ± 0.2°C). At 124 hours following Matrix withdrawal, gilts were sacrificed and ovaries were harvested and used for protein extraction and Western blot analysis for autophagy-induction related proteins. Ovarian protein abundance of BECN1 and LC3B-II were each elevated approximately 5-fold as a result of HS (P = 0.001 and 0.003, respectively). The abundance of the ATG12-ATG5 complex was decreased approximately 2-fold as a result of HS (P = 0.002), which is counterintuitive but could be due to a shift in ubiquitin-like conjugation pathways. These results suggest that induction of autophagy occurs in the ovary in response to HS during the follicular phase. Future investigations are required to determine the cell-specific changes responsible for HS-induced autophagy in the ovary and if autophagy is a mechanism which enables the ovary to mitigate the deleterious effects of HS on the follicle and developing oocyte.

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During cell division, chromosomes attach to spindle microtubules at sites called kinetochores, and force generated at the kinetochore-microtubule interface is the main driver of chromosome movement. However, despite this fundamental role for the kinetochore in modulating chromosome dynamics, a recent study reported the surprising discovery that kinetochores are not required for chromosome segregation on acentrosomal spindle in C. elegans female reproductive cells (oocytes) (1). The mechanisms driving chromosomes apart in these cells are not understood but are important to uncover, as they represent a new strategy for controlling chromosome dynamics during cell division. We have now discovered that lateral microtubule-chromosome associations established during prometaphase to facilitate congress remain intact during anaphase to drive separation, defining a novel form of kinetochore-independent segregation. Spindle pole broadening in anaphase creates microtubule channels that are open from pole to pole, and chromosomes segregate through these channels until they reach the microtubule minus ends; at this stage the channels close. Moreover, experiments with both bipolar and monopolar spindles revealed that chromosome dynamics during congression and segregation are controlled by opposing forces acting on the chromosomes. Plus-directed forces are mediated by a protein complex that forms a ring around the chromosome center and dynein on chromosome arms provides a counterbalancing minus-end force. At anaphase onset, ring removal coupled to the release of cohesion shifts the forces, triggering poleward movement along lateral microtubule bundles. This represents an elegant strategy for controlling chromosomal movements during cell division distinct from the canonical kinetochore-driven mechanism. (1) Dumont, et.al. NCB 12, 894-901
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Spermatogonial stem cells reside in specific local microenvironments, or niches. We use the Drosophila testis as model for studying stem cell niche biology in vivo, as it contains a single, relatively well-characterized niche comprised of a cluster of quiescent somatic support cells called the hub. Spermatogonial (or germline) and somatic stem cells (called cyst stem cells, or CySCs) adhere to the hub and cooperate to produce a lifetime supply of sperm. We recently found that the sexual identity of adult stem cells in this tissue is actively maintained by local niche signaling. The transcription factor Chronologically inappropriate morphogenesis (Chinmo), is expressed in response to niche signals. In turn, this putative transcription factor promotes the male identity of adult CySCs and their progeny, by promoting expression of the canonical male sex determinant DoublesexM (DsxM, which is the homologue of mammalian Doublesex and mab-3 related transcription factor 1, or DMRT1). DMRT1 is necessary and sufficient to promote male cell identity in the mouse gonad, and understanding the underlying mechanisms is of interest. Since Drosophila provides a useful system for complementary mechanistic studies, here we investigated Chinmo’s role in the Drosophila ovary. Chinmo is not expressed or required in the adult ovary, but ectopic expression of Chinmo within the adult ovarian somatic cells is sufficient to induce a male somatic identity in the adult ovary. This phenotype progresses over time, eventually completely disrupting oogenesis, but Chinmo is masculinizes the ovary in a DsxM-independent mechanism. The likely cellular targets of this sex transformation in the ovary are somatic stem cells (follicle stem cells), and competition between stem cells for their niche appears to be important in permitting sex transformation phenotypes to occur. We conclude that Chinmo is both necessary and sufficient to promote a male identity in adult gonadal somatic cells. The ability to reprogram the sexual identity of adult somatic cells in the adult Drosophila ovary as well as in the testis provides a unique opportunity to uncover the molecular mechanisms regulating both transdifferentiation of stem cells in vivo, and the highly conserved process of sex maintenance.

27. Transgenerational Inheritance of Centromere Identity in the Mammalian Oocyte.
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27. Transgenerational Inheritance of Centromere Identity in the Mammalian Oocyte.
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Centromeres control genetic inheritance by directing chromosome segregation, but centromeres in most species are not encoded by DNA sequence. Instead, centromeres are specified by the presence of Centromere Protein A (CENP-A), a histone H3 variant which replaces canonical histone H3 in centromeric nucleosomes. This epigenetic mechanism raises the question of how centromere identity is maintained during the extended prophase I arrest in mammalian oocytes. In contrast to DNA, how protein-based information is stably maintained is unknown. In cycling somatic cells, CENP-A is maintained by a cell cycle-coupled pathway, and there is no known mechanism to assemble new CENP-A nucleosomes outside of the G1-phase of the cell cycle. We tested whether centromere inheritance depends on a specialized meiotic loading pathway or on the retention of CENP-A nucleosomes loaded prior to the prophase I arrest. To eliminate a putative meiotic loading pathway, we generated mice in which the Cenpa locus is conditionally inactivated in oocytes in resting primordial follicles. We find that oocytes do not stockpile soluble CENP-A protein, and Cenpa mRNA is not present in Cenpa−/− oocytes. Moreover, we find no difference in centromeric CENP-A levels between Cenpa−/− and control oocytes at 12 months of age, indicating that a meiotic loading pathway does not contribute significantly to maintaining CENP-A nucleosomes. This evidence indicates that stability of CENP-A nucleosomes underpins centromere inheritance through the female germline.

28. A Requirement for Aurora Kinase B that is Independent from Aurora Kinase C in Female Meiosis.
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Meiosis differs fundamentally from mitosis because it involves two consecutive rounds of chromosome segregation without an intervening round of DNA replication. One way these differences are coordinated is through meiosis-specific signaling molecules. Aurora kinase C (AURKC) is primarily expressed in meiotic cells and arose through a genome duplication of Aurora kinase B (AURKB), a regulator of chromosome segregation in mitosis. The presence of AURKC is conserved in mammals suggesting an evolutionary requirement for having both protein kinases in meiosis. A predominant focus has been on establishing a role for AURKC in regulating kinetochore-microtubule connections. Therefore, whether AURKB is required for female meiosis remains a mystery. At metaphase of meiosis I AURKB localizes to the spindle and AURKC localizes to chromosomes suggesting that they have different meiotic functions. Published work on AURKB in Drosophila suggests that if AURKB is required during female meiosis or how much total AURKB/C activity is required we utilized oocyte-specific Cre drivers (ZP3 and Gdf9) to excise exons 2-6 in the murine Aurbk locus that were flanked by loxP sequences. These mouse strains were then crossed into an Aurkc−/− background to develop a genetic allelic series expressing different copy numbers of Aurbk and Aurkc. We compared phenotypes of WT mice to that of single knockouts and to those from mice containing 1 copy of Aurbk or Aurkc. These studies revealed that AURKB is required for meiosis. Oocytes lacking AURKB but not AURKC had significant increases in spindle morphology defects, consistent with its localization. Spindle abnormalities persisted in oocytes with a single copy of either kinase but were most severe in Aurbk−/− Aurkc−/− suggesting a specific role for AURKB in regulating the spindle. This requirement persisted in those oocytes that progressed to metaphase of meiosis II (Met II) both in vitro and in vivo. Aurbk−/− and Aurkb−/− Aurkc−/− Met II eggs were significantly more aneuploid than eggs from Aurkc−/− and Aurbk−/− Aurkc−/− mice suggesting a requirement for the physical presence of AURKB to maintain the spindle assembly checkpoint. These results phenocopy those seen in aged females, suggesting these mice exhibit premature reproductive aging. In support of this model, histology of Aurbk−/− ovaries revealed a significant decrease in the number of follicles. Furthermore, in fertility trials the first litters of Aurbk−/− females were consistently larger than WT controls but were followed by a rapid decline in the number of pups produced per litter suggesting a premature loss of follicular reserves. These data show for the first time that AURKB is not only required for female meiosis but that it has at least one function in regulating the meiotic spindle that AURKC is not capable of conducting. The early depletion of follicles leading to a rapid decline in fertility also suggests an AURKB-independent role for AURKB in oocyte development. Taken together these phenotypic analyses indicate
an unexpected division of labor between AURKB and AURKC during female meiosis that was not appreciated in mitotic cell lines ectopically expressing AURKC. This research was supported by a grant from the NIH (R00HD061657) and by RU institutional start-up funding.

29. Challenges of segregating chromosomes in oocytes.
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Mammalian oocytes and embryos are frequently affected by aneuploidy, caused by errors during chromosome segregation. The aneuploidy originated from meiosis or from the first cleavage cycles after fertilization is usually incompatible with embryonic development. In a few cases, when the extra chromosomes are tolerated, aneuploidy causes severe mental and developmental disorders, such as Down syndrome. The reason why chromosome segregation errors are more frequent in germ cells and embryos in comparison to somatic cells is unknown. We believe that the problem lies in less stringent chromosome segregation control mechanisms operating in这些 cells. Our study is focused on a function of a surveillance checkpoint mechanism called Spindle Assembly Checkpoint (SAC) in oocytes. Using micromanipulation and live cell confocal microscopy, we have performed a functional analysis of SAC in mouse oocytes and tested whether it is capable of arresting cell cycle in situations when univalent chromosomes or single chromatids are present in meiosis I. The same approach was used for monitoring the activity of SAC on individual kinetochores and for correlation of this activity with chromosome movements, spindle formation, onset of Anaphase Promoting Complex (APC) activation and polar body extrusion (PBE) at various time points during the first meiotic division. Our results show important and unexpected differences in SAC function in oocytes compared to somatic cells. The experiments have revealed that in contrast to the somatic cells, single chromatids, univalent and unaligned chromosomes are unable to mount functional SAC response and to prevent anaphase onset. Our results indicated that the checkpoint mechanisms involved in monitoring chromosome segregation, are in oocytes less capable to detect or respond to errors in this process, which might explain high incidence of aneuploidy in these cells.

30. Chromosome Segregation Errors in Meiosis and Mitosis—Similar, but Different.
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Chromosome segregation errors during early development cause embryo aneuploidy, which is considered detrimental for embryo fitness. Whereas segregation errors in oocytes cause embryos in which all blastomeres are aneuploid, errors in the early mitotic divisions cause embryos with mixtures of aneuploid and euploid cells – so-called ‘mosaic’ embryos. Our lab aims to explain these defects using a combination of live-cell time-lapse imaging and mechanistic intervention experiments. In this presentation I will discuss recent work aiming to unravel the causes of these errors, and determine whether meiotic and mitotic aneuploidies have common origins. Errors in meiosis-I are well-known to be exacerbated by advancing maternal age. I will discuss evidence that these age-related meiosis-I segregation errors may be caused not only by changes in structural aspects of the chromosome such as chromosome cohesion, but also by defective function of the spindle, the organelle responsible for chromosome segregation. In the early embryo I focus on the causes and consequences of lagging anaphase chromosomes, which are widely regarded as hazardous for the fidelity of chromosome segregation. Understanding the origin of errors in meiosis and mitosis cannot rely on information from traditional model systems, but demands that the mechanisms of chromosome segregation are directly examined in the unique cellular setting of early mammalian development.

31. Exploiting Centromere Asymmetry in Meiosis I to Probe Spatial Regulation of Kinetochore Microtubules.
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Accurate chromosome segregation in cell division depends on recognizing and destabilizing erroneous attachments of kinetochores to microtubules. Improper attachments are typically positioned off-center on the spindle and lack tension between kinetochores. Low tension is a widely accepted as a mechanism for recognizing errors, but it has been difficult to test whether chromosome position also contributes to regulating microtubule attachments. To experimentally uncouple position and tension, we established a meiotic system in which kinetochores attached to opposite spindle poles differ in their interactions with microtubules. Crossing mouse strains with different centromere strengths, manifested by unequal kinetochore protein levels, generates meiotic bivalents that are positioned off-center on the spindle in oocytes in meiosis I, while under normal tension. Using this system, we show that kinetochore microtubules are destabilized by proximity to spindle poles, and that stable attachments are restored by inhibiting Aurora A kinase at spindle poles. During the correction of attachment errors, detachment of kinetochore microtubules near spindle poles provides an opportunity for re-orientation and formation of correct attachments. Chromosome position on the spindle therefore provides spatial cues for the fidelity of cell division, complementary to tension-dependent regulation.

32. DNA Damage Induces Arrest Of Mammalian Oocytes In Meiosis I By Activating The Spindle Assembly Checkpoint.
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Extensive damage to maternal DNA during meiosis can cause infertility, birth defects and abortions. However, gametic strategies exist to mitigate the occurrence of these events. For example in the early phases of follicular growth, oocyte atresia follows DNA damage. This pathway disappears during growth, and it is therefore unknown if fully-grown oocytes have a mechanism to prevent creation of DNA-damaged embryos. Here we show that the DNA Damage Response (DDR) activates a pathway involving the Spindle Assembly Checkpoint (SAC) proteins Mps1 and Mad2. This provides a very effective block to Anaphase-Promoting Complex activity, and consequently the formation of mature eggs, in response to chemically induced double strand breaks, UVB and ionizing radiation. DNA induced SAC arrest in oocytes contrasts with somatic cells, where DNA damage fails to affect mitotic progression. However, it uncovers a new function for the meiotic checkpoint, which in the context of detecting microtubule-kinetochore errors has hitherto been labeled as weak or ineffectual in
mammalian oocytes. We propose that its essential role in the detection of DNA damage gives new light to its biological purpose in mammalian female meiosis.

33. RNF212 is a Meiosis-Specific SUMO E3-Ligase That Promotes Crossing-Over and Functions as a Meiotic Checkpoint Factor to Eliminate Defective Oocytes.
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Synapsis, recombination, and segregation of homologous chromosomes (homologs) are essential features of meiosis that ensure formation of gametes with balanced euploid genomes. Defects in synapsis and recombination cause gametocytes to be eliminated via poorly characterized checkpoint processes. In females, pre- and post-partum atrophy and atresia dictate the size of the resting oocyte pool and thus reproductive lifespan. Complete post-partum depletion of the oocyte pool is observed in a variety of mouse mutants that are defective for homologous recombination and/or chromosome synapsis. For example, Spo11 mutants fail to initiate recombination, show severe defects in homolog synapsis, and eliminate their oocyte pools within three months post partum. In Msh4 mutants, recombination is initiated, but its progression is blocked and homolog synapsis is defective. The oocyte pool of Msh4 mutants is depleted within four days following birth. Current evidence implies that two distinct branches of the meiotic checkpoint pathway respond to defects in recombinational repair or homolog synapsis, respectively.

We identified RNF212 as a novel component of the meiotic checkpoint machinery. Previously, we showed that RNF212 promotes crossing-over by selectively stabilizing pro-crossover factors at a subset of ongoing recombination sites. Unexpectedly, we now find that recombinational repair or homolog synapsis, respectively.

Following birth. Current evidence implies that two distinct branches of the meiotic checkpoint pathway respond to defects in recombinational repair or homolog synapsis, respectively. We identified RNF212 as a novel component of the meiotic checkpoint machinery. Previously, we showed that RNF212 promotes crossing-over by selectively stabilizing pro-crossover factors at a subset of ongoing recombination sites. Unexpectedly, we now find that Rnf212 mutation confers a striking restoration of the oocyte pool in both Spo11 and Msh4 mutant ovaries. The ovaries of Msh4/“Rnf212” double mutants contain more than 2000 follicles, which suggests that the Rnf212 mutation fully rescued the entire oocyte pool of the Msh4 mutant. Radiation-induced oocyte apoptosis is also compromised in the absence of RNF212. Irradiation at a 0.45-Gy dose eliminates essentially all primordial follicles from 10 dpf (days post-partum) ovaries five days after irradiation. In contrast, 1.3% of the primordial follicles in Rnf212 mutant ovaries survive from the irradiation at 5 dpf. Moreover, the enrichment of RNF212 in primordial follicles also suggests RNF212 functions in oocyte quality control.

Rnf212 encodes a RING-family E3-ligase that catalyzes protein modification by the ubiquitin-like molecule, SUMO. These data imply that RNF212-mediated SUMOylation is a novel component of the meiotic checkpoint-signaling pathway that leads to gametocyte apoptosis. Together, our results uncover a new aspect of meiotic checkpoint signaling and point to RNF212 as a central coordinator of chromosome synapsis, recombination, and meiotic progression.

34. Unified Theory for the Existence of a Single Checkpoint Mechanism During Meiotic Prophase I.
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Higher rates of aneuploidies contributed by oocytes and apparent tolerance to low levels of chromosome asynapsis during meiotic prophase I suggested less stringent meiotic checkpoints in females. Based on the phenotypic observations from various meiotic mutants it has been now widely accepted that abnormal oocytes can be eliminated by two independent checkpoint mechanisms operating at the end of prophase I. Persistent unrepaired meiotic DNA Double Strand Breaks (DSBs) activate the DNA-damage-dependent checkpoint. In the absence of those SPO11-catalyzed DSBs, second DNA-damage-independent surveillance mechanism responds to asynapsed chromosomes. DNA damage checkpoint is thought to be more stringent leading to complete oocyte depletion by 3 weeks of age as observed in recombination defective Trip13 mutant females. The putative synapsis checkpoint seems to be more permissive as few asynaptic oocytes survive until 2 months of age as described in Spo11 null females. We have recently described the meiotic DNA damage checkpoint and its key player CHK2 kinase. Deletion of Chk2 gene rescued Trip13 mutant oocytes from elimination. Surprisingly, Chk2 deficiency also rescued Spo11-/- oocytes suggesting that either CHK2 is also involved in the putative synapsis checkpoint or DNA damage of unknown origin triggers DNA damage response in Spo11 deficient oocytes. Recent reports provide evidence for non-meiotic DNA damage in Spo11-/- oocytes supporting the latter. Based on published observations and our own experiments we argue that asynapsis itself may not trigger meiotic checkpoint. Our results provide evidence for the existence of a single meiotic checkpoint sensing only DNA damage in a dose sensitive manner responsible for elimination of defective oocytes at the end of prophase I.

FUTURE SESSION 4: The Ultimate ART—How Far Can We Go with Assisted Reproduction and What Do We Gain?

35. Human Oocytes in Stem Cell Research and Reproduction.
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The past few years have seen significant progress in the manipulation of human oocytes for stem cell research and potentially for reproduction. Methods that have been established in animals have been translated to human cells. An important aspect of this progress has been the access to human oocytes for research. My laboratory investigates the use of nuclear transfer to generate stem cells from subjects with diabetes. We found that human oocytes have the ability to reprogram a somatic cell to a pluripotent state. The derivation of stem cell lines allowed us to compare embryonic stem cells to the reprogramming by induction with transcription factors. We found that at least with regard to gene expression and DNA methylation patterns, the two methods result in highly similar cell types. But both methods are associated with an increased rate of abnormalities in imprinted genes. Nuclear transfer can also be used to replace the oocyte genome with the genome of another oocyte. Because the oocyte genome (unlike a somatic genome) is prepared to undergo embryonic development, this method could be useful for reproductive treatments. Two applications are being considered, to restore developmental competence to
oocytes with low developmental potential, and to prevent the transmission of mitochondrial disease. We found that mitochondrial variants can effectively be replaced with nuclear transfer, and will provide an update on our attempts to translate this method to the clinic.

36. **Polar Body Transfer for the Prevention of Inherited Mitochondrial Diseases in the New Era of Reproductive Medicine.**
   Jenhong Zhu.

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   1Inherited mitochondrial DNA diseases transmit maternally and cause severe phenotypes. Large numbers of mitochondria DNA (mtDNA) deletions in brain and heart become fatal or in young adulthood with epilepsy, such as mitochondrial encephalopathy. Currently, there is no effective therapy for these diseases; however, nuclear genome transfer between patients' and healthy eggs to replace mutant mtDNAs holds promises. After many years of scientific and ethical debate, UK Parliament has decided this year to permit the use of mitochondrial donation to give families with serious mitochondrial disease the possibility of having their own healthy genetic children. This step could open a new era of reproductive medicine. However these progresses are also at the cutting edge of both science and of ethics. Globe researchers are working on new medical techniques that could allow women to avoid passing on genetically inherited mitochondrial diseases to their children. Considering that a polar body contains few mitochondria and shares the same genomic material as an oocyte, we perform polar body transfer to prevent the transmission of mtDNA variants. We compare the effects of different types of germline genome transfer, including spindle-chromosome transfer, pronuclear transfer, and first and second polar body transfer, in mice. Reconstructed embryos support normal fertilization and produce live offspring. Importantly, genetic analysis confirms that the F1 generation from polar body transfer possesses minimal donor mtDNA carryover compared to the F1 generation from other procedures. Moreover, the mtDNA genotype remains stable in F2 progeny after polar body transfer. To extend our findings in mouse models, we performed array comparative genomic hybridization (aCGH) to detect genomic aberrations between PB1 and its counterpart, the spindle-chromosome complex, in a human MII oocyte, PB2 and the female pronucleus in a human zygote at the single-cell level. Genomic integrity of human polar bodies is confirmed by aCGH. Our preclinical model demonstrates polar body transfer has great potential to prevent inherited mitochondrial diseases. But not too rush to move on from bench to clinic. Further research is needed to guarantee safety or efficacy before applied in the clinic. We want to know how it happened, and how we can control it.

37. **Conversion of epiblast type porcine induced pluripotent stem cells into ground state naïve pluripotent stem cells.**
   Ye Yuan1, Jin-Kyu Park1, Yuchen Tian2, Nicholas J. Genovese1, Lee D. Spate1, Randall S. Prather1, Toshihiko Ezashi1, R. Michael Roberts1.

   1Division of Animal Sciences, Columbia, MO, USA; 2Life Sciences Center, Columbia, MO, USA
   1Pigs are important agricultural animals and are widely used as biomedical models. Having authentic porcine embryonic stem cells (ESC) and fully reprogrammed transgene-free induced pluripotent stem cells (iPSC) available would enhance the value of pig models even further. We hypothesized that the culture conditions developed for naïve type mouse ESC and epiblast type human ESC may be insufficient to support the full activation of endogenous pig pluripotency networks in porcine iPSC and ESC. Therefore, the objective of this study has been to establish optimal naïve pluripotency culture conditions for porcine ESC and iPSC. To achieve complete reprogramming and a stable ground pluripotency state, first we attempted to adapt culture conditions recently described for maintaining naïve state human ESC and iPSC cultures to porcine stem cells. In particular, we examined whether or not a medium developed for naïve human iPSC and ESC could convert porcine epiblast/prime type iPSC into a naïve pluripotency state. Under these culture conditions, the porcine iPSC colonies had undergone a gradual transition to the morphologies analogous to those of naïve-type mouse and human ESC and iPSC, but they were negative for alkaline phosphatase (AP) staining, indicating that they had lost their pluripotency. We then tested the compounds in the medium developed for human cells that might have caused this loss of pluripotency in the pig cells and found that the presence of TGF-beta was detrimental. However, an optimized medium containing a combination of p38 inhibitor, JNK inhibitor, TGF-beta inhibitor, GSK3 inhibitor, MEK inhibitor, ROCK inhibitor, FGF2 and LIF was able to convert the flattened epiblast type porcine iPSC into dome shaped naïve type iPSC. After three passages in such new conditions, the epiblast type porcine iPSC formed compacted, dome shaped colonies, and became AP-positive. The converted, naïve type, porcine iPSC could be dispersed into single cells for passaging, i.e. clonally propagated, had shortened cell doubling time (from 17 h to 13.5 h), and relied on both FGF/ACTIVIN/NODAL and LIF/JAK/STAT signaling for cell self-renewal. They were positive for the pluripotency markers SSEA1, NANOG, POU5F1 and SOX2, as revealed by immunohistochemistry. More importantly, the expression of endogenous NANOG (determined by both immunohistochemistry and quantitative PCR) was significantly increased under the new culture conditions. This observation is in agreement with previous studies that the up-regulation of NANOG is the hall-mark of the establishment of naïve pluripotency. We also tested the ability of such culture conditions to derive porcine naïve type ESC from porcine blastocysts. Day 7 porcine IVF blastocysts were collected and cultured in medium without any inhibitors to form primary colonies. Once these were observed, the medium was changed to the newly formulated medium that included all the inhibitors described above. Dome-shaped, AP-positive colonies, phenotypically similar to naïve type mouse ESC developed. However, growth was slow, and the cells could not be maintained for beyond 3-4 passages in such conditions. We are now attempting to optimize the medium further with the goal of establishing self-renewing porcine ESC of the naïve type.

38. **Genetic rescue of Brucella abortus-positive Yellowstone bison via assisted reproduction.**
   Jennifer P. Barfield1, Will Falbo1, Alyssa Grossnickle1, Hayley M. Benham1, Zella Brink1.

   1Department of Biomedical Sciences, Colorado State University, Fort Collins, CO, USA
   1Bison from Yellowstone National Park (YNP) are valued for their unique genetics, but the disease brucellosis has limited conservation strategies that can be used to preserve these bison. The objective of this study was to develop a suite of assisted reproductive techniques that can be used to produce healthy offspring from bison that are sero-positive for *Brucella abortus*, the bacterium that causes brucellosis. Ovaries from 14 immature (IM), 61 pregnant adult (AP), and 15 non-pregnant (ANP) adult bison cows from YNP were collected at slaughter. Oocytes were immediately aspirated and shipped overnight in maturation medium to the laboratory at Colorado State
University where they were fertilized approximately 24 h after collection with frozen-thawed sperm from one of five YNP bulls and cultured to the blastocyst stage. Cleavage rates were obtained after the first 36h of culture, and blastocyst rates calculated on day 7 – 7.5. Grade 1 and 2 blastocysts were vitrified on cryotops using a two-step vitrification method: 5 min in 1.5M ethylene glycol in a chemically defined medium (CDM) followed by 45 sec in 7M ethylene glycol + 0.6M galactose + 18% Ficoll in CDM before being plunged into LN2. Embryos were warmed via successive incubation for 3 min in warmed HCDM-1 with 1 M, 0.5M, and lastly 0.25 M galactose. Embryos were then washed through a trypsin rinse per IETS guidelines prior to transfer to recipient bison. Spent media from the final embryo culture wells in addition to embryos that did not reach the blastocyst stage of development were cultured for Brucella abortus. Pierson Chi-Square test was used for statistical analyses. The mean numbers of oocytes retrieved (± SD) from IM, ANP, and AP bison cows were 23 ± 8.7, 32 ± 14.2, and 23 ± 10.5, respectively. Mean cleavage rates were 45 ± 24.3% for IM, 51 ± 23.4% for ANP, and 70 ± 25.9% for AP bison cows. Day 7 blastocyst production rates per oocyte and per cleaved oocyte were 8 ± 8.94 % and 12 ± 14.22% for IM, 9 ± 13.5% and 15 ± 22.61% for ANP, and 11 ± 11.6% and 16 ± 17% for AP. Of the bulls used for IVF, one resulted in no embryo development to the blastocyst stage despite a cleavage rate of 30 ± 19%. There were no statistical differences between the cleavage and blastocyst rates for the other 4 bulls. All spent media and undeveloped embryos were culture-negative for Brucella abortus. Eleven Brucella-negative bison recipients were synchronized with 100 µg GnRH (i.m.) and the insertion of a CIDR followed 7 days later by removal of the CIDR and 25 mg Lutalyse (i.m.). Embryos originating from Brucella-positive bison females were transferred to recipients 10 days after removal of the CIDR. One pregnancy was confirmed 4 months post-transfer and is on-going. This study demonstrates that reproductive material from Brucella abortus sero-positive bison females can be used to establish viable pregnancies via assisted reproductive techniques. Disease status of the offspring and recipient mother will be determined shortly after parturition. This research was supported by the Bernice Barbour Foundation and the Wildlife Conservation Society.

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Comprehensive chromosome screening (CCS) for aneuploidy in the human blastocyst has recently demonstrated the expected clinical benefit in 3 randomized controlled trials. CCS improves the success of in vitro fertilization (IVF) through selection of euploid embryos for transfer, increasing the implantation rate in the first attempt, reducing miscarriage rates of ongoing pregnancies, and increasing the effectiveness of elective single embryo transfer. However, ~30% of euploid embryos remain unsuccessful. Therefore, additional biomarkers of reproductive potential are needed to further enhance embryo selection and continue to improve IVF success rates. Given that approximately one third of the aneuploidy found in human embryos may be derived from postzygotic mitotic errors, many "euploid" embryos may actually possess mosaicism. That is, some embryos identified as euploid may have aneuploid cells in the remaining embryo or at low levels within the trophectoderm biopsy. This study evaluated two methods which may improve the identification of mosaic embryos and therefore enhance embryo selection. First, next-generation sequencing (NGS) based CCS was evaluated for its ability to provide enhanced sensitivity to detection of aneuploidy within a mosaic sample. Mixtures of cells from cell lines were used to model different levels of aneuploidy mosaicism within a trophectoderm biopsy. Detection was demonstrated to a level of 16% trisomy (chromosome 13, 15, or 18) or monosomy (X chromosome in female/male mixtures) within 6-12 cell samples (p<0.05), indicating that NGS based CCS may provide a more sensitive method of detecting aneuploidy within a trophectoderm biopsy. In the second phase of this study, time-lapse imaging was evaluated for its ability to distinguish embryos with mosaicism from those with a uniform chromosomal makeup. Fifty three discarded blastocysts with previously collected time-lapse data were classified as either uniform aneuploid or mosaic through NGS based CCS analysis of multiple sections. Twelve (22.6%) of the embryos displayed evidence of mosaicism (i.e. reciprocal aneuploidy in two different sections) while the remaining embryos were found to possess uniform aneuploidy. The time taken by mosaic embryos to achieve the first cytokinesis and blastocyst cavitation were both significantly longer compared to uniformly aneuploid embryos (p=0.02 and 0.03, respectively), indicating that these parameters may be predictive of postzygotic mitotic errors (i.e. mitotic nondisjunction) and general mosaicism. In conclusion, this study has led to the preclinical development of two tools which may provide increased sensitivity to the detection of mosaicism within the human blastocyst and enhance embryo selection to improve the success of IVF. Based on these data, future clinical trials will involve characterizing these methods’ predictive value for actual clinical outcomes.

40. Mapping the Route from Totipotency to Lineage Specification in Mammalian Development.
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Implantation is the critical phase in mammalian pregnancy during which the basic body plan is generated. At this stage, the founder tissue of the foetus - the epiblast - transforms into a polarized epithelial tissue and initiates the progression towards lineage specification. Given the small size and inaccessibility of the embryo at this stage, the mechanics underlying the observed architectural changes and how they are linked to the restriction of developmental plasticity are largely unknown. Here, by combining a new embryo culture method with 3D embryonic stem cell cultures, and dissection of embryos from the mother’s uterus, I will describe how embryo architecture and gene expression profiles evolve together upon implantation. These studies represent the basis to explore how changes in tissue shape may route the exit from pluripotency during implantation development.

41. Do We Need Y Chromosome for Successful Assisted Reproduction in the Mouse?
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The mammalian Y chromosome, once thought to be a genetic wasteland, is now known to encode a battery of genes, many of which are thought to be involved in male reproduction. A substantial amount of work has been done to define which genes are important for maintaining sperm function under normal, in vivo, conditions. In the era of assisted reproduction technologies (ART), however, it is possible to bypass several steps of normal fertilization using immotile, nonviable, or even immature sperm. Consequently, in the context of ART, the essential roles of the Y chromosome genes may become abrogated. We have shown before that live offspring can be obtained
from mice lacking the entire Y chromosome long arm, in which the Y chromosome was reduced from 89.5 Mb to ~3.5 Mb and encoded only 9 genes and 3 gene families. Subsequently, we demonstrated that live offspring can be generated using germ cells from mice with the Y chromosome contribution limited to only two genes, the testis determinant factor Sry and the spermatogonial proliferation factor Eif2s3y. Sry is believed to function primarily in sex determination during fetal life. Eif2s3y may therefore be the only Y chromosome gene required to drive mouse spermatogenesis, allowing for a formation of haploid germ cells that are functional in assisted fertilization. If so, what is the importance of the Y chromosome in male reproduction? The answer lies in defining the need. The Y chromosome genes are undoubtedly needed for many aspects of reproduction involving the development of mature sperm and its function in normal fertilization. However, when it comes to ART, the mouse Y chromosome contribution may be brought to a bare minimum of Sry and Eif2s3y. Indeed, it may well be possible to eliminate the Y chromosome altogether if appropriate replacements are made for those two genes. In my talk I will present our new data documenting further reduction of the mouse Y chromosome gene content and its effects on the outcome of ART. [Funded: NIH HD072380 and HCF 14ADV-64546].

42. Microgrooves and fluid flows in the bovine female tract provide preferential passageways for sperm over the pathogen Trichomonas foetus.
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Successful mammalian reproduction requires that sperm migrate through a long and convoluted female reproductive tract before reaching oocytes. For many years, fertility studies have focused on biochemical and physiological requirements of sperm. The objective of this study was to show that the biophysical environment of the female reproductive tract, specifically the presence of microgrooves and fluid flow, critically guides sperm migration while at the same time preventing the invasion of sexually transmitted pathogens. We developed a microfluidic device to mimic microgrooves and fluid flows found in the cervix and uterine portion of the bovine reproductive tract. The device was made of polydimethylsiloxane (PDMS) molded from a silicon wafer and mounted on a glass slide. It contained six parallel channels, some of which were lined with smooth walls and others with 20 µm × 20 µm microgrooves in the upper wall. A syringe pump provided controlled physiological flow rates of 0 – 3 µl/min. We found that (1) when bull sperm encountered a µ wall, they tended to travel along it (81±7%, total 210 sperm from 3 bulls), while a flagellated sexually transmitted bovine pathogen, Trichomonas foetus, did not (22±10%, N=97 cells from 3 cultures); (2) sperm traveled with higher directional persistence (net displacement divided by path length, 0.87±0.02, N=50) than T. foetus (0.58±0.04, N=50, P<0.001); (3) sperm were unaffected by a slow fluid flow (1 µl/min) and swam upstream against a faster flow (90±14% upstream against a 2 µl/min flow, N=50), while T. foetus were brought downstream by 1 µl/min (98±14% downstream, N=50). Furthermore, sperm were much more likely to enter the microgrooves and travel along them (98±14%, N=50) than T. foetus (8±14%, N=50). With the presence of a flow, sperm were able to utilize the microgrooves to swim upstream and avoid being swept away by a 3 µl/min flow through the main channel (32±6% swept downstream), while T. foetus did not enter microgrooves and were swept downstream (98±11%). We attribute the differences in abilities of sperm and T. foetus to swim against flow to their distinct types of locomotion; specifically, sperm swim using a posterior flagellum and behave as “pusher” type microswimmers (which are known to swim along walls), whereas, T. foetus swim primarily via three anterior flagella and demonstrate much lower affinity for walls. The addition of 0.7% long chain polyacrylamide to mimic the viscosity of estrous cervical mucus had no effect on the different responses of sperm and T. foetus to solid surfaces. This work highlights the importance of biophysical cues within the female reproductive tract in the reproductive process and provides insight into co-evolution of males and females to promote fertilization while suppressing infection. Furthermore, the results provide novel directions for the development of in vitro fertilization devices and contraceptives. NIH R01HD070038-01 to SS and MW.

43. The Proteome of Exosomes Isolated from the Seminal Fluid of Oligoasthenospermic Patients Emulate Cell Surface Protein Expression of Spermatozoa from Fertile Normozospermic Individuals.
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The spermatozoal population from fertile individuals contains many abnormal cells, therefore the characteristics of individual fertile sperm remains enigmatic. Furthermore, the communication between mature spermatozoa and its microenvironment, namely the seminal fluid, are not well understood. Specifically, the cell surface proteome of spermatozoa and exosomes from normal and abnormal semen have not been studied in depth. In this study, we have utilized a High-Throughput Flow Cytometry (HTFC) to investigate the expression of 375 cell surface proteins from spermatozoa and seminal fluid cell-derived exosomes. Motile spermatozoa and seminal fluids were obtained from 20 normozoospermic ejaculates from men with proven fertility and 14 infertile individuals. Exosomes were enriched from seminal fluid by a series of differential centrifugations and isolated by magnetic beads with CD9 Exo-Flow capture kit. HTFC was performed on spermatozoa and exosome.

Substantial heterogeneity of normal sperm was demonstrated with 96 proteins being expressed only on minor sperm sub-populations (1-20% of total spermatozoa expressed these proteins in a specific sample). Hundred and three proteins were expressed on most spermatozoa, the majority of them have not been reported before. Interestingly, spermatozoa from infertile patients expressed only 14 of these proteins (p<0.0001). We were able to identify 29 proteins which were highly expressed on the surface of normozoospermic spermatozoa, but were absent on spermatozoa from infertile individuals. Exosomes from infertile men expressed higher number of proteins (98 proteins with more than 20% expression) in comparison to exosomes from fertile individuals (19 proteins) (p=0.05). Remarkably, the proteins that were highly expressed on the exosomes of infertile individuals demonstrated high correlation with the highly expressed proteins from spermatozoa fertile men (59 shared proteins). The mirror phenomenon between the proteome of exosomes isolated from infertile individuals and spermatozoa of fertile males can be explained either by the inability of abnormal spermatozoa to uptake exosomes or failure to maintain
these proteins on the surface of altered spermatozoa. The results of this study provide deeper understanding of the cell surface proteome landscape of semen from fertile and infertile males and can be used for diagnostic, cell sorting and enrichment of fertile sperm, and better understanding of disease mechanisms.


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In humans, a number of maternal and fetal health risks are associated with Assisted Reproduction Technologies (ART), including stillbirth, preterm birth, low birth weight, abnormal placentation, and other pregnancy complications. Indeed, experimental studies in mice support that ART procedures, independent of infertility, can result in low birth weight, placentomegaly, and changes in imprinted gene expression. ART procedures entail multiple ex vivo manipulations, but the specific impacts of each manipulation on epigenetic profiles and phenotypic outcomes on offspring at term are unclear. In this study, placental histology and methylation profiles were simultaneously analyzed in mice generated by ART (n=10-20 pups per group, from a minimum of three different litters). In vitro fertilization was performed using donor sperm from B6SJLF1 males and eggs from superovulated CF-1 females. The resultant embryos were cultured under optimized conditions to the blastocyst stage before non-surgical transfer to pseudopregnant (2.5-3.5 dpc) CF-1 females. For natural controls, unstimulated CF-1 females were mated to B6SJLF1 males. To test the effect of the embryo transfer procedure, blastocysts were collected from unstimulated/naturally mated CF-1 females and immediately transferred to pseudopregnant CF-1 females. Mean fetal/placental weights and placental area were statistically compared by t-test, and differences in DNA methylation were analyzed by a variance ratio test. Although fetal weight was unchanged, absolute placenta weight was nearly doubled in the ART and embryo transfer-only groups by comparison to natural controls at 18.5 dpc. Histological analysis of the ART placentas showed disproportionate overgrowth of the junctional zone, encroachment of the junctional zone into the labyrinth, as well as a less distinct border between the two compartments compared with normal or embryo transfer-only groups. Placentas from the embryo transfer-only group were significantly larger, but in contrast to ART placentas, both the junctional zone and labyrinth were increased in size. Although global methylation levels were normal with ART a subset of imprinted genes (H19/ Igf2, Peg3, and Kcnq1ot1) were significantly hypomethylated in ART placentas by comparison to natural or embryo transfer-only groups. No significant differences in DNA methylation were observed in fetal brain or liver samples. Taken together, our results suggest that ART procedures increase the occurrence of epigenetic perturbations and morphological abnormalities in the placenta, specifically, abnormal overgrowth of the junctional zone. We also demonstrate that the embryo transfer procedure alone causes placentomegaly, suggesting that this phenotype is not due to superovulation, in vitro fertilization, or extended embryo culture. Rather, brief culture and non-surgical transfer of blastocysts can induce placentomegaly-- conditions intrinsic to ART treatment.

FOCUS SESSION 5: Uterine Receptivity to Implantation and Placentation.

45. Cyclic Remodeling of the Nonhuman Primate Endometrium: A Model for Understanding Endometrial Receptivity. Ot D. Slayden1

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Embryo implantation is often cited as a critical step limiting the success of assisted reproduction programs. During the implantation process, a synchronized molecular dialogue develops between maternal and embryonic tissues. This dialog is dependent on endometrial receptivity, which typically occurs only during a brief period in the secretory phase of the menstrual cycle. The receptive maternal state, or “window of implantation”, is initiated and maintained by the action of the ovarian hormones estradiol (E) and progesterone (P) on the endometrium. The receptive state involves expression of a large number of mediators including adhesion molecules, growth factors and cytokines. Comprehensive assessment of the morphological action of steroid hormones on the endometrium during the menstrual cycle is essential for determining and analyzing the factors associated with embryo implantation. In the follicular phase of the menstrual cycle, estrogen drives endometrial cell proliferation and increases expression of estrogen receptor 1 (ESR1) and progesterone receptors (PGR) in the functionalis zone. In the luteal phase of the cycle, P inhibits epithelial cell proliferation and stimulates secretory differentiation, which is essential for successful embryo development and implantation. Progesterone down-regulation of glandular ESR1 is a definitive physiological marker for the onset of endometrial receptivity. These actions of P are specific for the functionalis zone of the endometrium, as P does not fully inhibit ESR-1 in the glands of basalis zone. Paradoxically, during the secretory phase of the cycle, PGR is also minimal in the glandular epithelium of the progestin-responsive functionalis zone. Therefore, P action on the epithelium in the functionalis zone may be mediated by paracrine factors arising from the PR-positive cells in the stroma. Genomic analysis of the macaque endometrium has revealed numerous secretory phase genes that may contribute to differentiation of the endometrium into a receptive state. These include TGF beta, TNF alpha, IL6, uteroferrin and uroteroglobin. Due to the endometrium’s distinct zonal response to ovarian steroids, accurate histological characterization will remain necessary to interpret the exact nature and function of these putative factors in fertility. We conclude that nonhuman primates, especially macaques, can provide a valuable animal model for experimental testing of the role of P-regulated targets on endometrial receptivity.

46. Tweaking Maternal-Fetal Signals Through Atypical Chemokine Receptors. Kathleen M. Caron1, Brooke Matson2, Manyu Li2.

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Adrenomedullin (AM) is a peptide vasodilator that is elevated nearly 5-fold in normal human pregnancies but often blunted in pregnancies that are complicated by fetal growth restriction, gestational diabetes or preeclampsia. We have previously shown that polymorphisms in the genes encoding AM peptide or its G protein-coupled receptor CLR, are associated with poor pregnancy outcomes, including altered birth weight, gestational diabetes and preeclampsia (AJOG, 2013). Moreover, our current studies have demonstrated that low levels of MR-proAM (a proteolytic by-product and surrogate of AM peptide) are predictive of severe preeclampsia, equivalent to other common preeclampsia biomarkers, endoglin and PlGF (Placenta, 2014). Thus, maintaining high levels of AM peptide signaling during human pregnancy is critical (Trends Endo & Metab., 2012). Using gene targeted mouse models, we showed that haploinsufficiency for maternal AM leads to poor uterine receptivity, reduced pinopode formation and sub-fertility (BOR, 2008). Pregnant AM−/− females exhibit abnormal implantation, ectopic placentation and fetal growth restriction that is largely independent of fetal genotype (JCI, 2006). Our recent studies have determined a critical role for the dosage and function of the peptide within the uterine epithelium and stroma to mediate the tight and gap junction coupling of these cells, respectively. Thus a modest genetic reduction in maternal levels of AM is sufficient to cause pregnancy complications due to abnormal uterine receptivity. We have also shown that fetal AM is required for branching morphogenesis of the placential labyrinth layer and remodeling of maternal spiral arteries through a concomitant reduction in uNK cells and a significant change in the placental chemokine and cytokine profile of AM−/− placentas. Therefore, AM serves as a trophoblast- derived factor that is critical for fetal placental vascularization and for enabling the maternal vascular adaptation to pregnancy to protect against preeclampsia (JCI, 2013). Most recently, we have discovered that an atypical chemokine receptor, CXCR7, serves to balance the effects of AM dosage at the maternal-fetal interface, which may represent a broader paradigm for this unusual class of non- signaling innate immune G protein-coupled decoy receptors (Dev Cell, 2014). These data raise the compelling hypothesis that maternal administration of AM during implantation and/or early pregnancy may help improve fertility, protect against preeclampsia and ultimately result in improved pregnancy outcomes.

47. BMP Signaling through ALK3 Regulates Embryo Implantation.
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Despite the increased use of assisted reproductive technologies in the clinic, failure rates remain high: out of the 151,923 procedures performed in 2011, only 47,818 (about 31%) resulted in live-birth deliveries. Thus, understanding the biological pathways that coordinate embryo implantation is necessary to improve the success of current reproductive interventions. The luminal uterine epithelium is the first site of contact between the mother and the invading blastocyst. Proper implantation requires drastic structural transformation of the luminal epithelium including microvilli flattening, loss of apical cell polarity, focal adhesion disassembly and tight junction remodeling. Previous studies demonstrate that bone morphogenetic protein (BMP) ligands and their receptors are crucial for the establishment and maintenance of pregnancy. In this study, we deleted the BMP type 1 receptor, Alk3 (Bmpr1a), in the female reproductive tract by generating Alk3flox/flox females carrying the Progesterone receptor-Cre (Pgr-cre) allele (Alk3 conditional knockout (cKO) mice). We found that Alk3 cKO females are infertile, demonstrate implantation defects and have impaired decidualization. Furthermore, Alk3 cKO females demonstrate defects in the uterine luminal epithelium during the window of implantation as demonstrated by increased expression of the apical cell polarity markers, e-cadherin, acetylated-α-tubulin, and phosphorylated-ezrin-radixin-moesin. Electron microscopic analyses demonstrate increased abundance of microvilli, desmosomes and tight junctions in the luminal epithelium of Alk3 cKO females. Accordingly, the expression of genes involved in cell adhesion (Proteocadherin 17) and actin polymerization (Adducin 2) are more abundantly expressed in Alk3 cKO females compared to Alk3 controls. Further, the expression of the BMP-regulated lysine demethylase, Kdm6a, is increased in Alk3 cKO females, suggesting that chromatin remodeling during the peri-implantation period is perturbed in the mutant mice. The signaling pathway is likely conserved in humans as treatment of human endometrial carcinoma cells (ECC1) with recombinant BMPs induces phosphorylation of Smad1/5/8, and regulates the expression of the BMP target genes ID1, ID4, and SMAD7. Our studies indicate that BMP signaling via Alk3 directs the uterine epithelial cell remodeling required for embryo attachment. These studies were supported by the Institutional Research and Academic Career Development Award (IRACDA) K12-GM084897 and the Eunice Kennedy Shriver National Institute of Child Health and Human Development grant R01-HD32067.

48. Endometrial Ablation of FOXO1 Inhibits Embryo Invasion of the Luminal Epithelium.
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Implantation of the blastocyst in the uterus is a critical step in mammalian reproduction and can only occur during a very specific period of the reproductive cycle termed “the window of receptivity.” The Forkhead Box O1 (FOXO1) protein is a critical transcriptional regulator during the in vitro differentiation of human endometrial stromal cells in a process termed decidualization. It is known that nuclear localization of FOXO1 in decidual cells is partially limited by inhibitory phosphorylations regulated by the AKT/P3K pathway. We hypothesize that the spatiotemporal expression and subcellular localization of FOXO1 in the peri-implantation period may have a defining role in conferring endometrial receptivity. The objective of this study was to define the cellular and compartmental expression and role of FOXO1 in the endometrium. Immunohistochemical analysis of FOXO1 was conducted on the uteri of day 0.5 to day 4.5 pseudo pregnant female C37BL/6J mice (n=3 per time point). FOXO1 expression was found predominantly in the cytoplasm of the luminal and glandular epithelium. The expression in the cytoplasm increased until day 4.5 when the expression became localized to the nucleus. To test our hypothesis we conditionally ablated FOXO1 in the adult uterus with the PgrCre/+ allele (PgrCre/+ Foxo1f/f) mice were determined to be infertile when compared to the wild type Foxo1f/f mice (n=5 per group). Examination of pregnancy progression demonstrated that embryos made contact with the antimesometrial pole of the implantation chamber at day 4.5 in but were unable to penetrate the luminal epithelium at day 6.5 (n=5 per group). The incomplete attachment event was sufficient to trigger a decidual response in the underlying stroma that progressed normally despite absence of embryo invasion. In order to identify the mechanism by
which FOXO1 regulates implantation, direct targets of FOXO1 at the window of receptivity were identified by chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) in day 4.5 pseudo pregnant female C57BL/6J mice (n=4). Ontology analysis revealed that genes containing FOXO1 binding intervals near promoter regions were enriched in processes that regulate cell cycle, protein localization, transport and degradation, RNA processing, apoptosis and DNA damage response. Immunohistochemical analysis was conducted on full thickness endometrial sections from biopsies collected on all stages of the menstrual cycle to determine the cell-specific expression of FOXO1 in humans (n=5 per stage of the cycle). FOXO1 was found to be low in all endometrial compartments during the proliferative phase and increase only in the epithelium in the early secretory phase. During the mid-secretory/receptive phase FOXO1 accumulates in the nucleus of the glandular epithelium where it remains throughout the late secretory phase. Collectively, this evidence suggests an evolutionarily conserved role for endometrial epithelial FOXO1 at the window of receptivity. We propose FOXO1 is a marker of human endometrial receptivity and regulates processes that are critical in the preparation of the luminal and glandular epithelium for the invading embryo.

49. Potential Role for Intermedin to Facilitate Embryo Attachment.
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Embryo–endometrial interactions are critical for implantation and subsequent placental development. Bidirectional embryo–endometrial communication is thought to be critical for implantation and occurs via soluble or cell surface mediators. The effect of the human blastocyst in modifying the endometrial luminal/surface epithelium during implantation remains largely unexplored due to ethical limitations. In this study we demonstrate a potential role for Intermedin (IMD) peptide on attachment of 1° trimester trophoblastic spheroids to a monolayer of uterine epithelial cells (RL-95 cells) and provide a potential mechanism for its action in embryo-endometrial communication. **Hypothesis:** IMD facilitates embryo implantation by regulating the expression of MMP2 and MUC1 in trophoblast cells and uterine epithelial cells. **Objective:** Assess the effect of IMD on: 1) the attachment of HTR-8sv/neo (HTR) spheroids on a monolayer of RL-95 cells, 2) Spreading and outgrowth of HTR spheroid on collagen, 3) expression of MUC1 mRNA and protein in HTR spheroids, and 4) expression of MMP2 protein in HTR spheroids and, 5) expression of MUC1 immunoreactivity in monolayer of RL-95 cells. **Methods:** RL-95 and HTR cells were cultured in MEM and RPMI respectively, supplemented with 10%FBS. HTR cells were used for generating spheroids and RL-95 were cultured as monolayer. Spheroids were generated with 750 cells resuspended in 7ul of RPMI containing 2%FBS and 1% methylcellulose. For attachment studies 20 spheroids were co-cultured with a monolayer of RL-95 cells in presence or absence of IMD 10^{-8}M for 1 hr at 37°C followed by counting the number of attached spheroids. Two to four spheroids per well were transferred to lab-tak chambers coated with or without collagen in presence or absence of IMD 10^{-8}M a nd assessed for either MMP2 and MUC1 immunoreactivity or trophoblast spreading and outgrowth on collagen, respectively. **Results:** IMD treatment 1) increases the number of attached spheroids on collagen (P<0.05), 2) increases the spreading and outgrowth of trophoblast cells from the spheroids by 2.5 fold compared the control (P<0.05), 3) decreases the expression of MUC1 mRNA and immunoreactivity in trophoblast spheroids (P<0.05), 4) increases the expression of MMP2 immunoreactivity in trophoblast spheroids and, 5) increases the expression of MUC1 immunoreactivity in RL-95 cells (P<0.05). **Conclusion:** IMD induced increase in trophoblastic spheroids despite IMD induced increase in MUC1 expression in epithelial cells allows us to speculate a potential model for embryo-endometrium communication whereby, during apposition, IMD induced increases in MMP2 expression in trophoblast results in a shedase activity on the MUC1 expressed on the epithelial cells to facilitate attachment and embryo implantation.

50. The Role of Progesterone Receptor Signaling in Regulating Uterine Receptivity in the Peri-Implantation Period.
Francesco J. DeMayo1, John P. Lydon1.

The window of receptivity is the defined period of time in which the uterus allows embryo attachment and invasion. The coordination of the timing of the window of receptivity with ovulation and embryo development is governed by the ovarian hormones estrogen and progesterone. These hormones signal through their cognate receptors the estrogen (ESR1) and progesterone receptor (PGR). Both receptors are critical for the establishment and maintenance of pregnancy. We have focused on defining the molecular mechanisms governed by the PGR in regulating the uterine support of pregnancy. This has been accomplished by using Genetically Engineered Mouse models, GEM, and the primary culture of Human Endometrial Stroma cells (HES) in combination with transcriptomic and cistromic approaches. Using these approaches we have defined the molecular pathways regulated by PGR in the epithelium and stroma of the endometrium. In the pre implantation uterus the epithelial PGR is critical for initiating a cascade of epithelial to stroma signaling that primes the uterus for embryo attachment and stroma decidualization. At the window of receptivity the epithelial expression of the PGR is lost. Using GEMS allowing for the continued expression of the Pgr through the window of receptivity results in failure for embryo implantation. Using Chip Seq to identify regions of the genome in which the PGR binds we have identified Gata2 and Sox17 as Pgr regulated genes that bind similar regions of the genome with the PGR. Ablation of Gata2 in the uterus resulted in loss of Pgr gene expression and sterility. Ablation in the uterus of Sox17 resulted in normal Pgr expression but sterility due to loss of key PGR target genes. Investigation of the role of PGR in the HES cells shows that PGR is critical in regulating pathways governing HES cell differentiation. These analysis also demonstrated that the transcription factor FOXO1 is critical for regulating the ability of PGR to bind to and regulate a subset of PGR responsive genes. The application of transcriptomic and cistromic approaches in combination with GEM and HES cells allows for the identification of novel pathways regulating endometrial function in the mouse and human. This work was supported by R01 HD042311 and U54 HD07495.

51. Conceptus-Uterine Interactions During the Periods of Implantation and Placentation in Pigs and Sheep.
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Domestic animal models offer unique characteristics of pregnancy as compared to rodent or primate models. Studies of pigs and sheep have provided significant insights into the physiology of communication between the conceptus (embryo/fetus and associated placental membranes) and uterus including: 1) elongation of the blastocyst into a filamentous conceptus; 2) the protracted peri-implantation period of pregnancy when the conceptus is free within the uterine lumen requiring extensive paracrine signaling between conceptus and endometrium, as well as nutritional support provided by uterine secretions; 3) a protracted and incremental attachment cascade of trophoectoderm to endometrial epithelium during implantation; and 4) development of a true epitheliochorial or synepitheliochorial placenta, respectively, that utilizes extensive uterine and placental vasculatures for hemotrophic nutrition, and placental areolae for histotrophic support of the developing fetuses. This invited talk will begin with a discussion of conceptus-uterus interactions with respect to uterine gene expression in response to estrogens and interferons secreted from elongating conceptuses to signal pregnancy recognition. A description will follow of the physical changes that occur as the blastocysts of pigs and sheep elongate. As these blastocysts elongate they enter a protracted apposition and adhesion cascade for implantation, and the roles of integrins and the extracellular matrix protein, osteopontin, will be the focus of discussion. Conceptus adhesion transitions into placentation, characterized by increasing interdigitation between intact uterine and placental tissues. This process will be examined in the context of the emerging idea that tissues respond to mechanical forces that coordinate morphogenesis, and that mechanotransduction drives morphogenesis to develop folding and efficient contact between uterine and placental tissues of pigs and sheep. Finally, to link form to function, an overview of the cell-type-specific expression of various glucose and fructose transporters, as well as the polyol pathway enzymes necessary to convert glucose to fructose, will be described at the uterine-placental interface of pigs. Our understanding of the complex mechanistic events that underlie successful implantation and placentation across species has been, and will likely continue to be, advanced by studies of pigs and sheep as biomedical research models and for increasing reproductive success in animal agriculture enterprises providing high quality protein for humans.

52. Global transcriptomic analysis and profiling of the canine early pregnant, preimplantation uterus, and expression and distribution patterns of selected extracellular matrix proteins.

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Uterine differentiation and preparation for embryo attachment, implantation and trophoblast invasion require functional and structural tissue remodeling. This is initiated by progesterone and further modulated by the conceptus. The dog is the only domestic animal species devoid of an antiluteolytic signal in absence of pregnancy and exhibiting similar circulating hormone profiles in pregnant and non-pregnant animals. This implies the presence of mechanism of embryo-maternal communication which is different than that found in other species. Just recently, we showed the differential expression of several deciduation markers in the early pregnant canine uterus prior to implantation. Their expression was stimulated by the presence of free-floating embryos. Here, in order to gain new information concerning early embryo-maternal communication in the dog, global transcriptome analysis using a custom-designed Agilent microarray was performed on uteri from early pregnant dogs (days 10-12); dogs determined as non-pregnant served for controls. Among 433 differentially expressed genes (false recovery rate 10%), 332 were upregulated and 101 downregulated. Over-representation analysis revealed strongest enrichment for functional terms related to extracellular matrix proteins (ECM), cell signalling, positive regulation of cell motion and cell migration, and inflammatory response. Greater variety and fewer functional terms were found for downregulated genes. Following this, in view of the lack of information concerning the composition of ECM proteins in the canine uterus, expression and distribution of collagens (COL) 1, 3, -4, and of laminin 2 (LAMA2), fibronectin 1 (FN1) and ECM1 protein were evaluated in early pregnant uteri prior to implantation (days 10-12 confirmed by uterine flushings; dogs, which were inseminated but determined as non-pregnant served as controls), and after implantation and initiation of trophoblast invasion. Whereas COL1, -3 and -4 and LAMA2 were not modulated by the presence of free-floating embryos, ECM1 was significantly upregulated, contrasting with decreased expression of FN1. Following decidua formation and placentation, COL1, COL3 and LAMA2 were decreased at placentation sites, compared with interplacental sites (parts of the uterine wall not attached to the placenta). COL1 represents the tougher type of collagen while COL3 is characteristic of the reticular type of fibers indicating the proliferative activity of fibroblastic tissues. Together with COL3, the increased expression of LAMA2 indicates their roles in uterine proliferative activity in the second half of gestation. FN1 and LAMA2 were localized in the endometrial epithelial compartment; LAMA2 was represented more strongly than FN1 in uterine glands. Stromal stained strongly for COL1 and LAMA2 and only weakly for FN1. In the myometrium, COL1 and -3, FN1 and LAMA2 stained strongly. Displaying high individual variations, COL3 seemed to be more strongly represented in the outer uterine layers than in the lumen. Together with LAMA2, COL4 (characteristic of basal lamina) was additionally localized in the media of larger blood vessels. COL4 expression did not vary greatly between the groups. In conclusion, even though not directed towards suppression of luteolysis, the early free-floating canine embryo is apparently capable of transmitting signals to the uterus. Initiation of structural changes does not seem, however, to play a primary role in this early embryo-maternal communication. Instead, the early evolutionary signaling seems to be comprised of proliferative, secretory and, likely, immunomodulatory effects. Research supported by Swiss National Science Foundation (SNSF) grant 31003A_140947 to MPK. FG and EK contributed equally.

53. Pig Endometrium Expresses The Polyol Pathway Enzymes Necessary To Convert Glucose To Fructose Prior To Implantation With A Shift To Chorion Expression Post-Implantation.

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Glucose and fructose are abundant hexose sugars in pig endometria and conceptuses (embryo/fetus and associated placenta). While glucose is mostly catabolized for energy, in vitro studies implicate fructose as a substrate for glycoaminoglycan and phospholipid biosynthesis as well as a signaling molecule for the mechanistic target of rapamycin (MTOR) pathway. Pregnant gilt uterine flushings contain greater than 5 mM fructose and, as pregnancy progresses, so do the fetal fluids. Fructose cannot be detected in maternal blood, which suggests local fructose production. Fructose can be synthesized from glucose without using ATP via the polyol pathway. Glucose is converted to sorbitol by aldose reductase (AKR1B1) and sorbitol to fructose by sorbitol dehydrogenase (SORD). Fructose can then be transported across cell membranes by the solute carriers SLC2A5 and SLC2A8 and converted to fructose-1-phosphate by keto-hexokinase (KHK). Therefore, our hypothesis is that specific cell types in the endometrium and choioallantois utilize the polyol pathway to convert glucose to fructose for conceptus use. AKR1B1, SORD, KHK, SLC2A5, and SLC2A8 mRNAs and proteins were analyzed in the endometria and placentae of gilts from Days 9 to 85 of gestation and Days 5 to 17 of the estrous cycle using qPCR and in situ hybridization or immunohistochemistry. AKR1B1 mRNA increased on Days 9 to 11 in pregnant gilt endometrium and then decreased to levels similar to cyclic gilts by Day 15. AKR1B1 protein was detected in uterine luminal epithelium (LE) on Days 13 to 20 of pregnancy. However, after Day 20 of pregnancy, AKR1B1 protein was undetectable in LE, but detectable in the chorion, except for the areolae. SORD mRNA peaked in endometrium on Days 5 to 9 of the estrous cycle and pregnancy, and protein was localized to LE, uterine glandular epithelium (GE), and conceptus trophectoderm (Tr) throughout the peri-implantation period. Interestingly, by Day 20 of pregnancy, the majority of SORD protein was localized to the chorion, and to a lesser extent the GE. SLC2A8 mRNA increased on Day 9 in endometria from cyclic and pregnant gilts, and peaked on Day 11. SLC2A8 protein was detected in GE beginning on Day 9 of the estrous cycle and pregnancy, while LE expression became detectable on Day 13. After Day 20 of pregnancy, SLC2A8 protein was detected primarily in the areolae. SLC2A5 mRNA localized to the LE, GE, and chorion throughout pregnancy. Only low levels of KHK mRNA and protein were detected in endometrium, however, KHK protein was detected in Tr on Day 11 of pregnancy and expression increased through Day 15. KHK protein continued to be expressed in the chorion, especially in the tall columnar cells and areolae through Day 85. KHK protein localization in the Tr/chorion is particularly interesting since KHK catalyzes the conversion of fructose to fructose-1-phosphate, which can be used for synthesis of hexosamines, lipids, and ATP, as well as for regulating the glucokinase, MTOR, and proinflammatory pathways. These results are the first to establish that the molecular components for conversion of glucose to fructose, and fructose transport, are present in the LE and Tr/chorion. The intriguing shift in the expression of the polyol enzymes from LE to chorion during pregnancy suggests that the free-floating, elongating conceptuses are supported by LE synthesized fructose. However, after implantation is established at the maternal/conceptus interface, the chorion becomes self-sufficient for fructose synthesis and transport.

54. The Uterine-Based Kisspeptin-KISS1R Signaling System Promotes Embryo Implantation in the Mouse Potentially via the MAPKs ERK1/2 and p38.

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Kisspeptins (KPs) are a group of related peptides that are derived from a primary-translational product called KISS1. KPs signal via the receptor KISS1R (GPR54), to potently trigger GnRH release in the brain. Inactivating mutations in the genes that encode either KISS1 or KISS1R are associated with hypogonadotropic hypogonadism in humans and mice. We previously demonstrated that in Kiss1-/- female mice, acute replacement of gonadotropins and estradiol restores ovulation, mating, and fertilization; however, these mice are still unable to achieve pregnancy because embryos fail to implant. Analysis of the Kiss1-/- uterus revealed a reduced number of uterine glands and reduced expression of glandular leukemia inhibitory factor (LIF) on the day of implantation. We found that the administration of exogenous LIF, which is absolutely required for embryo implantation in the mouse, was sufficient to partially rescue implantation in the Kiss1-/- uterus. In the wild-type pregnant mouse, on the day of implantation (D4 of pregnancy), we confirmed that KP is expressed throughout the uterus while KISS1R is expressed in the mouse luminal epithelium. Experiments were then designed to determine whether there was a functional KP/KISS1R signaling system in the wild-type mouse uterus on D4 of pregnancy. Since KISS1R activation triggers the phosphorylation of the mitogen-activated protein kinases (MAPKs), p38 and ERK1/2, in a variety of cell types, we hypothesized that detection of phosphorylated p38 and ERK1/2 would confirm the presence of a functional KP/KISS1R signaling system in the uterus. To test our hypothesis, wild-type 8 week old 129S1/SvImJ mice were synchronized to D4 of pregnancy by inducing superovulation with 7.5IU PMSG followed 48 hours later by 7.5IU hCG and mating with proven 129 wild-type males. The following morning, only female mice that displayed a copulatory plug were considered to be on D1 of pregnancy. On the morning of D4 of pregnancy, mice were injected i.p. with KP (KP54, 100 nmol KP54/kg body weight in a final volume of 100 µl) or phosphate-buffered saline and uteri were recovered 30 minutes later. When antagonists were used, they were given 30 minutes before KP54. Uteri were immediately processed for the immunohistochemical detection of phosphorylated p38 and ERK1/2, using phospho-specific antibodies. Our results revealed low levels (basal expression) of phosphorylated p38 and ERK1/2 in the uterus of PBS-treated mice on D4 of pregnancy. Specifically, p-p38 was localized to almost all cells in the luminal and glandular epithelia while p-ERK1/2 were only localized to a subset of cells in the luminal epithelium. KP54 potently increased expression of p-p38 and p-ERK1/2 in the respective endometrial cells. An inhibitor of KISS1R signaling (peptide 234) completely inhibited KP-induced p-p38 expression while an inhibitor of NPFF, another receptor potentially capable of transmitting KP signals, was ineffective in blocking the p38 phosphorylation induced by KP54. Thus the majority of KP signaling appears to occur through KISS1R. Taken together, we suggest that superovulated Kiss1-/- mice are infertile due to a lack of KP/KISS1R/MAPK signaling in the luminal and glandular epithelia of the uterus during pregnancy. This study was funded by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC, RGPIN/327334–2011 (A.V.B.))
55. Reversible Epigenetics and Seasonal Neuroendocrine Plasticity.

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Epigenetic modification such as DNA methylation and histone acetylation, are important molecular events that act to regulate gene transcription. The general assumption is that epigenetic events are permanent. Seasonal rhythms are associated with extensive genomic plasticity in a diverse range of tissues and cellular phenotypes. Using a seasonally breeding species, this presentation will demonstrate light and hormone dependent regulation of DNA methylation in the adult hypothalamus and testes. In Siberian hamsters, a simple change in day length leads to marked variation in the levels of DNA methyltransferase 1 and 3b expression in the hamster hypothalamus and testes. One genomic region in particular, Type 3 deiodinase (DIO3) is one target of the light induced change in \(dnmt1/3b\) expression with greater levels of \(dio3\) promoter methylation in hamster hypothalami when housed in long days compared to short days. Studies that assessed the efficiency of melatonin revealed timed injections were able to reduce \(dnmt1/3b\) expression and \(dio3\) proximal promoter methylation. The development of refractoriness in hamsters was associated with a complete reversal in \(dnmt1/3b\) enzyme expression and \(dio3\) promoter methylation. The pattern of \(dnmt1/3a\) expression is completely opposite in the hamster testes with greater \(dnmt3a\) expression in SD compared to LD. \(Dnmt3a\) expression appears to be localized to spermatogonia B cells. Altogether, these data indicate that DNA methylation in the adult mammalian brain is dynamic and reversible.

56. Neural and Molecular Basis of Leptin Action in Reproduction.

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Many aspects of the reproductive physiology are energetically demanding (e.g., pregnancy, lactation, territoriality) and, therefore, the individual nutritional state is a critical factor for pubertal development and attainment of reproductive capacity. Metabolic cues are important players in this equation; they signal energy availability to the neuroendocrine reproductive axis and modulate the adequacy and timing of engaging in reproductive function. Leptin is an adipocyte-derived hormone with a permissive role in sexual maturation and key function in signaling energy sufficiency to the brain. However, the neuronal and molecular targets of leptin in reproductive control have been difficult to determine. In this symposium, I will present data collected using genetic tools and molecular brain mapping, including Cre conditional deletion and re-expression of relevant genes using mouse models and virus vectors. Against the initial predictions and expectations, we will demonstrate that leptin action in kisspeptin neurons is neither required nor sufficient for leptin’s effect in reproduction. Rather, alternative neuronal populations including the ventral premammillary nucleus, AgRP and nNOS neurons play a major role. Special attention will be also given to the role of GABAergic versus glutamatergic neurotransmission and the apparent inconsistencies generated from data obtained in developing and adult mice.

57. Reactive Oxygen Species Modulate Local L-type Calcium Channel Signaling in Gonadotropes.

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The binding of hypothalamic neuropeptide gonadotropin-releasing hormone (GnRH) to its receptor on gonadotrope cells in the anterior pituitary initiates signaling cascades that result in the activation of extracellular signal regulated kinase (ERK) and subsequently enhanced luteinizing hormone biosynthesis. Most dramatic is the sharp rise in LH secretion (the “LH surge”) that precedes and is necessary for final follicular maturation and ovulation. Previous work has established that Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels is necessary for ERK phosphorylation. More recently, we have identified discrete microdomains of GnRH induced Ca\(^{2+}\) influx (“Ca\(^{2+}\) sparklets”) mediated by L-type Ca\(^{2+}\) channels by using a combination of TIRF microscopy and electrophysiology. Next, we sought to examine molecular events that reside between the activation of the GnRHR and these biologically relevant localized subplasmalemmal Ca\(^{2+}\) signals. Reactive oxygen species (ROS) are recognized as cognate signaling molecules that regulate cell function. However, while the generation of ROS is a ubiquitous phenomenon, ROS signaling has not been examined at a cellular level in gonadotropes. We hypothesize that ROS play a role in GnRH signaling and local L-type Ca\(^{2+}\) channel function. To test our hypothesis, we first determined if GnRH receptor stimulation increased ROS production. Using TIRF microscopy and a cell-permeant ROS indicator (2',7'-dichlorodihydrofluorescein diacetate (DCF); 1 \(\mu M\)) to monitor subplasmalemmal DCF fluorescence, acute stimulation of the GnRH receptor (with GnRH; 3 nM) produced localized ROS “puncta” in gonadotropes. If the ROS contained in the puncta visualized near the plasma membrane modulate the activity of nearby L-type Ca\(^{2+}\) channels, then application of exogenous ROS should result in an increase in Ca\(^{2+}\) sparklets. Consistent with a stimulatory role, exogenous hydrogen peroxide (H\(_2\)O\(_2\); 100 \(\mu M\)), a physiologically relevant ROS, increased local Ca\(^{2+}\) sparklet activity within 5 min (control \(n\)Ps = 0.013 ± 0.005, H\(_2\)O\(_2\) \(n\)Ps = 0.30 ± 0.07; \(P < 0.05, n = 7\)). We next examined if endogenous ROS generators (e.g. \(NADPH\) oxidase) are activated with GnRH-dependent activation of L-type Ca\(^{2+}\) channels. To test the involvement of \(NADPH\)-derived ROS, we pharmacologically inhibited \(NADPH\) oxidase activity with apocynin (25 \(\mu M\) pretreatment for 5 min) followed by GnRH (3 nM). Apocynin pretreatment abolished stimulation of L-type Ca\(^{2+}\) channel sparklets by GnRH (\(P > 0.05, n = 11\)). Cells treated with catalase (500U/mL), an enzyme to decompose intracellular H\(_2\)O\(_2\), also decreased GnRH-induced Ca\(^{2+}\) sparklet activity compared to GnRH control (\(P > 0.05, n = 6\)). Thus, GnRH produced localized ROS generation, H\(_2\)O\(_2\) was sufficient for stimulating localized Ca\(^{2+}\) influx, and inhibition of endogenous ROS generation decreased GnRH-induced Ca\(^{2+}\) influx. Collectively, these data provide strong evidence that ROS signaling plays an important role in GnRH-dependent Ca\(^{2+}\) channel activity in gonadotropes. This research was supported by NIH 5R01HD065943 and the Pew Charitable Trusts.

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Gonadotropin-releasing hormone (GnRH) plays a key role in the vertebrate reproductive system by stimulating biosynthesis and secretion of pituitary gonadotropins. Although a wealth of knowledge has accumulated on transcriptional control of gonadotropin subunit genes, the potential involvement of microRNAs (miRNAs) has still to be explored. During the last decade, miRNAs emerged as critical regulators of gene expression by modulating target mRNA availability (degradation or inhibition of translation) through their capture into the RNA-induced silencing complex (RISC). In this study, we investigated the role of two miRNAs that target the same transcripts, miRNA-132 and miRNA-212 on the GnRH-induced Follicle-Stimulating Hormone (FSH) expression. Both miRNAs are encoded by the same intronic sequence and are activated by GnRH. We first showed in rat pituitary cells that blocking miR-132/212 action by locked nucleic acid overexpression reduced the activation of FSH secretion by GnRH. It also abolished the GnRH stimulation of Fshb mRNA steady state level. The mechanism of this mediation was then explored in mouse gonadotrope LβT2 cells. The GnRH stimulation of Fshb mRNA was reproduced in these cells by overexpressing one or both miRNAs and was prevented by blocking both miRNAs together. Sirt1 deacetylation is a potential target of miR-132-212. We showed that GnRH treatment induced a capture into RISC of miR-132 and Sirt1 mRNA, resulting in a lowered level of SIRT1 deacetylation and a concomitant increase in the acetylated form of FOXO1 (FOXO1), a transcriptional repressor of Fshb. Blocking miR132/212 prevented GnRH-induced FOXO1 acetylation. Over-expression of an acetylated-mimicking mutant of FOXO1 induced an increase in Fshb mRNA expression, likely via its observed exit from the nucleus and consequently, the release of the inhibitory action on Fshb promoter. Overall, we show that GnRH increases miR-132/212 to target Sirt1 mRNA into the RISC complex. The lower level of SIRT1 deacetylation results in an increase in the acetylated form of FOXO1 and a decrease in its transcriptional inhibitory action on Fshb subunit gene. This is the first demonstration of an obligatory microRNA pathway in the GnRH-regulated expression of a gonadotropin gene. Lannes J. is a recipient of a PhD grant from Université Paris-Diderot.

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59. The tale of the tail: Novel insights into the evolution of GnRH receptor signaling.

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The mammalian type-I GnRH receptor (GnRHR) is unusual among G protein coupled receptors (GPCRs) in lacking an intracellular C-terminus (C-tail). The C-tail in many GPCRs mediates agonist-induced desensitization and receptor internalization. Indeed, the lack of a C-tail prevents homologous desensitization and slow internalization of mammalian GnRHRs. It was previously hypothesized that the loss of the GnRHR C-tail during mammalian evolution may have allowed for the generation of protracted luteinizing hormone (LH) surges in response to the preovulatory GnRH surge. This idea is challenged, however, by the observation of robust LH surges in non-mammalian vertebrates, like chickens, whose GnRH receptors possess C-tails. To better understand the functional significance of the loss of the C-tail in mammals, we generated knock-in mice expressing a chimeric GnRHR. In this model, the chicken GnRHR C-tail was fused in-frame with the C-terminus of the endogenous murine GnRHR (hereafter GnrhrCtail/Ctail). Neither serum LH nor pituitary LHβ (Lhb) subunit mRNA levels differed between adult GnrhrCtail/Ctail and wild-type males. In contrast, both serum FSH and pituitary FSHβ subunit (Fshb) expression were decreased by about 50% in GnrhrCtail/Ctail males. Pituitary expression of the gonadotropin α subunit (Cga) and Gnrhr was also decreased in these mice. In contrast, gonadotropin subunit and Gnrhr mRNA levels did not differ between intact GnrhrCtail/Ctail and wild-type females on metestrus. Following bilateral ovariectomy (2 weeks), pituitary Fshb, Lhb, and Cga mRNA levels as well as serum LH increased significantly in females of both genotypes; however, the response was blunted in GnrhrCtail/Ctail mice compared to their wild-type littermates. GnrhrCtail/Ctail females exhibited abnormal estrous cyclicity and were subfertile. Reduced litter sizes could derive from impaired FSH induced follicular maturation and/or from altered LH surge dynamics. We are currently exploring both of these possibilities. However, it is notable that intact wild-type and GnrhrCtail/Ctail females showed equivalent increases in serum LH in response to the potent GnRH agonist, buserelin. In males, the response was actually enhanced in GnrhrCtail/Ctail mice relative to wild-type. Collectively, these observations suggest that the addition of a C-tail creates a signalling bias that preferentially hinders GnRH regulation of FSH, at least in intact animals. Consistent with this idea, GnRH regulates Lhb synthesis via an ERK1/2-dependent pathway. ERK1/2 signaling in gonadotropes in vivo appears to be less critical for Fshb, Cga, and Gnrhr, than Lhb, expression. In heterologous cells, the chimeric GnRHR retained the ability to stimulate ERK1/2 phosphorylation in response to GnRH. We are currently exploring how the addition of a C-tail alters murine GnRHR signaling. Based on the data collected thus far, we propose that the loss of the C-tail in GnRH evolution conferred a selective advantage by enhancing GnRH’s regulation of FSH rather than by enabling the LH surge. Supported by FQRNT Team Grant PR-174948 and CIHR MOP-123447 to DJB and DB. CT and JF contributed equally to this work.

60. Gonadotropin Re-routing and Evolution of Estrus Cycles.

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The two pituitary gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) are synthesized in the same cell, gonadotrope. But, their intracellular trafficking and secretion pattern are distinct. LH is released as pulses via the regulated trafficking pathway, whereas FSH in many species is mostly constitutively secreted. The basis for this hormone-specific secretion pattern and whether the target organ, ovary responds distinctly to a specific hormone release pattern is not understood. We identified a gonadotrope-specific regulated pathway sorting determinant in vivo that is uniquely present on LH. Our recent studies on gonadotropin re-routing will be discussed that will allow us to provide a molecular framework for the evolution of distinct patterns of gonadotropin secretion and the origin of estrus cycles in mammals.

61. Mechanisms of FSH Synthesis and Donald Rumsfeld: Revisiting the 'KnownKnowns'.
histology. Treatment with isolated zuclomiphene at high dose has a deleterious effect on LH, FSH and testosterone levels and results in testicular and other tissue pathology. Repros Therapeutics Inc. is currently using enclomiphene, the trans-isomer of Clomid, in clinical trials in men with secondary hypogonadism This study provides evidence supporting the use of enclomiphene over zuclomiphene.

62. Phage-Peptide Constructs for Elucidation of Humoral Immune Responses Against Gonadotropin Releasing Hormone.

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Bacteriophages (phages) are viruses that infect bacteria. Phages are not pathogenic for animals, including mammals. They consist of an outer protein capsid enclosing genetic material. Phage particles can be genetically re-engineered and utilized as carriers for delivery of peptides with desired immunogenic characteristics. The focus of this study was on development of phage-peptide constructs that stimulate production of antibodies against gonadotropin releasing hormone (GnRH). GnRH acts as a master reproductive hormone via regulation of the release of major gonadotrophic hormones. Neutralizing GnRH antibodies can inactivate endogenous GnRH. This leads to the reduced release of gonadotropic hormones and suppression of gonadal function, including related production of sex hormones.

Consequently, this approach can be used in contraception of animals. Here, phage-GnRH constructs were generated via selection from a phage display library using several types of GnRH antibodies as selection targets. Phage constructs selected from the library that displayed GnRH-like peptides were characterized for frequency of their occurrence in selection rounds, sequence similarities to GnRH, and specificity of binding to GnRH antibodies in ELISA. Five of the constructs with suitable characteristics were tested in mice for anti-GnRH antibody production. Mice (10/group) were immunized with phage at 5x10^11 virions/mouse using subcutaneous administration. In each test group, mice were immunized with one phage-peptide construct. Additionally, two groups of mice, one injected with PBS and the other with phage vector, were used as negative controls. Phage constructs were tested in mice as a single dose plain vaccine (no adjuvants or other immune enhancers were used). Serum samples were collected from mice before and after the immunization at multiple time points and compared for the presence of anti-GnRH antibodies. Measurements of antibody titers were performed in duplicate and are presented as means ± SD. While all of the phage-GnRH constructs stimulated production of specific GnRH antibodies, their titer and duration were different in different treatment groups. The highest antibody responses were in mice injected with phage bearing EPTSHWSA peptide (amino acids similar or identical to GnRH peptide sequence EHWYGLRPG are highlighted). The most long lasting antibody responses (no titer decrease in 10 weeks, duration of experiment) were detected in mice injected with phage carrying EHPYGLAPA peptide. Thus, phage-peptide constructs can be used to induce a sustained, GnRH-specific immune response. Administration of phage to mice produced no visible adverse effects, local or systemic. Importantly, phages, as bacterial viruses, can be easily obtained in large quantities from bacterial cultures. This makes the cost of phage preparations much lower than the cost of peptides vectored in mammalian viruses or the cost of production of synthetic peptides.

The cost of phage preparations makes the cost of phage prepaarations much lower than the cost of peptides vectored in mammalian viruses or the cost of production of synthetic peptides. To achieve optimal immune responses for desired applications, phage-GnRH constructs can be modified with respect to flanking sequences of GnRH-like peptides displayed on phage. Anticipated therapeutic effects, including immunoncontraception, also might be attained using optimized phage doses, a combination of several constructs in a single treatment, and application of adjuvants and advanced phage delivery systems. Supported by Found Animals Foundation D0910-S10 and Scott-Ritchey Research Center, Auburn University.

63. Differential Effects of Isomers of Clomiphene Citrate on Mice.

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Clomid (clomiphene citrate) is approved and widely used in women for induction of ovulation for several conditions, especially PCOS. It is often used off label in men who have low testosterone (T) to raise that hormone and is useful for the restoration of sperm counts in men who have used exogenous testosterone to relieve symptoms of secondary hypogonadism. Clomid is a mixture of two structural isomers, clomiphene citrate and enclomiphene citrate. Enclomiphene has anti-estrogenic properties and in men with low T appears to block the negative feedback inhibitory effects of estradiol resulting in increased LH levels which stimulates endogenous testosterone production. Zuclophene has been shown to be an agonist in some uses and has a longer biological half-life than enclomiphene and thus may persist in the body. It is our hypothesis that the accumulation of the estrogenic clomid isomer may further suppress the production of FSH and LH and have negative effects on male fertility whereas the anti-estrogenic enclomiphene isolomers REServe LH and FSH from the pituitary possibly through the hypothalamus. We report that treatment with the isolated isomers have differential effects in mice. Treatment with the isolated enclomiphene isomer has positive effects on testosterone production and no effects on testicular histology. Treatment with isolated zuclophene at high dose has a deleterious effect on LH, FSH and testosterone levels and results in testicular and other tissue pathology. Repros Therapeutics Inc. is currently using enclomiphene, the trans-isomer of Clomid, in clinical trials in men with secondary hypogonadism.
64. Over-expression of Estrogen Receptor Alpha in Pituitary of ERα-null mice Results in Lower LH but Cystic Ovaries.

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Polycystic ovarian syndrome (PCOS) affects nearly 5 million women in the United States and is a major cause of female infertility. The primary diagnostic features of PCOS include cystic ovaries, excess androgen production, and ovulatory dysfunction. Many women with PCOS also have high LH and an elevated ratio of LH/FSH. It is well known that mice lacking estrogen receptor alpha (ERα) are anovulatory and develop cystic hemorrhagic ovaries thought to be due to persistently high circulating levels of LH. Global deletion of ERα in the ERα knock-out (αERKO) mouse model has shown disruption of the hypothalamus-pituitary-gonadal (HPG) axis resulting in hormonal dysregulation where the αERKO mice have elevated E2, LH, and T levels in their serum. However, the role of estrogen receptor in the pituitary is still controversial as most research has focused on the hypothalamic ERα contribution and models of pituitary specific ERα deletion have shown inconsistent results. Therefore, we developed a mouse model where ERα is specifically expressed in the mouse pituitary on the background of a global ERα knockout (called the PitERtgKO). We hypothesized that expressing ERα in the pituitary only would restore normal serum LH levels and therefore the mice would lack the cystic ovarian phenotype seen in the global knockout. As we hypothesized, serum E2 and LH levels in PitERtgKO females were comparable to WT serum levels and significantly lower than levels seen in the αERKO. The serum testosterone (T) level in PitERtgKO mice was still significantly higher than WT, but slightly lower than that of the αERKO.

Surprisingly, the ovaries of the PitERtgKO adult mice displayed a more overt cystic and hemorrhagic phenotype when compared to their αERKO littermates. We evaluated the expression of LH-responsive genes in the ovary and found increased expression of Ptgs2, Btc, and Areg in PitERtgKO adult mice while little expression was seen in the WT littermates. Following the supervolution regime in the WT mouse, the ovarian expression of these genes are up-regulated 4 hours after hCG treatment and return to a low basal level by 20 hours post hCG. In order to elucidate the disconnect between the seemingly normal serum LH levels yet signs of LH stimulation (LH induced gene expression and cystic ovaries), further studies are underway to determine whether the female PitERtgKO mice have LH pulsation that occurs throughout the day similar to the profile of LH secretion in the male. The PitERtgKO model will be useful in the study of clinical cases of PCOS where the classical elevated serum levels of LH are not present, but ovarian cystic morphology still persists.

65. The Evolution of Sex: Rethinking the Pristine X and Rotting Y Chromosomes.


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The mammalian X and Y chromosomes arose from an ordinary pair of autosomes during the past 200-300 million years. Following the emergence of the sex-determining gene, SRY, on the Y chromosome, a series of inversions on the Y chromosome suppressed X-Y crossing over. Suppression of X-Y crossing over liberated the X and Y chromosomes to radically differentiate; they remain identical only in the pseudoautosomal regions, where X-Y crossing over still occurs. Our recent sequencing of the mouse Y chromosome (Cell 159:800 [2014]) added several twists to this story. In contrast to theories that Y chromosomes are heterochromatic and gene poor, the mouse Y chromosome is 99.9% euchromatic and contains about 700 protein-coding genes. Only 2% of the mouse Y chromosome derives from the ancestral autosomes that gave rise to the mammalian sex chromosomes. Instead, all but 45 of the mouse Y chromosome’s genes belong to three acquired, massively amplified gene families that have no homologs on primate Y chromosomes but do have acquired, amplified homologs on the mouse X chromosome. On both the mouse X and mouse Y chromosomes, the acquired, amplified gene families are expressed predominantly or exclusively in testicular germ cells. We speculate that lineage-specific (rodent-specific) convergent acquisition and amplification of X-Y gene families was fueled by antagonism between acquired X-Y homologs, which are engaged in a protracted sex-linked meiotic drive battle on behalf of their respective (X and Y) chromosomes. These findings bring to light a paradoxical combination of divergence and convergence between the X and Y chromosomes in mouse sex chromosome evolution. They also raise the question of whether the mouse sex chromosomes are exceptions among mammals. We are presently seeking to answer this question through studies of other mammalian X and Y chromosomes.


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Understanding the molecules mediating reproduction has been a major focus of developmental and reproductive biology. A recurring observation in the study of reproductive molecules is diversity. For example, the human egg coat protein ZP2 is among the 5% most divergent molecules between humans and rodents. My lab has focused on adaptive protein evolution, with a particular focus on the function and evolution of reproductive proteins. Our studies are highly integrative, using proteomics, genomics, biochemical and
computational approaches. We work on a variety of taxonomic groups, including abalone, *Drosophila* and primates. I will use the abalone system of sperm lysin mediated egg vitelline envelope dissolution to demonstrate how evolutionary analyses can be used to guide functional analysis of sperm-egg interaction. In particular, I will discuss how the co-evolution of interacting proteins can be used to predict functional interactions. This approach could be informative in the study of mammalian fertilization since the identification of interactions between sperm and egg molecules during fertilization have proven elusive. Study of the function and evolution of molecules involved in fertilization will provide invaluable information to help advance studies of fertility and contraception. Rapidly evolving reproductive molecules could lead to a mismatch in sperm-egg proteins, which could contribute to infertility.

**FOCUS SESSION 7: Germline Transmission of Epigenetic Effects.**

67. **Intergenerational Epigenetic Inheritance in a Mouse Model of Undernutrition.**
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Adverse prenatal environments can promote metabolic disease in offspring and subsequent generations. Animal models and epidemiological data implicate epigenetic inheritance, but the mechanisms remain unknown. In an intergenerational developmental programming model affecting F2 mouse metabolism, we demonstrate that the in utero nutritional environment of F1 embryos alters the DNA methylome of F1 adult male sperm in a locus-specific manner. Differentially methylated regions are hypomethylated and enriched in nucleosome-keeping regions. A substantial fraction is resistant to early embryo methylation reprogramming, potentially impacting F2 development. Importantly however, differential methylation is not maintained in F2 tissues, yet locus-specific expression is perturbed. Thus, in utero nutritional exposures during critical windows of germ cell development can impact the male germline methylome and associated with metabolic disease in offspring; but DNA methylation is not the long term heritable memory maintained in the subsequent F2 generation.

68. **Genomic Imprinting: Mechanisms and Environmental Sensitivity.**
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Imprinted genes are expressed from a single parental allele and most reside in clusters that are located throughout the mammalian genome. The clusters typically contain an imprinting control region (ICR), which harbors allele-specific methylation and governs the imprinting of the entire domain. Although most imprinted clusters use long non-coding RNAs to regulate imprinted gene expression, a few are regulated by CTCF and allele-specific insulator function. One such cluster harbors the *H19* and *Igf2* imprinted genes, and is controlled by an ICR that contains multiple CTCF binding sites. Gain of maternal methylation and loss of paternal hypermethylation of the *H19/IGF2* ICR are associated with the human growth disorders Beckwith-Wiedemann Syndrome and Silver-Russell Syndrome, respectively. Using gene targeting and genome editing, we have generated ES cells, iPS cell lines and mice to study the mechanisms of imprinting for these imprinted loci and its model the epigenetic mutations in human syndromes. We have also developed SNV-FISH to study the dynamics of allele-specific gene expression at the single cell level in cell lines and tissues with loss of imprinting.

69. **Assessing the Transmission of an Altered Epigenotype and Phenotype Following Exposure to Endocrine Disrupting Compounds.**
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Fetal exposure to endocrine disrupting compounds (EDCs) results in aberrant developmental outcomes and increased disease susceptibility in adult life. Not only does exposure to EDCs in utero affect the developing fetus, but these effects can also be transmitted across multiple generations. Although the precise mechanisms by which these compounds act remain to be elucidated, it has been proposed that epigenetic pathways mediate their effects. Exposure to the EDCs bisphenol A (BPA) and di(2-ethylhexyl) phthalate (DEHP) have been shown to alter DNA methylation, an epigenetic regulatory mechanism critical for proper development. DNA methylation is also a well-established mechanism of imprinted gene regulation. The purposes of our studies are to understand how EDC exposure during early mammalian development affects epigenetic regulation in the developing mouse embryo as well as determining whether these altered epigenotypes and/or phenotypes can be transmitted to subsequent generations of offspring. In our mouse model, oral exposure to BPA (0 μg/kg, 10 μg/kg, or 10 mg/kg body weight/day) during the late stages of oocyte development in the F0 dam and early stages of F1 embryonic growth significantly disrupts imprinted gene regulation in a gene- and tissue-specific manner in the F1 offspring. Additionally, aberrant allelic expression corresponds with altered DNA methylation at regulatory elements of imprinted genes. In adulthood, BPA-exposed male offspring (n = 8 – 14 animals, from 3 – 5 litters, per treatment group) exhibit increased body fat, impaired glucose homeostasis, and altered glucose-stimulated insulin secretion. The significance of differences among groups was examined using analysis of variance, student t test, or chi-squared analysis. No significant gross phenotypic changes were detected in BPA-exposed female offspring. Interestingly, these phenotypic changes are transmitted to the next (F2) generation, which were exposed as germ cells in the developing F1 embryo. At the molecular level, misregulation of *Igf2*, a growth-promoting imprinted gene, is associated with the observed phenotype. Total expression of the *Igf2* gene and methylation at its corresponding Differentially Methylated Region (DMR) 1 are altered in the F1 and F2 offspring. Using a genetically engineered mouse with *Igf2* overexpression, a subset of metabolic changes observed in the BPA-exposed mice could be phenocopied, suggesting a role for *Igf2* in the adverse metabolic changes following perinatal BPA exposure.
Because humans are rarely exposed to a single EDC at once, it is critical to assess the synergistic and/or antagonistic effects of combinatorial exposures. Using a similar paradigm, our preliminary data demonstrate that fetal exposure to the combination of BPA and DEHP produces a greater perturbation in the allelic expression of imprinted genes in the F1 placenta as compared to single compound exposures (n = 15 – 40 animals, from 2 – 6 litters, per treatment group), suggesting an additive or synergistic effect. Taken together, our results suggest that early life exposure disrupts the developmental trajectory of not only the developing fetus but can affect its offspring as well. Identifying the detrimental effects of early-life EDC exposure on fetal and postnatal development across multiple generations and determining their mode of action will ultimately improve human health risk assessments of these compounds.

70. Developmental genome-wide DNA methylation asymmetry between mouse placenta and embryo.
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Following fertilization, the majority of the DNA methylation patterns found across the embryonic genome are removed and reacquired in the peri-implantation period by a wave of genome-wide epigenetic reprogramming. The methylation of repetitive elements, sequences that constitute a large portion of the mammalian genome, is important for genomic stability and accounts for the majority of methylated DNA. Although early reports suggested that levels of DNA methylation are not equally distributed between the trophectoderm and the inner cell mass, the types of sequences involved and the potential roles of such differences in development of the placenta and the embryo are unclear. To better understand this developmental asymmetry, we compared sequence-specific and genome-wide DNA methylation patterns between mouse E10.5 placenta (n=4) and embryo (n=4). Examination of methylation across chromosome 9 by quantitative analysis of methylation by qPCR indicated that all intergenic sites examined were hypomethylated in the placenta as compared to the embryo. Initial genome-wide results using restriction landmark genomic scanning (RLGS), which assesses methylation at approximately 3000 loci in the mouse genome, confirmed this overall hypomethylation of extra-embryonic tissue; 83 regions showed decreases while 24 showed increases in methylation. To further investigate the discrepancy in methylation we used reduced representation bisulfite sequencing (RRBS), a higher resolution genome-wide technique. With a 15-fold sequencing coverage cutoff, 113,449 100-bp tiles were found to be differentially methylated between placenta and embryo. Similar to RLGS results, the vast majority of these differences were found to be decreases in methylation (109,899 hypo tiles vs. 3,550 hyper tiles). Overall, intergenic regions made up 49.5% of the differentially methylated tiles (DMTs) while intron, exon and promoters accounted for 46.9% (32.9%,12.2% and 1.8% respectively). LINEs (43.6%) accounted for the majority of the repetitive elements found to be differentially methylated between tissues, with LTR and SINE families accounting for 31.2% and 19.7%, respectively. Interestingly, when looking solely at tiles that showed increased methylation in the placenta, the percentage of tiles found in intergenic regions decreased to 33.1%, while intron, exon and promoters increased to 59.7% (36.6%, 15.7% and 7.4%, respectively). We next followed the methylation patterns of the DMTs between placenta and embryo using publicly available sequencing data from gametes (oocyte and sperm), early pre-implantation (zygote, 2-cell, 4-cell and 8-cell) as well as E6.5 and E7.5 embryos. While unique patterns were observed in both female and male gametes, early pre-implantation embryos revealed low levels of methylation in the regions found to be differentially methylated between placenta and embryo. A gradual increase in DNA methylation was observed as embryos developed from E6.5-10.5. We propose that the active pre-implantation methylation sequence patterns found within genes in the placenta may play a role in the function of this tissue. In contrast, possibly since the placenta is a short- lived tissue, widespread hypomethylation, mainly found in repetitive elements and away from genes, is tolerated without adverse effects that may be seen in somatic tissues. (Supported by the Canadian Institutes of Health Research)

71. Profiling Polycomb Repressive Complex 2 during germ-line development.
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During development, a small pool of embryonic cells give rise to primordial germ cells (PGCs), which ultimately form oocytes and sperm and pass an individual’s genetic and epigenetic information to the following generation. Epigenetic modifications involve chemical modifications to the chromatin (DNA and associated proteins) that facilitate DNA packaging and mark or “flag” genes for expression and repression in each cell type. With each generation, the epigenetic information contained in the genome is erased or reacquired during fetal germ cell development. New epigenetic modifications are established during spermatogenesis and oocyte development, and this epigenetic information influences gene expression and cell differentiation in the next generation. However, with the exception of DNA methylation, many of the epigenetic modifications and regulators that control germ cell development and epigenetic programming of the germ -line remain unknown. Polycomb Repressive Complex 2 (PRC2) is involved in the epigenetic regulation of developmental genes during embryogenesis. PRC2 is comprised of 3 core components: Enhancer of Zeste 1/2 (EZH1/2); Suppressor of Zeste 12 (SUZ12) and ‘reprogrammed’ during fetal germ cell development. New epigenetic modifications are established during sperm and oocyte development, expression and repression in each cell type. With each generation, the epigenetic information contained in the genome is erased or reacquired in the peri-implantation period by a wave of genome-wide epigenetic reprogramming. The methylation of repetitive elements, sequences that constitute a large portion of the mammalian genome, is important for genomic stability and accounts for the majority of methylated DNA. Although early reports suggested that levels of DNA methylation are not equally distributed between the trophectoderm and the inner cell mass, the types of sequences involved and the potential roles of such differences in development of the placenta and the embryo are unclear. To better understand this developmental asymmetry, we compared sequence-specific and genome-wide DNA methylation patterns between mouse E10.5 placenta (n=4) and embryo (n=4). Examination of methylation across chromosome 9 by quantitative analysis of methylation by qPCR indicated that all intergenic sites examined were hypomethylated in the placenta as compared to the embryo. Initial genome-wide results using restriction landmark genomic scanning (RLGS), which assesses methylation at approximately 3000 loci in the mouse genome, confirmed this overall hypomethylation of extra-embryonic tissue; 83 regions showed decreases while 24 showed increases in methylation. To further investigate the discrepancy in methylation we used reduced representation bisulfite sequencing (RRBS), a higher resolution genome-wide technique. With a 15-fold sequencing coverage cutoff, 113,449 100-bp tiles were found to be differentially methylated between placenta and embryo. Similar to RLGS results, the vast majority of these differences were found to be decreases in methylation (109,899 hypo tiles vs. 3,550 hyper tiles). Overall, intergenic regions made up 49.5% of the differentially methylated tiles (DMTs) while intron, exon and promoters accounted for 46.9% (32.9%,12.2% and 1.8% respectively). LINEs (43.6%) accounted for the majority of the repetitive elements found to be differentially methylated between tissues, with LTR and SINE families accounting for 31.2% and 19.7%, respectively. Interestingly, when looking solely at tiles that showed increased methylation in the placenta, the percentage of tiles found in intergenic regions decreased to 33.1%, while intron, exon and promoters increased to 59.7% (36.6%, 15.7% and 7.4%, respectively). We next followed the methylation patterns of the DMTs between placenta and embryo using publicly available sequencing data from gametes (oocyte and sperm), early pre-implantation (zygote, 2-cell, 4-cell and 8-cell) as well as E6.5 and E7.5 embryos. While unique patterns were observed in both female and male gametes, early pre-implantation embryos revealed low levels of methylation in the regions found to be differentially methylated between placenta and embryo. A gradual increase in DNA methylation was observed as embryos developed from E6.5-10.5. We propose that the active pre-implantation methylation sequence patterns found within genes in the placenta may play a role in the function of this tissue. In contrast, possibly since the placenta is a short- lived tissue, widespread hypomethylation, mainly found in repetitive elements and away from genes, is tolerated without adverse effects that may be seen in somatic tissues. (Supported by the Canadian Institutes of Health Research)
levels in spermatogonia and spermatocytes, but was not detected during spermiogenesis. In females, H3K27me3 was detected in E11.5 germ cells at the time of commitment to oogenesis, partially enriched during fetal oocyte development and further enriched during oocyte maturation. Together these results indicate that PRC2 regulates H3K27me3 at key stages of germline development and gametogenesis.

72. Chromatin Inheritance and Dynamics at the Onset of Life.
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In mammals, totipotent embryos are formed by fusion of differentiated gametes. In mice, acquisition of totipotency concurs with remodeling of chromatin states at parental genomes, major changes in the maternal transcriptome and proteome, and activation of the parental genomes. It is unknown to what extent reprogramming during gametogenesis and embryogenesis versus the intergenerational inheritance of chromatin states at specific genome regions contribute to successful early embryonic development. I will discuss our approaches and recent findings in addressing this question.

73. Epigenetics and Gamete Biology in Rare and Endangered Species.
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Gamete biology studies help to increase fundamental, comparative knowledge on rare and endangered species that, in turn, can be used to implement programs for managing animal populations in captivity or in the wild. While the theory appears straightforward, the actual practice of studying sperm or oocyte biology in rare and endangered species is extraordinarily complex. This is primarily due to the fact that there are as many differences in gamete morphology, ultrastructure, and functionality as there are species. Another difficulty is the enormous lack of scientific knowledge in these species that prevents from making progress as fast as in conventional animal models (laboratory and livestock species). So far, most gamete studies in rare genotypes have focused on the integrity of DNA sequences (prevalence of DNA fragmentation) and sometimes on DNA ‘packaging’ (protamine composition in spermatozoa for instance). Main epigenetic factors and patterns are expected to be conserved across species but this still has to be confirmed. Beyond the need to better understand the mechanisms in multiple species, one of the first priorities is to measure the impact of environmental factors as well as the various effects induced by non-physiological conditions (handling, preservation) on key areas of the epigenome that regulates gamete quality of individuals and the fertility of entire populations. For almost four decades, our laboratory has been studying the impact of cryopreservation and in vitro culture on gonadal tissues and mature forms of gametes using different comparative models (from carnivore to invertebrate species). Even though epigenetic studies have been limited, recent results in germinal vesicle histone acetylation/methylation have clearly demonstrated the fundamental role of non-traditional models for comparison and better translation to other species including humans. Integrating epigenetics in the study of gamete biology also will result in more solutions in fertility enhancement or contraception for many species that we manage in captivity or in the wild.

74. Environmental influences on sexually dimorphic DNA methylation patterning in the American alligator, an organism displaying temperature-dependent sex determination.
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Unlike traditional lab models, the American alligator is a long-lived apex predator that displays high site fidelity and undergoes temperature-dependent sex determination. These characteristics provide an ideal model in which to examine the effects of environmental factors, such as long-term, chronic exposures to complex mixtures of contaminants, on epigenetic patterning. When compared to their counterparts living in relatively pristine environments, alligators living in environments contaminated with endocrine-disrupting contaminants display a suite of reproductive disorders. Among these, the expression of CYP19A1 (Aromatase), a key gene within the estrogen synthesis pathway, is misregulated in animals originating from contaminated environments. We have previously shown that the promoter of CYP19A1 undergoes sexually dimorphic DNA methylation patterning, with males displaying hyper-methylation when compared to females. Here, we employ reduced representation bisulfite sequencing to further explore the sexually dimorphic methylome in embryonic gonads, and identify numerous sexually dimorphic methylated regions within the alligator genome. In addition, we use targeted bisulfite sequencing on the Illumina platform to examine methylation patterning at the CYP19A1 promoter and other loci within male and female embryos across sites with varying contamination levels. We find that sexually dimorphic CYP19A1 promoter methylation is significantly abated in embryos originating from a contaminated site (Lake Apopka) when compared to females from a relatively pristine site (Lake Woodruff). This reduction in sexual dimorphism is due to elevated gonadal methylation within females from Lake Apopka. We next examined the effect of embryonic estrogen exposures on CYP19A1 and report these findings. These results suggest that DNA methylation patterning may play an integral role in mediating the effects of incubation temperature on sex determination and that endocrine disrupting contaminants may exert their effects by interfering with sexually dimorphic epigenetic patterning occurring during early development.

75. Influence of Microwave-Assisted Dehydration and Supra-Zero Temperature Storage on Key Epigenetic Factors in the Cat Germinal Vesicle.
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The germinal vesicle (GV) is an alternative target to the whole oocyte for new genome preservation approaches, including storage at supra-zero temperatures. Using the domestic cat model, we have demonstrated the ability to reach an optimal moisture content of 0.01 gH2O/gDW by microwave-assisted dehydration. This moisture level allows achieving and maintaining a glass state (via trehalose exposure) below 26°C. Results to-date have revealed limited DNA damage after dehydration and 8 wk storage at ambient or cool
temperature. The present study was designed to explore the next step by examining key epigenetic factors critical to the functionality of the GV. Our objective was to assess the influence of dehydration and storage on two histone lysine methylations, histone H3 trimethylated at lysine 4 (H3K4me3) and dimethylated at lysine 79 (H3K79me2), both of which have been linked earlier to GV developmental competence in the cat. Oocytes were collected from antral follicles of excised adult ovaries. In each replicate, some of the fresh, untreated oocytes were controls, and the rest processed by: 1) permeabilization followed by exposure to 1.5 M trehalose for 10 min; 2) transfer onto conjugate-release filters and dehydration using a CEM SAM 255 microwave system for 30 min; and 3) storage in Dri-Shield moisture barrier bags at ambient temperature (22.8-26.3°C) or 4°C for 1 wk. After each of these treatment steps, a subset of oocytes was recovered to assess presence of H3K4me3 (n = 218 total oocytes, 3 replicates) or H3K79me2 (n = 250 total oocytes, 3 replicates) in the GV by immunostaining. Oocyte-free, control filters were processed with each replicate to assess moisture content immediately after dehydration or storage using volumetric Karl Fischer titration to ensure that appropriate moisture level was achieved. The moisture content (mean ± SD) decreased (P < 0.05) from 1.21 ± 0.09 to 0.10 ± 0.02 gH2O/gDW after microwave-assisted dehydration and then remained unchanged (P > 0.05) after storage at either ambient temperature or 4°C for 1 wk. The proportion of GVs containing H3K4me3 was unaffected (P > 0.05) by trehalose exposure alone (19.6%) or dehydration (20.0%) but decreased (P < 0.05) after 1 wk storage (0.0% at ambient temperature; 4.3% at 4°C) compared to fresh controls (18.2%). The proportion of GVs containing H3K79me2 was not affected (P > 0.05) by trehalose exposure alone (60.7%) and increased (P < 0.05) immediately after dehydration (85.4%) compared to fresh controls (65.3%). After 1 wk storage at ambient temperature or 4°C, all of the GVs contained H3K79me2. In summary, certain epigenetic patterns in the cat GV appear resistant to desiccation and supra-zero temperature storage, whereas others are affected adversely. The regulating mechanism is most likely expressed through perturbed demethylase and/or methyltransferase activities. Both may serve as targets for identifying mitigating methods to ensure retention of both epigenetic pattern and functionality for GVs preserved using this alternative approach.

76. Investigating transcriptional silencing during mouse spermiogenesis.
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Spermiogenesis, the differentiation of haploid round spermatids into mature functional spermatozoa, involves several dramatic changes in the structure and composition of the nucleus. It has been known for 40 years that an early nuclear event in spermiogenesis is the global down-regulation of transcription. Several studies have used immuno-staining approaches to study this process, however, higher resolution analyses have not been performed and mechanistic insights into this process have remained elusive. We have combined RNA-Seq and ChIP-Seq analyses of purified spermatid populations to capture a snapshot of the transcriptional dynamics during differentiation. We find that while the vast majority of genes are indeed transcriptionally silenced during the differentiation of round spermatids into elongating spermatids, a small fraction of genes escape from this silencing. We are analyzing the sequence characteristics that distinguish these genes from the others and are examining chromatin features that may play a role in their continued expression. We are also analyzing the localization of proteins involved in transcription at those genes that are silenced to examine where within the multi-step process of transcription this down-regulation occurs. These analyses will provide insights into possible mechanisms governing this relatively under-studied process. This work has been supported by EMBO (ALTF-253-2011 to M.E.G.) and the Swiss National Science Foundation (to A.H.P.)

FOCUS SESSION 8: Emerging Roles of Developmental Signaling Pathways in the Adult Gonad.

77. Non-Canonical WNT Signaling and Its Role in Ovarian Follicle Development.
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Recent studies have established the WNT family of secreted glycoproteins as key modulators and coordinators of ovarian follicular responses to the gonadotropins. Of the three major pathways that transduce WNT signals, the WNT/CTNNB1 pathway (also known as the canonical pathway) appears to play a central role in the ovary. Here, the binding of a WNT to a cognate Frizzled (FZD) receptor results in the stabilization of the signaling effector CTNNB1 (β-catenin), which then translocates to the nucleus and associates with a number of transcription factors to alter the transcriptional activity of target genes. In granulosa cells, CTNNB1 is required for FSH-stimulated Cyp19a1 and Lhcgr expression and follicle growth. Likewise, LH acts via CTNNB1 to regulate Star expression in granulosa-lutein cells. In addition to being regulated by WNTs, CTNNB1 stability is also regulated by the gonadotropins themselves via PKA, which affects its phosphorylation at a number of sites to enhance its stability and transcriptional properties. Whereas most WNTs preferentially (or exclusively) activate the canonical pathway, WNT5a and WNT11 are associated with non-canonical pathways in mammalian cells. The latter are expressed in mouse, bovine and human granulosa cells, but very little is known of their physiological functions in the ovary. Using conditional gene targeting, we found that granulosa cell-specific inactivation of Wnt5a in mice resulted in a 52% decrease in female fertility. This was associated with decreased numbers of healthy antral follicles, increased atresia, an accelerated depletion of the ovarian reserve, and a reduced ovulatory rate. Microarray analyses of cultured granulosa cells treated with Wnt5a were conducted to identify its transcriptional targets. Wnt5a was found to down-regulate a large number of FSH-responsive genes associated with granulosa cell differentiation, proliferation and steroidogenesis, and these same genes were found to be up-regulated in the granulosa cells of Wnt5a conditional knockout mice. Unexpectedly, we found that Wnt5a does not exert its gene regulatory functions by signaling via non-canonical pathways, but rather through a rapid and drastic down-regulation of CTNNB1. Loss of CTNNB1 was attributed to a loss in PKA activity, resulting in the loss of CTNNB1 phosphorylation at a site required for its stability. This loss of PKA activity also resulted in the suppression of granulosa cell responsiveness to both FSH and LH. Unlike Wnt5a, conditional knockout of Wnt11 had no effect on female
fertility, follicle development or ovarian gene expression, and concomitant inactivation of both Wnt5a and Wnt11 had no effect beyond that of inactivation of Wnt5a alone. Taken together, our findings indicate that Wnt5a is required for normal follicle development and female fertility. It acts, at least in part, to suppress gonadotropin responsiveness by downregulating CTNNB1 signaling and PKA activity. We hypothesize that Wnt5a functions to prevent premature or excessive responses to FSH and/or LH, thereby providing an autocrine/paracrine level of control of gonadotropin action.

78. The Hedgehog-Patched Signaling Pathway and Its Function in the Mammalian Ovary.
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During ovarian follicular development, granulosa (GC) and theca cell (TC) proliferation and differentiation are influenced by gonadotropins, insulin-like growth factors (IGF), and numerous intraovarian factors secreted by both the oocyte and the surrounding somatic cells, including potential paracrine mediation by components of the hedgehog (Hh) family. Since their first identification in the early 1990s, members of the Hh family proteins have been shown to be expressed at epithelial-mesenchymal boundaries during embryogenesis and regulate the growth, differentiation, and morphogenesis of various mammalian organs. Three Hh proteins, Indian (Ihh), Desert (Dhh), and Sonic (Shh) hedgehog, are capable of binding to the Patched 1 (Ptc1) receptor leading to signal transduction via derepression of its co-receptor, Smoothened (Smo) and activation of the transcription factor, glioma-associated oncogene homolog 1 (Gli1). Importantly, the Ihh sequence is highly conserved among mammalian species; for example, the nucleotide sequence of bovine and human Ihh is 87% identical. Studies have also demonstrated the importance of the Hh signaling in many tissues during postnatal life. This presentation will summarize evidence for a potential role of Hh proteins within the mammalian ovary. In rodents, Ptc1 and Gli1 mRNA are primarily expressed in TC whereas Ihh and Dhh mRNAs are predominately located in GC. Immunostaining of Ptc1 was found in both TC and GC of mice, and GC respond to Shh in vitro with increased proliferation. In PMSG-treated mice, hCG treatment in vivo decreased whole ovarian Ihh and Dhh mRNA, indicating changes in the Hh signaling system may be hormonally regulated. A recent study in mice indicates that growth differentiation factor-9 (GDF9)-induced Ihh production by GC is required for TC formation during oogenesis. Studies from our laboratory indicate that Shh stimulates bovine TC proliferation while enhancing IGF1-induced TC androstenedione production without affecting progesterone production, identifying a specific role of Hh proteins in ovarian TC function of mammals. We also discovered that Ihh mRNA in GC of estrogen-active bovine follicles increases between day 3 and 5 of an estrous cycle, and that dizygotic twinning is associated with less Ptc1 mRNA abundance in TC. We have further shown that estradiol increases whereas IGF1 decreases Ihh mRNA in bovine GC, and LH, IGF1 and bone morphogenetic protein-4 (BMP4) treatments alone decrease Ptc1 mRNA in TC, but GDF9 and activin have no effect; GC Dhh mRNA was not altered by hormones. In addition, we have discovered that Shh increases abundance of mRNA for the anti-differentiation factor fibroblast growth factor-9 (FGF9) in bovine GC. We hypothesize that as follicles grow and develop, increased free IGF1 may suppress production of Ihh by GC and Ptc1 by TC, and these effects are regulated by estradiol and other intra- and extra-gonadal factors. In another study, we found that Ihh mRNA abundance is less in GC of cystic vs. normal dominant follicles of cattle, and studies of others have revealed that the hedgehog signaling pathway is activated in human ovarian carcinomas calling for a need to understand what role the hedgehog pathway plays in the development of ovarian pathologies. In summary, current evidence implicates Ihh as an important regulator of ovarian follicular growth and steroidogenesis, and in regulating single vs. multiple ovulations.

79. Gonadotropins Regulate the Hippo Pathway in the Murine Ovary.
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Hippo is an evolutionarily conserved pathway known for its roles in regulating organ size and tissue growth in mammals. The pathway is composed of a protein kinase cascade that becomes activated by intercellular contact, which leads to the phosphorylation of LATS1/2, which in turn phosphorylate the signaling effectors YAP and TAZ, resulting in their retention in the cytoplasm. Absence of intercellular contact inhibits LATS1/2 activity, allowing YAP and TAZ proteins to translocate to the nucleus and bind to transcription factors of the TEAD family. This results in the modulation of the transcriptional activity of a variety of target genes in a cell type- and context-specific manner. Recent studies have begun investigating Hippo’s potential implications in ovarian follicle development. Kawamura, et al. revealed that mechanical disruption of Hippo signaling in the ovary stimulates follicle growth and oocyte maturation. However, regulators of the Hippo pathway and their effects on ovarian processes in the mouse have yet to be discovered. Our objectives are 1) to describe gonadotropin regulation of the Hippo pathway in the ovary in vivo and in vitro and, 2) to understand the mechanism of Hippo activity in murine granulosa cells by identifying key Hippo pathway target genes. To elucidate the in vivo effects of follicle-stimulating hormone (FSH) on Hippo signaling, immature female mice were treated with equine chorionic gonadotropin (eCG) in a timecourse experiment and their granulosa cells isolated for analyses. Expression of total and phospho-LATS1 and phospho-YAP protein expression after 4 hours. Peak of LATS1 activity was followed by a decrease in the mRNA levels of Hippo target genes Ctgf, Nov, and Areg. Similarly, the in vitro treatment of granulosa cells with FSH for 1 hour resulted in increased Nov and Areg mRNA levels, which was reversible by treatment with a YAP/TAZ transcriptional inhibitor, verteporfin. Our results strongly suggest that eCG/FSH inhibits the Hippo kinase cascade, resulting in an increase in the expression of Hippo pathway target genes, which may be associated with granulosa cell proliferation in growing follicles. The in vivo effects of luteinizing hormone (LH) on Hippo signaling were investigated with eCG-primed immature female mice, which were treated with human chorionic gonadotropin (hCG) in a timecourse experiment. HCG treatment induced an increase in total and phospho-LAT1 and phospho-YAP protein expression after 4 hours. Peak of LAT1 activity was followed by a decrease in the mRNA levels of Hippo target genes Ctgf, Nov, and Areg. Likewise, the in vitro LH timecourse revealed a peak of total and phospho-LAT1 expression at 15-30 minutes post-LH. Verteporfin inhibited the LH-induced expression of Areg, Nov, and Ibg2. These findings suggest that LH/hCG turns on the Hippo pathway at 4h post-hCG, followed by the downregulation of key Hippo pathway target genes, which may be related to the halting of granulosa cell proliferation. These results provide novel insights into the Hippo pathway as a regulator of murine folliculogenesis and evidence that LH and FSH may act in part via the Hippo pathway to regulate the expression of target genes including Areg, Ctgf, Nov, Wisp2, and Ibg2 in murine granulosa cells.
Selective theca mutant (tPtenMT) mice for phosphatase and tensin homolog deleted from chromosome 10 (Pten) exhibit androgen excess, anovulation, antral follicle accumulation, enlarged ovaries and early loss of female fertility phenotype, mimicking human polycystic ovary syndrome (PCOS). Genome-wide association screenings in multiple ethnic groups have identified that YAP, a pivotal component of the Hippo signaling pathway, is one of susceptibility genes for PCOS. The Hippo pathway is known to be an important signaling framework relevant for cell growth and organ size control through a kinase cascade that ends up influencing transcriptional regulator YAP activity. Disruption of the Hippo signaling pathway activates YAP by reducing its phosphorylation and subsequently retaining it in the nuclei. It is not yet clear that the pathophysiologically significant of the Hippo signaling pathway might contribute to the PCOS phenotype. The current study examined protein levels, phosphorylation status and subcellular location of YAP and other core components of the Hippo pathway in the ovaries of tPtenMT mice. Ovaries (n = 3) from 2.5-month old tPtenMT and wild type (WT) littermates were prepared for Western immunoblot (WB) and immunohistochemistry (IHC). WB results demonstrated that phosphorylation of YAP at both serine 127 and 397 sites, but not total YAP protein levels was significantly decreased in tPtenMT ovaries. IHC showed that YAP is mainly localized in ovarian granulosa cells. More abundant nuclear immunostaining of YAP was observed in granulosa cells of tPtenMT ovaries as compared to WT littermates. Furthermore, WB analyses of upstream and downstream components of the Hippo signaling pathway revealed that the regulatory protein SAV1 levels and phosphorylation of the cofactor MOB1 in mutant ovaries were remarkably reduced while total protein levels of MOB1, other kinases including MST1, MST2 and LATS1 and YAP binding transcription factor TEAD were comparable to WT animals. These results suggest that selective ablation of Pten in theca cells disrupts the Hippo signaling pathway that leads to a sustained activation of YAP in granulosa cells. Dysregulation of Hippo signaling may contribute to uncontrolled granulosa cell proliferation and follicle growth which expands the size of the ovary as seen in tPtenMT mice. The mechanism by which selective deletion of Pten in theca cells alters the activity of Hippo signaling in granulosa cells is being investigated in the laboratory.

81. The Hippo Pathway Plays a Role in Oocyte Maturation and Differentiation of the Cumulus-oocyte Complex During Ovulation.
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Oocytes and somatic cells undergo dramatic changes during the ovulatory transition. For example, cumulus cells cease to proliferate, undergo cumulus expansion and produce progesterone. Meanwhile, fully grown oocytes complete meiosis I and arrest at metaphase II until fertilization. The hippo signaling pathway has been recently recognized as a regulator of proliferation, differentiation and apoptosis in a wide variety of tissues. Activation of the hippo signaling leads to phosphorylation and inhibition of the transcriptional co-activators YAP/TAZ, whereas inhibition of the hippo signaling leads to nuclear translocation and activation of various regulatory functions of YAP/TAZ including binding to TEAD proteins to promote proliferation and survival. Previously, we found that verteportin (VP), a small molecule inhibitor of YAP-TEAD interaction, suppresses granulosa cell proliferation stimulated by oocytes, suggesting that oocytes regulate the proliferation of granulosa cells through YAP-TEAD. However, the possible role of YAP-TEAD complex in cumulus cell differentiation and oocyte maturation has not been explored. In this study, we hypothesized that inhibiting YAP-TEAD interaction would promote both cumulus cell differentiation and oocyte maturation. First, we investigated the effect of VP (200nM or 1µM) on cumulus cell differentiation by examining the concentration of expansion and differentiation transcripts in COCs (cumulus-oocyte complex) cultured with or without VP for 16 hours. Interestingly, VP induced expression of expansion markers (Hsd3b1 by 2 fold; Ptgs2, Prx3, Tnfaip6 by 10~20 fold) at a dose of 1µM, but not 200nM (n≥6, P<0.05). Moreover, 89.5% of VP (1 µM) treated COCs underwent what appeared to be morphologically normal cumulus expansion whereas none of control COCs underwent cumulus expansion. Similarly, 1 µM but not 200 nM VP increased mRNA expression of steroidogenic markers such as Star, Cyp19a1 (to 7~9 fold) and Cyp11a (to 19 fold) in cumulus cells (n=3~10, P<0.05) while Hsd3b1 and Lhcgr mRNA were unchanged (n=3~9). Consistent with an increase in Star mRNA, progesterone was higher in the COCs treated with VP (1µM or 5µM) compared with control (n=2). Therefore, we concluded that YAP-TEAD inhibitor promotes cumulus cell proliferation while preventing premature differentiation. Although VP promotes cumulus cell differentiation, there was a detrimental effect on oocyte maturation. For example, VP treatment decreased oocyte meiotic progression to metaphase II by 28.6% at 200nM and by 31.8% at 1µM (≥100 oocytes/treatment, P<0.05). Contrary to our hypothesis, this result suggests that inhibiting YAP-TEAD interaction attenuates oocyte maturation in denuded oocytes, which indicates a novel role for YAP-TEAD in this process. Together, these results suggest that YAP-TEAD, the regulatory module of the hippo pathway, prevents cumulus cell differentiation before ovulation, while in the oocytes, YAP-TEAD is required for completion of the MI to MII transition during maturation. Gene expression data were analyzed by ANOVA, followed by Tukey test; oocyte maturation data were analyzed using one-way Chi square comparison, and a P<0.05 was considered significantly different. This work was supported by start-up funds from The Pennsylvania State University to FJD.

82. Notch Juxtacline Regulation of Ovarian Follicle Formation and Development.
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The mammalian ovary is central to female reproductive function, providing the source of crucial secreted hormonal factors and serving to nurture the female germ cell through ovulation. Communication between germ cells and somatic support cells of the ovary is critical for formation of follicles and for establishment of the follicular niche in which the oocyte develops to maturity. Substantial
emerging evidence supports a role for Notch signaling in this intra-follicular cell communication. The Notch pathway is one of the most conserved signaling systems in multi-cellular organisms, and Notch signaling has a fundamental role in cell fate determination during development. The Notch pathway involves contact-dependent, or juxtacrine, signaling between families of membrane-bound ligands and receptors. Using a Notch-responsive transgenic reporter as well as assessment of Notch receptor, ligand, and effector RNA and protein expression, we found that Notch signaling is active in the embryonic mouse ovary and is strongly upregulated at birth in somatic cells that surround germ cell syncytia, coincident with the resolution of these syncytia and establishment of the primordial follicles pool. Live imaging is being used to explore the relationships between Notch-active cells and germ cells in the ovary, while cell sorting using fluorescent reporter lines is being applied to identify and quantify the population of Notch active cells in the ovary. Although several Notch receptors and ligands are expressed in the neonatal ovary, our studies have focused on the ligand Jagged1, which is expressed in the oocyte, and the receptor Notch2, which is localized in granulosa cells, indicating a likely signal from germ cell to somatic cell of the follicle. Our earlier data demonstrated that pharmacologic inhibitors of Notch signaling delayed the resolution of germ cell syncytia and the formation of primordial follicles. Current studies using conditional knockout mice in which Jag1 is disrupted in the oocyte, or Notch2 in the granulosa cell, reveal a prominent phenotype of “multi-oocytic follicles”, in which multiple oocytes are found within a single follicular boundary, consistent with a role for Notch signaling in the resolution of syncytia and the formation of normal primordial follicles. We also observe reduced proliferation and increased death in the granulosa cells of maturing follicles in the Notch knockout mice. The Jag1 KO mice are subfertile and exhibit reduced ovulation as well as defects in meiotic maturation, while the Notch2 KO mice have largely normal fertility. Ongoing studies are focused on investigating mechanisms through which Notch signaling impacts granulosa cell gene expression and function, examining the fertility defects in the Jag1 KO mice, generating new genetic models to explore aspects of Notch function in the ovary, and understanding how Notch signaling is integrated with other signaling pathways that regulate follicle function, particularly activin and gonadotropin signaling. These studies support the idea that Notch signaling plays important roles in regulating follicle formation, growth, and function in the mouse ovary. Supported by P01 HD021921 to KEM from the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

83. Regulation of the Spermatogonial Stem Cell Niche by NOTCH signaling.
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Stem cells are influenced by their surrounding microenvironment, or niche, which provides the factors necessary for their development. In the postnatal testis, Sertoli cells are the main component of the spermatogonial stem cell (SSC) niche and express factors regulating SSC self-renewal and differentiation, such as GDNF, CYP26B1 and KITL. Because germ cells and Sertoli cells are in close contact within the seminiferous epithelium, they must communicate to adapt the size of the stem cell pool and maintain homeostasis, possibly using juxtacrine receptors/signals such as NOTCH receptors and their ligands. We found NOTCH pathway activated exclusively in Sertoli cells within the seminiferous epithelium. Using conditional gain- and loss-of-function mouse models, we demonstrated that NOTCH signaling in Sertoli cells is critical for the delivery of proper amounts of niche factors by down-regulating their expression through the HEY1 and HEYL transcriptional repressors. Further, we also have evidence that germ cells, through expression of NOTCH ligands such as JAG1, regulate NOTCH signaling in Sertoli cells by cell-cell contact. Therefore, germ cells are part of a negative feedback mechanism that controls their own numbers, and alterations of NOTCH signaling in Sertoli cells can lead either to infertility or germ cell hyperplasia. This research was supported by NIH HD068989 and HD081244 (MCH), and by a NIH T32 ES007326 fellowship (TG).

84. GDNF Expression in Sertoli Cells is Modulated by the Activity of the HES/HEY Basic Helix-loop- helix Transcriptional Repressors.
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Stem cells are influenced by their surrounding niche microenvironment. In the testis, Sertoli cells are the key niche cells directing the population size and differentiation fate of germ cells including spermatogonial stem cells (SSCs). Our previous studies with Sertoli-cell specific Notch gain- and loss-of- function mouse models complementarily demonstrated that Notch signaling in Sertoli cells is crucial for the proper regulation of germ cell fate. Since Sertoli cell expression of the Hes/Hey genes in these mutant mice was inversely correlated with the expression levels of Gdnf, and the HES/HEY proteins in general function to inhibit the expression of genes by forming complexes with corepressors, we hypothesized that the differences in expression of Gdnf in these mutant mice were directly related to the differential expression of the Hes/Hey genes. To this end, we used primary cultures of wild-type Sertoli cells treated with and without the ligand to the Notch receptor, JAG1. In some experiments, the cells were also transfected one day after ligand induction with dicer-substrate siRNAs targeting control (scramble), Hes1, Hey1, or HeyL transcripts for degradation through the RNAi pathway. We found that activation of Notch signaling in cultured Sertoli cells, as measured by the induction of Hes/Hey gene expression, could be achieved through culture of the cells in the presence of immobilized JAG1. Ligand- induced increases in Hes/Hey gene expression were associated with significant decreases in Gdnf expression and when Hes1, Hey1, or HeyL transcripts were knocked down through siRNAs, the expression levels of Gdnf were significantly increased. Our results confirm that the effects upon Hes/Hey gene expression are directly attributable to Notch receptor activation and activity in Sertoli cells. In conclusion, we were able to confirm the regulatory role Notch signaling confers upon Gdnf expression in Sertoli cells, and determine, by directly manipulating the level of individual Hes/Hey genes, that the changes in Gdnf expression are in fact due to the changes in the expression in Hes/Hey genes downstream of Notch signaling activation. Future studies will be geared towards identifying the critical HES/HEY binding sites within the Gdnf promoter that mediates their transcriptional repressor activity. Funded by R01 HD081244.

85. Notch2 Receptor Disruption During Gonadotropin-Dependent Follicle Development in the Mouse Ovary May Result in Reduced Granulosa Cell Function.

2015 Abstracts - Page 32
Ovarian follicle assembly and maturation are critical developmental events that dictate female fertility and reproductive health. The continual spermatogenesis that generates millions of genetically unique gametes daily relies on the activities of an undifferentiated spermatogonial population that consists of rare spermatogonial stem cells (SSCs) and numerous progenitor spermatagonia. During steady-state conditions, self-renewal by SSCs maintains a pool from which progenitors arise periodically and then

FOCUS SESSION 9: Male Germ Cell Biology.

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The continual spermatogenesis that generates millions of genetically unique gametes daily relies on the activities of an undifferentiated spermatogonial population that consists of rare spermatogonial stem cells (SSCs) and numerous progenitor spermatagonia. During steady-state conditions, self-renewal by SSCs maintains a pool from which progenitors arise periodically and then
transiently amplify in number before committing to a pathway of terminal differentiation. Also, SSCs are capable of regenerating spermatogenesis following transplantation into a recipient testis or cytotoxic damage that eliminates the majority of the germline. A foundational SSC pool develops from a subset of prospermatogonial precursors during early neonatal life, a process that is obligatory for continuity of the spermatogenic lineage, yet the underlying kinetics and mechanisms regulating it are undefined. Our previous studies demonstrated that regenerative capacity in spermatogonia is linked to expression of inhibitor of DNA binding 4 (ID4) and we generated an Id4-Gfp reporter mouse line to study the population in more detail. We have found that the ID4+ population is a minor subset of spermatogonia prior to birth and the pool is established during a defined period of 0-3 days of postnatal life in mice. In addition, we have found that ID4 physically interacts with retinoblastoma protein (RB) in SSCs and RB expression in prospermatogonia is required for the formation of the SSC pool during neonatal development. However, formation of the primary progenitor and differentiating spermatogonial populations are not impaired in the absence of RB. In more recent studies, we have discovered that RB is dispensable for the formation of prospermatogonia but expression in primordial germ cells (PGCs) is required for the initial formation of all postnatal spermatogonial subtypes. Collectively, our findings and those of other research groups support a model in which a subset of prospermatogonia are programmed in prenatal development to be the seed population, i.e. Golden Boys, for establishment of the SSC pool in neonatal development. This research was supported by grant HD061665 awarded to J.M.O. from the National Institutes of Health.

88. Filling the Spermatogonial Stem Cell Pool One Cell at a Time.
Brian P. Hermann

Spermatogenesis and male fertility are dependent upon formation of a pool of spermatogonial stem cells (SSCs) in the mammalian testis. SSCs arise from prospermatogonia, but only some prospermatogonia become foundational SSCs, suggesting there are subtypes of undifferentiated germ cells with discrete molecular signatures that predispose distinct fates. We recently used single-cell gene expression approaches to demonstrate considerable heterogeneity in mRNA and protein abundance of a panel of germ cell and stem cell genes in the P6 mouse testis. Among the 172 genes examined, 27 exhibited bimodal patterns of mRNA abundances among Id4-eGFP+ spermatogonia (i.e., present/absent), suggesting these genes mark two or more discrete cell subpopulations of Id4-eGFP+ undifferentiated spermatogonia. Co-staining for three cell surface markers found among the bimodal transcripts (TSPAN8, EPHA2 and PVR) using flow cytometry demonstrated colocalization in ~10% of Id4-eGFP+ cells. In addition, mRNA levels for a panel of bimodal genes were largely enriched among TSPAN8-high and EPHA2-high sorted subpopulations of P6 Id4-eGFP+ spermatogonia, validating the single-cell gene expression data. Additional flow cytometry analyses demonstrated that the TSPAN8-high/low and EPHA2-high/low subpopulations of Id4-eGFP+ spermatogonia do not differ significantly in cell cycle state. These results suggest that the genes which exhibit bimodal transcript abundance patterns among individual P6 Id4-eGFP+ spermatogonia mark phenotypically-distinct subpopulations. Thus, gene expression heterogeneity among undifferentiated spermatogonia likely reflects the existence of distinct cell subtypes with differing functional capacities, including stem and progenitor spermatogonia fates.

89. A long non-coding RNA interacts with GDNF receptor Gfra1 and maintains self-renewal of mouse spermatogonial stem cells.
xin wu1, lufan li1, min wang1, mei wang1, xiaoxi wu1, lei gen1

Spermatogonial stem cells (SSCs) support life-long sperm production and male fertility. Knowledge of mechanisms governing self-renewal and proliferation of SSCs is essential for the understanding of spermatogenesis and of potential consequences of pathogenic insult. Long non-coding RNAs (lncRNA) have been identified as key regulators of stem cell fate; however, their role in SSCs has not been explored. Here, we report that a novel spermatogonia-specific lncRNA (LncRNA033862) is essential for the survival of murine SSCs. LncRNA033862 is expressed in early spermatogonia including SSCs and was among 805 lncRNAs identified by global expression profiling as responsive to glial cell derived neurotrophic factor (GDNF), a growth factor required for SSC self-renewal. LncRNA033862 is an antisense transcript of the GDNF receptor alpha1 (Gfra1) that lacks protein coding potential and regulates Gfra1 expression levels by interacting with Gfra1 chromatin. Importantly, LncRNA033862 knockdown severely impairs SSC self-renewal and their capacity to repopulate recipient testes in transplantation assays. Collectively, our study demonstrates for the first time that long non-coding RNAs (lncRNAs) are important regulators of SSC cell fate. Our data provide novel insight into the biology of SSCs, and establish a foundation for further exploration of long non-coding RNAs involved in the regulation of not only SSCs, but other adult stem cells.

90. Testis Expressed Actin-like 7b (Actl7b) Is Associated with the Golgi Derived Developing Acrosome and Required for Acrosome Attachment, Spermatid Morphogenesis, and Fertility.
Tracy Clement1, Chris Geyer2, Garrett Warren3, Eugenia Goulding4, William Willis1, Mitch Eddy1

Human male infertility is often associated with a high incidence of abnormally shaped sperm heads, suggesting that cytoskeletal regulation may be important for male fertility. The involvement of filamentous actin (F-actin) has been suggested for several aspects of spermatid differentiation, including acrosome formation and attachment to the nucleus. Although the structural components and morphological changes associated with spermiogenesis have been described in detail, relatively little is known about the mechanisms that drive these structural changes. Actin-like 7b (Actl7b) is an orphan actin related protein (ARP) family member that we have shown is required for spermatid development and fertility. Immunochemistry and indirect immunofluorescence reveal ACTL7B is localized in the cytoplasm of round and elongating spermatids in mouse and human. It co-localizes with phalloidin-labeled F-actin in or around the forming acrosome, suggesting a role in acrosome formation. Actl7b knockout mice develop to adulthood normally and knockout males mate, but are infertile due to severe and variable teratozoospermia and a lack of sperm motility. Electron microscopy observations reveal that the primary defects are disruption of acrosome matrix localization and acrosomal attachment beginning in early spermiogenesis. To determine the mechanism of action of ACTL7B, protein structure, overexpression, and protein associations are being investigated. ACTL7B has a conserved actin domain with striking sequence and predicted structural similarity to beta-actin. In whole testis
protein extracts, ACTL7B consistently associates with the insoluble fraction. ACTL7B also has a unique N-terminal 60 aa sequence. In transfected COS7 cells, overexpressed GFP-ACTL7B fusion protein is localized to the Golgi. Together our data indicate 1. Actl7b is required for spermatid morphogenesis, sperm motility, and male fertility, 2. ACTL7B is localized to the Golgi-derived acrosome, likely by its unique N-terminal domain, and 3. ACTL7B is either an acrosomal F-actin or required for proper F-actin assembly in spermatids. This research was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

91. Dynamic control of FGFR-mediated self-renewal signaling in adult spermatogonial stem cells.
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Spermatogonial stem cells (SSCs) together with the somatic cells in the mammalian testis maintain tissue homeostasis through carefully orchestrated, dynamic changes in signaling. In addition to canonical factors, such as glial derived neurotrophic factor (GDNF), other signals including fibroblast growth factors (FGFs) produced by somatic cells, contribute to SSC self-renewal. However, it remains unclear how these signals regulate SSC fate even under stressful conditions. Recently, a clustered distribution of pathogenic mutations was found in the normal adult human testis of older men, suggesting a clonal, stem cell-based process. Thus, positive selection of SSCs was proposed to explain the higher frequency of some disorders (e.g., Apert syndrome) among children of older fathers, a correlation referred to as the paternal age effect (PAE). Using a long-term, in vitro culture system and transplantation analysis, to study adult mouse SSCs, we recently demonstrated for first time that SSCs bearing a mild gain-of-function Apert syndrome-associated mutation in FGFR2 (referred to as AS SSCs) exhibit enhanced competitiveness in vivo and in vitro. Our data showed that the competitive advantage of AS SSCs is contingent upon the dose of growth factor, indicating that the stem cell niche can greatly influence whether a phenotypic effect of the mutation manifests. Interestingly, PAE disorders identified to date do not include strong gain-of-function (i.e., oncogenic mutations), suggesting that hyperactivation of growth factor signaling is not compatible with gene transmission to the offspring in humans. Herein, we have addressed the detailed mechanisms of dynamic FGFR-mediated signaling in SSCs. We hypothesize that SSC cell fate specification depends in part on activation of the FGF/FGFR pathway in conjunction with tight negative feedback control to prevent excessive output of the pathway. To test this hypothesis, we studied adult SSCs in culture in varied growth factor conditions. We found that AS SSCs exhibited increased sensitivity to very low concentrations of FGF2, manifested by enhanced MAPK signaling, which is consistent with a gain-of-function of the mutated protein. Moreover, AS SSCs maintained cell proliferation in suboptimal GDNF conditions in the presence of reduced FGF2, suggesting that enhanced FGF signaling is sufficient to preserve stem cell activity when growth factors are scarce. Paradoxically, when we characterized canonical signaling pathways for adult SSC maintenance, we observed an FGF2 dose-dependent inhibition of self-renewal-associated pathway activation. We also profiled mRNA in SSCs at varying chronic doses of FGF2 and found increased expression for certain stem cell markers at lower doses. Moreover, we observed sustained activation of MAPK signaling only with lower FGF doses, a response consistent with the distinct transcriptional changes related to cell fate specification observed in other cellular systems. Additionally, we identified canonical and non-canonical signals that mediate negative feedback downstream of FGFRs in SSCs. These data suggest that FGF/FGFR signaling must be tightly regulated to preserve SSCs. Research supported in part by NIH 1DP2HD080352.

92. The Multiple Roles of Retinoic Acid in Male Germ Cell Development.
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Spermatogonial stem cells (SSCs) must balance self-renewal with production of transit-amplifying progenitors that differentiate in response to retinoic acid (RA) before entering meiosis. This self-renewal vs. differentiation spermatogonial fate decision is critical for maintaining tissue homeostasis, as imbalances cause spermatogenesis defects that can lead to human testicular cancer or infertility. A great deal of progress has been made towards understanding how the SSC population is maintained. In contrast, there is currently very little known about the program of differentiation initiated by retinoic acid (RA), and the pathways and proteins involved are poorly defined. A major impediment to this progress is that only a very small number of genes have been identified whose steady-state mRNA levels differ significantly during differentiation. The best-described role of RA in germ cells is to activate transcription of target genes such as Stra8, whose promoters contain RA response elements. We recently discovered an additional role for RA in stimulating the PI3/PDK1/AKT/mTOR kinase signaling pathway during spermatogonial differentiation. This led to translational activation of mRNAs such as Kit, Sohlh1, and Sohlh2 that encode essential regulators of differentiation and are present but repressed in undifferentiated spermatogonia. This suggests that activation of a pool of repressed mRNAs is a major driving force behind spermatogonial differentiation. Here, we examined the requirement for mTOR complex 1 (mTORC1) in mediating the RA signal to direct spermatogonial differentiation. We found that in vivo inhibition of mTORC1 by rapamycin blocked spermatogonial differentiation, which led to an accumulation of undifferentiated spermatogonia. In addition, rapamycin treatment also prevented the RA -induced translational activation of mRNAs encoding KIT, SOHLH1, and SOHLH2 without affecting expression of STRA8. These findings highlight dual roles for RA in germ cell development – transcriptional activation and kinase signaling to stimulate translation of repressed mRNAs.

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Despite the father transmitting half the heritable information to the embryo the focus on preconception health has largely been on the mother. New studies highlight the role of the father in disease transmission via non-genetic inheritance, through epigenetic mechanisms. Epigenetic mechanisms include, DNA methylation, post-translational modifications of histones and noncoding RNAs. Paternal effects have been linked to developmental abnormalities and complex diseases such as cancer, diabetes and obesity. Studies in humans and animals have linked epigenetic inheritance to the transmission of environmentally induced phenotypic traits from the father to
the developing embryo and these have been associated with altered gene expression and developmental abnormalities in first and second offspring generations. However when in germ cell development environmental signatures are established and by what mechanism remains unknown. To address this gap in knowledge we have been investigating the fundamental processes governed by epigenetic mechanisms in the supplier cells of spermatogenesis, the spermatogonial stem cells (SSC). Using transgenic mouse models in combination with cell enrichment methods we have been investigating the mechanisms involved in the establishment of the epigenome of spermatogonia and the consequences of disruption of this process.

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Proper maintenance and differentiation of spermatogonia are essential for male reproduction. To better understand this process, we have developed a novel flow cytometry (FACS)-based approach by combining two cell surface markers (α6-integrin that marks undifferentiated spermatogonia with stem cell capacity and c-Kit that marks differentiating spermatogonia) with a GFP reporter driven by the promoter of stimulated by retinoic acid gene 8 (Stra8). Transcriptional activation of Stra8 is perhaps the earliest event known to date during meiosis initiation in both sexes, so we used its promoter activity to monitor early spermatogonial differentiation. This strategy allows separation of undifferentiated and early differentiating spermatogonia at 6 consecutive stages. Specifically, within the integrin-α6-high c-Kit- negative undifferentiated spermatogonia compartment, we identified the GFP-negative population (P1) and the GFP-positive population (P2). We considered P1 to represent the most undifferentiated spermatogonia because it is negative for Stra8 promoter activity and P2 to represent the earliest differentiating spermatogonia, in that, while the Stra8 promoter is turned on, c-Kit expression still remains negative. Within the integrin-α6-low c-Kit-positive differentiating spermatogonia compartment, we identified 4 distinct populations (P3 – P6) based on their progressively decreasing levels of GFP expression with P3 exhibiting the highest GFP and P6 the lowest. We considered P3 – P6 as differentiating spermatogonia at consecutive stages towards maturation. Gene expression analysis coupled with immunocytochemistry staining of the isolated P1 – P6 cells further supported that these cells are undifferentiated spermatogonia (P1), early differentiating spermatogonia (P2 – P4), preleptone-miotic spermatocytes (P5) and meiotic spermatocytes (P6). Importantly, we found that transcription of p53, one of the most critical cell cycle regulators under both genotoxic stress and physiological conditions, is turned on during early spermatogonia differentiation between P2 and P3 stages. After we introduced the pStra8-GFP reporter allele into p53 knockout mice, dynamic changes in the frequencies of P1 – P6 populations showed that p53 deletion drove spermatogonia out of the undifferentiated state by causing a slight decrease in the P1 population and a consistent expansion of differentiating spermatogonia and preleptone spermatocytes from P2 to P5 populations. However, the meiotic spermatocyte population (P6) is remarkably decreased by 27.7%. These data are further confirmed by histological analysis, through which we found a 55.7% increase in Stra8-expressing stage VII seminiferous tubules, but a significant 40.2% decrease in tubules containing Symp3-positive spermatocytes undergoing meiotic recombination. Mechanistic study revealed that p53 loss augmented the mTORC1 activity, as shown by increased levels of phosphorylated-ribosomal protein S6 (p-PS6, a downstream target of mTORC1) expression. Taken together, our FACS analysis of spermatogonia differentiation represents a powerful tool to analyze progressive gene expression changes during this process and to elucidate their function. By using this approach, we characterized a critical role for p53 in spermatogonia maintenance. Such information may provide novel insight into germline stem cell biology and disease pathogenesis of infertility. (Supported by NIH R00-AG039512 and MGH Vincent Department of OB/GYN)

95. Cullin-Family Ubiquitin Ligase CUL4B is Required for Mouse Spermatogenesis.
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Ubiquitin ligase CUL4B is a member of the cullin-RING ubiquitin ligase family, the largest E3 ligase family which facilitates ubiquitination of specific substrates for protein degradation by the ubiquitin-proteasome system or modulation of protein activities. The CUL4 protein shares extensive sequence identity and structural similarity with the other CUL4 species, CUL4A. We have previously reported that the two Cull4 genes exhibit distinct expression pattern in the mouse testis, and revealed that genetic ablation of Cull4a in the mouse leads to male, but not female infertility, due to aberrant meiotic progression. In the present study, we generated Cull4b germ cell–specific conditional knockout as well as Cull4b-global knockout mice to investigate its role in spermatogenesis. Germ cell-specific deletion (Cull4bVasa) led to male infertility, despite normal testicular morphology. Mutant males produced comparable numbers of spermatozoa; however, sperm mobility was significantly impaired. Reduced mitochondrial activity and loss of mitochondrial membrane potential, as well as reduced levels of glycolysis were observed in the majority of the mutant spermatozoa, accompanied by low, if any, sperm ATP production. Furthermore, Cull4bVasa spermatozoa exhibited defective arrangement of axonemal microtubules and flagellar outer dense fibers. Mass spectrometry analysis identified a list of potential CUL4B substrates in male germ cells. Cull4b-global knockout (Cull4bSox2) males lost their germ cells in an age-dependent manner, in addition to a similar immotile sperm phenotype compared to the conditional mutants. These results indicate that CUL4B is indispensable for spermatogenesis. It functions cell-autonomously in male germ cells to ensure spermatozoa motility, and non-cell-autonomously in somatic cells to maintain spermatogonial stemness.

96. A defect in the mitotic/meiotic switch contributes to testicular germ cell tumorigenesis in the 129 inbred mouse strain.
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In the 129 inbred mouse model of human testicular germ cell tumors (TGCTs), tumors initiate between embryonic days (E)13.5 to 15.5, which coincides with the mitotic/meiotic switch: germ cells of both sexes lose pluripotent capacity, female germ cells (oogonia) commit to meiosis, and male germ cells (gonocytes) enter mitotic arrest. We previously discovered that genes expressed in pre-meiotic oogonia and adult male germ cells, including cyclin D1 (CenD1) and stimulated by retinoic acid 8 (Stra8), are prematurely expressed in
TGCT-susceptible gonocytes, and in rare instances induced gonocyte entry into meiosis. The inappropriate initiation of the meiotic program in TGCT-susceptible gonocytes suggests that a signal normally restricted to the developing ovary and adult testis, is aberrantly active at embryonic time-points in the TGCT-susceptible testis. Retinoic acid (RA) signaling precedes Ccnd1 and Stra8 expression in oogonia and induces the meiotic program, while RA is catabolized by CYP26B1 in the embryonic testis. Because TGCT-susceptible gonocytes express components of the meiotic program, we hypothesized that aberrant RA signaling in the TGCT-susceptible testis contributes to a breakdown of the mitotic:meiotic switch and TGCT initiation. Culturing testes from E14.5 TGCT-resistant embryos in medium containing RA induced Ccnd1 expression in gonocytes. Interestingly, gonocytes from RA-cultured testes maintained expression of pluripotency genes (e.g., Nanog), suggesting that gonocytes may be competent to remain pluripotent when exposed to RA. Additionally, evidence from TGCT-resistant embryonic testes heterozygous for the Cyp26b1m1bhr knockout allele suggests that RA induces Ccnd1 expression in gonocytes in vivo. Importantly, we also found by using the Ccnd1m1bhr knockout allele on the high TGCT risk 129-Chr19MOLF/Ei (M19) background that Ccnd1 deficiency significantly reduces TGCT incidence by 62% (p<0.001) compared to wild-type and heterozygous mice. Together, the above evidence suggests that RA signaling is capable of inducing Ccnd1, an important modifier of TGCT incidence. Interestingly, our results indicate that male differentiation signaling from somatic cells, namely Fgf9, is not differentially expressed between resistant and tumor-susceptible strains. However, expression of male-specific genes (e.g., Nodal, Nanos2) induced by FGF9 and inhibited by RA is decreased in TGCT-susceptible strains. The reduced expression of Nodal and Nanos2 in TGCT-susceptible strains suggests that normal FGF9 signaling and inappropriate RA signaling are competing in the embryonic TGCT-susceptible testis to determine germ cell fate. We propose three outcomes for TGCT-susceptible gonocytes determined by the balance of this interaction: (A) Most TGCT-susceptible gonocytes respond to FGF9 signaling and undergo male germ cell specification; (B) A rare subset of TGCT-susceptible gonocytes respond to RA and initiate meiosis; (C) A portion of TGCT-susceptible gonocytes respond to normal gonocyte developmental signals (FGF9) as well as abnormal RA. This process disrupts gonocyte entry into mitotic arrest and repression of pluripotency, leading to TGCT initiation.

FOCUS SESSION 10: How to Turn-On an Egg and Make an Embryo.

97. **Calcium Influx: A Window into Egg Activation.**
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Initiation of mammalian embryonic development depends upon a series of fertilization-induced rises in intracellular calcium. Complete egg activation requires influx of extracellular calcium; however, the channel(s) that mediate this influx remain unknown. In this talk, I will review evidence that calcium influx-mediated signaling is necessary for polar body emission but not cell cycle resumption in mouse eggs. I will then present both genetic and chemical inhibitor-based data documenting the requirement for specific calcium channel subtypes to support calcium influx during oocyte maturation and following fertilization.

98. **Egg Activation In Drosophila: Calcium Waves And Macromolecular Changes.**
Marina F. Wofliner⁰, Caroline V. Sartain⁰, Taro Kaneuchi⁰, Amber Krauchunas⁰, Zijing Zhang², Vanessa L. Horner², Norene A. Buehner², Satomi Takeo¹, Toshiro Aigaki¹

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Egg activation is the essential process by which a mature oocyte becomes capable of supporting embryo development. During egg activation, the oocyte’s nucleus is released from divisional arrest and completes meiosis, the oocyte’s transcriptome and proteome change to forms capable of supporting embryogenesis, and the membranes and envelopes around the oocyte are modified to prevent polyspermy and to protect the embryo. In many species, the fertilizing sperm induces activation by triggering calcium waves involving release of intracellular calcium stores in the oocyte. Arthropod eggs, in contrast, activate without fertilization. Insects like Drosophila thus offer the unique possibility to focus solely on maternally-specified molecules and events that characterize egg activation. Moreover, model systems like Drosophila permit genetic identification of conserved macromolecules that mediate egg activation. We therefore characterized the triggers, effects, and regulators of egg activation in flies. Our results show that Drosophila egg activation is triggered by ovulation. Specifically, mechanical forces that impact the egg during ovulation cause it to take up calcium from the external environment at the egg poles. This calcium uptake triggers a wave of increased calcium that progresses across the egg by release of calcium from intracellular stores through the action of IP3 receptors. These receptors as well as various calcium-regulated proteins play conserved roles essential for egg activation in Drosophila and other animals. We have therefore used genetic and biochemical screens in Drosophila to discover and characterize molecules that are candidates to mediate the egg-to-embryo transition in all animals including mammals.

99. **Regulator of G-protein Signaling 2 (RGS2) Suppresses Premature Calcium Release in Mouse Eggs.**
Miranda L. Bernhardt¹, Katie M. Lowther¹, Caitlin E. McDonough¹, Alexei V. Esvikov¹, Elizabeth Padilla-Banks¹, Tracy F. Uliasz², Carmen J. Williams¹, Lisa M. Mehlimann²

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During oocyte maturation, capacity and sensitivity of Ca²⁺ signaling machinery increases, preparing the metaphase II (MI)-arrested egg for fertilization. Upon sperm-egg fusion, Ca²⁺ release from IP3-sensitive endoplasmic reticulum stores results in a series of cytoplasmic Ca²⁺ oscillations that drive the events of egg activation and initiate early embryo development. Premature calcium release can cause parthenogenetic activation prior to fertilization; thus, preventing inappropriate Ca²⁺ signaling is critical to ensuring robust MI arrest. Here, we show that regulator of G-protein signaling 2 (RGS2) suppresses Ca²⁺ release in MI eggs exposed to G-protein-coupled
receptor ligands. Rgs2 mRNA was highly expressed in oocytes and recruited for translation during oocyte maturation, resulting in increased protein levels in MII eggs and early stage embryos. RGS2-depleted oocytes matured to MII; however, they had increased sensitivity to low pH and acetylcholine (ACh), which caused inappropriate Ca^{2+} release and premature egg activation. When matured in the absence of exogenous stimuli, RGS2-depleted eggs underwent spontaneous Ca^{2+} increases sufficient to promote cortical granule exocytosis. These findings suggest that RGS2 functions as a brake to suppress premature Ca^{2+} release in eggs that are poised on the brink of development.

100. Evidence Supporting an Intracellular Zinc Fluctuation Following Fertilization in the Mouse Zygote.

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Recent studies have shown that zinc regulates meiotic progression in the mammalian oocyte and upon fertilization undergoes periodic zinc exocytosis events termed “zinc sparks,” which involves the loss of 12-15 billion zinc atoms. By manipulating zinc availability in the oocyte using the zinc chelator (TPEN) and a zinc ionophore (Zn-pyridhione), we demonstrated that these zinc fluxes are required for proper progression from oocyte to egg to embryo. Furthermore, utilizing a recently synthesized zinc probe, ZincBY-1 (Que, et al., Nat. Chem. 2015), we discovered an intracellular “zinc wave” occurring immediately after the zinc spark at the time of egg activation. The zinc wave is observed to persist over a much longer time period than calcium oscillations and the zinc sparks. We utilized existing and newly invented cell-permeable, small molecules to manipulate and monitor zinc in the female gamete; however, they act both intracellularly and extracellularly and are not targetable to particular regions inside a cell. Thus, we do not know the origin of the observed “zinc wave” nor do we know if it is contained within vesicles or diffusing through the cytosol. In order to address the technical gap associated with this family of agents, we have designed a new generation of fluorescent zinc probes to measure the zinc status of the oocyte in a targeted manner. The goal of these studies is to test whether zinc fluxes that follow fertilization occur predominantly in the cytosol or if other compartments. Herein we will present our preliminary results in both understanding the properties of the zinc wave and the development of a new zinc sensor. Funding: This work was supported by the W. M. Keck Foundation and National Institutes of Health Grants P01HD021921, U54CA143869.

101. Protein kinase-mediated control of chromatin structure during the pronuclear stage.


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The PTK2b kinase becomes localized to the oocyte cortex and activated at the site of sperm-oocyte interaction where it plays an important role in sperm incorporation and remodeling of the cortical actin cytoskeleton. The purpose of this study was to determine whether PTK2b also played an important role in the later stages of the egg activation process. The approach used was to inhibit PTK2b activity during the pronuclear (PN) stage (after sperm incorporation was completed) and observe the effect on embryonic development. Two methods of PTK2b inhibition were employed. In one approach, zygotes obtained from mated females were incubated for 12hr with the PTK2b inhibitor PF4594755 (10, 25, and 50uM). A second approach was to express a dominant-negative construct encoding the N-terminal PERM domain in GV stage oocytes, allow maturation to proceed, and activate the mature oocytes by ICSI. Both treatments blocked (PN) congression of pronuclei remaining in close proximity and nuclear envelopes intact. The arrested pronuclei exhibited abnormally condensed chromatin indicating a possible role for PTK2b signaling in controlling chromatin structure at this stage of development. In an effort to identify what signaling pathways during pronuclear development were sensitive to PTK2b inhibition, we performed an unbiased kinase analysis of early and late PN stage oocytes in the presence and absence of the PTK2b inhibitor. Fertilized oocytes obtained from mated females (1700/group) were collected at 2-5hrs post fertilization (EPN sample), or were cultured for an additional 12 hrs (LPN sample) in the absence or presence of 25uM PF4597455. Kinome analysis was performed by Kinexus Bioinformatics Corp (Vancouver, BC) using a high density antibody array screening 850 protein kinases, phosphatases, or protein kinase targets. Samples were analyzed in duplicate and normalized against internal controls. Output was expressed as the Z-score ratio of Late PN stage / Early PN stage signal intensities. The results showed that 88 protein kinases/phosphatases were significantly (Z-score ratio >2.0) up or down-regulated during the progression from early to late PN stage. Suppression of PTK2b with 25uM PF4594755 caused significant disregulation of 29 kinases / phosphatases within this group. Ingenuity Pathway analysis revealed that 15 of the most highly impacted targets were involved in regulating DNA transcription directly or indirectly. ABL, HDAC4, MEF2, PAX25894 and Rb3608 exhibited the most significant responses to PF4597455 treatment. The potential role of these PTK2b targets in histone modification suggested that PTK2b suppression might lead to abnormal changes in chromatin structure during the PN stage. This was tested by analyzing the effect of PF4597455 on total oocyte acetyl-lysine content. Early PN stage oocytes were incubated with or without 25uM PF4597455 for 12hr, then solubilized and subjected to western blot analysis with an anti-acetyl-lysine antibody. The results demonstrated that inhibition of PTK2b activity caused a significant decline in acetyl-lysine content. The observation that PTK2b appears to regulate several proteins with the capacity to control histone modification raises the possibility that this kinase may play an important role in the development of a new zinc sensor. Funding: This work was supported by the W. M. Keck Foundation and National Institutes of Health Grants P01HD021921, U54CA143869.

102. New Roles for Old Players in Oocyte Maturation.

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Abstract not submitted.

103. Activation of Cell Volume Regulation in the Oocyte and Early Embryo.

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Early preimplantation embryos are prone to developmental arrest when subjected to stress. One stress that effectively causes blocked preimplantation development is increased osmolarity. Since animal cells regulate their volumes osmotically, dysfunction of cell volume-regulatory mechanisms was implicated, leading us to investigate the mechanisms of cell volume regulation in oocytes and preimplantation embryos. Early preimplantation mouse embryos use the same mechanism as somatic cells to quickly counter decreased cell volume. This involves the rapid activation of sodium-hydrogen exchangers (NHE) coupled to bicarbonate-chloride exchangers, which together mediate the uptake of NaCl and increase intracellular osmolarity. However, early embryos appear to need to replace a portion of the intracellular inorganic ions with “organic osmolytes” using mechanisms apparently unique to embryos and not used by somatic cells. Organic osmolytes are a broad range of benign, uncharged compounds used by many cell types to provide intracellular osmotic support. The two main organic osmolytes used by early preimplantation mouse embryos are glycine and betaine (N,N,N-trimethylglycine). Both glycine and betaine are initially accumulated in mouse oocytes during meiotic maturation. The mechanisms of accumulation differ, however, with glycine transported into the oocyte by the specific transporter, GLYT1 while betaine appears to be synthesized via the enzyme choline dehydrogenase. When maximal levels of organic osmolytes have been achieved, their levels are maintained and adjusted in response to perturbations of cell size by specific transporters – GLYT1 for glycine and SIT1 for betaine. All these processes are developmentally regulated and coordinated during oocyte development and early preimplantation embryo development, and are needed for healthy embryo development.

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104. Evidence That ORC4 Plays a Functional Role in Polar Body Extrusion.

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INTRODUCTION: During meiosis, two divisions occur that reduce the chromosome number to half. In mammalian female meiosis, this is accompanied by two asymmetric divisions so that most of the cytoplasm remains in the oocyte that will eventually be fertilized. In both divisions, one set of chromosomes is extruded in much smaller cells called polar bodies. In assisted reproductive technology, polar bodies provide critical information to detect chromosomal and genetic abnormalities of the offspring. Several laboratories have characterized the cytoskeletal proteins that cause the mitotic plate to migrate to the oocyte cortex in the first meiotic division, and then cause the asymmetric division in which the discarded chromosomes are extruded in the much small polar bodies. We have recently reported that DNA replication licensing protein, the origin recognition complex subunit 4 (ORC4), preferentially surrounds the chromatin that will be extruded during anaphase in both meiotic divisions but not the chromatin that remains in the oocyte (Nguyen et al., 2015). Here, we test two functional aspects ORC4's involvement of polar body extrusion. METHODS AND RESULTS: Treatment of brefeldin a (BFA), an inhibitor of Golgi-based membrane fusion, causes mouse oocytes that are activated to divide equally into two cells, rather than asymmetrically into a larger oocyte and a smaller polar body. We treated germline vesicle (GV) and metaphase II (MII) oocytes, supplemented with 10 mM SrCl2 for 6 hours, with 10μM BFA for 16 hours. We tested whether either set of chromosomes was still encapsulated with ORC4. We found that when oocytes were activated in the presence of BFA, the twelve out off twenty of oocytes divided symmetrically and neither set of chromosomes were encapsulated by ORC4. We next tested whether ectopic sperm polar bodies could be induced to generate ORC4 capsules around the sperm chromatin. During normal mammalian fertilization, the decondensing sperm cell forms an “abortive polar body” which is then reabsorbed into oocyte. When sperm are treated first with ionic detergents to prevent activation, and then injected into oocyte, the sperm chromatin is then extruded as an ectopic polar body. In our previously published observations, we demonstrated that sperm DNA is not normal encapsulated by ORC4. In this work we found that when sperm induced to be extruded as ectopic polar bodies, an ORC4 capsule surrounds the sperm chromatin, just as is the oocyte polar body chromatin. DISCUSSION: These data suggest demonstrate that the ORC4 shell is only associated with chromosomes that are ejected from the oocyte in a polar body. This suggests that ORC4 may play functional role in polar body extrusion in addition to its role DNA origin licensing. This is the first chromatin-associated protein that has been asymmetrically associated polar body extruded chromatin. This work was supported by NIH Grant no. HD060722.

105. Evidence support a potential role for the AKT signaling pathway in mediating embryotrophic actions of follistatin on bovine early embryonic development.

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Poor oocyte quality limits in vitro embryo production efficiency in cattle and women. Our previous studies demonstrated an essential role for oocyte-derived follistatin in bovine early embryonic development and stimulatory actions of exogenous follistatin treatment on early cleavage, embryonic development to 8- to 16-cell and blastocyst stages and blastocyst cell lineage allocation to trophectoderm. Follistatin is a transforming growth factor β (TGFβ) superfamily binding protein and may exert its embryotrophic effects through modulation of one or more of the SMAD (SMAD2/3, SMAD1/5) or non-SMAD (e.g. AKT, JNK, ERK and P38) signaling pathways regulated by TGFβ superfamily ligands. AKT is a second messenger regulated kinase essential for oocyte maturation and early embryonic development in the mouse, but its regulatory role and link to follistatin action in early bovine embryos are not known. The objectives of the present studies were 1) to investigate the effects of treatment with the AKT inhibitor SH6 on development of bovine early embryos and AKT phosphorylation, 2) to determine the effect of follistatin supplementation on AKT phosphorylation and 3) to determine whether follistatin treatment can rescue development of AKT inhibitor treated embryos. To elucidate the effect of AKT inhibition of indices of development progression in early bovine embryos, presumptive zygotes derived from in vitro fertilization were cultured in the presence of 0, 25, 50 or 75 μM SH6 for 72 h, then washed and cultured in fresh culture media lacking SH6 until d 7 (n = 4 replicates; 25-30 embryos/treatment). Treatment with 25, 50 and 75 μM SH6 totally blocked early cleavage at 30 hour post insemination (hpi). No
significant effects on total cleavage (48 hpi) and development to the 8-16 cell (72 hpi) and blastocyst stages (d 7) were observed in response to both 25 and 50 μM doses compared to diluent treated controls. However, total cleavage rates and percentage of embryos reaching the 8- to 16-cell and blastocyst stages were reduced in response to 75 μM SH6 treatment. Western blot analysis was performed to confirm inhibitory effect of SH6 treatment on abundance of phosphorylated form of AKT (pAKT) in early bovine embryos. Treatment of bovine zygotes (n = 20/treatment) with 50 and 75 mM SH6 for 10 h caused a 50% reduction in pAKT levels relative to controls. To determine whether follistatin treatment can rescue inhibitory effects of AKT inhibition, presumptive zygotes (n = 25-30 zygotes/treatment; n = 4 replicates) were cultured in the presence or absence of 75 mM SH6 and increasing concentrations of follistatin (0, 1, 10 and 100 ng/ml). Treatment with 10 ng/ml follistatin reversed the inhibitory effects of SH6 treatment on early cleavage, total cleavage and development to the 8- to 16-cell stage and partially rescued development to the blastocyst stage. Western blot analysis was performed to determine effects of 24 h follistatin treatment (0 versus 10 ng/ml; n = 20 zygotes/treatment; n = 5 replicates) on AKT phosphorylation in early bovine embryos. Follistatin treatment caused a significant increase in pAKT levels in bovine embryos. Collectively, results suggest a potential requirement of AKT for bovine early embryonic development, and suggest that trophic actions of follistatin on bovine early embryos may be mediated, at least in part, by modulation of AKT signaling. Supported by NIH grant HD072972 (GWS, JK) and Egyptian government’s ARE grant JS2687 (MA).

106. Regulation of nuclear size in mouse embryos.
Elina Tsichlaki1, Greg FitzHarris2.

Regulation of nuclear size is important for maintaining cellular health, and changes in the size of the nucleus can be associated with cellular pathology. However, the mechanisms that dictate nucleus size are largely mysterious. Here we set out to understand the relationship between nuclear size and cell size in the mouse embryo, with a view to understanding the underpinnings of a healthy early embryo. First, to begin to probe the mechanisms by which nucleus size is regulated in embryos, we generated three-dimensional confocal images of preimplantation mouse embryos to determine Nuclear/Cytoplasmic (N/C) volume ratio. As expected, nuclear size decreases with successive cleavage divisions from 1-cell stage to blastocyst. N/C ratio was relatively constant at any given developmental stage, alluding to a direct relationship between nuclear and cell size. Experimental cytoplasmic removal significantly reduced nuclear size (P<0.01), further suggesting that cell size directly influences nuclear volume. However, the N/C ratio increases progressively through development (from 0.057 in 2-cells to 1.728 in blastocysts), revealing that factors other than cell volume also influence nuclear size. Consistent with this, nuclei in experimentally-generated embryos with ‘double-sized’ blastomeres were of normal size, further indicating that nuclear size is controlled by a developmental programme. We therefore next set out to determine what might comprise this developmental program. Changes in nuclear import rates have previously been suggested to play such a role in other systems. However, using Fluorescent Recovery After Photobleaching (FRAP) of GFP-tagged nuclear localization signal (GFP-NLS) we found that import rates were identical throughout preimplantation development. Alternatively, in some cell types the composition of the nuclear lamina can influence nucleus size. Intriguingly, we find that whereas the nuclear lamina is rich in Lamin A in early embryos (~2-8 cells) nuclear Lamin B1 is more abundant in the lamina of later stage embryos. Moreover, experimentally eliminating lamin B1 expression using RNAi arrests embryo development. Together our experiments suggest that nucleus size regulation in the early embryo comprises cell-size-dependent and developmentally-regulated factors, and provide indirect evidence that differential expression of nuclear lamina components might contribute to nucleus size establishment.

FOCUS SESSION 11: Pregnancy: Signaling, Placentation, and Parturition.

107. Can We Make the Pig Placenta Work Better?
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The number of piglets born alive at each parity contributes to the efficiency of swine production. Moreover, piglet birth weights affect both survival to weaning and future growth rate. Litter size and birth weight are influenced by placental function. The pig placenta is classified as diffuse epithelial-chorial because no erosion of fetal or maternal tissue occurs. Because of the superficial placenta, there is a tendency to think about the pig placenta as only that portion that originates from the fetus. However, pig placental function originates from components of both maternal (i.e., the uterus) and fetal (i.e., the “placenta”) origin. Uterine space is a primary determinant of placental function, which has been demonstrated by the relationship between conceptus survival and uterine length per conceptus. Although this relationship has been known for decades and uterine length is highly variable in pigs, little is known regarding the physiological mechanisms that control this trait. We performed a genomic analysis for uterine length in ~1,000 post-pubertal gilts from a commercial maternal line as part of a National Pork Board-funded study. Preliminary genomic analysis of these data revealed at least 5 quantitative trait loci for uterine length (ranges of effects of 30 to 67 cm in length). These loci may be exploited to increase uterine length, potentially improving placental size and function if the number of embryos is held constant. Further improvements in placental function require an understanding of how the placenta works. It is helpful to divide placental function into structural factors that contribute to nutrient transport generally and nutrient-specific factors that promote transport of individual nutrients or classes of nutrients. Structural factors include the microscopic architecture of the maternal-fetal interface in relationship to maternal and fetal blood flow. Previous results indicated that placental microarchitecture is altered in the placenta of small fetuses in ways that are consistent with improvement of nutrient transport but that these beneficial modifications may be limited by placental stromal tissue. From these results, we proposed the concept of
feeding the placental stroma as a way to improve placental function. A primary component of placental stroma is hyaluronic, which is made up of glucuronic acid and glucosamine. Supplementation of diets of pregnant gilts with glucosamine in late gestation tended (P = 0.09) to improve litter size and resulted (P = 0.05) in beneficial changes in placental microarchitecture, suggesting that our concept may be correct. We have also performed a transcriptomic analysis of fetal placenta trophoblast cells using RNA-seq. This resulted in a comprehensive list of nutrient transporter genes along with their expression levels. The two most expressed sugar transporters (SLC2A genes) were SLC2A12 and 13, corresponding to GLUT12 and proton-coupled myoinositol transporter (HMIT). Immunohistochemical analysis for HMIT confirmed its presence within the maternal-fetal interface. This result suggests active transport of inositol by the pig placenta for reasons that are currently unclear. Nevertheless, inositol represents a potential further avenue to explore that could improve placental function and is an example of many possible interventions that might come from an improved understanding of specific nutrient transport mechanisms.

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Infertility and subfertility are issues relevant to humans, domesticated animals and endangered species. Pregnancy in ruminants is established in an environment of transient secretion of a unique, type I interferon, interferon tau (IFNT). IFNT rescues the corpus luteum (CL) through local actions on the endometrium and also initiates a cascade of expression of interferon-stimulated genes across the entire uterine wall and extending to circulating immune cells and the CL. Our working hypothesis is that pregnancy and IFNT induce changes in immune cells that promote tolerance, tissue remodeling and angiogenesis. Twenty dairy heifers were randomly allocated to Day 17 cyclic (n=5-9), Day 17 pregnant (n=5-7) and Day 20 pregnant (n=4-5) treatment groups and uteri, lymph node, spleen and blood were collected at slaughter. Flow cytometry, immunofluorescence (IF) labeling and qPCR were used to determine effects of pregnancy on endometrial immune cells expressing markers for natural killer (NK) and cytotoxic (CD8+) T cells and for macrophages/dendritic cells. In addition, mRNA abundance of pro-inflammatory (Th1) and anti-inflammatory (Th2) cytokines and other regulatory molecules was determined in the endometrium. Early pregnancy was accompanied by a marked increase (P<0.05) in the proportion of endometrial CD45+ cells expressing markers for natural killer (NK) cells and cytotoxic T cells (CD8) and an increase (P<0.05) in MHCII+ cells, particularly around shallow glands of the endometrium of pregnant heifers on Day 20. Quantitative PCR analysis showed increased abundance of mRNA for interleukin (IL)-15, an NK cell growth factor, and IL-10, a tolerogenic cytokine, in the endometrium during early pregnancy. Furthermore, expression of indoleamine 2,3 dioxygenase (IDO) was ~15 fold greater in pregnant compared to cyclic heifers at Day 17, but then IDO mRNA and protein abundance declined by Day 20 of pregnancy to amounts similar to Day 17 cyclic heifers. IDO converts tryptophan to kynurenine, which is thought to alter immune function by activation of the aryl hydrocarbon receptor (AHR). Endometrial expression of the AHR was detected, but there were no effects of day or pregnancy status. Pregnancy induced expression of CD172a, a marker for immature dendritic cells, and for inhibitory proteins PDL1 (Status * Day; P<0.05), LAG3 (Status; P=.06) and CTLA4 (Status; P<0.05). It is interesting that expression of IDO, IL10 and several other immune cell regulators decreased between Day 17 and 20 of pregnancy, suggesting a strong induction and then repression of selected responses. Overall, a picture is emerging that early pregnancy signaling at the fetal-maternal interface involves immune cell activation as well as induction of key regulatory molecules known to mediate tolerance. We show here a pattern of induction and then repression of key mediators of immune function, which we postulate serves as a developmental switch to promote an immune privileged niche and tissue remodeling in the endometrium during early pregnancy. These results should help better define the mechanisms that promote establishment of pregnancy and formation of the placenta in ruminants.

109. Mechanotransduction Drives Morphogenesis to Develop Folding at the Uterine-Placental Interface of Pigs.
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Pigs develop an epitheliochorial placenta in which two intact epithelial layers are present at the uterine- placental interface; the uterine luminal epithelium (LE) and the placental chorionic epithelium (CE). To optimize nutrient transport across this significant tissue barrier, complex folds develop at the uterine- placental interface. An emerging idea is that tissues respond to mechanical forces that coordinate morphogenesis. The porcine uterine endometrium is exposed to increasing mechanical forces from growing fetal membranes and fluids as well as increasing blood flow. Therefore, we hypothesized that these increasing mechanical forces coordinate development of uterine/placental folds by driving morphological changes at the uterine-placental interface. In Study 1, we examined: 1) changes in the length of uterine-placental folds on Days 20, 25, 35, 40, 50 and 60 of pregnancy; and 2) expression of mechanotransduction-implicated molecules in uterine-placental tissues from Days 24, 35, 40 and 60 of pregnancy including molecules responsible for focal adhesion (FA) assembly, cytoskeletal dynamics, and connective tissue remodeling. In Study 2, we measured changes in the size of subepithelial blood vessels within the endometrium of uterine-placental tissue from Days 11, 13, 17, 24 and 35 of pregnancy. In Study 3, we supplemented the diet of pregnant pigs from Days 14 to 25 with 0 or 0.4% arginine (an amino acid that increases blood flow) per day, hysterectomized pigs on Day 60 of pregnancy, and examined effects on length of uterine-placental folds. We observed that: 1) the length of uterine-placental folds increased between Days 25 and 35, remained constant from Day 35 through 50, then increased significantly between Days 50 and 60; 2) osteopontin (OPN), talin, and FA kinase (FAK) were detected at the uterine-placental interface and co-localized into aggregates; 3) actin-binding proteins including filamin, actin related protein 2 (ARP2), and F-actin were enriched at the top of the uterine-placental folds from Days 35 through 60 of pregnancy; 4) vimentin-expressing fibroblasts within the endometrial stroma acquired alpha smooth muscle actin (α-SMA) expression by Day 35 of pregnancy; 5) the size of the blood vessels beneath the uterine LE increased on Days 24 and 35 of pregnancy; and 6) supplementation with arginine increased uterine-placental fold length. These results indicate that mechanical forces from
110. Uterine ALK5 is Crucial for Placental Development and Pregnancy Maintenance.
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The transforming growth factor β (TGFβ) is the largest superfamily of secreted growth factors in mammals. Although previous studies have demonstrated members of the growth factor family are critical regulators in most developmental and physiological processes, the in vivo roles of the ligands and receptors in female reproduction still remain uncertain. Activin receptor-like kinase 5 (ALK5) is the major type 1 receptor for TGFβ ligands. Loss of ALK5 leads to early embryonic lethality because of the severe defects in vascular development. In this study, we conditionally ablated uterine ALK5 using progesterone receptor-cre (PR-Cre) mice to define the physiological roles of ALK5 in female reproduction. Despite normal ovarian functions, embryo implantation and response to artificial decidualization in the cKO female mice, deletion of uterine ALK5 resulted in dramatically reduced female reproduction due to some striking abnormalities observed in placenta, including disorganization of the trophoblast cells, deletion of the uterine natural killer (uNK) cells, and defective remodeling of the spiral arteries. Microarray analysis demonstrated that genes involved in cytokine-cytokine receptor interaction and NK cell mediated cytotoxicity were downregulated in the cKO decidua. Results from flow cytometry confirmed the deletion of uNK in the cKO decidua, but other immune cells showed no change. According to these data, we hypothesize that TGFβ acts on decidual cells via ALK5 to induce expression of many cytokines, which are key regulators in uNK maturation and trophoblast development during early placenta. Our findings do not only generate a mouse model to study the TGFβ signaling in female reproduction, but also provides significant insights into the pathogenesis of many pregnancy complications in human, including spontaneous abortion, preeclampsia, intrauterine growth restriction (IUGR), and preterm birth. These studies were supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development grant R01-HD33438.

111. Blocking Leukemia Inhibitory Factor impairs trophoblast invasion and leads to abnormal placentation and pregnancy loss in mice.
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Introduction: The placenta forms the interface between the maternal and fetal circulation and is critical for healthy pregnancy. Placenta involves extravillous trophoblast (EVT) invasion to remodel maternal uterine arteries and establish utero-placental blood flow. Abnormal placentation is as a major cause of pregnancy pathologies including pregnancy loss, among others. Leukemia inhibitory factor (LIF) is produced by the placenta and EVT in women and promotes primary human first trimester EVT adhesion in vitro, suggesting a role in placenta. However, this has not been investigated in vivo in mice, as LIF null female mice are infertile due to implantation failure. We hypothesized that LIF alters EVT invasion and is required for normal placentation in mice and women. We aimed to determine the effect of LIF/LIFR inhibition on placenta and pregnancy outcome in mice and on primary human first trimester EVT invasion. Methods: LIF and LIF-receptor(R)-α were localized in mouse implantation sites at mid-gestation (E10-13) and co-localized with cytokertatin to detect EVTs. Mice were injected intraperitoneally with our unique PEGylated LIF antagonist (PEGLIA) or PEG daily (n=4/gp; 500µg/injection) at E8-10, E10-13 or E10-17 of pregnancy and sacrificed at the end of treatment. Placental morphology was assessed by immunohistochemistry and gene targets by PCR arrays. Primary human first trimester placental villous explants cultured on collagen drops were treated with LIF (100ng/ml) ± PEGLIA (100ng/ml) or control for 48h. The area of outgrowth (mm2) was quantified (n=6/gp). Results & Discussion: At mid-gestation in mice, LIF/LIFRα localized to invasive EVTs, decidual cells, placental trophoblast and fetal endothelial cells in the placental labyrinth. Temporally blocking LIF action during placentation resulted in reduced trophoblast invasion (cytokeratin) and impaired spiral artery remodeling (α-SMA). PEGLIA dramatically altered the labyrinth (site of fetal-maternal exchange) with a 35% reduction in maternal blood sinusoid area (isolecitin-B, p<0.01) and 38% reduction in fetal vessel area (CD31, p<0.01) compared to PEG control. PEGLIA reduced activated STAT3 but not ERK in the placenta and altered gene targets regulating angiogenesis and oxidative stress at E13. Cleaved caspase-3 staining highlighted increased trophoblast apoptosis at all time points in PEGLA treated mice. At E17, pregnancy viability was significantly compromised following PEGLA treatment from E10-17 (PEG 9.0 implantation sites ± 0.4 vs. PEGLA 4.2 ± 0.95, p<0.05). In humans, LIF promoted placental outgrowth (144 ± 28%, p<0.01) compared to control (100%). PEGLA treatment alone reduced outgrowth compared to LIF (55 ± 15%, p<0.05) and totally blocked LIF-mediated outgrowth (p<0.01). Conclusion: This is the first study to demonstrate that LIF is critical for trophoblast function and placenta formation in vivo. Blocking LIF with our unique inhibitor resulted in impaired trophoblast invasion in vitro and in vivo and lead to compromised placentation development in mice. LIF inhibition during placentation resulted in significant pregnancy loss, demonstrating that LIF is required for placenta and pregnancy viability. Research supported by the National Health and Medical Research Council of Australia.

112. Choriodecidual Signaling Network During Human Labor.
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The pathway of human parturition is a complex process that appears as a sequence of events initiated by myometrium contractions, then the cervix ripens, the fetal membranes rupture and the fetus and placenta are expelled. The mechanisms underlying the
onset and progression of normal spontaneous labor remain unclear. Increasing evidence shows that some components of the inflammatory pathway are involved in normal term labor and in preterm labor. Efforts to characterize the mechanisms of activation of this process have signaled the choriodecidual microenvironment during late gestation, a key player in the coordination of reproductive tissues for labor. The choriodecidualia is strategically located, as it represents an area of direct contact between maternal (decidua) and fetal tissues (chorion). Several groups have demonstrated phenotypic and functional modifications in the cellular components in the maternal/fetal interphase, that include the active secretion of cytokines and chemokines by local choriodecidual cells, which results in the recruitment and activation of highly specific leukocytes subpopulations. Leukocyte recruitment at choriodecidual and other reproductive tissues has been proposed as the first phase in preparation for labor. We identified the secretion of specific chemokines in association to time of human labor and the resulting attraction of cells populations to the choriodecidual space. Main chemokines include IP-10, MIP-1a, MIP-1b and IL-8. On the other hand, attracted cells include among others, two T-cell subtypes: "memory-like CD4+ T cells" and "memory-like double-negative T cells". Arrival of this adaptive immune system cells opens the participation of the adaptive immune system in the control of labor induction. Once peripheral leukocytes extravasates to the choriodecidualia, they conditionate a very specific microenvironment composed by a signaling network that acts as a two-waves cascade. Primary components include paracrine and autocrine signals, mainly pro-inflammatory cytokines such as IL-1 and TNF-α, eliciting the local secretion of secondary mediators such as prostaglandins that act as uterotonins and matrix metalloproteinases (MMPs) such as 92 kDa type IV collagenase (MMP-9) which in turn is able to degrade the extracellular matrix (ECM) degradation in these tissues associated to increased mechanical forces generated by the contracting uterus. We have found that in addition to proinflammatory signals, T-lymphocytes residing in the choriodecidualia during labor, contribute with massive enzyme of MMP-2 and MMP-9, two key enzymes required for extracellular matrix degradation. Secretion and activation of these enzymes in synchronization with other events of labor is dependent of direct interaction between arriving leukocytes and local cells.

113. **Endogenous Retroviruses Rewire the Placenta.**
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The mammalian placenta is remarkably distinct between species, suggesting a history of rapid evolutionary diversification1. To gain insight into the molecular drivers of placental evolution, we compared biochemically predicted enhancers in mouse and rat trophoblast stem cells (TSCs) and found that species-specific enhancers are highly enriched for endogenous retroviruses (ERVs) on a genome-wide level. One of these ERV families, RLTR13D5, contributes hundreds of mouse-specific histone H3 lysine 4 monomethylated (H3K4me1)- and histone H3 lysine 27 acetylation (H3K27ac)-defined enhancers that functionally bind Cdx2, Eomes and Elf5—core factors that define the TSC regulatory network. Furthermore, we show that RLTR13D5 is capable of driving gene expression in rat placental cells. Analysis in other tissues shows that species-specific ERV enhancer activity is generally restricted to hypomethylated tissues, suggesting that tissues permissive for ERV activity gain access to an otherwise silenced source of regulatory variation. Overall, our results implicate ERV enhancer co-option as a mechanism underlying the extensive evolutionary diversification of placental development.

114. **Differential regulation of myometrial contractile activity as a function of FSH receptor density.**
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Previous studies have reported an association of SNPs in intronic regions of the human FSHR gene with preterm birth, findings that are inconsistent with ovarian FSHR functions. However, recently we reported the novel expression of FSHR protein in extra-gonadal reproductive tissues including myometrium. We further reported FSH mRNA expression in myometrium and an upregulation of FSH receptor in human term myometrium relative to non-pregnant, suggesting a role for local FSH/FSHR signaling. In light of previous findings that granulosa cells with low vs high numbers of recombiant FSHR respond to FSH with increased cAMP vs cAMP as well as 1,4,5-triphosphinositol (IP3), we hypothesized that the balance between FSH/FSHR-stimulated cAMP-mediated quieting vs IP3-mediated activation may determine myometrial contractile activity. To examine signaling in myometrial cells as a function of FSHR density, we transduced hTERT-HM cells, an immortalized line of human non-pregnant myometrial cells that do not express FSHR, with increasing MOIs of adenovirus-FSHR. cAMP and IP3 production stimulated by a maximal concentration of FSH were measured as a function of cell surface FSHR density. As predicted, the IP3 response to FSH required greater densities of FSHR than the cAMP response. Mouse and human myometrial tissues were then used to determine relative FSHR protein expression and contractile responses to FSH during different stages of pregnancy. In non-pregnant human myometrium with low FSHR, FSH quieted contractile activity. Notably, in human term myometrium with ~10-fold higher FSHR, FSH stimulated contractile activity. In mice, myometrial FSHR density was low in non-pregnant mice, increased gradually on days 7, 11, and 16 and markedly (~7-fold) on day 19. In the non-pregnant or early pregnant myometrium, FSHR receptor densities were relatively low and a quieting of myometrial contractions was observed (50% quieting in non-pregnant myometrium to 20% at day 11). As pregnancy advanced to term, there was an increase in FSHR density with a concomitant FSH-stimulated excitation of myometrial contractions (20% excitation at day 16 to 40% at day 19). Taken altogether, our data provide a potential basis for the reported association between certain intronic SNPs of the human FSHR gene and preterm birth. Although not yet determined, the intronic locations of these FSHR SNPs suggest that they would more likely alter FSHR expression, not function. Consistent with this, our study demonstrates that uterine contractile activity is dependent upon the relative expression levels of myometrial FSHR. In the future, these data could provide insights into the mechanism underlying preterm birth as well as potential risk identification and/or novel therapies.
115. A Hypoxia/HIF/Kdm3a Pathway Controls Trophoblast Stem Cell Lineage Decisions and Organization of the Hemochorial Placenta.

Damayanti Chakraborty1, Wei Cui2, Regan Scott1, Pramod Dhakal1, Stephen Renaud1, Gracy Rosario3, Jay Vivian4, Makoto Tachibana5, Mohammad K. Rumi4, Michael Soares6.

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The hemochorial placenta develops from the coordinated multi-lineage differentiation of trophoblast cells. Specific trophoblast cell lineages are organized within the placentation site to perform specialized functions. The invasive trophoblast lineage remodels uterine spiral arteries to convert them to flaccid low resistance vessels, facilitating the flow of nutrients to the placenta and fetus. Failure of trophoblast invasion and vascular remodeling is associated with pathological conditions such as preeclampsia, intrauterine growth restriction, and preterm birth. Oxygen delivery represents an environmental stimulus with an instructive role influencing trophoblast cell differentiation and organization of the hemochorial placenta. In this study, we perform a series of in vitro and in vivo experiments delineating a hypoxia-activated regulatory pathway controlling hemochorial placentation. Key downstream events are delineated using rat trophoblast stem (TS) cells and tested in vivo using trophoblast-specific lentiviral gene delivery and genome editing. Initially, DNA microarray analyses were performed on rat TS cells exposed to ambient or low oxygen (0.5%). Upregulation of genes characteristic of an invasive/vascular remodeling/inflammatory phenotype and a marked downregulation of stem state-associated genes were observed. Among the upregulated genes were a histone H3K9 demethylase (Kdm3a) and a matrix metalloelastase (Mmp12). Upregulation of these transcripts was dependent upon the transcription factor, hypoxia inducible factor (HIF). We hypothesized that Kdm3a was a mediator of hypoxia-directed trophoblast cell lineage differentiation and that Mmp12 was a key downstream target responsible for the trophoblast cell invasive and vascular remodeling phenotype. Consistent with the hypothesis, knockdown of Kdm3a in rat TS cells inhibited the expression of a subset of the hypoxia/HIF-dependent transcripts, including Mmp12, and altered locus specific as well as global H3K9 methylation status. Consistently ectopic expression of Kdm3a upregulated a subset of hypoxia/HIF-dependent transcripts, including Mmp12, and this upregulation was dependent on Kdm3a enzymatic activity. Furthermore, Kdm3a knockdown decreased hypoxia-induced trophoblast cell invasion in both in vitro and in vivo experiments. Mmp12 possesses the capacity to degrade elastin and modify the structure of arterial blood vessels. To further explore the functional importance of Mmp12 in trophoblast cell-directed uterine spiral arterial remodeling, we generated an Mmp12 mutant rat model using TALEN-mediated genome editing. A rat model was established with a 609 bp deletion targeting exon 2 of Mmp12. Homozygous mutant rats showed reduced hypoxia-dependent endovascular trophoblast invasion and impaired trophoblast-directed uterine spiral arterial remodeling. In summary, we have discovered a hypoxia/HIF/Kdm3a pathway modulating trophoblast cell lineage development, leading to acquisition of the invasive trophoblast cell phenotype, including upregulation of the extracellular matrix-modifying enzyme Mmp12 and subsequent uterine spiral arterial remodeling.

116. Endometrial gene expression in lactating and dry Holstein cows and Holstein heifers on Day 19 of pregnancy.

Stefan Bauersachs1, Niamh Forde2, Jochen Bick1, Stefan Krebs1, Helmut Blum3, Eckhard Wolf3, Patrick Lonergan2.

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The compromised fertility of high producing dairy cows may be caused by negative energy balance. However, the point at which this metabolic state impacts on impaired fertility is not known. We have previously shown that different types of embryos elicit different transcriptomic responses from the endometrium. The aim of this study was to test the hypothesis that lactation alters the ability of the endometrium to respond appropriately to the developing conceptus. Endometrial gene expression on Day 19 of pregnancy in a model of metabolic stress was analyzed using RNA sequencing. Immediately after calving, primiparous Holstein cows with similar production and fertility estimated breeding values (EBVs) were randomly divided into two groups, standard lactation (“Lact”) or dried off immediately (“Dry”, i.e., never milked). Pregnancy was established by transferring grade 1 embryos recovered from superovulated Holstein heifers (“Heif”) with similar EBVs for production and fertility into lactating and nonlactating recipients (n=1 per recipient). A control group of Holstein heifers (“Heif”), with similar EBVs for production and fertility as groups 1 and 2, was artificially inseminated. Endometrial tissue samples were collected after slaughter and recovery of a conceptus on Day 19 of pregnancy. Total RNA was isolated from intercaruncular endometrium. Strand-specific RNA-Seq libraries (Heif n=4, Lact n=5, Dry n=8) were produced using the Encore Complete RNA-Seq DR Multiplex System (NuGEN, San Carlos, CA) and run on an Illumina HiSeq 1500. Obtained 100 bp single-end reads (28-65 Mio reads per sample) were processed using Trimomatic (version 0.32.1) and mapped to the bovine genome assembly Btau_4.6.1 (bosTau7) with TopHat2. Mapped reads were counted per gene by the use of QuasR qCount. Statistical analysis with DESeq2 revealed 17 differentially expressed genes (DEG) between Dry and Heif (FDR 5%) and 135 DEG (FDR 5%) between Lact and Heif. The comparison of Lact and Dry did not reveal differences at a Dry threshold of 5%. Overall, gene expression differences were rather small and lower than two-fold. Multi-dimensional scaling plots and principal component analysis confirmed the more pronounced differences in gene expression between lactating cows and heifers and showed that gene expression in the endometrium of dry cows is intermediate compared to the Lact and Heif groups. Furthermore, higher variation of gene expression in the Dry group was observed for the genes differential between Heif and Lact. Functional annotation of the DEG between Lact and Heif revealed, e.g., endosome, cytoplasmic vesicle, endocytosis, regulation of exocytosis, and cytokine receptor activity as overrepresented. The functional categories related to vesicle-mediated transport were specifically enriched for genes with higher expression in lactating cows and defense response for genes with higher expression in heifers. In conclusion, our data suggest that parity (cow vs. heifer) and metabolic condition (negative vs. normal energy balance) modulate the ability of the endometrium to respond to a high-quality embryo on the level of the transcriptome. This research was supported by the European Union (7th Framework Program, KBBE.2012.1.3-04: “Optimised terrestrial farm animal reproduction systems and/or technologies”, project FECUND).
FOCUS SESSION 12: Sex Determination from Fish to Mammals.

117. A Picture of Sex Determination, Viewed from Studies on Teleost Fish, Medaka.
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In teleost fish, medaka, the expression of Y chromosome gene, DMY/dmrt1bY, in somatic cells determines the sex. We have revealed that the germ cell regulation is also critical for sex determination in the pathway downstream of sex determination gene and dysregulation of germ cell number causes sex-reversal against the direction of sex determination gene. Recently we have proved presence of the intrinsic mechanism of sex determination in germ cells through identifying the critical factor. Disruption of the factor causes change of the sex of germ cells and, as the result, development of numerous functional sperm in the ovary. This indicates that downregulation of this factor in the germ cells is critical for initiation of spermatogenesis. We will discuss the regulation of the factor in the context of the mechanism of sex maintenance in mammals.

118. Determination of Sexual Fate in the Temperature-Dependent Red-Eared Slider Turtle, Trachemys scripta elegans.
Blanche Capel, Michael Czerwinski, Anirudh Natarajan, Lindsey Mork, Loren Looger.
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In mammals, sex determination is controlled by antagonistic signaling pathways that are activated by expression of the male sex-determining gene on the Y-chromosome, Sry. In contrast to this strong genetic mechanism, the incubation temperature of the egg determines sexual fate in many reptilian species, including the red-eared slider turtle, Trachemys scripta elegans. In this species, incubation at 27°C during the initial stages of gonad formation results in >95% male development, while incubation at 31°C results in >95% females. Incubation at the threshold, or pivotal, temperature (PvT) results in an even ratio of males and females, and rarely produces an intersex individual. The primary advances in understanding TSD in T. scripta and related species have come through a candidate-based approach in which genes identified as important for mammalian GSD were studied in TSD species. While this approach has identified some common elements of the pathways, it cannot uncover novel mechanisms or TSD-specific genes. Recently next-generation sequencing has become a cost effective method to analyze transcriptome-wide gene expression even in non-model organisms without a comprehensive genome assembly. Using next-gen sequencing and a Hidden Markov Model based clustering approach, we identified the sequential activation and repression of all genes expressed in the gonad across 4 stages spanning the entirety of the temperature dependent sex determination process in T. scripta. By comparing these results with a similar dataset for genetic sex determination in the mouse, we identified genes and pathways that are (or are not) conserved between species, and points where modules are temporally shifted. These data allow us to better understand the differences between GSD and TSD and may point to cause and effect relationships between specific genetic modules and molecular and cellular events during sex determination. This work was funded by grants to BC from the National Science Foundation and NICHD.

119. Identification and Evolution of Gecko Sex Chromosomes.
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Evolutionary studies of sex determination and sex chromosomes have been hindered by a lack of information on the types of sex-determining mechanisms that occur among different species. This is particularly problematic in groups where most species lack visually heteromorphic sex chromosomes, such as amphibians, reptiles, and fish, because conventional cytogenetic analyses will fail to identify the sex chromosomes in these species. I will describe the use of next-generation DNA sequencing to identify sex-specific genetic markers and subsequently determine whether a species has male or female heterogamety (XY or ZW sex chromosomes). I use this high throughput DNA sequencing to identify the sex chromosome systems in twelve gecko species. Geckos are ideally suited to test hypotheses about sex chromosome origins and evolution because they have multiple, independent transitions among sex determining mechanisms. Combining these newly generated gecko data with data from the literature, I reinterpret the evolution of sex-determining systems in lizards and snakes (Squamates). I discover between 17 to 25 transitions among gecko sex-determining systems. This is roughly ½ to ⅔ of the total number of transitions observed among all lizards and snakes. The large number of transitions observed in geckos provides an excellent set of replicate evolutionary “experiments” to examine the origin and evolution of sex chromosomes and sex-determining systems. Identifying multiple examples of independently derived XY and ZW taxa is important to distinguish factors common to all XY (or ZW) clades from factors unique to each individual group and can help answer the many outstanding questions about the origins and evolution of sex chromosomes. Next-generation DNA sequencing technologies will undoubtedly prove useful in evaluating other species for male or female heterogamety, particularly the majority of fish, amphibian, and reptile species that lack visibly heteromorphic sex chromosomes, and will significantly accelerate the pace of biological discovery.

120. Exposures to differing thermal dynamics during alligator embryonic development generates intra- and inter-sexual variation in gonadal gene expression.
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The unique ability of the embryonic gonad to develop into either a testis or an ovary sets the stage for one of the most critical events in vertebrate development. The outcome of this decision bears important fitness consequences for offspring by influencing multiple levels of organization ranging from cellular and molecular dynamics, physiology, morphology and behavior. Many reptiles including all crocodilians studied to date employ a form of environmental sex determination (ESD) where the temperature experienced during
embryonic development determines the fate of a bipotential gonad. Numerous studies have shed light on how incubation temperature influences characteristics of reptiles, specifically with respect to gross morphology and growth rates. Here, we take a closer look at the more subtle influences of temperature on sex. One key feature of alligator sex determination is that both males and females can develop from multiple incubation temperatures allowing for exploration of how traits differ among same sex individuals derived from different incubation temperatures. To address the questions of intra-sexual variation, eight clutches of alligator eggs were collected from the wild and assigned to one of four incubation temperatures until just before hatch. Growth measures, blood, and tissues were collected from all individuals and used to sex animals and assess inter- and intra-sexual variation. Our findings thus far suggest that with increasing incubation temperature (towards a temperature that produces a male biased sex ratio), the transcript levels of genes important for testicular differentiation are elevated leading to an increase in the magnitude of sex biased gene expression. In addition, we assess transcript levels of factors involved in steroidogenesis and circulating hormone levels to determine whether the observed differences in gonadal gene expression patterns impact steroidogenic capacity. The design presented here provides a framework for further tests of inter-sexual and intra-sexual variation and explorations of how changes in the magnitude of sex biased gene expression ultimately influence the expression of sexually dimorphic phenotypes.

121. Hormone-Independent Sexual Dimorphisms in Mammals.
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Despite some initial resistance, new observations over the last 25 years of hormone-independent sexual dimorphisms have gradually and unequivocally overturned the dogma, arising from Jost’s elegant experiments in the mid-1900s, that all somatic sex dimorphisms in vertebrates arise from the action of gonadal hormones. Although we know that Sry, a Y-linked gene, is the primary gonadal sex determinant in mammals, our observations in marsupials, and more recent analyses of mice and finches have highlighted numerous sexual dimorphisms that are evident well before the differentiation of the testis and which cannot be explained by a sexually dimorphic hormonal environment. In marsupials, scrotal bulges and mammary primordia are visible long before the testis has differentiated due to the sexual dimorphisms that are evident well before the differentiation of the testis and which cannot be explained by a sexually dimorphic sex determinant in mammals, our observations in marsupials, and more recent analyses of mice and finches have highlighted numerous sexual dimorphisms that arise from the action of gonadal hormones. Although we know that

122. Autocrine/Paracrine Regulation of Somatic Cell Fate in the Mouse Fetal Testis.
Humphrey H. Yao2.
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Transformation of the gonadal primordium into a testis or an ovary defines the first morphogenetic event in mammalian sex determination. The Y-chromosome-derived SRY gene and its downstream regulator SOX9 initiate the testicular program and gear bipotential somatic cells toward a Sertoli cell fate, rather than an ovarian granulosa cell destiny. Once established and differentiated, Sertoli cells require the transcription factor DMRT1 to maintain their identities, and without DMRT1, Sertoli cells transdifferentiate into FOXL2-positive granulosa cells. These evidence support the model that establishment and maintenance of the Sertoli cell lineage occurs cell autonomously, via a coordinate action of transcription factors. However in the case of freemartinism, where a female twin shares a placenta with a male twin, the ovary of the female twin develops testis types and structures. This phenomenon led us to hypothesize that somatic cell fate in the fetal testis is sensitive to secreted factors with hormonal properties. Candidates for such factors are anti-Müllerian hormone (AMH) and activin B, both hormones produced by Sertoli cells. Fetal mouse testes lacking either one of these two genes developed normally; however, Amh/activin B double knockout testes exhibited progressive sex reversal with the disappearance of SOX9-positive Sertoli cells, appearance of FOXL2-positive granulosa cells, and disintegration of testis cord structures. When Foxl2, the fate maintenance factor for granulosa cells, was inactivated in the Amh/activin B double knockout testes, the sex reversal phenotypes were prevented. These genetic evidence reveal another level of somatic cell fate regulation in the fetal testis: Sertoli cell-derived AMH and activin B act in an autocrine/paracrine manner to maintain Sertoli cell fate by suppressing Foxl2 expression and the consequent emergence of the granulosa cell program. This study was supported by NIH Intramural Research Program.

123. Regulation of Gonadal Macrophase Activity is Critical for Sexual Differentiation and Vascular Remodeling of the Fetal Testis.
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Gonad development is a morphogenetic process that is orchestrated among many different cell types: primordial germ cells, somatic supporting cells, interstitial/mesenchymal cells, and vascular endothelial cells tightly regulate their interactions to generate the mature testis and ovary. The XY mouse fetal gonad undergoes massive cellular rearrangement between E11.5 and E12.5 to form testis cords, the basic units of the testis that give rise to seminiferous tubules in the adult organ. The Sertoli cell, the male -specific supporting cell lineage, is traditionally considered the main driving force in testis development. However, recent work from our lab suggests that Sertoli cells are not sufficient for cord formation, and that other cell types (e.g., interstitial and endothelial cells) play essential roles in
driving cord morphogenesis. One cell type that has not been well-appreciated in the development of the fetal reproductive system is the macrophage, which is a mononuclear phagocyte belonging to the myeloid immune cell lineage. Macrophages are nearly ubiquitous within developing and adult organs, but their roles in fetal organogenesis are poorly understood. In the postnatal and adult testis, macrophages constitute a large part of the interstitial compartment and play important roles in Leydig cell development and steroidogenesis; however, macrophages have not been detected or functionally characterized in the fetal testis prior to or during sexual differentiation. Our analyses reveal that primitive macrophages arising from the yolk sac are present in the region of the gonad-mesonephros primordium prior to sex determination. Macrophages interact with several gonadal cell types but are predominantly localized to nascent vasculature, suggesting a close functional relationship between blood vessels and macrophages during gonad development. Specific ablation of macrophages using a Cre/Rosa26-DTA system results in reduced testis-specific vascular remodeling and abnormal testis cord formation, while in Maf/−/Mafb/− mutant embryos which contain supernumerary macrophages we observe hypervascularization and disruption of testis cord structure. Our findings indicate that macrophage activity is regulated to promote normal testis architecture and vascularization. These studies reveal a previously unidentified role for gonadal macrophages in testis differentiation, consistent with a broader role for macrophages in fetal development and organogenesis. This research was supported by: NIH HD058433, March of Dimes (#5- FY14-32), a Cincinnati Children’s Research Innovation and Pilot Funding grant, and Cincinnati Children’s developmental funds to TD; and NIH HD039963 and March of Dimes (FY10-355) to BC.

124. Epigenetic Regulation of Sex Determination in the Mouse Fetal Gonad.
Danielle M. Maatouk1, Christopher Futtner1, Sara Alexandra Garcia-Moreno3, Isabella M. Salamone1.
1Department of Obstetrics and Gynecology/Northwestern University, Chicago, IL, USA
Sex determination is the first developmental process that distinguishes males from females by directing the fetal gonad to differentiate into a testis or ovary. Defects in sex determination result in disorders of sex development (DSDs), inherited congenital conditions where disparities exist between an individual’s chromosomal, gonadal and phenotypic sex. Research on human DSD patients and mouse models of sex determination has led to the discovery of over 35 genes involved in sex determination that can lead to DSDs upon disruption. However, mutations in these genes explain less than 20% of DSD cases and the remaining mutations likely lie in unknown sex-determining genes or in the regulatory elements of these genes. Disruption of several chromatin remodeling enzymes can lead to DSDs in mice and humans; therefore, chromatin remodeling is critical for sex determination. To understand the epigenetic changes that mediate this developmental transition we are investigating changes in several aspects of chromatin. Open chromatin, or regions of nucleosome depletion, mark genomic features including enhancers, repressors, promoters and insulators. XX and XY gonad progenitor cells are bipotential and have the ability to differentiate into pregranulosa or preSertoli cells, regardless of their chromosomal sex. Therefore, we hypothesized that regions of open chromatin would be similar at the bipotential stage (E11.5), but be remodeled during sex determination, leading to distinct patterns of open chromatin in pregranulosa and preSertoli cells (E13.5). Using genome-wide approaches, we mapped open chromatin regions for FACS purified preSertoli and pregranulosa cells isolated from E13.5 mouse fetal gonads. Surprisingly, our preliminary analysis found that patterns of open chromatin were overwhelmingly similar in XX and XY differentiated cells. This suggests that, just after sex determination, global patterns of open chromatin are not remodeled, and these open chromatin regions are likely bound alternatively by activating or repressive transcription factors in differentiated cells. We further investigated changes in histone modifications, specifically H3K27me which is critical for Polycomb-mediated gene silencing. ChIP-qPCR on small numbers of FACS purified cells (under 1000 cells per reaction) found similar patterns of H3K27me3 in E10.5 progenitor cells and sexually dimorphic patterns in E13.5 pregranulosa and preSertoli cells. These results suggest that prior to sex determination, bipotential gonad progenitor cells are epigenetically identical and during sex determination global patterns of nucleosome organization remain unchanged while histone modifications at gene promoters are altered to facilitate sexually-dimorphic gene expression patterns. Our results further our understanding of how chromatin is remodeled during cellular differentiation and identify gene targets of the Polycomb-repressive complex in gonadal cells. Additionally, our data identifies the genome-wide locations of critical regulatory elements for sex-determining genes enabling future studies to address the mechanisms of sex-determining gene regulation and potentially aiding in the identification of non-coding mutations that lead to unexplained cases of DSDs.

125. Serum Replacement Alters Somatic and Germ Cell Development in Ex-Vivo Mouse Fetal Gonad Culture.
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Identifying optimal culture conditions for fetal gonads is paramount for elucidating gonadal development under the most physiologically relevant circumstances. Knock-out serum replacement (KSR) has been widely applied in culture medium as a means to produce defined culture conditions by replacing fetal bovine serum (FBS). However, KSR is an animal product that is not fully chemically defined. In addition, KSR increases the conversion efficiency of fetal germ cells to pluripotent embryonic germ cells and improves derivation and maintenance of induced pluripotent stem cells. These observations indicate that KSR alters germ cell development but the mechanisms it affects remain unknown. We studied the functional effects of KSR compared to FBS, in whole male gonads cultured ex vivo and examined key developmental outcomes in germ and somatic cells. Gonads, dissected from embryonic day (E)12.5 male mouse fetuses carrying a germ cell specific GFP transgene, were cultured for 72h in DMEM:F12K medium containing either 0, 2 or 10% FBS, or KO DMEM medium containing 0, 2, or 15% KSR, conditions commonly used for FBS and KSR. After culture, gonads were either processed for flow cytometry to study cell cycle dynamics (n=3/condition), or were FACS purified into GFP +ve (germ cell) or GFP −ve (somatic cell) populations for gene expression analyses (n=5/condition). ANOVA plus post-hoc analyses were used to calculate statistical significance. In flow cytometric analyses, DNA synthesis was measured via EdU incorporation and propidium iodide used to identify DNA content, thus distinguishing the G0/G1, S and G2/M stages of the cell cycle. After 72h gonad culture, DMEM:F12K containing FBS had no effect on cell cycle parameters compared to DMEM:F12K alone. In contrast, KO DMEM containing KSR resulted in a significant dose-dependent increase in germ cells (mean KO DMEM 6.4% vs. KO DMEM +15% KSR 23.6%; P<0.01) and Sertoli cells (mean KO DMEM 13.4% vs. KO DMEM +15% KSR 28.9%; P<0.001) undergoing S-phase and a reciprocal drop in cells residing in G0/G1 (P<0.01), without
Regulated in germ cells of gonads cultured in 15% KSR, including Mulin Xiong1, Ianina Ferder1, Yasuyo Ohguchi1, Ning Wang1. Maintenance by Suppressing mTORC1.

94. Quantitative Analysis of Male Germline Stem Cell Differentiation Reveals a Critical Role for p53 in Spermatogonia

Supported by FQRNT Team Grant PR-174948 and CIHR MOP-123447 to DJB and DB. CT and JF contributed equally to this work.

GnRHR evolution conferred a selective advantage by enhancing GnRH’s regulation of FSH rather than by enabling the LH surge.

We have developed a novel flow cytometry (FACS)-based approach by combining two cell surface markers (6-integrin that marks 6-high c-Kit-positive differentiating spermatogonia compartment, we identified 4 distinct populations (P3 – P6) based on their progressively decreasing levels of GFP expression with P3 exhibiting the highest GFP and P6 the lowest. We considered P3 – P6 as differentiating spermatogonia at consecutive stages towards maturation. Gene expression analysis coupled with immunocytochemistry staining of the isolated P1 – P6 cells further supported that these cells are undifferentiated spermatogonia (P1), early

95. Quantitative Analysis of Male Germline Stem Cell Differentiation Reveals a Critical Role for p53 in Spermatogonia

Maintenance by Suppressing mTORC1.

Mulin Xiong1, Ianina Ferder1, Yasuyo Ohguchi1, Ning Wang1.

Proper maintenance and differentiation of spermatogonia are essential for male reproduction. To better understand this process, we have developed a novel flow cytometry (FACS)-based approach by combining two cell surface markers (6-integrin that marks undifferentiated spermatogonia with stem cell capacity and c-Kit that marks differentiating spermatogonia) with a GFP reporter driven by the promoter of stimulated by retinoic acid gene 8 (Stra8). Transcriptional activation of Stra8 is perhaps the earliest event known to date during meiosis initiation in both sexes, so we used its promoter activity to monitor early spermatogonia differentiation. This strategy allows separation of undifferentiated and early differentiating spermatogonia at 6 consecutive stages. Specifically, within the integrin-66-high c-Kit- negative undifferentiated spermatogonia compartment, we identified the GFP-negative population (P1) and the GFP-positive population (P2). We considered P1 to represent the most undifferentiated spermatogonia because it is negative for Stra8 promoter activity and P2 to represent the earliest differentiating spermatogonia, in that, while the Stra8 promoter is turned on, c-Kit expression still remains negative. Within the integrin-66-low c-Kit-positive differentiating spermatogonia compartment, we identified 4 distinct populations (P3 – P6) based on their progressively decreasing levels of GFP expression with P3 exhibiting the highest GFP and P6 the lowest. We considered P3 – P6 as differentiating spermatogonia at consecutive stages towards maturation. Gene expression analysis coupled with immunocytochemistry staining of the isolated P1 – P6 cells further supported that these cells are undifferentiated spermatogonia (P1), early
115. A Hypoxia/HIF/Kdm3a Pathway Controls Trophoblast Stem Cell Lineage Decisions and Organization of the Hemochorial Placenta.

Damayanti Chakraborty1, Wei Cui2, Regan Scott1, Pramod Dhakal1, Stephen Renaud1, Jay Vivian4, Makoto Tachibana5, Mohammad K. Rumi4, Michael Soares2.
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The hemochorial placenta develops from the coordinated multi-lineage differentiation of trophoblast cells. Specific trophoblast cell lineages are organized within the placenta site to perform specialized functions. The invasive trophoblast lineage remodels uterine spiral arteries to convert them to flaccid low resistance vessels, facilitating the flow of nutrients to the placenta and fetus. Failure of trophoblast invasion and vascular remodeling is associated with pathological conditions such as preeclampsia, intrauterine growth restriction, and preterm birth. Oxygen delivery represents an environmental stimulus with an instructive role influencing trophoblast cell differentiation and organization of the hemochorial placenta. In this study, we perform a series of in vitro and in vivo experiments delineating a hypoxia-activated regulatory pathway controlling hemochorial placenta. Key downstream events are delineated using rat trophoblast stem (TS) cells and tested in vivo using trophoblast-specific lentiviral gene delivery and genome editing. Initially, DNA microarray analyses were performed on rat TS cells exposed to ambient or low oxygen (0.5%). Upreregulation of genes characteristic of an invasive/vascular remodeling/inflammatory phenotype and a marked downregulation of stem state-associated genes were observed. Among the upregulated genes were a histone H3K9 demethylase (Kdm3a) and a matrix metalloelastase (Mmp12). Uregulation of these transcripts was dependent upon the transcription factor, hypoxia inducible factor (HIF). We hypothesized that Kdm3a was a mediator of hypoxia-directed trophoblast cell lineage differentiation and that Mmp12 was a key downstream target responsible for the trophoblast cell invasive and vascular remodeling phenotype. Consistent with the hypothesis, knockdown of Kdm3a in rat TS cells inhibited the expression of a subset of the hypoxia/HIF-dependent transcripts, including Mmp12, and altered locus specific as well as global H3K9 methylation status. Concurrently ectopic expression of Kdm3a upregulated a subset of hypoxia/HIF-dependent transcripts, including Mmp12, and this upregulation was dependent on Kdm3a enzymatic activity. Furthermore, Kdm3a knockdown decreased hypoxia-induced trophoblast cell invasion in both in vitro and in vivo experiments. Mmp12 possesses the capacity to degrade elastin and modify the structure of arterial blood vessels. To further explore the functional importance of Mmp12 in trophoblast cell-directed uterine spiral artery remodeling, we generated an Mmp12 mutant rat model using TALEN-mediated genome editing. A rat model was established with a 609 bp deletion targeting exon 2 of Mmp12. Homozygous mutant rats showed reduced hypoxia-dependent endovascular trophoblast invasion and impaired trophoblast-directed uterine spiral artery remodeling. In summary, we have discovered a hypoxia/HIF/Kdm3a pathway modulating trophoblast cell lineage development, leading to acquisition of the invasive trophoblast cell phenotype, including suppression of the extracellular matrix-modifying enzyme Mmp12 and subsequent uterine spiral artery remodeling.

126. Antagonism Of Gonadotropin Responsiveness By WNT5a In Granulosa Cells Is Mediated By Suppression Of Canonical WNT Signaling. 

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The WNTs are a large family of secreted signaling molecules that regulate a variety of physiological and developmental processes in a range of tissues including the mammalian ovary. WNT signaling occurs via β-catenin dependent (canonical) and -independent (non-canonical) pathways. Whereas the role of canonical signaling is well established in granulosa cells (GCs), the roles of non-canonical WNTs and their mechanisms of action are unclear. WNT5a and WNT11 are known to activate the non-canonical planar cell polarity (PCP) and WNT/Ca2+ pathways in mammalian cells. We found the expression of both Wnt5a and Wnt11 rapidly increases in GCs following the LH surge, suggesting that they may play redundant roles in the ovary. The objective of this study was to elucidate the physiological roles of Wnt5a and Wnt11 in follicle development and to determine their mechanisms of action. Conditional disruption of Wnt5a in the granulosa cells of Wnt5a[flox/-];Amhr2[cre/+] (Wnt5a cKO) mice resulted in reduced female fertility (total litters and pups/litter over a 6-month span) and ovulation rate. Conversely, (Wnt11)[flox/-];Amhr2[cre/+] mice showed no overt fertility issues or follicle development defects. To test for potential functional redundancy between Wnt5a and Wnt11 in granulosa cells, Wnt5a+/+ conditional knockout mice were generated. The fertility of the latter was comparable to Wnt5a cKO animals. Follicle counting experiments in 5 day, 42 day and 8 month-old animals determined that Wnt5a cKO mice have less healthy follicles and increased rates of follicular atresia at all stages of follicle development. WNT5a is therefore...
required for normal follicle development and female fertility, whereas WNT11 appears entirely dispensable and does not function in a redundant manner with WNT5a. To determine the mechanism of action of WNT5a, granulosa cells from immature mice were placed in culture and treated or not with recombinant WNT5a, and global changes in gene expression analyzed by microarray and confirmed by RT-qPCR. Results notably indicated that WNT5a decreases the expression of genes associated with granulosa cell differentiation (Lhcgr, Prlr, Cyp19a1, Fshr, Inhbb). Expression of the latter genes was found to be increased in the granulosa cells from Wnt5a conditional knockout mice, confirming their identity as WNT5a transcriptional targets. To determine the intracellular signaling mechanisms whereby WNT5a regulates its target genes, primary mouse granulosa cell cultures were treated with WNT5a on a time course, and the expression of mediators of various WNT signaling pathway effectors was assessed. WNT5a did not stimulate the non-canonical pathways, but rather decreased β-catenin expression, as well as levels of β-catenin phosphorylation at a PKA site that regulates its stability. As this suggested that PKA activity may be regulated by WNT5a, we determined if WNT5a affects gonadotropin responsiveness. Pretreatment of granulosa cells with WNT5a abrogated the ability of FSH and LH to induce CREB phosphorylation or to increase FSH target gene (Cyp19a1, Fshr, Lhcgr and Prlr) and LH target gene (Ereg, Areg, Star, Cyp11a1) expression. Together, these data indicate that WNT5a is required for normal female fertility, follicle development and ovarian steroidogenesis, and is an antagonist of gonadotropin responsiveness that acts via the suppression of canonical WNT signaling.

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Uterine leiomyomas (UL), also known as fibroids, are benign neoplasms arising from smooth muscle cells of the uterus. They are clinically diagnosed in 25% of women and are often associated with dysmenorrhea, infertility, and miscarriage. Although fibroids are the most common gynecological tumors, the etiology of fibroids remains elusive. About 40% of tumors have a range of cytogenetic abnormalities, such as deletions of 7q and rearrangements of 6p21, 12q14 and 12q15 loci. Whole exome approaches have identified heterozygous somatic mutations in the mediator complex subunit 12 (Med12) in about 70% of leiomyomas. All of the identified mutations occur in exon 2 of Med12 with c.131G>A being the most common non-synonymous SNP amongst them. MED12 protein is part of a large mediator complex and is involved in transcriptional regulation of POLII. We hypothesized that the Med12 exon 2 mutations are causative of tumor formation and hence generated a mouse model where we conditionally floxed Med12 c.131G>A cDNA and inserted into the ROSA locus to generate Med12Rmt/+ mice. Amhr2-cre was used to drive the expression of the mutant Med12 from the ROSA locus either in the presence (Med12Rmt/+ Amhr2-cre) or absence (Med12Rmt/− Amhr2-cre) of X-chromosome wild type Med12 in the uterine mesenchyme. About 50% and 77% of female mice (nulliparous and multiparous), respectively, developed leiomyomas. To understand the underlying mechanisms of mutated Med12 -driven tumor development, we investigated the genomic landscape of the mouse tumors by performing array comparative genomic hybridization (aCGH) using the Agilent SurePrint G3 mouse Genome array kit. Genomic “test” DNA from uteri of multiparous and nulliparous Med12Rmt/− Amhr2-cre females was compared to “reference” DNA from uterus of littermate controls without cre. All tumors showed a range of chromosomal abnormalities (40-50 per tumor), with chromosomes 2, 7, 14 and 17 being most frequently affected. The regions of aberrations often consisted of genes targeting cell cycle checkpoints or multiple tumor pathways such as Ras, Wnt/β catenin, Tp53/Rb, NF-kappaβ and Tgfβ signaling. Chromothripsis was also observed among the chromosomes of mouse tumor samples. We additionally, mapped the mouse aberrations to the human genome (hg19) to determine syntenic regions of chromosomal aberrations between mouse and human. Approximately 50% of the mouse aberrations mapped to human chromosomes, with several regions having been previously reported in human leiomyomas. Interestingly, we observed 17qA3.3 locus to be duplicated in all mouse tumors, which maps to the human 6p21 locus. This data suggests that Med12 exon 2 mutations are precursors to genomic rearrangements and therefore can cause an unstable genome and drive tumor progression. Thus the Med12 mouse model we have developed will provide further insights into the earliest steps in the genesis of genomic instability and resultant leiomyoma formation.

128. The ERK-signaling pathway regulates junctional protein complexes in the initial segment of the mouse epididymis.
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The initial segment (IS) of the mouse epididymis is functionally and structurally distinct from other epididymal regions and is essential for male fertility. The integrity of epithelial cells in this region is highly dependent upon the establishment of elaborate junctional complexes, including tight-junctions (TJ) and gap-junctions (GJ). Epithelial cell differentiation in the IS is under the control of the MEK1/2-ERK1/2 signaling pathway, which is stimulated by testicular luminal factors. We tested here the hypothesis that the high level of pERK in the IS is essential for the maintenance and organization of the TJ proteins ZO-1-2-3, Claudins (CLDN-1-3-4) and Occludin (OCLN), and the gap junction protein connexin 43 (Cx43). Western blotting (WB) and immunofluorescence analysis showed that treatment of mice with the MEK1/2 inhibitor PD325901 (10mg/kg/day, i.p twice daily) induced a progressive decrease in the level of ERK phosphorylation after 24h and an almost complete disappearance of pERK after 36 hours, while total ERK remained unaffected. This was accompanied by a decrease in ZO-2 expression and an increase in ZO-3 expression, while ZO-1 expression remained unaffected after 36 hours of treatment versus control. In control mice, CLDN-1, -3 and -4 were located in the lateral membrane of epithelial cells in addition to TJs, as previously described. A stronger CLDN-1 and -4 labeling was detected in basal cells compared to principal cells. While CLDN-1 and -4 expression was significantly reduced after PD325901 treatment, CLDN-3 remained unaffected. These results were confirmed by WB. OCLN was not detected in the IS of control mice, in agreement with previous studies (Daniel G. Cory et al., 1999). However, PD325901 induced a significant increase in OCLN labeling in ZO-1-positive TJs. WB analysis showed an increase in OCLN protein levels after treatment, although OCLN mRNA, assessed by real-time PCR, remained unchanged. Lastly, double-labeling for ZO-1 and Cx43, showed that while Cx43 was mainly located in punctate structures along the lateral membrane of epithelial cells in control mice, PD325901 treatment induced a significant relocalization of this protein in TJs, where it co-localized with ZO-1. In recovery experiments, ERK phosphorylation and the localization and expression of junctional proteins returned to normal levels after 72 hours. Our results show differential regulation of ZO-2, ZO-3, CLDN-1, CLDN-4, OCLN and Cx43 by the ERK1/2 pathway, while ZO-1 and CLDN-3 remained...
unaffected. Taken together, our results suggest that ERK phosphorylation plays a key role in the regulation of TJ and GJ dynamics in the IS of the mouse epididymis. This research was supported by NIH grant DK085715.

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Viviparity is not confined to mammals. It has evolved many times in non-mammalian vertebrates, including reptiles, amphibians and fish (but not in birds) and in some instances is supported by a primitive form of placentation in which the developing conceptus draws directly on resources provided by the mother, rather than egg yolk. The placenta, once regarded as the “hallmark” of mammals, was for a while thought to afford a basis for mammalian taxonomy. It provides a series of common functions across mammalian taxa, but, despite such apparent conservation of function, the placenta is arguably the most diverse of mammalian organs and displays a range of gross morphologies and much variability in the extent to which the fetal trophoblast penetrates the uterine wall and interacts with the maternal blood supply. I shall outline the range of placental structures found in all mammals, and contrast two of the more extreme types encountered in eutherian mammals. The hemochorial type occurs in rodents and primates and is the commonest form across all species, while the non-invasive epitheliochorial type, as found in pigs and ruminants, is arguably the most efficient. Although the epitheliochorial type relies extensively on uptake of histotrophe from uterine secretions and was once regarded as the most primitive form of placentation, it is now recognized as a derived form and that the ancestral mammalian placenta was hemochorial and discoid in gross morphology. I shall discuss the advantages and drawbacks of both these forms and the structural impositions each placentation type places on fetal-maternal signaling and acquisition of nutrients, such as iron. I shall then discuss the evolution of the eutherian placenta in molecular terms, including the conservation of some transcription factors and the astonishing diversification of placenta-specific genes that tend to be clustered in related taxonomic groups, but not across orders, and usually exist as members of large gene families that have diversified at unusually high rates. Examples include the prolactin/placental lactogen gene family, the interferon-tau family, the pregnancy-associated glycoprotein family, and the syncytons, which have evolved as products of retroviral envelope genes and are involved in formation of syncytiotrophoblast regions of the placenta. Various mechanisms appear to underpin this expansion of gene families, which may be a reflection of the greater promiscuity in genetic tampering allowed by the placenta relative to other organ systems.

There is much discussion about what processes may be driving the rapid morphological/physiological adaptations of the placenta. Potential mechanism include genetic conflict and an on-going “arms race” between paternal genes favoring the conceptus and maternal genes seeking to constrain exploitation, particularly in the provisioning of the conceptus where genetic imprinting has been considered to play a major role. Maternal-fetal conflict may also be responsible for the uneasy equilibrium that exists between the maternal immune system and the fetal trophoblast, although this battleground remains poorly understood, in part because it is undoubtedly multi-faceted and mechanistically diverse. Finally, I shall discuss a relatively conserved aspect of placentation, namely the events leading up to the divergence of trophoblast from the inner cell mass, first evident at the blastocyst stage. Here I shall focus, not on the embryo, but on a cell model, namely the formation of trophoblast from pluripotent stem cells and the role that bone morphogenic proteins, e.g. BMP4, may play in this transition. Supported by NIH grants HD067759, HD069979, and HD077108.

130. The Self-Direction of Human Evolution, from Darwin's Rabbits to CRISPR.
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Breakthrough technologies such as CRISPR-cas9 are touted has having great promise for treating and preventing disease. But some advocates ask why we should stop there. We have the opportunity—even the duty—they say, to wrest control of human evolution from the blind, cruel forces of nature. The self-direction of human evolution is an enduring fantasy. Charles Darwin’s cousin Francis Galton first distinguished what we now call the germline from the somatic line and proposed a system by which the British germ plasm could be steadily improved. He called his plan “eugenics.” Eugenics has a long and sordid career. So why are some commentators trying to revive it in the twenty-first century? Today’s new eugenicists argue that molecularization, combined with today’s strongly individualist political and cultural climate, solves eugenic’s ethical problems. So long as eugenics does not involve coerced sterilization and state-controlled efforts to create a master race, they say, it is benevolent. If people are free to manage their own reproduction, there is no ethical problem. Conscientious scientists, however, are wary of such talk. In this lecture, I will explore the history and motivations behind human genetic improvement, as well as what’s at stake in these debates. Taking the long view suggests that the urge to control our own evolution—and our sense of how close we are to doing so—is independent of either technology or knowledge.

HERITAGE LUNCHEON

131. M.C. Chang - Grandfather of IVF.
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Min Chueh Chang, affectionately called “Chang” or “Mac”, was born in a farming village of China. He graduated from Tsing-Hua University in Beijin in 1933 before obtaining his Ph.D. degree from Cambridge University in 1941. In 1944 he joined the newly started Worcester Foundation for Experimental Biology (WFEB), Shrewsbury, Massachusetts a year before the end of World War II (WW
II). He went there to learn IVF technique from Gregory Pincus who previously claimed successful IVF in the rabbit. Chang did not return to China because of the internal conflict in his home country which started immediately after the end of WW II. Chang helped Pincus in the development of oral contraceptive, carrying all animal experiments before the clinical trials were executed in Puerto Rico. Chang continued basic studies of reproduction at the WFEB until his death in 1991. He preferentially used rabbits as model animals. His numerous, groundbreaking discoveries and technological breakthroughs include: (1) the first aerial transport of preimplantation embryos, (2) discovery of the importance of a temporal synchrony between the developmental stage of animal embryo and that of the regional environment within the female tract, (3) discovery of sperm capacitation and decapsulation, and (4) the first production of live (rabbit) offspring by IVF (1959) almost 20 years ahead of the first human IVF baby born (1978). He mentored many young fellows who continued and extended his research.

FOCUS SESSION 13: Epigenetics and Clinical Considerations.

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Persistent Organic Pollutants (POPs) are organochlorine chemicals that include PCBs, dioxins and legacy insecticides. POPs are a health concern because they bioaccumulate and biomagnify in the food chain. POPs are therefore, restricted according to the Stockholm Convention, although they remain ubiquitous worldwide. Because they are transported to the Polar Regions via natural weather currents, the Arctic food chain is particularly contaminated with POPs and the Inuit have high body burdens.

There is a major health discrepancy between Inuit people in Arctic regions and non-Aboriginal Canadians, including poor fetal growth, placental abnormalities, stillbirths, congenital defects, diabetes and mental health issues. Consequently, the life expectancy of Northern Aboriginal people is about 10 years shorter than that of other Canadians. Although health status is multifactorial, it possible that exposure to the POPs contributes to this health discrepancy. Children are particularly vulnerable to the POPs since they are readily eliminated from the mother to her fetus and during breastfeeding. We hypothesized, therefore, that early exposure to Arctic POPs affects the paternal epigenome and the health of his future generations. To test this hypothesis, Sprague-Dawley female rats (F0) were gavaged for 5 weeks with an environmentally-relevant concentration of POP mixture or corn oil (Control) and mated to untreated males. Gavage continued until parturition of F1 litters. Adult F1 males were mated to untreated females to generate F2 fetuses and pups; F2 development was followed until 90 days of age. F3 and F4 generations were similarly produced. After weaning, all pups were fed commercial chow without direct exposure to the POPs. To determine if prenatal POP exposure alters the paternal epigenome, F1 sperm were analyzed by reduced representation bisulfite sequencing (RRBS) to obtain genome-wide information on DNA methylation. RRBS libraries (n=6) were used in paired-end sequencing in 1 lane of a HiSeq 2000 sequencer (Illumina). Analysis and statistics for differentially methylated regions (≥ 20%) were conducted using Methyldet software. RRBS data were validated by pyrosequencing. F2 and F3 litters sired by F1 or F2 POP-exposed males had more preimplantation loss vs. Controls (P < 0.05); no treatment difference occurred in F4 litters sired by the F3 fathers. Both placenta and pups in the F2 litters by the F1 POP-exposed fathers were smaller than Controls and this body weight discrepancy persisted throughout life (P < 0.05). Moreover, 38% of those F2 pups died before postnatal day 2 (P < 0.05). Litters sired by the F1 or F2 POP-exposed males appeared to be normal, although 13% pups died before weaning (P < 0.05). The severe congenital birth defect, situs inversus was observed in F2, F3 and F4 pups sired by F1, F2 and F3 POP-exposed fathers, although the F4 litters appeared to be otherwise normal. RRBS revealed that early-life exposure to the Arctic POPs mixture altered F1 sperm DNA methylation, which was confirmed by pyrosequencing. Moreover, >200 genes in the POPS-exposed sperm from the F1 males were differentially methylated, including those in regions involved in embryo development. Pathologies linked to the altered genes include metabolic, neurological, psychological and cardiovascular disorders, cancers and reproductive dysfunction, many of which are either documented to be related to prenatal exposure to organochlorines or are of concern to Inuit populations. Sperm from the F2, F3 and F4 generations are currently being assessed. In conclusion, our results confirm our hypothesis, indicating that early-life paternal exposure to environmentally-relevant Arctic POPs induces reproductive dysfunction as well as developmental pathologies in their offspring, possibly due to epimutations of the sperm DNA. Financed by FQRNT & CIHR.

133. Folate Supplementation Impacts Heritable Epigenetic Information in Sperm.
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There are sex-specific differences in the timing of epigenetic patterning, specifically DNA methylation, in male and female germ cells. Such sex-specific differences suggest that males and females will be susceptible to the induction of epigenetic defects in their germ cells at different times in their lives, with the possibility of passing such defects on to their offspring. In the male, the major period of DNA methylation acquisition (de novo methylation) occurs before birth in male germ cells of the fetal testis; postnatally, the patterns must be maintained (maintenance methylation) during the continuous, lifelong, cell divisions that occur in the male germ line stem cells (spermatogonial stem cells) that produce sperm. In male germ cells a small amount of additional methylation on a minority of sequences occurs as germ cells develop from spermatogonia to spermatocytes, when meiotic recombination occurs. Dietary folate is a key source of methyl groups for DNA methylation. Folate deficiency, either due to diet or folate pathway enzyme defects and folate supplementation are important clinical situations in which methyl donors required for DNA methylation are either lower or higher than normal. High dose folate
(4-5mg/day) supplementation is currently used clinically in the treatment of women at high risk for neural tube defects and pregnancy complications, such as preeclampsia, and in the treatment of men with infertility. The potential adverse effects of high dose folate on the male germ cell epigenome have not been examined. We hypothesize that folate status will perturb DNA methylation in male germ cells that are exposed at the key prenatal and postnatal times (susceptibility windows) when DNA methylation patterns are being acquired. We are using sensitive next generation sequencing based assays to identify DNA methylation defects in sperm associated with altered folate status in mouse and human models. In the mouse we have used administered folate supplemented diets to model prenatal and postnatal exposures and have found evidence of intergenerational adverse reproductive effects and sperm DNA methylation defects. In men with idiopathic infertility who received supplements of 5mg/day of folic acid for 6 months, DNA methylation at imprinted loci remained unchanged, however, unexpected loss of methylation was observed across the sperm epigenome, effects that were exacerbated in men homozygous for a common polymorphism in the folate pathway enzyme methylenetetrahydrofolate reductase (MTHFR). Thus, both the mouse and human data indicate that folate supplementation can impact the sperm epigenome. In addition, the mouse studies suggest that DNA methylation defects in male germ cells associated with folate supplementation may be heritable and have adverse effects on future generations. (Supported by CIHR).

134. Alterations in the epigenetic landscape of the adult mouse uterus following neonatal estrogen exposure.
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Neonatal exposure to diethylstilbestrol (DES) results in abnormal reproductive tract morphology, female infertility and uterine cancer in mice. This exposure also causes altered gene expression in the female reproductive tract that persists into adulthood. The permanent up-regulation of estrogen-related homeobox 1 homolog (Six1) in the uterus is of particular interest because this gene has a key role in organ differentiation and is upregulated in numerous cancers. Coordinate with aberrant expression of Six1 is permanent association of active transcription marks (histone H3K4me3, H3K9acetyl and H4K5acetyl) near the TSS of Six1 and no apparent difference in association of the repressive mark (H3K27me3). These findings indicated that epigenetic modifications likely play a role in the permanent up-regulation of Six1 expression and that neonatal estrogens alter the epigenetic landscape of uterine genes. To further explore on a genome-wide basis how neonatal estrogen exposure results in permanent epigenetic changes, we performed RNA-seq and ChIP-seq analyses of DES-treated and control mice. CD-1 mice were treated on postnatal days (PND) 1-5 with DES (2 µg/pup/day) or corn oil as a control; uterine tissues were collected on PND5, PND22 (prepubertal) and in ovariectomized adults (ovx + Estradiol 24h; E2). RNA-seq analysis resulted in 4,514, 46, 264 and 164 differentially expressed genes on PND5, PND22, ovx vehicle and ovx+E2 respectively (>1.5 fold difference; FDR <0.05). ChIP-seq was performed on uterine samples from PND5, PND22 and ovx ± E2 using H3K4me3 (active mark), H3K27acetyl (active mark/enhancer) and H3K27me3 (repressive mark) as the precipitating antibodies. Global analysis of all 2,028 TSSs showed increased association of H3K4me3 and H3K27acetyl and decreased H3K27me3 at the TSS (-2kb to +2kb) as expected. In addition, there was a global increase in H3K4me3 and decrease in H3K27me3 at the TSS between PND5 and PND22 samples but no difference between treatment groups suggesting more transcription in general as the mice age. Interestingly, there was an increase in H3K27acetyl at the TSS in both PND5 and PND22 DES-treated samples compared to their respective controls suggesting this mark may be involved in differential gene expression. Uterine samples from ovx ± E2 are currently being analyzed for similar global effects. In addition to these global effects, preliminary data suggests that there is differential H3K27acetyl association outside of the TSSs of several differentially expressed genes (presumed enhancers) further suggesting that this mark may play an important role in permanently altering gene expression. These findings indicate that neonatal exposure to estrogenic chemicals permanently alters the epigenetic landscape of the adult uterus and that both global and specific gene loci may be altered in this process.

135. Treatment with trichostatin-A and 5-aza-2’-deoxycytidine improves the developmental competence, quality and epigenetic status and alters the gene expression in cloned buffalo (Bubalus bubalis) embryos.
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Incomplete reprogramming of the nuclei of somatic cells is believed to be the primary reason responsible for limiting the success of somatic cell nuclear transfer (NT). We have previously shown that treatment of donor cells with trichostatin A (TSA), a histone deacetylase inhibitor, and 5-aza-2’-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, promotes reprogramming and improves the development and quality of NT buffalo embryos (Saini et al., 2014 Reprod. Fertil Dev. doi: 10.1071/RD14176). The present study was aimed at examining the effects of treatment of both donor cells and reconstructed embryos with 50 nM TSA+7.5 nM 5-aza-dC on the developmental competence, quality, epigenetic status and gene expression in cloned buffalo embryos, which were produced by Hand-made cloning (HMC) as described earlier (Saini et al., 2014). Treatment with TSA+5-aza-dC increased (P<0.05) the blastocyst rate (43.1±3.4 vs 71.8±2.4%) and decreased (P<0.05) the apoptotic index of blastocysts (19.5±2.1 vs 7.4±1.3) compared to that of untreated controls whereas the apoptotic index in IVF blastocysts was 6.0±0.8. However, the total cell number of blastocysts was not significantly different between control and treatment groups (191.1±20.9 vs 268.4±41.3). The treatment increased (P<0.001) the global level of H3K18ac and decreased (P<0.05) the global level of H3K27me3. The relative transcript level of DNMT1 and DNMT3a was lower (P<0.05) and that of SOX2 and MASH was higher (P<0.001) in the blastocysts of treatment group than that in control and IVF blastocysts. The relative expression of OCT4 and NANOG was not significantly different between the blastocysts of treatment and control groups but was lower (P<0.05) than that in IVF blastocysts whereas the differences among the three groups were not significantly different in case of HDAC1, P33 and FGF4. The expression level of CDX2 was similar between IVF and cloned embryos of the treatment group but was lower (P<0.05) than that in the
untreated control group. These results show that treatment of donor cells and reconstructed buffalo embryos with TSA and 5-aza-dC improves the developmental competence, quality and epigenetic status, and alters the gene expression in cloned embryos produced by NT.

136. Expression Profile of EZH2 isoforms in Endometriosis.
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Introduction: Endometriosis is a gynecological hormonal-dependent disease that affects 1 in 10 women during their reproductive years causing incapacitating pelvic pain, dysmenorrhea, and often infertility. Genetic, inflammatory, environmental, and, recently, epigenetic factors have roles in the pathogenesis of this disease. Histone methylation is an important epigenetic modification that regulates different cellular process via modulation (activation or silencing) of the transcriptional machinery by changing chromatin structure. Tri-methylation at lysine residue 27 at histone 3 (H3K27me3) is a histone mark associated with gene repression. EZH2, the catalytic subunit of the polycomb repressive complex 2 (PRC2), is the histone methyltransferase (HMT) responsible for catalyzing the addition of three methyl groups to lysine residue 27 at histone 3. Although this HMT and its resulting histone mark are well studied in cancer, little is known about the expression profile of EZH2 in endometriosis. We have previously shown that endometriotic lesions are characterized by higher levels of H3K27me3; thus, we hypothesized that EZH2 expression will be higher in endometriotic epithelial cells as well as in endometria from women with endometriosis (patients) than in endometria from endometriosis-free women (controls). Objective: To elucidate the EZH2 expression profile in ectic and ectopic endometrium. Methods: Western blot analysis was conducted in 1) ovarian hormone-treated endometriotic and non-endometriotic epithelial cells and 2) endometrial tissues from patients and controls using an antibody against EZH2. Results: Endometriotic epithelial cells express EZH2 which is upregulated by progesterone compared to non-endometriotic epithelial cells. Interestingly, we found not only the most studied EZH2 isoform, EZH2α, but also other EZH2 isoforms (e.g., β, γ, and δ) were detected in endometria from patients and controls. In this context, EZH2 alpha and EZH2 delta were expressed more robustly in endometria from patients whereas only EZH2 delta was expressed robustly in endometria from controls. Conclusions: We described here for the first time the protein expression profile of EZH2 isoforms in endometriotic cells and endometrial tissues. These studies will help to better understand the role of histone methyltransferases in endometriosis and may support the use of novel histone methyltransferase inhibitors (HMTi) for the treatment of this enigmatic disease. This research was supported by R25GM096955, MCHDRO1HD050509, NC1U56CA126379-01, MMSGN082406.

137. Egg Quality Resulting From Ovarian Stimulation Possibly Causing Epigenetic Perturbations.
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Purpose: To see whether ovarian stimulation in human IVF causes poorer egg quality than no ovarian stimulation, possibly through an epigenetic perturbation. Methods: In one study from 2008 to 2013, 1,219 women underwent minimal stimulation for IVF, and 895 underwent conventional stimulation for IVF. The average number of eggs retrieved with minimal stimulation was 3.8±4.4. The average number of eggs with conventional stimulation was 12.1±2.3. Implantation, pregnancy, and live baby rates were determined for both groups, as well as the average number of eggs required to make a baby. Selection criteria for minimal stimulation patients was over 40 years of age or low ovarian reserve (<10 AFC), and for conventional stimulation was normal ovarian reserve regardless of age. In a second, larger collaborative study, 21,455 women underwent IVF with no stimulation whatsoever (natural cycle IVF) with single embryo transfer. 15,743 eggs were thus retrieved. Again, pregnancy rate, implantation rate, live baby rate, and average number of eggs it requires to make a baby was determined. This was compared to this average number of oocytes it takes to make a baby with conventional ovarian stimulation. Results: In the first study with minimal stimulation, pregnancy rates averaged almost as high for low ovarian reserve patients as for high ovarian reserve patients (43.1% vs. 49.4%). The implantation rate per egg with Mini-IVF was almost twice as high at any age than with conventional stimulation (age <35, 8.4% vs. 5.9% (p< .01) and for 35-39, 7.9% vs. 5.5% (p< .01). For women over 40 this superiority of minimally stimulated eggs was even more dramatic (implantation rate per egg age 40-42 was 4.2% vs. 2.2%; age 43+, it was 3.1% vs. 0.0%). The actual pregnancy rate per egg by age was twice as high for conventional stimulation, and at any age under 35, 8.0% vs. 4.2%, and age 35-39, 8.4% vs. 5.0% (p< .01) for mini-IVF as compared to conventional stimulation. In the second larger collaborative study involving NO stimulation at all and single embryo transfer, the results were even more dramatic. The mean number of eggs to make a FHB was 5.9, and to make a live baby was 6.7. For conventional stimulation, the mean number of oocytes to make a FHB was 25.1, and for a live baby was 29.5. Thus comparing natural cycle to standard ovarian stimulation, it required an average 5 times more eggs to make a baby using conventional ovarian hyperstimulation (25.1 vs. 5.9, and 29.5 vs. 6.7). Discussion: While natural cycle IVF or minimal stimulation IVF produces more eggs per cycle, these eggs are clearly of poorer quality. It can be in practical IVF that obtaining larger numbers of eggs will overbalance the poorer quality of those eggs and produce higher pregnancy rates, which could discourage practicing Ob-Gyn doctors from using natural cycle or minimal stimulation. However, this may come at the cost of poorer quality eggs and later problems with offspring. Epigenetic aberrations has been offered as one such hypothesis.

138. Impact of Superovulation on Genomic Imprinting.
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Genomic imprinting is a specialized transcriptional mechanism that employs epigenetic modifications to silence one parental allele while the other copy is expressed. DNA methylation is one epigenetic mark that regulates imprinted gene expression and is generally associated with gene repression. During gametogenesis, imprinted DNA methylation undergoes a cycle of erasure and acquisition. This methylation is maintained during early embryogenesis when genome-wide demethylation takes place. Gamete and embryo manipulations for the purpose of assisted reproduction are performed during these reprogramming periods and may lead to the disruption of genomic
imprinting. Recent studies point to the role of maternal effect proteins, such as zinc-finger protein 57 (ZFP57), in imprinting gene regulation, but the effects of assisted reproductive technologies on their function are unknown. Using a mouse model system, we investigated the effects of superovulation on genomic imprinting. Contrary to our expectation, imprinted methylation acquisition in oocytes was not affected by superovulation. Instead, we found that superovulation disrupted imprint maintenance in blastocyst stage embryos. To determine whether maternal epigenetic proteins were misregulated by superovulation, we investigate ZFP57. While we observed no change in transcript abundance between control and superovulated oocytes and embryos, our data showed greater ZFP57 protein from the 2-cell to blastocyst stage in the superovulated group compared to controls. At the global level in control 1- and 4-cell embryos, ZFP57 protein was localized to the cytoplasm and nucleoplasm. Beginning at the 8-cell stage, there was a reduction in cytoplasmic staining of ZFP57, with nuclear only staining in morula and blastocysts. In comparison to controls, 1- and 4-cell embryos in the superovulated group possessed primarily cytoplasmic localization of ZFP57. Furthermore, 8-cell, morula and early blastocysts stage embryos displayed aberrant cytoplasmic staining. This indicates that superovulation led to aberrant ZFP57 localization with delayed or defective nuclear import until the 8-cell stage combined with increased ZFP57 protein stability. Using small scale ChIP analysis to assess ZFP57 binding at imprinting control regions, we found that blastocysts in the superovulated group had reduced enrichment at imprinting control regions compared to controls. These results indicate that superovulation disrupts protein level and localization of a key maternal effects factor, ZFP57, necessary for imprint maintenance during early embryogenesis. No conflict of interest to declare.

139. Human Placental Imprinted Gene Expression Is Established Early and Is Stable Across Gestation.
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Genomic imprinting is an essential epigenetic mechanism of gene regulation that results in parent-of-origin-specific monoallelic expression. However, monoallelic expression of imprinted genes can vary in a species-, tissue-, cell-type-, and developmental-stage-specific manner. Loss of imprinting (LOI), which refers to the re-expression of the silenced allele, occurs frequently in cancer cells and also has been linked to aberrant placentation and adverse pregnancy outcomes. First trimester human placentas have been reported to show biallelic expression of some imprinted genes that are monoallelic at term delivery, suggesting that silencing of the imprinted allele might be developmentally regulated across gestation. To examine this possibility in more detail, we developed a multiplexed assay to both genotype and quantify allele-specific expression (ASE) in order to verify the imprinted status of 19 known and putative imprinted genes in first trimester and term human placentas from uncomplicated pregnancies. First trimester placentas were obtained following elective termination (N=20; median gestational age 8.5 weeks, range 5-13 weeks) and term placentas were obtained at delivery (N=56; median 39 weeks, range 37-41 weeks). Twenty single nucleotide polymorphisms (SNPs) were used to genotype and measure ASE in two sets of 10-plexes using the Sequenom MassArray system. Genomic DNA (gDNA) and RNA were isolated from the same placental homogenate and two independent reverse-transcription reactions were tested for each sample. ASE ratios were normalized to mean gDNA allele ratios from heterozygous individuals to correct for assay bias. The number of placentas heterozygous for each SNP ranged from 11 to 42. We verified that 11 imprinted genes showed monoallelic expression (DLK1, H19, IGF2, KCNQ1OT1, MEG3, MEST, PEG3, PEG10, PHLD42, PLAGL1, SNRPN); 3 genes showed skewed ASE in a parent-of-origin-specific manner (KCNQ1, PHACTR2, SLC22A18), and 5 genes showed biallelic expression (CD44, EPS15, SLC22A3, STX11, TP73) in both first trimester and term placentas. Because all SNPs were genotypeyped in each sample, maternal cell contamination was apparent when heterozygous placentas showed marked skewing of gDNA allele ratios or when homozygous placentas showed expression of two alleles. We found that maternal cell contamination was more common in the first trimester placentas (8/20, 40%) compared to the term placentas (3/56, 5%). In samples with no apparent maternal cell contamination, possible LOI was observed in three first trimester samples for H19 (n=2) and MEG3 (n=1), and for MEST in a single term placenta.

Nevertheless, the imprinted allele was still largely repressed with allele ratios on average of 86:14. Our results indicate that imprinted gene expression in the human placenta is established early in gestation and that LOI occurs infrequently in uncomplicated pregnancies. In addition, maternal cell contamination of placentals samples was readily detected using this multiplexed assay and if not recognized, could be mistaken for LOI. This research was supported by the Indiana CTSI, funded in part by grant UL1 TR000006 from National Center for Advancing Translational Sciences, NIH; Sequenom assays were carried out in the Center for Medical Genomics, partly supported by the Indiana Genomics Initiative (INGEN), which is supported in part by the Lilly Endowment.

140. Functional Role of CHD1 During Bovine Early Embryonic Development.
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Fertility is poor in dairy cows (e.g. pregnancy rate to a single insemination typically < 40%). Around 40% of embryonic loss occurs during the first week after insemination, making early embryonic mortality a major cause of female infertility. However, molecular regulation of bovine early embryonic development is not well understood. The Chd family of proteins is characterized by the presence of chromodomains (chromatin organization modifier) and SNF2-related helicase/ATPase domains, which alter gene expression by modification of chromatin structure. Chd1-null embryos arrest at the peri-implantation stage in mice. However, the functional role of CHD1 during preimplantation embryonic development remains unknown given maternal CHD1 may mask the essential role of CHD1 during this stage in traditional knockout models. Hence, the objectives of the present studies were 1) to examine CHD1 expression during bovine early embryogenesis, 2) potential relationship of CHD1 transcript abundance with oocyte quality and 3) effects of CHD1 knockdown on preimplantation development. Oocytes (GV and MII) and early embryos (pronuclear, 2-, 4-, 8- and 16-cell, morula and blastocyst stages) were collected and quantitative RT-PCR analysis was performed. Results showed that CHD1 mRNA is elevated after meiotic maturation and remains increased through 16-cell stage followed by a sharp decrease at morula to blastocyst stage. To determine if CHD1 mRNA is present in early embryos is of maternal origin, presumptive zygotes were treated with α-amanitin (RNA polymerase II inhibitor) for 52 h (until 8-cell stage), or served as untreated controls (n=4 pools of 10
embryos/group). QPCR analysis showed that CHD1 mRNA level was partially decreased in response to α-amanitin treatment, suggesting CHD1 mRNA in 8-cell embryos is of both maternal and zygotic origin. To examine the relationship between CHD1 transcript abundance and oocyte quality, QPCR analysis was conducted on oocytes of abattoir origin subjected to Brilliant Cresyl Blue staining, a well-established model of oocyte quality linked to subsequent embryo developmental potential. Results indicated CHD1 mRNA was lower in oocytes negative for BCB staining (indicative of reduced oocyte quality) than oocytes that had completed the growth phase and were BCB positive, suggesting maternal CHD1 levels may influence embryogenesis. Thus, to determine the functional role of CHD1 in bovine early embryonic development, siRNAs targeting CHD1 were produced and validated procedures for siRNA-mediated ablation of endogenous CHD1 expression performed. Microinjection of CHD1 siRNA greatly reduced endogenous CHD1 mRNA level by >90% in bovine embryos collected at 42-44 hours post insemination (hpi) compared to either uninjected control or negative control siRNA-injected embryos. Corresponding reduction in CHD1 protein in CHD1 siRNA injected embryos was demonstrated by Western blot analysis. Presumptive zygotes were then injected with CHD1 siRNA, a negative control siRNA or served as uninjected controls and effects on total cleavage (48 hpi) and development to 8- to 16-cell (72 hpi) and blastocyst stages (d 7) were examined (n = 25-30 embryos/treatment; n = 6 replicates). Results demonstrated that percentage of embryos developing to 8- to 16-cell and blastocyst stages were both significantly reduced in response to CHD1 ablations. Results support a functional requirement of CHD1 for bovine preimplantation development.

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### 141. Combined effects of DNA methyltransferase 1o-deficiency and ovarian stimulation on embryonic outcome and epigenetic patterning at mid-gestation.

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The use of assisted reproductive technologies (ARTs) has been linked with an increased incidence of growth and genomic imprinting disorders in children, shown in some cases to be the result of aberrant DNA methylation. DNA methylation is a well-characterized epigenetic mechanism catalyzed by DNA methyltransferases (DNMTs). Mouse studies have shown reduced expression of DNMTs in the oocytes of aging females. We propose that factors related to underlying infertility (i.e. reduced expression of DNMTs) will increase offspring susceptibility to aberrant DNA methylation patterning, exacerbated by ART. We assessed the effects of maternal deficiency in an oocyte-specific DNMT1 (DNMT1o) model in combination with ovarian stimulation on offspring DNA methylation and development. Blastocysts were collected from superovulated control and Dnmt1o-heterozygote females (5.0 IU PMSG/hCG) and transferred non-surgically to recipients. Mid-gestation embryos and placentas were collected and assessed for developmental delay and morphological abnormalities. DNA methylation was examined at imprinted genes by pyrosequencing and global DNA methylation patterns by Reduced Representation Bisulfite Sequencing (RRBS), where a cutoff of 15-fold coverage was applied for analysis. Presently, 40 embryos have been collected from control superovulated donors and 39 from heterozygotes, with similar rates of pre- and post-implantation loss observed between groups. A trend towards an increased proportion of delayed and abnormal embryos was observed, from 8% in control to 18% in the Dnmt1o -heterozygotes. DNMT1o deficiency had no apparent effect on methylation of imprinted genes H19 and Snrpn in all embryo and placenta examined, as assessed by pyrosequencing (n=8-22/group). RRBS analysis from female placenta (n=4/group) revealed a total of 1685 100bp-tiles exhibiting DNA methylation changes of at least 20%, with both loss and gain of methylation observed (815 and 870 tiles, respectively). Fifty percent of these differentially methylated tiles (DMTs) mapped to intergenic regions, 32% to introns and 13% to exons, with highly significant changes occurring in each of these regions. Altered tiles were distributed across all chromosomes of the genome, including the X chromosome, where many genes responsible for placental function are found. Interestingly, greater methylation variability was observed in the placentas of Dnmt1o-heterozygotes compared to controls. RRBS analysis of female embryos (n=4/group) revealed 537 DMTs, with tiles exhibiting both loss and gain of methylation (267 and 270 tiles, respectively); 47% of these DMTs mapped to intergenic regions, 39% to introns, and 8% to exons. Of all regions identified as differentially methylated due to Dnmt1o deficiency (genotype effect), only 15 DMTs were identified as common in both placentas and embryos. These common DMTs were predominantly in intronic and intergenic regions, and the direction of change, either gain or loss, was variable between tissue types. Finally, in both the embryo and placenta, nearly half of the DMTs found in repeat regions mapped within LINE elements (46% in embryo, 52% in placenta), consistent with the distribution of all repeat elements covered by RRBS. These preliminary results indicate that DNMT1o deficiency exacerbates genome wide DNA methylation abnormalities induced by ovarian stimulation and may play a role in mediating poor embryonic outcome. (Supported by the Canadian Institutes of Health Research)

### 142. The Role of the Orphan Nuclear Receptor, Liver Receptor Homolog-1 (LRH-1, NR5A2), in the Transcriptional Program for Periovulatory Remodeling in the Ovary.

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Following the LH surge, the ovarian pre-ovulatory follicle enters an extensive program of cellular differentiation, orchestrated by major changes in the transcriptional profile of granulosa cells, ultimately triggering ovulation and luteinization, processes essential for...
Nuclear receptor Nr5a2 (also known as Liver receptor homolog-1) is an orphan nuclear receptor expressed in granulosa cells. It plays a crucial role in folliculogenesis, particularly in the pre-ovulatory stage. In mice, Nr5a2 is specifically expressed in granulosa cells, and its deletion results in infertility due to defective oocyte quality and reduced ovulation rates. Recent studies have shown that Nr5a2 regulates the expression of genes involved in oocyte maturation, cumulus expansion, and luteinization. The essential role of Nr5a2 in granulosa cell function is supported by the observation that Nr5a2 knockout mice exhibit reduced ovulation rates and immature oocytes.

The role of Nr5a2 in the development of antral follicles in primates is less well understood. Recent studies have shown that Nr5a2 is expressed in the granulosa cells of antral follicles in humans and non-human primates. This suggests that Nr5a2 may also play a role in the development of antral follicles in primates, similar to its function in mice. However, further studies are needed to fully understand the role of Nr5a2 in antral follicle development in primates.

143. Gonadotropin and Local Control of Folliculogenesis in Primates.
Richard L. Stouffer, Jing Xu.

Controlled ovarian stimulation, using exogenous gonadotropin hormones to generate multiple mature oocytes for assisted reproductive technologies, provides some understanding of antral follicle development in primates. However, the events and control of early follicular development in primates, up to the antral stage, remain poorly understood. We recently developed a three-dimensional (3-D) culture system, in which individual primate follicles are placed in alginate beads, that supports the growth of macaque (monkey) follicles from the primary and secondary (preantral) to small antral (1mm diameter) stages, with achievement of steroidogenic (estrogen) and gametogenic (oocyte meiotic maturation) function (Xu, J et al. Adv Exp Med Biol 761:43-67, 2013). Numerous factors influence the survival, growth rate and functional activity of the follicle cohort, including monkey age, stage of the menstrual cycle at ovary collection, culture conditions (e.g., O2 milieu) and presence of hormonal factors (gonadotropins, insulin). Four subgroups of follicles were noted: (a) those that did not survive in culture, (b) those that survived but did not grow (no-grow), (c) those that grew slowly over 5 weeks to twice their original diameter (slow-grow), and (d) those that grew to > 4-times their original diameter (fast-grow). This culture system permits studies exploring the actions of autocrine and paracrine factors (e.g., steroids and anti-mullerian hormone, AMH) on early follicular development in vitro. Local steroid action appears critical since suppression of steroid synthesis (using a 3-β-hydroxysteroid dehydrogenase inhibitor, Trilostane) significantly reduced follicle survival, antrum formation and growth rate, plus oocyte quality. Add back experiments indicated that androgen (testosterone T; dihydrotestosterone, DHT) and estrogen (estradiol, E2) promote follicle survival. Both T and E2 promoted follicle growth and antrum formation, but T’s action appears indirect due to aromatization, since DHT did not exhibit this effect. Notably T and DHT, but not E2, suppressed AMH production and only DHT inhibited E2 production. Both androgens and estrogen promoted oocyte quality, growth and meiotic maturation. In contrast, progestin (R5020) add back had little effect, except to promote oocyte degeneration at high (intrafollicular) doses. This culture system can also be used to evaluate the effects of in vivo manipulation on early folliculogenesis in vitro. For example, administration of a high-fat, western-style diet (WSD), for 1.5 years, alone and with testosterone (T) treatment, resulted in secondary follicles with poor survival, reduced E2 production, and smaller oocytes, compared to age-matched controls. Moreover, follicles from WSD + T treated animals produced less AMH and vascular endothelial growth factor (VEGF-A), compared to those of controls or WSD treated monkeys. The 3-D culture system applied to preantral follicles provides a valuable means for investigating the endocrine and local control of early follicular development in primates. Initial studies suggest that local androgen action promotes early follicular development but suppresses the activity of preantral (AMH) and small antral (E2) follicles.

US4 and P50HD071836, R21RR030276, P51OD011092, K12HD043488

144. The p44 subunit of general transcription factor IIIH (GTF2H2) regulates oocyte-to-embryo transition in mice.
You-Qiang Su, Xinyue Zhang, Lanying Shi, Hong Yin.

The production of a normal oocyte competent to complete meiosis and undergo fertilization and preimplantation development requires precise control of gene expression and the maintenance of genomic integrity within the oocyte. However, factors that specifically regulate these oocyte quality-determining events remain largely undiscovered. Here, using the “ENU-mutagenesis”-based forward genetic approach, we revealed that general transcription factor IIIH (TFIHH), a protein with remarkable dual roles in the control of transcription and DNA nucleotide excision repair (NER), is indispensable for oocytes to acquire full meiotic and developmental competence in mice. We found that mutation of Gtf2h2, the gene encoding p44 subunit of TFIHH, causes infertility in female, but not male, mice. Gtf2h2 mutant females ovulate the similar number of mature oocytes embedded in the well-expanded cumulus mass as by the wild type controls. However, when the ovulated Gtf2h2 mutant eggs were inseminated by normal spermatozoa in vitro, few of them were activated and formed 2-cell stage embryos, and no mutant embryos developed beyond the 2-cell stage. Furthermore, we found that Gtf2h2 is robustly expressed by oocytes as compared to granulosa cells and other types of tissues. These data, therefore, indicate that GTF2H2 is a novel maternal factor essential for oocyte-to-embryo transition. Consistent with TFIHH’s general nuclear role in transcription and NER, we also found that GTF2H2 localizes to the nucleus of the GV-stage oocyte with punctuate distribution beneath the nuclear lamina, and co-localizes with spindles at MI- and MII-stage oocytes with more enrichment at the spindle poles. Although the number of ovulated oocytes having the first polar body is similar between wild type and mutant females, there are more oocytes in mutant failed to progress to...
metaphase II and displayed abnormal conformations of spindles and chromosomes. These suggest that GTF2H2 also regulate meiotic progression in oocytes, agreeing well with the cell-cycle regulating function of the cyclin-dependent kinase activating kinase (CAK) subunits of TFIH. Taken together, our data indicate that GTF2H2 is a key intrinsic factor determining oocyte quality, and point to the existence of an oocyte-specific pathway regulating transcription and genomic integrity. [Supported by the Major Basic Research Program (grant # 2014CB943200, 2013CB945500) and the National Natural Science Foundation of China (grant # 31471351, 31271538) to YQS].

145. Oocyte-secreted Factors (OSFs) in *Bos taurus* are identified using Bioinformatics. Beatriz E. Castro-Valenzuela¹, M.Eduvigis Burrola-Barraza², Joel Dominguez-Viveros³, Concepción Martínez-Lozoya¹.¹ Facultad de Zootecnia y Ecologia, Universidad Autónoma de Chihuahua, Chihuahua, Chihuahua, Mexico; ²Facultad de Zootecnia y Ecologia, Universidad Autónoma de Chihuahua, Chihuahua, Chihuahua, Mexico; ³Facultad de Zootecnia y Ecologia, Universidad Autónoma de Chihuahua, Chihuahua, Chihuahua, Mexico

Advances in assisted reproductive systems have significantly influenced animal breeding in cattle production systems. However, the efficiency of in vitro embryo production remains low, with only 40% of oocytes undergoing maturation up to the blastocyst stage during fertilization compared to 80% in vivo. Oocyte quality greatly impacts early embryonic survival and it is acquired during folliculogenesis. During this process, oocytes are able to regulate their own microenvironment through bidirectional communication with follicular cells, and this communication is mediated by direct cell contact and oocyte- secreted factors (OSFs). OSFs regulate important functions in granulosa and cumulus cells, including cell growth, regulation of metabolism, cell expansion, modulation of steroidogenesis, and prevention of cell death. In cattle, only a few OSFs have been identified. These include GDF9 and BMP15, which have improved the rate of in vitro oocyte maturation up to the blastocyst stage from 40% to 60%. Until this day, many OSFs remain unknown. However, given their role in folliculogenesis, is important to identify potential OSFs and investigate their capacity to promote the culture and maturation of oocytes in vitro, with the goal of achieving higher rates of viable blastocyst formation. Therefore, the aim of this study was to identify OSFs using a bioinformatic analysis method that consisted of three phases. First, Expressed sequence tag (ESTs) from protein of bovine oocytes were obtained from the GenBank database of the National Center for Biotechnology Information (NCBI). Furthermore, EMBOSS Transeq server was used to predict protein sequences in ESTs with no associated protein. Second, secreted proteins were predicted using SignalP, SecretomeP, TargetP, and TMHMM servers. Third, proteins of the secretome were classified using the following NCBI databases: InterProScan, KEGG Brite, KEGG Pathway, and BLASTP. Of the 2167 EST sequences analyzed, 1237 sequences represented different proteins, and SignalP predicted 87 of these to be classical secreted proteins. The remaining non-classical secreted proteins (n = 1150) were processed by SecretomeP and 89 non-classical secreted proteins were predicted. Both sets of classical and non-classical secreted proteins (n = 176) were then analyzed by TargetP to predict mitochondrial proteins. Results showed that only three of the proteins have a mitochondrial origin; also, 22 proteins have a predicted localization different from the mitochondria and 52 proteins have no predicted localization at all. The remaining 99 proteins were analyzed by TMHMM to predict transmembrane proteins. Twenty-nine proteins were predicted to contain one or more transmembrane helices. Of the 70 secreted proteins only eighteen sequences identified were subsequently found to belong to: SDF2L1, CARTPT, OOSP1, TIP-1, MMP1, TNFAIPG, PTGS2, POSTN, P4HA3, TNCCSTK, CTSK, PTX3, PMP2, PSAP, SEPINEZ, SRGN, INHBA, and SRPX. These proteins potentially represent OSFs in cattle, and further studies are necessary to determine whether they affect the maturation and fertilization of oocytes. For these studies, the proteins of interest would be recombinantly expressed and purified, and then they would be applied to in vitro cultures of oocytes.

146. Development of orally active positive allosteric modulators of follicle stimulating hormone receptor for infertility treatment in assisted reproductive technology. Stephen S. Palmer¹, Selva Nataraja¹, Henry Yu¹.¹ TocopheRx, Inc., Burlington, MA, USA

Follicle-stimulating hormone (FSH) is one of the primary glycoprotein hormones involved in mammalian reproduction and is necessary for follicular development in female and spermatogenesis in males. FSH is prescribed for controlled ovarian hyperstimulation (COH) in patients undergoing either ovulation induction followed by intrauterine insemination (OI-IUI), or by in vitro fertilization (COH-IVF) cycle. One COH treatment cycle involves repeated subcutaneous injections of FSH that adds to the significant stress associated with fertility treatment. The availability of an orally active FSH receptor agonist (FSHR; MW<600) for COH equipotent to injectable FSH would: a) meet patients and physicians requests for more effective therapies to be available earlier in the fertility journey; b) reduce the average number of treatment cycles required to become pregnant, and c) reduce the proportion of patients that dropout from fertility treatments because of repeated failures with less effective treatments (clomiphene) or following multiple injections of FSH. Although several attempts have been made to develop an oral FSH agonist, none has yet been successful. Following a campaign of high throughput screening, a rational drug discovery strategy employing combinatorial chemistry was adopted to identify oral FSHR mimetics. TocopheRx (TOP) discovered a highly potent FSHR allosteric agonist, TOP002, with good potency through this effort and it is being further evaluated as a candidate for clinical development. TOP002 stimulated cAMP in CHO cells expressing hFSH with an EC50 of 0.98±0.31 nM, while it was less effective in stimulating CHO cells with hTSHR (EC50=176±58 nM) and hLHR (EC50=34±13 nM). In the physiologically relevant rat granulosa cells, TOP002 stimulated estradiol production with an EC50 of 37±18 nM in the presence of 0.02 nM recombinant FSH, and 103±33 nM in the absence of FSH. In human granulosa cells, TOP00002 dose dependently increased estradiol secretion in the media with EC50 at 44±16 nM in the absence of FSH. TOP002 is a more selective agonist for the FSHR than for the LH receptor. In primary cultures of rat Leydig cells, TOP002 (in absence of added LH) increased testosterone production at much higher concentrations (EC50 = 1980 nM). TOP002 is an orally active FSH agonist in traditional immature rat ovarian stimulation animal models. Five groups of immature female rats were provided TOP00002 orally, at doses ranging from 0.5 mg/kg to 20 mg/kg, and other groups received as controls, low dose or high dose of hFSH by injection. TOP00002 showed dose dependent increase in the number of eggs released into the oviduct 18 hours after hCG administration to induce ovulation. Injection of hCG alone caused release of fewer than 5 oocytes. TOP002 was effective over the dose range from 1 to 20 mg/Kg body weight. At 5 mg/kg TOP002, the number of oocytes obtained (46±5.3) was similar to the response obtained with the maximally effective injected dose of FSH (4.6 IU total dose, 4 injections; 47.6±5.6). The lowest detectable
response to FSH (1.1 IU total dose, 4 injections; 6.6 ± 1.5 oocytes) is slightly greater than the response obtained with hCG alone (4-5 oocytes). This molecule is currently being evaluated in preclinical safety and toxicology studies to develop as a clinical candidate.

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The emergence of CRISPR (Clustered regularly interspaced short palindromic repeat)/Cas (CRISPR associated) system has opened the window for the next generation targeted mutagenesis. The combination of Cas9 protein with single guide RNA (sgRNA) could reconstitute RNA-based nucleases, and thus prepared Cas9/gRNA complex recognizes the target 20 nucleotides with PAM (NGG) sequence and causes double strand break (DSB). The subsequent error-prone non-homologous end joining (NHEJ) would result in small indels. If ssODN (single stranded deoxyoligonucleotide) or dsDNA (double stranded DNA) are co-introduced, the designed mutation could be introduced into the target locus via homology dependent repair (HDR). Here we present our recent results of CRISPR/CAS mediated mutagenesis in mice. We first established the validation system for sgRNA mediated Cas9 cleavage in mammalian cells. In brief, the plasmids expressing hCas9 and gRNA were prepared by ligating oligos into BbsI site of pX330 (http://www.addgene.org/42230/). The reporter plasmid was prepared by placing the ~500bp target region between N- and C- terminal EGFP coding regions in pCAG-EGxFP (http://www.addgene.org/50716/). Once the target region was cleaved in the co-transfected cells, reconstitution of EGFP expression cassette by HDR would give us the green fluorescence. When we injected the pX330 plasmids into pronucleus of the zygote at 5 ng/ul, 52.9 ± 22.3% (100/196) of pups carried NHEJ mutations. When we co-injected ssODN at 100 ng/ul, HDR mediated point mutations were introduced about one third of the pups (17/50). We conclude that combination of our sgRNA validation system and subsequent pX330 plasmid injection into zygote provides a simple, efficient, and cost-effective way for mammalian gene editing that is applicable for the study of reproductive system in mammals.

148. HENMT1 Is Required for piRNA Stability and Both Male and Female Fertility. Moira O'Bryan1, Shu Ly Lim1.
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The Piwi-interacting RNA (piRNA) pathway is an RNA silencing pathway that represses the expression of transposable elements (TE) in spermatozoa via binding of piRNAs to their complimentary RNA targets. Mammalian piRNAs are 26–31 nt in length and are 2′–O–methylated at their 3′ termini. The role of piRNAs in adult male germ cells types, however, wherein the majority of piRNA sequences are not complementary to TE sequences, remains poorly defined. To address this question we have used a unique mouse model of Henmt1 dysfunction (Henmt1Pim/Pim), and ultimately piRNA instability, to define the effects of piRNA depletion on both male and female fertility. Henmt1 is an RNA methyltransferase that acts to add stabilizing 3′ methyl groups to piRNAs. The loss of piRNAs in adult male germ cells results in male sterility characterized by TE over-expression, the precocious expression of haploid germ cell transcripts in meiotic cells and a catastrophic deregulation of the haploid germ cell program. Our data strongly suggests a role for piRNA in promoting a heterochromatin state during meiosis and their necessity to set an appropriate spermatogenic gene expression program. Further our data show Henmt1 dysfunction in female mice leads to a sub-fertility phenotype reminiscent of premature ovarian failure in humans. Specifically, Henmt1Pim/Pim females are fertile while young, but have depleted ovarian reserves by six months-of-age. Further, even when young the quality of oocytes from Henmt1Pim/Pim females is significantly compromised. Collectively these data suggest a role for HENMT1 and piRNAs in oocyte survival and the regulation genes critically involved in adult oocytes function.

149. Seipin Deficiency Increases Chromocenter Fragmentation and Spermatid Apoptosis Leading to Male Infertility. Ahamed E. El Zowalaty1, Claudia Baumann2, Rong Li3, Weiqin Chen4, Rabindranath De La Fuente5, Xiaoqin Ye1.
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Seipin is encoded by Berardinelli-Seip congenital lipodystrophy 2 (Bsel2) gene. It is an integral endoplasmic reticulum membrane protein with known functions in adipogenesis. Bsel2−/− males were infertile but had normal mating behavior. Bsel2−/− cauda epididymis sperm count was ~20× less than control and Bsel2−/− sperm had impaired motility. Testis histology and vimentin immunofluorescence indicated that the Bsel2−/− seminiferous tubules had relatively normal presence of Sertoli cells, spermatogonia, and spermatocytes, but reduced spermatids and sperm. Additionally, the Bsel2−/− spermatids were often seen disoriented throughout the seminiferous epithelium. In situ hybridization in the Bsel2−/− tests from postnatal day 15 (PND15) to adult demonstrated low level of expression at PND15 but prominent Bsel2 transcriptional activity in the spermatocytes with a plateau reached ~PND28. The spatiotemporal expression of Bsel2 mRNA suggested its expression in both pachytene spermatocytes and secondary spermatocytes. However, immunohistochemistry revealed seipin protein localization in the postmeiotic spermatids, suggesting translational repression of Bsel2 mRNA in spermatocytes. BrDU (5-bromo-3-deoxyuridine) labeling detected comparable germ cell proliferation but ISEL+ (in situ end-labeling plus) detected increased spermatid apoptosis in the Bsel2−/− tests compared to control. Immunofluorescence of marker proteins SYCP3, SYCP1 and H3K9me3 in germ cell spreads detected normal meiotic chromosome pairing and homologous chromosome synapsis in the Bsel2−/− spermatocytes. However, there were significantly increased percentages of round spermatids with chromocenter fragmentation, and late spermatids and sperm with chromatin vacuoles, indicating impaired chromatin integrity and defective chromatin condensation in the Bsel2−/− spermatids. Protamins 1 and 2 are involved in chromatin compaction during spermiogenesis and are translationally repressed in the round spermatids. Their transcripts were downregulated in the Bsel2−/− tests. In addition, Bsel2−/− sperm had abnormal acrosomes compared to the control. These data demonstrate a novel role of seipin in spermatid chromatin integrity and compaction during spermiogenesis. Increased spermatid apoptosis and defective chromatin condensation contributed to decreased sperm production and impaired sperm motility that resulted in Bsel2−/− male infertility. (Supported by NIH R15HD066301 and NIH R01HD065939 to XY, NIH 2R01-HD042740 and the Georgia Cancer Coalition to RDLF)
In the testis, germ cell differentiation is accompanied by dramatic changes in Golgi apparatus structure and function. These events coincide with mitosis of spermatagonia, meiosis of spermatocytes, acrosome formation by early spermatids, and Golgi migration in late spermatids undergoing extensive changes in plasma membrane and cell shape remodeling. At the last step of spermiogenesis, step 19 spermatids, isolated Golgi cisternae congregate into the forming cytoplasmic droplet where they are retained as sperm pass through the epididymis. The protein complement of germ cell Golgi apparatus during the course of spermatogenesis is unknown, and such information could help reveal functions of the Golgi apparatus as it relates to germ cell differentiation. To address this, abundant proteins were characterized quantitatively by tandem mass spectrometry of germ cell Golgi fractions isolated from adult rat whole testis homogenates. From 1318 proteins assigned to 22 functional categories, antibodies were generated or obtained to 20 of these proteins and examined by light microscope immunohistochemical analysis. The most abundant protein was GL54D, a protein of unknown function not previously uncovered but shown here to be a Golgi localized type II integral membrane glycoprotein and germ cell-specific. It was first detected in the Golgi apparatus of pachytene spermatocytes at stage VII of the cycle coincident with glucose transporter 3 (GLUT-3) suggesting a functional role in connection with this protein. Another protein of unknown function, TM9SF3, was a new universal Golgi marker for both somatic and germ cells, with expression in the latter from spermatagonia up to step 17 spermatids. Two other TM9 protein family members were identified and predicted to be Golgi markers based on extensive organelle sub-fractionation. The TMED7/p27 Golgi protein was also expressed from spermatagonia to spermatids but was retained up to step 19, while TMED4/p25 was maintained only up to step 17 spermatids. Selective expression of MG160 was noted in spermatocytes. During acrosome formation, different Golgi localized proteins were segregated to the Golgi apparatus (TMED7/p27, TMED4/p25, GL54D, TM9SF3) or were shared with the forming acrosome (GRASP55, GPP34, GBF1, ManIX, Man2alpha 1, LAMAN) from steps 1-7 spermatids. The former Golgi proteins retained Golgi identity as the Golgi apparatus migrated away from the acrosome after its formation, while the latter remained trapped in the acrosome, without expression in the Golgi apparatus. Based on the maturation model for acrosome formation, such segregation is unexpected. Additionally, unexpected proteins (UBXD8, PDILT, Fam3C, sapreticulin and HSP70.2) localized to both the Golgi apparatus and acrosome from steps 1-7, but were only retained in the acrosome beyond step 8 spermatids. While not expressed early in spermatogenesis, sortilin first appeared in the Golgi apparatus at step 8 spermatids and was maintained up to step 19. Also unexpected was the finding of selective expression of Golgi proteins (TMED2/p24 and carboxypeptidase D) to the forming cytoplasmic droplet in step 19 spermatids, enriched in unstacked Golgi cisternae of unknown function. Several proteins, localized to the Golgi apparatus of earlier germ cells, revealed renewed expression at step 19, but with the exception of TMED7/p27, Golgi proteins were not abundant. When the localization of the 20 Golgi localized proteins were compared with 30 proteins characteristic of plasma membrane, endosome, endoplasmic reticulum, coats and cytosol, organelle based molecular signatures were uncovered for all 63 morphologically distinct germ cells undergoing spermatogenesis. In addition, besides well-characterized Golgi proteins, there were over 230 proteins of unknown function enriched in germ cell Golgi fractions. Thus, a marker-based resource with insight from the cognate protein functions has been established to uncover new mechanisms and proteins in Golgi membrane trafficking and fate during germ cell differentiation. (Supported by CHIR).

FOCUS SESSION 15: Oocytes Unscrambled.

152. Establishing and Maintaining a Pool of High Quality Oocytes.
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How is the length of the female fertile lifespan regulated? The answer to this question is not yet clear and the suite of factors that influence the duration of female fertility are likely to be complex in nature. However, two key parameters involved in the regulation of reproductive longevity have been identified: i) the number of available oocytes, which are stored in the ovary as primordial follicles, and ii)
the quality of those oocytes that survive the process of folliculogenesis and eventually ovulate. Growing evidence indicates that the intrinsic apoptosis pathway, which is controlled by the relative levels and activities of the members of the B-cell lymphoma-2 (BCL-2) family, plays an important role in regulating the number of primordial follicles established in the ovary at birth as well as the number of primordial follicles maintained throughout reproductive life. Because of this, the pro- and anti-apoptotic BCL-2 family of proteins are emerging as key determinants of the length of the female fertile lifespan. This talk will cover the relationship between the intrinsic apoptosis pathway, primordial follicle number and length of the female fertile lifespan. This talk will also include a discussion regarding the importance of apoptosis for ensuring that only the highest quality oocytes are available for ovulation and perpetuation of the next generation.

153. Programming the Oocyte with Zinc.
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Oocyte quality refers to the ability of the oocyte to support maturation, fertilization, and embryo development. The quality of the oocyte is established during oogenesis and is supported by multiple interactions with the somatic cells of the follicle and by endocrine and metabolic processes. Many environmental and physiologic factors can influence the course of oogenesis and hence can alter the quality of the oocyte. Nutritional factors are among the most important determinants of oocyte quality, but the mechanisms by which specific nutrients regulate oocyte development are just now coming into focus. Recent work by our lab and others using in vitro methods show unequivocally that the mineral zinc has profound effects on oocyte maturation and the production of high quality oocytes. Zinc is tightly regulated during oocyte maturation. In GV-stage oocytes, free intracellular zinc is low, but increases dramatically after maturation. This abrupt increase in zinc is regulated by cumulus cells which secrete a putative zinc inhibitory factor (ZIF) that prevents an influx of zinc before maturation. However, ovulatory signals abolish ZIF activity and allow zinc to increase in the oocyte. Blocking this increase in zinc with a metal chelator, such as TPEN, causes severe spindle defects and meiotic arrest before metaphase II in a high proportion of oocytes. However, even oocytes that make it through meiosis are less competent to undergo fertilization and preimplantation embryo development with fewer oocytes forming 2-cell and blastocyst embryos. Importantly, our subsequent in vivo studies show that similar meiotic and preimplantation defects are observed when female mice were fed a zinc-deficient diet for 3-5 days before ovulation. This shows that in vitro effects of zinc deficiency are recapitulated in vivo. Unexpectedly, developmental defects of acute preconception zinc deficiency continue after implantation. For example, trophoblast cells are less able to differentiate in vitro (outgrowth assay) when the mothers were fed a zinc deficient diet preconception. Indeed, we also observed that the resulting placentas later in pregnancy were much smaller, mostly due to a decrease in development of the fetal side of the placenta. Finally, fetal growth was slowed by preconception zinc deficiency and we observed a high proportion of fetuses with neural tube defects as late as embryonic day 16.5. The mechanisms responsible for the defects caused by preconception zinc deficiency are likely complex because zinc binds to over 300 proteins, but one pathway that is critically impacted is the generations of s-adenosylmethionine (SAM), a universal methyl donor required for DNA and histone methylation. SAM is lower in live of mothers fed a zinc deficient diet and this is associated with a global decrease in histone H3K4 trimethylation and DNA methylation in GV-stage oocytes. Supplementing zinc deficient oocytes with exogenous SAM during in vitro maturation improves fertilization rate indicating that SAM synthesis is one pathway altered by zinc deficiency in the oocyte. Thus, acute preconception zinc deficiency causes profound meiotic and developmental defects, but much more work is needed to gain a comprehensive understanding of the zinc-dependent pathways regulating oocyte development and function.

154. TAF4b directs an oocyte-specific gene regulatory network required for primordial follicle assembly.
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The condition of premature ovarian aging, or primary ovarian insufficiency (POI), affects 1% of women under 40, and can be induced by ovarian follicle disruption or depletion. However, little is known about the developmental or molecular etiology of this condition and its association with infertility in women. TAF4b is a gonad-enriched subunit of the general transcription factor TFIID that is implicated in promoting healthy ovarian aging and female fertility in mice and in women. TAF4b-deficient mice experience hallmarks of POI, including premature follicle depletion, poor oocyte quality and infertility. While this phenotype is well-established and model several aspects of POI in women, the molecular mechanisms underlying these aspects of infertility remain unknown. To better understand the developmental timing of TAF4b in promoting long term ovarian health and fertility, we sought to elucidate the earliest ovarian defects in the context of the TAF4b-deficient mice. This recent analysis revealed an unexpected role of TAF4b in the regulation of primordial follicle assembly. While TAF4b-deficient oocytes contain a normal complement of Tra98-positive oocytes at embryonic day (E)18.5, the majority of these oocytes are excessively depleted by caspase-dependent apoptosis at birth when a dramatic increase in activated caspase 3-positive germ cells are observed. Additionally, germ cell loss in these oocytes can be prevented by ex vivo culture of embryonic TAF4b-deficient oocytes in the presence of the pan- caspase inhibitor ZVAD-FMK, further implicating apoptosis as a primary mechanism for excessive germ cell loss. Finally, neonatal TAF4b-deficient mice experience delayed cyst breakdown, resulting in large cysts of germ cells during a time when control ovaries have begun forming a robust primordial follicle pool. To better understand the mechanistic functions of TAF4b in regulating primordial follicle assembly and post-natal oocyte survival, we have utilized RNA deep sequencing to uncover gene expression changes in fetal TAF4b-deficient ovaries at E18.5, prior to germ cell loss, compared to control ovaries. We have found that at E18.5, TAF4b-deficient ovaries display significantly reduced mRNA expression of critical oocyte-expressed genes that are well known to function in meiosis, transcription, and piRNA-regulating processes. These affected genes include Dazl, Sycp3, MSY2, Lhcx8, Nobox, and Mov10I. In addition to observing differential mRNA expression, we have confirmed the reduced expression of Dazl, MSY2, and Nobox protein in neonatal TAF4b-deficient oocytes by immunofluorescence. As a number of these proteins are known to function in meiosis, we observe a concomitant increase in meiotic double strand breaks and diplotene meiotic delay. We are currently using chromatin immunoprecipitation assays with TAF4b-specific antibodies to test the hypothesis that TAF4b acts as a master regulator of oocyte transcription and development during the embryonic-to-neonatal transition. This is the time at which the ovarian reserve is being
established, and as part of a germ cell-specialized version of the TFIID complex, TAF4b regulates the precise expression of key oogenic and meiotic genes. Our research serves the dual purpose of helping to better understand the ways in which general transcription factor variants can work in specific developmental contexts, especially in the germline. It also helps to refine our understanding of the pathological mechanisms underlying premature ovarian aging. Moreover, a better understanding of the molecular and developmental mechanisms responsible for establishing the oocyte reserve in the mouse ovary will be useful in better managing conditions of infertility and in promoting long term ovarian health and fertility in women.

155. Protein SUMOylation is essential for oocyte development and female fertility.  
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The reproductive lifespan in women spans puberty to the menopause. In about 1-2% of women, early menopause occurs. This negatively impacts a woman's health and well-being, and can result in increased cardiovascular disease, bone loss, sexual dysfunction, hot flashes, or even early death. Menopausal age is largely determined by the number of oocytes found in the non-growing pool of primordial follicles that make up the ovarian reserve. In mice and humans, the ovarian reserve appears to be set by birth. Mouse models have demonstrated that oocyte-specific transcription factors are required for the formation, activation, and development of oocytes within the ovarian reserve. Deletion of these genes, which include newborn ovary homeobox gene (Nobox), spermatogenesis and oogenesis basic helix-loop helix gene (Sohlh1 and Sohlh2), and factor in the germline alpha (Figla), causes sterility and premature oocyte loss during the postnatal period. Furthermore, mutations in some of these genes, including NOBOX and FIGLA, are associated with premature ovarian failure in women. While these transcription factors are necessary for oocyte development, surprisingly little is known about how their activity is regulated. We performed an in silico analysis of these oocyte-specific transcription factors and found that they contain a conserved consensus site for a post-translational modification called SUMOylation. SUMOylation controls protein stability, localization, and activity, but its function in the developing oocyte is unknown. Therefore, we generated a novel oocyte-specific knockout of Ubc9, a central component of the SUMOylation cascade. Ubc94lox4lox Gdf9-icre female mice are sterile and have almost complete depletion of oocytes by two months of age. Prior to germ cell depletion, these mice have compound ovarian defects in follicle development, ovulation, and oocyte meiotic maturation. Localization assays demonstrate that SUMOylation regulates oocyte-specific transcription factors through several different mechanisms, including altered nucleo-cytoplasmic shuttling. This novel mouse model provides a key discovery tool for uncovering candidate pathways involved in premature ovarian failure and ovarian dysfunction, and establishes SUMOylation as an essential process during oocyte development.

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Germ cells develop in a microenvironment generated by the somatic compartment of the gonad. In female mammals, the follicular granulosa cells that surround the oocyte constitute its microenvironment and provide signals that regulate homeostatic processes and its developmental progression of the oocyte. Many of these signals are transmitted through gap junctions that link the oocyte and granulosa cells, and impaired gap junctional communication prevents normal oocyte development. As the oocyte grows, however, it secretes an extracellular matrix termed the zona pellucida that separates it from the granulosa cells and imposes a physical barrier to contact and communication. This barrier is bypassed by means of narrow cytoplasmic extensions, termed transzonal projections (TZPs), that extend from the granulosa cells, traverse the zona and contact the oocyte. The tips of TZPs harbor the essential gap junctions. TZPs have been identified in all mammalian species studied to date and, as the sole means by which the granulosa cells establish contact-dependent communication with the oocyte, are indispensable for oocyte development. However, despite their crucial role in fertility, the mechanism by which TZPs are generated is completely unknown. We found that the TZPs increase in both number and density as the oocyte grows, both in vivo and in vitro. This indicates that, as oocytes grow, an unknown mechanism in the surrounding granulosa cells actively generates new TZPs that establish new sites of contact and gap junctions with the oocyte. Myo10, Daam1 and fascin are actin-associated proteins that play key roles in the formation of filopodia. We found that granulosa cells express all three factors. Moreover, Myo10 and Daam1 are localized in foci located at the plasma membrane on the zona-facing side of the granulosa cells immediately adjacent to the oocyte. Removing the oocyte from granulosa-oocyte complexes decreases the amount of mRNA encoding fascin in the remaining shell of granulosa cell, and a similar reduction occurs when intact GOCs were treated with an inhibitor of the SMAD signaling pathway. Based on these results we propose a new model according to which TZPs are specialized filopodia that are elaborated by granulosa cells and attracted by oocyte-secreted factors to the oocyte, where they establish contact and communication required for normal oocyte development. This work suggests a novel mechanism by which oocytes establish contact and communication with the somatic microenvironment and identify a key parameter to monitor in future work aimed at growing oocytes in vitro to preserve human and animal fertility.

157. The Program of Maternal mRNA Translation During Mouse Oocyte Meiosis: Complex Interactions and Multiple Inputs Control Protein Synthesis.  
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In most species, oocyte growth is associated with the accumulation of large amounts of mRNAs that are stable and not translated. Translation of these maternal mRNAs is activated during oocyte maturation and the early stages of embryo development. In some species, embryo development up to gastrulation depends exclusively on translation of these mRNAs accumulated during growth, in the absence of gene transcription. The mechanisms underlying this post-transcriptional program have been extensively studied in model
organisms but it is unclear whether the same regulations function in mammalian oocytes. We have used a genome-wide strategy to investigate patterns of maternal mRNA translation in the fully grown mouse oocyte and found that approximately 1/3 of the maternal mRNAs are recruited to translating polysomes during oocyte progression through the cell cycle, and 1/3 are released from the polysomes and often degraded, while the rest are constitutively translated. By analyzing the properties of the co-regulated mRNAs we found that binding elements for the Deleted in Azoospermia Like (DAZL) protein are enriched in a subset of transcripts whose translation is activated during maturation. DAZL plays a pivotal role during primordial germ cell development but its function in fully grown oocytes was not investigated. Loss of function studies confirmed that DAZL protein plays a role in translation of these mRNAs and the function of this RNA binding protein (RBP) is indispensable for oocyte maturation and early embryo development. Using a candidate approach, we determined that DAZL is required to recruit ribosomes to these mRNAs, that binding of multiple DAZL protein molecules is necessary for translational activation, and that accumulation of DAZL protein is necessary for progression through the meiotic cell cycle. Thus, DAZL is a major regulator of translation in fully grown oocytes. DAZL is also required for the CPEB1-dependent translation of a subset of maternal mRNAs. CPEB1 is an RBP required for polyadenylation of mRNAs, and, in frog, it regulates the translational activation that is in turn necessary to reenter the cell cycle. However, CPEB1-dependent activation of translation in mouse oocytes requires prior MPF activation and occurs well after oocyte reentry into the cell cycle. Thus, although the same proteins are involved, their regulation differs in mouse and frog oocytes. Finally, we found that meiotic resumption is necessary for translational activation, but that these regulations are not exclusively oocyte cell autonomous. Inputs from somatic cells surrounding the oocyte contribute to activation of translation of a subset of mRNAs and this somatic cell-dependent component is necessary for the developmental competence of the oocyte. Thus, multiple mechanisms of translational control are active in mouse oocytes and are required for completion of meiosis and for generation of an egg able to sustain embryo development. (Supported by NIH RO1 GM097165, RO1 GM115241 and P50 HD055764)

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The ability to develop human oocytes from the earliest follicular stages through to maturation and fertilisation in vitro could revolutionise fertility preservation practice. This has been achieved in mouse where in vitro grown (IVG) oocytes from primordial follicles have resulted in the production of live offspring. However, developing IVG systems to support complete development of human oocytes has been more difficult because of differences in scale of timing and size. The aim of our work is to determine whether complete oocyte development can be achieved from human ovarian tissue grown in a multi-step culture system. We have developed a dynamic 3 step culture system that supports the activation of primordial follicles (In Vitro Activation (IVA) step 1) growth of multi-laminar follicles (In Vitro Growth (IVG) step 2) and oocyte growth out with the large follicular environment (step 3). Using this system a population of oocytes capable of reaching Metaphase II can be obtained. This system also allows us to test the role of various regulatory factors such as components of the PI3K pathway (e.g. PTEN, mTOR1) on human oocyte development. This presentation will focus on the challenge that lies ahead to improve quantity and quality in vitro grown human oocytes and will discuss the effect of various regulatory factors and age on human oocyte development in vitro.

159. LHX8 maintains meiosis arrest during ovarian folliculogenesis.
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LHX8 is a LIM homeodomain protein, preferentially expressed in oocytes beginning at E18.5, and throughout folliculogenesis. LHX8 is essential for early folliculogenesis and global knockouts to early ovarian failure due to block in primordial and primary follicle formation. In mammals, oocytes are arrested at diplotene stage of prophase I of meiosis circa E18.5, until luteinizing hormone (LH) stimulates meiotic resumption of oocytes in preovulatory follicles. Upon resumption of meiosis, the chromatin becomes condensed and accumulates in contact with the germlinal nuclear/vesicle envelope, which subsequently break down (GVBD). By using of a Zp3Cre induced conditional knockout of LIM homeobox protein 8 (Lhx8), we depleted LHX8 from primary oocytes. We found that the loss of Lhx8 in oocytes of primary follicles caused premature condensation of chromatin, associated with enhanced trimethylated lysine 9 of histone H3 (H3K9me3). Some primary oocytes at postnatal day 21 (PD21) had chromat in configuration, which usually happens at the beginning of GVBD, and showed discontinuous nuclear envelope surrounding the condensed chromatin granules, as revealed by nuclear envelope marker Lamin A/C. The transcription of genes necessary to maintain meiosis arrest was significantly down-regulated in PD16 Lhx8Zp3Cre oocytes, and included Gpr3 (4-fold), Wec2 (3-fold) and Myt1 (2-fold). We also detected down-regulation of other meiotic genes, including Cdc25b (4-fold) and Ccnb1 (4-fold), but not Cdc25a or Cdk1. However, spindle-shaped microtubules were not observed in the premature oocytes, although the microtubule network was reorganized compared with the uniform distribution throughout the ooplasm in control oocytes. Furthermore, we found that pericentric heterochromatin structure was disrupted in Lhx8Zp3Cre primary oocytes, as indicated by major satellite DNA FISH and its reduced transcription, which may shake kinetochore stability and impairs kinetochore-microtubule attachment. Finally, the premature oocyte died in an apoptosis independent way. We conclude that LHX8 protects oocytes from premature meiosis resumption and therefore, premature aging.

160. Luteinizing Hormone Signaling Rapidly Reduces Cyclic GMP Levels in Rat Ovarian Follicles by Dephosphorylation of NPR2 and Phosphorylation of PDE5A, Leading to Meiotic Resumption.
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2015 Abstracts – Page 63
In mammals, meiotic arrest of a fully-grown oocyte is maintained by the diffusion of cGMP through gap junctions from the surrounding granulosa cells, where it is produced by the guanylyl cyclase natriuretic peptide receptor 2 (NPR2). In response to luteinizing hormone (LH), cGMP levels fall rapidly in both the granulosa cells and the oocyte, triggering meiotic resumption. However, the mechanisms by which LH causes this decline in cGMP are not fully understood. A recent study from our lab has established that LH signaling mediates rapid deadenylation of NPR2 in rat follicles, reducing its guanylyl cyclase activity by ~50% within 30 minutes. However, preventing NPR2 deadenylation did not fully inhibit the decrease in cGMP after LH. Furthermore, the kinetics of the decrease in cGMP were more rapid than the decline in NPR2 activity, suggesting that other factors might also contribute to the LH-induced cGMP reduction. These data prompted us to identify the phosphodiesterase(s) (PDEs) that hydrolyze follicle cGMP, and to examine whether LH stimulates their activity. Assays of PDE activity showed that ~56% of the total activity in untreated rat follicles is calcium/calmodulin-dependent (implicating PDE1), and ~44% is calcium-independent. A qRT-PCR analysis in rat granulosa cells revealed that, among mRNAs encoding cGMP-degrading PDEs, Pde1a and Pde5a were expressed at the highest levels (~56% and ~16% of the total, respectively). Correspondingly, cGMP ELISA measurements showed that incubation of rat follicles with inhibitors of either PDE1 (PF-04822163, 3 µM) or PDE5A (sildenafil, 100 nM) elevated the basal cGMP concentration (5- and 3-fold, respectively), and partially inhibited the cGMP decrease in response to LH. Incubation of follicles with both inhibitors in combination elevated basal cGMP ~10-fold compared to untreated follicles, and attenuated the LH-induced decrease much more than either inhibitor alone. Nevertheless, some reduction occurred, due either to incomplete inhibition of PDE1A or PDE5A activity, or to the function of another PDE or cGMP transporter. These results show that both PDE1A and PDE5A contribute to cGMP hydrolysis in rat follicles, but do not indicate whether the activity of these PDEs increases with LH signaling. PDE5A activity can be stimulated by phosphorylation of Ser92 by PKA, and because PKA is activated by LH, we investigated whether LH signaling promotes PDE5A phosphorylation. Phos-tag gel electrophoresis of lysates from rat follicles treated with LH for 0, 10, or 30 minutes and immunoblotting with an anti-PDE5A antibody showed an LH-stimulated increase in PDE5A phosphorylation; this was confirmed with a PDE5A antibody specific for phosphorylated Ser92. Furthermore, treatment of follicles with forskolin (100 µM for 30 minutes) resulted in PDE5A phosphorylation similar to LH, strengthening the evidence for PKA involvement. These results demonstrate that LH signaling rapidly reduces follicle cGMP by two concurrent mechanisms: (1) dephosphorylation and inactivation of PDE1, and (2) phosphorylation of PDE5A, which based on previous studies should stimulate its activity. As follicle cGMP is reduced, cGMP in the oocyte decreases by equilibrating through gap junctions with the large volume of the surrounding follicle, allowing meiosis to resume.


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Maternal mRNAs in oocytes are remarkably stable. In mouse, oocyte maturation triggers a transition from mRNA stability to instability, which is a critical event in the oocyte-to-embryo transition, in which the differentiated oocyte loses its identity as it is transformed to totipotent blastomeres. We previously demonstrated that phosphorylation of MSY2, an RNA-binding protein, and mobilization of mRNAs encoding the DCP1A-DCP2 decapping complex contribute to maternal mRNA destruction during meiotic maturation. We now report that Cnot7, Cnot6l, and Pan2 and key components of deadenylation machinery are also dormant maternal mRNAs that are recruited during oocyte maturation as determined by immunoblotting. In addition, luciferase assays following injection of cRNA constructs encoding luciferase fused with the 3' UTR of these transcripts also indicates that these transcripts are recruited during maturation. The average length of the poly(A) tail of eight transcripts analyzed ranged from 50-100 nucleotides, which was substantially shortened following maturation. When the maturation-associated increase in CNOT7 (or CNOT6L) was inhibited using an siRNA approach mRNA deadenylation was substantially attenuated such that on average the length of the poly(A) tail of the transcripts was 68% ± 6% the length in GV oocytes. In addition, the intensity of the signal was markedly increased, being on average 55% ± 3% that in GV oocytes. Inhibiting the increase in PAN2 had little effect deadenylation. Reciprocally, expressing CNOT7 (or CNOT6L) in oocytes prevented from resuming meiosis initiates deadenylation of mRNAs. On average the length of the poly(A) tail of the transcripts and signal intensity in the CNOT7-expressing oocytes was 53% ± 6% and 47% ± 3%, respectively, that in GV-intact oocytes. These effects on deadenylation are also observed when the total amount of poly(A) is quantified. Last, inhibiting the increase in CNOT7 protein results in an ~70% decrease in transcription in 2-cell embryos. Taken with our previous work on MSY2 and DCP1A/DCP2 the picture that emerges is that mRNAs are stable in oocytes because the activity of the RNA degradation machinery is intrinsically low with mRNAs further protected from degradation by their association with MSY2. Maturation triggers recruitment of critical components involved in RNA degradation from both the 5' and 3' ends, with phosphorylation of MSY2 making mRNAs more susceptible to the degradation machinery. These changes initiate the oocyte-to-embryo transition, which entails transforming a highly differentiated oocyte into totipotent blastomeres, and loss of oocyte identity.
Piwi-interacting RNAs are a diverse class of small non-coding RNAs implicated in the silencing of transposable elements and the safeguarding of genome integrity. In mammals, male germ cells express two genetically and developmentally distinct populations of piRNAs at the pre-pachytene and pachytene stages of meiosis, respectively. Pre-pachytene piRNAs are mostly derived from retrotransposons and required for their silencing. In contrast, pachytene piRNAs originate from about one hundred genomic clusters and their biogenesis and function remain enigmatic. We previously reported that MOV10L1 is required for biogenesis of pre-pachytene piRNAs (Zheng et al., PNAS 2010). Subsequently we have demonstrated that conditional inactivation of the putative RNA helicase MOV10L1 in mouse spermatocytes produces a specific loss of pachytene piRNAs, significant accumulation of pachytene piRNA precursor transcripts, and unusual polar congregation of Piwi proteins with mitochondria. Pachytene piRNA-deficient spermatocytes progress through meiosis without derepression of LINE1 retrotransposons, but become arrested at the post-meiotic round spermatid stage with massive DNA damage (Zheng and Wang, PLoS Genet 2012). Our results demonstrate that MOV10L1 acts upstream of Piwi proteins in the primary processing of pachytene piRNAs and suggest that, distinct from pre-pachytene piRNAs, pachytene piRNAs fulfill a unique function in maintaining post-meiotic genome integrity. Our studies have demonstrated that MOV10L1 is a master regulator of the piRNA pathway in mammals. Recently we have shown that MOV10L1 exhibits 5'-to-3' directional RNA-unwinding activity in vitro and that a point mutation that abolishes this activity causes a failure in primary piRNA biogenesis in vivo. We demonstrate that MOV10L1 selectively binds piRNA precursor transcripts and is essential for the generation of intermediate piRNA processing fragments that are subsequently loaded to Piwi proteins. Multiple analyses suggest an intimate coupling of piRNA precursor processing with elements of local secondary structures such as G quadruplexes. Our results support a model in which MOV10L1 RNA helicase activity promotes unwinding and funnelling of the single-stranded piRNA precursor transcripts to the endonuclease that catalyzes the first cleavage step of piRNA processing (Vourekas et al., Genes Dev 2015). Funded by NICHD R01 HD069592.


163. The Chromatoid Body and Small RNAs.
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Male germ cell differentiation is orchestrated by accurate, spatially and temporally controlled gene expression patterns. Meiotic and early postmeiotic RNAs have an exceptionally diverse transcriptome that includes mRNAs and their isoforms, but also a considerable number of non-coding RNAs and intergenic transcripts. The complex transcriptome of male germ cells creates a high demand for efficient post-transcriptional regulation. During the high level of high transcriptional activity, an unusually large, male germ cell-specific ribonucleoprotein (RNP) granule, a chromatoid body (CB), appears in the cytoplasm of round spermatids. We have shown that the CB a dynamic structure that moves actively and makes contacts with the nuclear envelope and the Golgi complex. High resolution imaging revealed a close association of the CB with the endoplasmic reticulum and cytoplasmic vesicles that were also found to be embedded in the cavities of the CB. We developed a protocol to isolate CBs from mouse testes and identified its molecular composition by mass spectrometric analysis and RNA sequencing. Our results showed that the CB accumulates a multitude of different RNA-binding and RNA-processing proteins as well as a diverse set of RNAs including mRNAs, intergenic non-coding RNAs and PIWI-interacting RNAs (piRNAs). The piRNA pathway represents the predominant functional pathway in the CB, and we demonstrated that piRNAs are unlikely to be produced in the CB but are targeted to the CB for downstream actions. Given the constant flow of cellular RNAs into the CB and its molecular composition, our results suggest that the CB has an important role in the control and coordination of the male germ line transcriptome.

164. Circulating microRNAs as biomarkers of early embryonic viability in cattle.
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Embryonic mortality (EM) is considered to be the primary factor limiting pregnancy success in cattle and occurs early (< day 28) or late (≥ day 28) during gestation (day 0 = estrus). In cattle, early EM accounts for 25% of pregnancy loss, while late EM generally accounts for less than 10% of pregnancy losses. While real-time ultrasonography can be used to diagnosis pregnancy as early as day 28, methods for earlier diagnosis (e.g. by day 17) would dramatically increase our understanding of reproductive efficiency. Recently, microRNA packaged into small vesicles called exosomes, that are released by cells, have been shown to be excellent blood borne biomarkers of disease conditions and different physiologic states. We therefore set out to test the hypothesis, that circulating exosomal-derived miRNA could be identified that may differentiate pregnant versus non-pregnant cows during early (<28 d) gestation. In this study, cows were randomly assigned to be artificially inseminated (AI) with high fertility (live) semen (n=36; treatment group) or heat-treated (dead) semen (n=8; control group) on d 0 (day of estrus). Blood was collected from all animals on d 17 and 24 followed by pregnancy diagnosis via ultrasonography on d 30. Following diagnosis of pregnancy on d 30, cows were retrospectively classified within the AI assignment as pregnant (n=17), non-pregnant (n=19), or control (n=8; AI with dead semen). In addition, expression of IFN-stimulated genes (ISG-15, Mx2 and OAS-1) were measured in each group to select cows that had low IFN-stimulated gene expression at d 0 followed by either high message at d 17 (pregnant and non pregnant) or low message (control). Cows with increased IFN-stimulated gene expression on d 17 but not pregnant on day 30 were considered to have experienced EM. Exosomes were isolated via high-speed ultracentrifugation, quantified via nanoparticle tracking analysis and western blot analysis (CD 81 Positive). Exosomal miRNAs were extracted from the d 17 and d 24 samples and miRNA sequencing was performed on the Illumina HiSeq2500 Sequencing System (pregnant n=4/day; non-pregnant n=4/day;
165. Requirement for adenosine deaminase domain containing proteins in male germ cell development.
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Adenosine deaminase, RNA-specific (ADAR) proteins are the only known drivers of adenosine to inosine (A-to-I) RNA editing. Murine ADARs (encoded for by Adar, Adarb1, and Adarb2) contain two conserved domains: an adenosine deaminase (AD) domain, which catalyzes A to I conversion, and one or more double-stranded RNA binding motifs (dsRBM). While expression of Adarb1 and Adarb2 is confined to neural tissue, Adar is observed in a wider range of tissues, including the testis. In addition, the testis expresses two closely related AD domain-containing proteins, Adad1 and Adad2. Both carry amino acid substitutions in critical regions of the AD domain, suggesting they do not have catalytic activity, although this has not been formally proven. Both ADADs contain dsRBMs similar to those found in ADARs, implying they may bind a similar set of targets. Expression profiling in isolated testicular cell types, throughout testis development, and in germ cell ablated mutant models demonstrated both Adad1 and 2 are expressed exclusively in the meiotic and post-miotic germ cell populations while Adar is expressed in germ and somatic cells. The extent of RNA editing in the testis was determined by applying a computational pipeline to high throughput RNA sequence data of isolated testicular cell types. This analysis demonstrated A to I editing in both the germ line and soma, with a much higher number discovered in Sertoli cells as compared to germ cells. To address the functional role of RNA editing in the testis and the specific requirement of AD-domain containing proteins in male germ cell development, we generated germ cell and Sertoli cell-specific knockout models of Adad1 and Adad2, respectively. Despite the occurrence of editing in both cell types, germ cell or Sertoli cell ablation of Adar had no appreciable impact on germ cell development. In contrast, mutation of either Adad1 or 2 resulted in male-specific sterility. Tolerance for germ cell ADAR loss demonstrates ADAR-mediated editing is not essential for male fertility. However, the absolute requirement of both Adad1 and Adad2 for male fertility confirms a fundamental role of AD-domain containing proteins in germ cell development. Whether ADADs catalyze or regulate RNA-editing events in the germ line or have evolved essential functions outside of RNA editing is unknown. Current studies are aimed at distinguishing between these disparate hypotheses.

166. Human Follicular Fluid-Derived Non-Coding RNAs are Associated with Competent Oocytes.
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The developmental competence of the oocyte is acquired during follicle maturation in an orchestrated process that involves the egg, the surrounding follicular cells, and the follicular fluid (FF). Exosomes and extracellular RNAs are known to participate in cell-cell communication and thus can influence and potentially predict oocyte developmental competence. To further characterize exosomal and non-exosomal RNAs in select individual human follicles and correlate it to pregnancy outcome, we performed Small RNA Deep Sequencing and validated a focused group of miRNA using a commercial Human Cell Development & Differentiation miRNA PCR array. This study was approved by the University of Toronto Research Ethics Board. FF from consenting patients (n = 24) was collected by individual follicular puncture. The quality and pregnancy outcomes from each oocyte in these follicles of interest were recorded. Three pooled FF samples (3 patients each) from pregnant and non-pregnant patients each were analyzed. Exosomes were enriched using the commercial reagent, ExoQuick, and subjected to small RNA isolation. cDNA libraries were made and sequenced using the 2x75bp paired-end Illumina Next-Generation Sequencing (NGS) platform. Select candidate miRNAs were validated using the Human Cell Development & Differentiation miRNA PCR array with 6 individual patient samples. The analysis from the pooled samples representing pregnant versus non-pregnant patients produced 5000 reads. We found various classes of small non-coding RNA, including asRNA (30%), lncRNA (23%), RNA and tRNA-like (3%), miRNA (2%), and piRNA (1%). Interestingly, in the pregnant group, we found linc-ABHD6, linc-CDR2 and GDF9-antisense to be over-expressed with no detectable expression in the non-pregnant group. Conversely, we found that linc-ACP1, linc-TEKT4-1, linc-TAOK, and NFKBIE-antisense were over-expressed in the non-pregnant group, with no detectable expression in the pregnant group (p value <0.05). The expression of FF-derived miRNAs, the potential target genes were predicted, and functional annotation and pathway analyses revealed that most of these pathways are known regulators of follicular development and oocyte growth, including purine and pyrimidine metabolism, oocyte meiosis, and progesterone-mediated oocyte maturation. In conclusion, FF extracellular vesicle small RNAs can be used as non-invasive measures of oocyte developmental competence, and can complement other morphological and morphokinetic parameters to ultimately improve embryo selection with the objective of successful single embryo transfers.

167. Compartmentalizing Sperm RNAs.
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In essence, sperm are transcriptionally and translationally silent having purged the majority of their cyto/nucleoplasm as they reduce their total cellular volume to approximately 1/13 that of the oocyte nucleus. The reduction in nuclear volume to an almost crystalline inert nucleus appears independent of the varied range of species-specific protamination. Interestingly, the diverse pool of RNAs that are delivered to the oocyte upon fertilization remain encapsulated within the silent spermatozoan. Their complexity appears to reflect their method of isolation. The technology to define this contribution continues to evolve to now include Next Generation Sequencing. This has provided a window to understanding the continuum of RNAs that are delivered to the oocyte that has revealed a complex steady state population of messenger RNAs, epigenetic RNAs and small non-coding RNAs. We have proposed that this suite of paternally delivered transcripts are primed for function then put into use by hijacking the maternal machinery. Perhaps, they confront – consolidate genomes, provide signals for the first division and determine early lineage. Though the precise location of RNAs retained within the sperm cell remains largely unknown early evidence has suggested that many were embedded within the nucleus. To define the global pattern of compartmentalization, total RNA was extracted from whole mouse spermatozoa and detergent demembranated sucrose gradient fractionated nuclei. Isolated RNAs were subjected to RNA-sequencing (RNA-seq) and their abundance used to infer localization. Surprisingly, transcripts enriched in the unfractionated cells were representative of those RNAs associated with the outer membranes. These included transcripts related to the production and function of exosomes. Their absence in the nucleus was suggestive of an origin other than sperm and contributes to the growing evidence of RNA-rich sperm bound exosomes. The majority of the remaining sperm RNAs were associated with the nucleus with the abundant fragmented ribosomal transcripts likely persisting between the nuclear envelope and the perinuclear theca. The spermatozoal inner-nuclear compartment was also enriched in repetitive transcribed sequences. This included LINE elements and simple repeat sequences both of which have been shown to contribute to chromatin structure in other cell types. Perhaps they serve parallel roles in the spermatozoan.

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168. Role of Small Noncoding RNAs in Fertility Control.
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My talk will cover two topics: 1) control of male and female fertility by the miR-449/miR-34 family of miRNAs, and 2) the physiological role of sperm-borne/paternal miRNAs and endo-siRNAs. I will present our latest data on a knockout mouse line lacking two miRNA clusters encoding five miRNAs (miR-449a, 449b, 449c, 34b and 34c) that share the same “seed sequence”. The KO mice are completely infertile, but the causes for male and female infertility may be different. Moreover, sperm-borne miRNAs and endo-siRNAs have been identified for many years, but the physiological role of these paternal miRNAs remain elusive. Our recent data suggest a critical role of maternal miRNAs and/or endo-siRNAs in the control of transcriptomic homeostasis in fertilized eggs, zygotes and 2-cell embryos.

169. Role of Dicer Haploinsufficiency in Aggressive Endometrial Cancer in Mice and Women.
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Endometrial cancer is the most common gynecologic malignancy, affecting 1 out of every 38 women in the United States. Activation of the PI3K pathway through loss or mutation of the tumor suppressor PTEN accounts for 80% of these tumors. Late-stage and recurrent disease carries poor 5-year survival. Decreased Dicer expression is associated with poor prognosis endometrial cancers. Dicer is the RNase responsible for processing the precursor miRNA hairpin to generate 2 single-stranded mature miRNA forms, the complementary miRNA-5p and miRNA-3p forms. In general, the miRNA-5p form is more abundant and thought to be more functionally active. RNA binding proteins are critical for regulating Dicer’s miRNA processing function, including strand selection of miRNA-5p and miRNA-3p strands. Published in vitro studies have shown that Dicer−/− cells have a switch in production from the abundant miRNA-5p form to the miRNA-3p form. Based on our interests in Dicer processing and miRNA function, we mined the Cancer Genome Atlas (The Cancer Genome Atlas) endometrial cancer datasets to discover that 16% of PTEN mutant (PTENmut) endometrial cancers also contained heterozygous mutations in Dicer (Dicer−/−). Translationaly important, gene signatures associated with PTENmut/Dicer−/− tumors showed significantly worse survival compared to PTENmut only (P<0.02). This is the first human tumor with Dicer haploinsufficiency to show worse prognosis. Our analysis shows 164 mature miRNAs differentially expressed (fold change 1.25, P<0.05) between PTENmut/Dicer−/− and PTENmut genotype TCGA tumors. Examination of let-7a, a miRNA known to be dysregulated in Dicer−/− cells in vitro, showed a specific dysregulation of let-7a-3p but not let-7a-5p. Integration with PTENmut/Dicer−/− gene signature showed enrichment of let-7a-3p targets, potentially targeting 181 genes out of 2441 genes in our PTENmut/Dicer−/− gene signature (Fisher’s exact test, P=0.0002). To investigate the molecular mechanism of Dicer haploinsufficiency in endometrial cancer, we created both in vivo (mouse) and in vitro (human endometrial cancer cell line) model systems of Dicer haploinsufficiency. On a Pten deleted background, Kaplan-Meier survival analysis showed protection from loss of 2 alleles of Dicer and more aggressive endometrial cancer from loss of 1 allele of Dicer (P<0.05) compared to Pten cKO. Median survival for Dicer haploinsufficient mice was 150 days, 240 days for Pten cKO, and 315 days for double cKO. By 12 weeks, invasion through the myometrium occurred in 85% of cases for Dicer haploinsufficient tumors compared to 30% for Pten cKO. Additionally, Dicer haploinsufficient tumors showed an increase in let-7a-3p expression. This is the first in vivo evidence in both mice and women that the miRNA-5p to miRNA-3p miRNA processing switch occurs with Dicer haploinsufficiency, leading to significant changes in gene expression and more aggressive tumors. In vitro, heterozygous deletion of Dicer resulted in a 65% increase in cellular proliferation. In summary, our model systems will allow us to understand why loss of 1 allele of Dicer leads to biologically aggressive endometrial tumors and discover novel therapeutic targets for aggressive endometrial cancers or other cancers that are Dicer−/−.
170. Androgen Regulates Phagocytic Clearance of Apoptotic Germ Cells by Sertoli cells via miR-471-5p-DOCK1-RAC1 GTPase Signaling and Autophagy Component Proteins.
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Introduction: Phagocytic clearance of apoptotic germ cells and residual cytoplasm by Sertoli nurse cells is necessary for the proper male germ cell development and differentiation. We show that androgen regulates Sertoli cell phagocytosis by controlling the expression of microRNA-471-5p (miR-471-5p) and its targets guanine nucleotide exchange factor DOCK1-RAC1 GTPase as well as autophagy proteins ATG12, BECN1 and TECPR1. Methods: We generated transgenic mice expressing miR-471-5p driven by the Sertoli cell-specific promoter Rhox5P. We performed fertility, histological, germ cell apoptosis and phagocytosis assays to characterize reproductive phenotype of miR-471 mice. Results: Using an in vivo transgenic mouse model, we demonstrate that expression of androgen responsive miR-471-5p in the Sertoli nurse cells is critical for adjacent male germ cell development and differentiation. Mice overexpressing miR-471-5p suffered from increased germ cell apoptosis, abortive germ cell meiotic progression, impaired spermatid differentiation, compromised Sertoli cell-Sertoli cell adhesion at the blood-testis barrier and impaired fertility. Interestingly, miR-471-5p transgenic mice showed defective Sertoli cell phagocytosis. Transgenic mice overexpressing miR-471-5p in the Sertoli cells showed reduced levels/activity of DOCK1/RAC1 GTPase, ATG12, BECN1 and TECPR1, proteins known to play critical roles, in autophagy and phagocytosis. In addition, miR-471-5p transgenic mice showed overall lower levels and altered ratio of LC3-II to LC3-I, microtubule associated protein that is critical for the induction of autophagy/phagocytosis, in the Sertoli cells, suggesting defective turnover/maturation of phagosomes. Importantly, our analysis revealed that level of autophagy proteins was similarly altered in the testis of androgen suppression mouse models. Conclusion: Our study is first to suggest that LC3 associated phagocytosis (LAP) is an androgen dependent event and miR-471-5p transgenic mice represent a unique genetic model for studying the function of androgen-regulated events in Sertoli–germ cell communication including phagocytosis and maintenance of optimal male fertility.

171. Small RNAs and their modifications in sperm-mediated intergenerational transmission of acquired metabolic disorder.
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The discovery of sperm-borne RNAs has opened the possibility of additional paternal contributions aside from providing the DNA. Recently, the sperm-borne RNA mediated transmission of phenotypes has drawn increasing attention as an important type of non-genetic (epigenetic) inheritance. However, the exact carrier of such RNA-mediated information remains elusive. By analyzing small RNA deep-sequencing data, we recently revealed that the mature sperm contain a unique subset of highly enriched small RNA families derived from 5' halves of mature tRNAs, mostly being 29-34nt in length, which are named tsRNAs (tRNA-derived small RNAs). We further found that the sperm-borne tsRNAs not only carry the sequence of their tRNA predecessors, but also the various kinds of RNA modifications. The sperm tsRNAs as well as their RNA modifications are sensitive to diet-induced paternal metabolic disorder, and the injection of which into normal zygote could change the metabolic phenotype of the offspring. Since tsRNAs are evolutionarily conserved across most known vertebrate species, our data suggested that tsRNAs act as an ancient epigenetic “carrier” that responds to environmental changes and paternally influences future progeny.

FOCUS SESSION 17: Parental Diet and Gamete/Embryo Development.

172. Mitochondria, the Source of Good or Evil: Is Mitochondrial Function in the Embryo a Key Determinant of Fetal Growth Trajectory and Adult Onset Disease?
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Developmental programming was initially thought to occur primarily during in-utero development; however it is now evident that the peri-conceptual period is also a key time for programming adult burden of disease. There are now several examples of in vivo exposures (such as diet) and in vitro induced stresses (oxygen, pH) during preimplantation embryo development that program offspring for ill health later in life. Currently the underlying mechanism for how this peri-conceptual programming occurs is unknown. We have proposed that mitochondrial function is a key determinant not just for the viability of the embryo but also in setting the developmental trajectory for the fetus. Unlike any other cell, early embryos prior to compaction rely exclusively on mitochondria for energy production, as well as for cellular homeostasis. However due to the lack of regulatory mechanisms in the pre-compacting embryo, mitochondria at this stage are uniquely vulnerable to their environment (e.g. oxidative, pH, ionic and osmotic) and appear to be an early target for cellular dysfunction. Due to the significant role that mitochondria play in not only energy production but also calcium regulation, reactive oxygen species generation, production of intermediary metabolites and apoptosis, any damage to mitochondria caused by exposure to suboptimal environmental conditions can have significant downstream repercussions for cellular homeostasis. Using our animal models we have shown that a direct impairment of mitochondrial function during this pre-implantation window impairs fetal growth in utero and alters the transcriptome of the placenta and fetal brain. Further, when these offspring develop into adults they display altered glucose and insulin
regulation and altered body composition. How reduced mitochondrial output results in a permanent programming change in the embryo and altered health trajectory of offspring is not known, nor is whether this mitochondria-led developmental programming can be reversed. However in our animal models we have shown that mitochondrial stimulants in an aged model can improve pregnancy and fetal growth. We will provide some emerging evidence for the link between mitochondrial function and epigenetic regulation of the preimplantation embryo as a result of in vivo and in vitro stresses. Unraveling how mitochondrial function links to molecular signalling in the early embryo and can set the trajectory for lifelong development will facilitate strategies for prevention and/or correction of stresses and provide the basis and guidelines for human based studies.

173. **Maternal Protein Nutrition and Embryo Developmental Programming.**

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Research across different mammalian species including the human indicates the periconceptional (PC) period is vulnerable to environmental influences which may change the developmental programme and cause lasting effects into adulthood associated with health and disease risk. Analysis of the effect of maternal undernutrition (low protein diet, LPD; 9% vs 18% in control diet) during the PC period in mice has provided a suitable model. Restricting maternal LPD to just the preimplantation period with normal nutrition for the rest of gestation and postnatal life (Emb-LPD) leads to increased risk of adult cardiovascular, metabolic and behavioural disease in a gender-specific manner. We have found a sequence of events underlies adverse long-term programming initiating through Emb-LPD to comprise compensatory responses within the extra-embryonic cell lineages (trophectoderm; primitive endoderm) to promote nutrient retrieval during gestation and thereby to support fetal growth. Such responses include increased proliferation, Rho-A regulated increase in endocytosis, and enhanced motility of affected tissues with evidence indicating modulation is controlled by epigenetic changes. In contrast, embryonic lineages show evidence of increased apoptosis and reduced survival within stem cell pools. Embryonic stem cell lines derived from blastocysts from diet-treated mothers retain cellular and epigenetic programming characteristics over several passages and permit mechanistic analyses and reduced use of animals. Emb-LPD compensatory responses induced within extra-embryonic lineages cause increased perinatal growth. However, neonatal weight in programmed offspring correlates positively with disease risk in later life. A continuum of biological processes therefore exists between maternal PC dietary composition and adult disease risk. Funded by BBSRC, NICHD, EU FP7 EpiHealth and EpiHealthNet.

174. **Significant reduction of nutrients in culture medium supports embryo development and improves cell allocation, suggesting improved viability.**

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Metabolomic analyses reveal that embryos use a limited amount of the substrates provided to them in vitro. Our objective was to determine the effect of reducing nutrient (carbohydrates, amino acids, vitamins and EDTA) concentrations in culture media on resulting mouse embryo development and quality. In vivo matured mouse eggs were fertilized in vitro and zygotes were placed into sequential, defined culture medium containing 100% (control), 75%, 50%, and 25% of control nutrient concentration (5 replicates, ≥147 embryos/treatment). Embryos were cultured for 112 hrs, with a media change at 48 hrs. There were no significant differences between treatments in embryo cleavage. However, development to the blastocyst stage at 96 hr was significantly lower in 25% compared to 50%, 75%, and 100% (39.8±3.4%, 67.1±3.9%, 63.3±4.0%, 61.2±4.0% per zygote, respectively). Additionally, development to hatching blastocyst was significantly lower in 25% compared to 50%, 75%, and 100% at 112 hr (43.2±3.5%, 63.8±4.0%, 61.9±4.0%, 62.6±4.0% per zygote, respectively). Resultant blastocysts were analyzed for cell number and allocation. Blastocysts developing in 25% differed from all other treatments, with significantly lower number of trophectoderm (TE) and total cells. There was no difference between treatments in the number of inner-cell mass (ICM) cells, but blastocysts cultured in 25% tended (p=0.08) to have a higher percentage of ICM cells than those in 100% and 50% (12.13±1.4%, 9.13±0.7%, 9.4±0.7% respectively). There were no significant differences in ATP content. Embryos were also cultured individually (20 embryos/treatment) in the EmbryoScope™ (Vitrolife A/S) to evaluate developmental kinetics. Embryos cultured in 50% reached the 8-cell stage significantly faster than all other treatments. Embryos cultured in 25% were significantly slower than those in 50% and 100% in time to start of cavitation and hatching, and significantly slower than all treatments in time to blastocyst. In a second experiment, EDTA, glucose, alanyl-glutamine, and pyruvate/lactate were individually increased to 50% concentration in the 25% nutrient media in an attempt to rescue embryo development (4 replicates, ≥99 embryos per treatment). Only the addition of pyruvate/lactate significantly increased development compared to 25%, to blastocyst at 96 hr (68.7±4.7%, 29.4±4.1% per zygote, respectively), and hatching blastocyst at 112 hr (63.6±4.9%, 28.6±4.0% per zygote, respectively). The addition of pyruvate/lactate significantly increased total cell number compared to 25% (115.0±8.2, 86.5±11.3, respectively), as well as the number of ICM cells (15.6±1.4, 10.5±1.4, respectively), and tended to increase (p=0.07) the number of TE cells. In summary, culture of mouse zygotes in nutrient concentrations half those typically supplied is not detrimental to embryo development or quality. A nutrient concentration of just 25% can successfully support embryonic development when only pyruvate and lactate are restored to 50% concentration. Of particular interest, embryos cultured in 25% nutrient concentration supplemented with pyruvate and lactate had the highest number of ICM cells across both experiments, resulting in an improved ICM:TE ratio (1.62) compared to standard (100%) culture medium (1:9.8). Reducing the concentration of nutrients in culture media to more closely approximate what the embryo uses may improve embryo viability.

175. **A Diet Enriched In Linoleic Acid Enhanced Endometrial Expression Of Genes Associated With Embryo-maternal Cross-talk On Day 14 Post-insemination.**

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In cattle, up to 40% of total embryonic losses occur between days 8 and 17 post-insemination, indicating that early embryonic mortality is the main reason for reproductive failure. Studies have shown that certain dietary long chain unsaturated fatty acids (LCFA) positively influence early embryo development and reduce pregnancy losses. However, the underlying mechanisms are yet unknown. Therefore, the aim of this study was to investigate the effects of different dietary LCFA on embryo elongation and gene expression in the pregnant endometrium on day 14 post-insemination. Twenty-one non-lactating Holstein cows were blocked by parity and body weight, and equally assigned to one of three diets enriched in three different sources of LCFA: canola seed (CAN, 62% oleic acid), sunflower seed (SUN, 73% linoleic acid) or flax seed (FLX, 57% α-linolenic acid). After receiving the diets for at least 40 days, cows were superovulated, artificially inseminated twice, and slaughtered 14 days post-insemination. Reproductive tracts were removed immediately after slaughter, transferred to the lab on ice, flushed, and embryos evaluated morphologically within 3.5 hours. Samples of the caruncular (CR) and intercaruncular (IC) regions of the endometrium were collected, snap frozen in liquid nitrogen and stored at -80°C until analysis. Total RNA was isolated from CR and IC samples (n=3/treatment) using Trizol reagent, q-PCR reactions performed with Fast SYBR Green Master Mix and data normalized with geometric mean of B-actin and H2AFZ as housekeeping genes. The mRNA expression of genes involved in immune modulation (CD81, BST2, PTX3, RSAD2, TGF-β, ISG15, ISG20, IRF9, OAS1, MX1, MX2 and CXCL10), apoptosis (BAX), tissue remodeling (CLDN4 and 10), angiogenesis (NR2F2 and EPAS1), cell adhesion (AGR7 and TGM2), cell proliferation and growth (EIF4E and USP18), embryonic development (DKK1), IFNT signaling (IFNTR1 and 2) and luteolysis (OXTR) was determined in the CR and IC regions of the endometrium. Feeding LCFA did not affect (P>0.10) superovulatory response (CL number: 22.0±4.9; anovulatory follicle number: 3.0±1.5), total embryo production (6.0±1.8) and percentage of elongated embryos (39.8±5.3%). However, feeding SUN resulted in higher expression of genes responsible for tissue remodeling and angiogenesis in both IC and CR regions. Moreover, the increased expression of DKK1 and IFNTR1 in the IC region of SUN fed cows implies improved embryo-maternal communication.

176. Paternal obesity shifts founder sperm microRNA profile, implicating it as a candidate epigenetic mechanism underlying paternal programming.

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Obesity and related comorbidities are continually increasing globally prevalent. We have previously demonstrated that the consumption of a paternal high fat diet (HFD) programs two subsequent generations of mice for reproductive and metabolic dysfunction. We have now demonstrated that a paternal HFD concomitantly shifts the microRNA profile of sperm, with 25 sperm borne microRNAs displaying differential expression. Interestingly 2 of these microRNAs are sperm specific (not detectable in oocytes; but are present in zygotes) and their homologs are amongst the most abundant microRNAs in human sperm. Pathways that contain experimentally validated microRNA targets of these altered microRNAs include embryonic development, carbohydrate metabolism, adipogenesis, nervous system and neurological disorders, metabolic disease and cancer.

Post fertilisation experimentally validated mRNA targets of sperm microRNAs that are altered in abundance due to a HFD, have altered expression in zygotes sired by HFD males, including key developmental genes (Oct4, Sox2). A subset of sperm borne microRNAs that are altered by a paternal HFD are also dysregulated in the pancreas of adult male mice, albeit in an inverse relationship and despite being separated by a chasm of developmental time. Promisingly the alteration to sperm microRNA content caused by HFD feeding can be somewhat restored by interventions that include target obesity, such as diet and/or exercise. Intervention based restoration of the sperm microRNA profile occurs concomitantly with partial restoration of paternal obesity programed subfertility and metabolic impairment observed in F1 males. Overall these studies implicate sperm borne microRNAs as part of an epigenetic mechanism that is sensitive to the dietary/metabolic state of a male and which is capable of having molecular consequences in the preimplantation embryo. This potentially triggers a molecular cascade that not only impairs embryo development, but also programs the F1 generation for reproductive and metabolic dysfunction, remaining evident as an altered microRNA profile in adult offspring tissue. As the reproductive and metabolic disturbances are further transmitted to the F2 generation, the gametes of the F1 generation may also be burdened with an altered epigenetic makeup. Thus representing a potential for intergenerational amplification of subfertility and metabolic derangements in humans, especially in light of the current global obesity epidemic. Understanding how a father’s nutritional status programs offspring health may ultimately provide a significant as yet unrealised window of opportunity for intervention, thereby improving the health of the future generations and reducing the long term burden of health.

177. Effects of Periconceptional Nutrition on Human Developmental Epigenetics.

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Transient nutritional exposures during critical ontogenic periods can cause persistent changes in gene expression, metabolism, and risk of disease. We have been investigating whether such ‘developmental programming’ occurs via nutritional influences on developmental epigenetics. Our studies in agouti viable yellow and axin fused mice showed that developmental establishment of DNA methylation at ‘metastable epialleles’ is especially sensitive to maternal nutritional status around the time of conception. To identify human
metastable epialleles, we have been using a multiple-tissue screen for systemic interindividual variation in DNA methylation. Genomic regions we have identified exhibit all the characteristics of metastable epialleles; DNA methylation at these loci is established stochastically, affected by maternal nutrition around the time of conception, consistent across multiple tissues, and stable for many years. Our most recent studies using genome-wide bisulfite sequencing have identified candidate metastable epialleles associated with human disease, providing exciting opportunities for epigenetic epidemiology. In particular, we have performed detailed characterization of VTRNA2-1, a genomically imprinted small non-coding RNA that appears to be a tumor-suppressor in several types of cancer.

178. One-Carbon Metabolism: Linking Nutritional Biochemistry to Epigenetic Programming of Long-Term Development. Kevin Sinclair1, Juan Xu1, Wing Yee Kwong1
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One-carbon (1C) metabolism consists of an integrated series of metabolic pathways that include the folate cycle and methionine remethylation and trans-sulfuration pathways. Most, but not all, 1C metabolic enzymes are expressed in somatic cells of the ovary, mammalian oocytes and in preimplantation embryos with subtle differences in expression existing between species. The metabolic implications of this, with regard to the provision of methyl donors, are not fully understood but mathematical models developed in house predict consequences for intra-cellular trans-methylation. These predicted effects are currently being tested experimentally both with ovarian somatic cells and zygotes cultured in vitro. However, we demonstrated previously in sheep that physiologically relevant reductions in the dietary supply of vitamin B12, folate and methionine around the time of conception can epigenetically modify DNA in their progeny and lead to sex-biased insulin resistant and hypertensive offspring. Epigenetic alterations to DNA methylation in genes involved in key pathways associated with insulin signalling and endoplasmic reticulum stress have been confirmed. Furthermore, we’ve observed similar sex-biased effects in offspring of rats fed folate, choline and methionine deficient diets. Our most recent data indicate that in utero exposure to cigarette chemicals induces sex-specific disturbances in human fetal-liver 1C metabolism (obtained from elective terminations between 11 and 21 weeks of gestation) linked to altered DNA methylation in key imprinted genes and at the glucocorticoid receptor. Focus has now turned to consider the contribution of polymorphic variances in genes encoding 1C enzymes, where initial studies have reverted back to the outbred sheep as a model species. Preliminary findings from these investigations will be presented. Current research supported by BBSRC-IPA (BB/K017810/1) with EBLEX, HCC and AgriSearch.

179. Functional Roles of Fructose: Induction of Proliferation and Adhesion of Ovine Trophectoderm Cells via O-Linked Glycosylation Mediated Phosphorylation of the Akt/PKB-TSC2-MTOR Cell Signaling Cascade. Xiaoqiu Wang1, Kathrin A. Dunlap1, Guoyao Wu1, Fuller W. Bazer1
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During pregnancy, the placentae of ungulates (e.g., cows, sheep, and pigs) convert glucose into fructose, which is the most abundant hexose sugar in fetal fluids and blood. However, the role of fructose, the most enigmatic component of carbohydrate metabolism in fetal-placental tissues, is largely ignored since there is no evidence for its metabolism via the glycolytic pathway or the Krebs cycle as an energy source. In this study, we used ovine trophectoderm (oTr1) cells to investigate functional roles of fructose and the underlying mechanism of action. Fructose increased oTr1 cell proliferation in a dose-dependent manner with maximum stimulation at 4 mM (P<0.05), and fructose also increased (P<0.05) intracellular concentrations of glucosamine-6-phosphate (GlcN-6-P), the precursor of UDP-N-acetylgalacosamine (UDP-GlcNAc), as did glucose. Inhibition of glutamine-fructose-6-phosphate transaminase 1 (GFPT1) with 2 μM azaserine blocked (P<0.05) the ability of fructose to stimulate oTr1 cell proliferation and GlcN-6-P production. Quantitative immunofluorescence analyses revealed that fructose stimulated (P<0.05) the phosphorylation of TSC2 and MTOR and that those effects were abrogated (P<0.05) by inhibition of GFPT1. In addition, UDP-GlcNAc directly increased (P<0.05) oTr1 cell proliferation. Further measurement of cell adhesiveness demonstrated increases (P<0.05) in oTr1 cell adhesion by UDP-GlcNAc at 1 h, as well as by fructose and glucose at 20 min and 1 h. Quantitative immunofluorescence analyses revealed that UDP-GlcNAc activated (P<0.05) the Akt-TSC2-MTOR signaling cascade. Results from the inhibition of O-linked N-acetylglucosamine transferase (OGT) by alloxan indicated that the fructose-induced increase in cell proliferation was mediated via O-linked glycosylation (O-GlcNAcylation). Furthermore, western blot analyses revealed that fructose activated (P<0.05) the Akt-TSC2-MTOR signaling cascade via O-GlcNAcylation-mediated phosphorylation. Collectively, these results demonstrate critical roles for fructose that are mediated via the hexosamine biosynthesis pathway to increase O-GlcNAcylation, and activation of the Akt-TSC2-MTOR signaling cascade which induces proliferation and adhesion of ovine conceptus trophectoderm cells. This research was supported by Agriculture and Food Research Initiative Competitive Grant no. 2011-67015-20028 from the USDA National Institute of Food and Agriculture.

180. Maternal fructose consumption is associated with increased fetal loss and impaired endometrial stromal cell decidualization: a potential role for oxidative stress and altered BMP2 signaling. Jessica L. Saben1, Laura Lawrence1, Julie Rhee2, Zeenat Asghar3, Joan Riley1, Kelle Moley1
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According to the Centers for Disease Control, roughly one-third of US adults suffer from metabolic syndrome. The most significant increases in metabolic syndrome over the previous decade occurred in women of reproductive age, which is especially alarming given that metabolic syndrome is associated with reproductive problems. Increased fructose consumption is often observed in individuals with metabolic syndrome, and several studies have concluded that excess intake of carbohydrates, specifically fructose, may contribute to metabolic syndrome development. Metabolic syndrome increases the risk for early pregnancy loss, which can occur if the uterus fails to become receptive to implantation. It is well known that successful implantation requires that the stromal fibroblasts of the uterus differentiate into specialized secreting cells in a process termed endometrial decidualization. What is unknown, however, is the extent to which metabolic syndrome affects decidualization and contributes to the increased incidence of early pregnancy loss in this population. Furthermore, it is unclear what role the consumption of a high-fructose diet has on uterine receptivity. Here, we hypothesized that feeding mice a high-fructose diet (HFbD) prior to and throughout pregnancy would lead to sub-fertility in mice that results from
impaired endometrial decidualization. To study endometrial decidualization, induced deciduomas were used. Additionally, embryos were cultured in 4mM D-fructose or L-glucose (two-cell to blastocyst) and transferred into chow-fed normoglycemic dams (CON) to examine possible effects of HFrD on embryonic competence. HFrD fed mice did not exhibit obesity, insulin resistance or dyslipidemia but were glucose intolerant. Pregnancy rate and litter size were significantly decreased in HFrD-fed mice compared with CON. Induced deciduoma weights were reduced by 50% in HFrD mice and showed significantly lower BMP2 signaling. Furthermore, western blot analysis of the deciduomas revealed significantly lower forkhead box-O1 and mitochondrial antioxidant manganese superoxide dismutase levels suggesting that antioxidant defenses may be impaired and could contribute to the elevated fetal loss in the HFrD-fed mice. Conversely, implantation rates were similar between D-fructose (36%) and L-glucose exposed embryos (30%), suggesting that reduced litter size in HFrD mice may be a consequence of impaired uterine receptivity as opposed to altered embryonic competence. In summary, our findings suggest that increased consumption of fructose prior to and throughout pregnancy leads subfertility that is caused by impaired endometrial decidualization and a pro-oxidative uterine environment.

181. Methyl donor supplementation during in vitro bovine early embryo development profoundly influences on genome-wide DNA methylome, transcriptome and embryonic phenotype.

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Mammalian early embryos react to their immediate microenvironment and it is believed that conditions provided by maternal nutrition or stress can induce short (transcriptome) and long term (epigenome) adaptation. It was hypothesized that early bovine embryos would react both at the transcriptomic and epigenomic levels when placed in a microenvironment rich in methyl donor. The impacts of supraphysiological S-Adenosyl methionine (SAM) supplementation, as the global methyl donor, on bovine early embryonic development was tested in vitro. The culture medium was supplemented with SAM starting at the 8-cell stage and cultured with the treatment up to the blastocyst stage. Day-7 blastocysts were collected and analyzed in parallel for genome-wide DNA methylation and transcription using our dedicated arrays. The SAM treatment induced a phenotypic response by significantly increasing embryonic hatching and skew in the sex ratio in favor of male embryos. The cellular allocation (ICM vs. TE) were similar between treated and controls. Transcriptomic analysis identified 504 transcripts including ncRNAs. DNA methylome analyses identified 4,016 differentially methylated regions (DMRs). The distribution of DMRs showed enrichment for exonic regions, promoters and CpG islands of high and intermediate density. DNA methylome pathways analysis revealed that he most affected differentially methylated genes involved in ESCs pluripotency and BER pathways. Overlaying the transcriptomic and DNA methylome information enabled to identify a sublist of 10 most of which are known for their involvement in embryonic development or in epigenetic modulation. In conclusion, this study shows that methyl donor (SAM) supplementation during in vitro bovine early embryo development profoundly influences embryonic gene expression and DNA methylation as well as developmental phenotypes.

182. Germline Epigenome and Sex Chromosome Inactivation.

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The germline is the only heritable lineage across generation. It remains unknown how the germline epigenome is distinctly established from that of somatic lineages. We recently demonstrated that genes commonly expressed in somatic lineages and spermatogenesis-progenitor cells (termed somatic/progenitor genes) undergo repression in a genome-wide manner during late stages of the male germline, and identify underlying mechanisms. SCML2, a germline-specific subunit of a Polycomb repressive complex 1 (PRC1), establishes the unique epigenome of the male germline through two distinct antithetical mechanisms on autosomes and sex chromosomes, respectively. In the stem cell phase of spermatogonial, SCML2 works with PRC1 and promotes RNF2-dependent ubiquitination of H2A, thereby marking somatic/progenitor genes on autosomes for repression. This repression of somatic/progenitor genes during meiosis and postmeiosis is associated with formation of a novel class of bivalent domains that allow for the recovery of the somatic/progenitor program after fertilization. Our results suggest that bivalent H3K27me3 and H3K4me2/3 domains are not limited to developmental promoters (which maintain bivalent domains that are silent throughout the reproductive cycle), but also underlie reversible silencing of somatic/progenitor genes during the mitosis-to-meiosis transition in late spermatogenesis. On the other hand, Induction of late spermatogenesis genes is facilitated by poised chromatin established in the stem cell phases of spermatogonia, Importantly, during spermatogenesis, mechanisms of epigenetic regulation on sex chromosomes are different from autosomes because of meiotic sex chromosome inactivation that is regulated by DNA damage response pathways. X-linked somatic/progenitor genes are suppressed by meiotic sex chromosome inactivation without deposition of H3K27me3. Furthermore, SCML2 has another independent function and prevents RNF2-dependent ubiquitination of H2A on sex chromosomes during meiosis, thereby enabling unique epigenetic programming of sex chromosomes for male reproduction. Taken together, our genome-wide studies reveal epigenetic principles during the mitosis-to-meiosis transition in spermatogenesis.

183. Regulation of Human Fetal Ovarian Germ Cell Development.

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The formation of an appropriately sized cohort of primordial follicles during fetal life is essential for normal human female reproductive lifespan. To reach this stage, primordial germ cells must proliferate sufficiently, commit to differentiation and progress through the first meiotic prophase as far as the diplotene stage, before finally being assembled into a follicle. Whilst our understanding of
the pathways that regulate primordial germ cell proliferation is extensive, we know comparatively little about the signals that regulate germ cell behaviour during the first meiotic prophase, and what triggers their subsequent assembly into primordial follicles. We have focussed on the role of the TGF-beta superfamily member activin A in the regulation of human fetal oogenesis. Activin is produced by meiotic germ cells just prior to the onset of follicle formation, and signals primarily to neighbouring somatic cells to regulate the production of reciprocal signals, influencing germ cell fate. Through candidate gene and genome-wide transcriptional approaches we have identified a number of activin-regulated genes in the human fetal ovary, many of which are involved in regulating other signalling pathways known to be important for follicle formation, as well as the structure of the extracellular matrix and the bioavailability of TGF-beta superfamily ligands themselves. This positions activin signalling as a key regulator of the microenvironment around germ cells, and reveals a complex network of extrinsic factors regulating germ cell development and differentiation in the human fetal ovary. Elucidating how these external signals are integrated with the intrinsic repertoire of transcription factors within germ and somatic cells in the fetal ovary is a key focus of our current work.

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The endothelial cell lining the blood vessels is not just a passive cell barrier which just allows passage of various biological substances to pass from the circulation to tissues. It also actively prevents substances passing from the circulation to the tissues as in the brain where it functions as the “blood brain barrier”. The endothelial cells are also known to convert biologically inert substance to bioactive substances like the conversion of Angiotensin I to Angiotensin II by the angiotensin converting enzyme (ACE) which exists on the endothelial surface. ACE is also responsible for inactivating bioactive substance like bradykinin. Endothelial cells also synthesize bioactive substances like prostacyclin, thromboxane A₂, as well as endothelin. Some of these substances can act on the luminal surface to prevent adhesion of circulating cells to the surface of endothelial cells. These substances also act on the abluminal side on the vascular smooth muscle to cause either relaxation or constriction. The very simple methods utilized to detect and identify these substances will be described along with the identification of an entirely new substance synthesized by the endothelial cells and the potential role in normal and abnormal pregnancy.

POSTERS
It is recommended that the itinerary builder be used for your personal poster schedule: http://tinyurl.com/pxob8bf Posts are organized by focus topic area; every third Poster will be presented on either Friday, Saturday, or Sunday, Sessions A, B, and C, respectively. First Headers below indicate the poster topic area; subsequent headers indicate the individual topic area and program number range.

Trainee Research Competition: Poster Presentations
The following posters are finalists in this year’s Trainee Research Poster Competition. When not being presented within their topic area (see abstracts in these sections), they will be on display at the front of the poster room.

- Feiyang Diao, Magee-Womens Research Institute, Pittsburgh, Pennsylvania, USA. Abstract 651 (Poster Session B).
- David Fleck, RWTH Aachen University, Aachen, Germany. Abstract 537 (Poster Session B).
- Lan Hai, Southern Illinois University, Carbondale, Illinois, USA. Abstract 618 (Poster Session B).
- Faezeh Koohestani, University of Kansas Medical Center, Kansas City, Kansas, USA. Abstract 482 (Poster Session A).
- Ying Yang, University of Missouri-Columbia, Columbia, Missouri, USA. Abstract 191 (Poster Session A).
- Jun Zhou, University of Georgia, Athens, Georgia, USA. Abstract 317 (Poster Session A).

Poster Topic Area 1: Pregnancy & Uterine receptivity and placentation

185. The Angiogenic Role of Hyaluronic Acid in Embryo Implantation.
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Implantation, a critical step in the establishment of pregnancy, is immediately followed by a marked increase in the permeability and density of the uterine blood vessels. Hyaluronic acid (HA) has been reported to participate in the regulation of vascular development in a number of physiological processes. Specifically, high molecular weight HA has been shown to inhibit angiogenesis, whereas its enzymatic degradation products are by nature pro-angiogenic. On the basis of this information we hypothesized that HA may be involved in vascular modifications associated with implantation. Our experiments revealed interesting alterations in HA distribution in the endometrium from the onset of implantation. In addition, we found an increase in HA fragmentation during early pregnancy. Moreover, substantial changes in the expression profile of genes encoding for HA synthesis and degrading enzymes, as well as in the distribution of their protein products, were observed in the implantation site during early pregnancy. Such gene and protein modifications were also noticed in the HA receptors as well as in some specific ECM stabilizing proteins. Functional MRI inspection of HA synthesis inhibitor 6-diazo-5-oxo-1-norleucine (DON)-treated pregnant mice, on embryonic day 6.5, showed a marked increase in decidua vessel permeability and accumulation of blood in close proximity to the implanting embryo. Moreover, MRI inspection of mice pregnant with embryos over-expressing hyaluronidase, the HA degrading enzyme, in their trophoblast cells, showed defective implantation along with increased permeability of blood vessels immediately surrounding the embryo. Taking these observations into account, we suggest that HA uterine metabolism has a pivotal role in vascular development and remodeling during embryo implantation in mice. Our study will potentially shed light on ECM participation in vascular events involved in successful pregnancy further deciphering some pathological processes responsible for implantation failure.

186. Reversing the reduced endometrial GLUT4 expression in polycystic ovary syndrome: a mechanistic study of metformin action.
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Conflicting results have been reported regarding whether or not insulin-regulated glucose transporter 4 (GLUT4) is expressed in human and rodent endometria. There is an inverse relationship between androgen levels and insulin-dependent glucose metabolism in women, and hyperandrogenemia, hyperinsulinemia, and insulin resistance are believed to contribute to endometrial abnormalities in women with polycystic ovary syndrome (PCOS). However, it has been unclear in previous studies if endometrial GLUT4 expression is regulated by androgen-dependent androgen receptors (ARs) and/or the insulin receptor/Akt/mTOR signaling network. In this study, we demonstrate that GLUT4 is expressed in normal endometrial cells (mainly in the epithelial cells) and is down-regulated under conditions of hyperandrogenemia in tissues from PCOS patients and in a 5α-dihydrotestosterone-induced PCOS-like rat model. Western blot analysis revealed reduced endometrial GLUT4 expression and increased AR expression in PCOS patients, but the reduced GLUT4 level was not always associated with an increase in AR in PCOS patients when comparing non-hyperplasia with hyperplasia. Using a tissue culture system, we investigated the molecular basis by which GLUT4 regulation in endometrial hyperplasia tissues is affected by metformin. We show that specific endogenous organic cation transporter isoforms are regulated by metformin, and this suggests a direct effect of metformin on endometrial hyperplasia. Moreover, we demonstrate that metformin induces GLUT4 expression and inhibits AR expression and blocks insulin receptor/Pi3K/Akt/mTOR signaling in the same hyperplasia tissues. These findings indicate that changes in endometrial GLUT4 expression in PCOS patients involve the androgen-dependent alteration of AR expression and changes in the insulin receptor/Pi3K/Akt/mTOR signaling network.

187. Involvement of endometrial epithelial cell MRP4 in embryo implantation.
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Embryo implantation is an intricate and rate-limiting step for human reproduction. Failure in embryo implantation accounts for nearly 75% pregnancy losses in humans. However, the molecular mechanism underlying embryo implantation remains elusive. It has been established that upon the attachment of embryo to the endometrium in the uterus, prostaglandin E2 (PGE2) is largely produced and released by endometrial epithelial cells, which in turn initiates signaling transduction in stromal cells that are required for a successful implantation. How PGE2 is transported from the epithelial cells during embryo implantation, however, is less studied. Multidrug resistance-associated protein 4 (MRP4) is a typical ATP-binding cassette transporter, which is known to transport PGE2. The present study, therefore, was carried out to investigate the involvement of MRP4 in embryo implantation. Western blot assay shows that MRP4 is highly expressed in the mouse uterus during embryo implantation period. Immunofluorescence staining shows that MRP4 is located at the basolateral membrane of endometrial epithelial cells, where they are in the interface with stromal cells. Intratroine injection with a selective inhibitor of MRP4, MK-571 (0.5, 1, 5 mM) or siRNAs specifically targeting MRP4, resulted in a significant reduction in embryo implantation rate in mice. Moreover, in a human endometrial surface epithelial cell line (HEK), inhibition or siRNA-based knock-down of MRP4 significantly reduced the levels of PGE2 released from the cells. Taken together, these results have suggested that MRP4, by mediating the transport of PGE2 from endometrial epithelial cells, may play an important role in embryo implantation.

188. Effect of lipopolysaccharide on villous trophoblast invasion from human first trimester placenta.
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It has been shown that adverse obstetrical outcomes such as pre-eclampsia and fetal growth restriction correlate with intrauterine infection. However, the mechanisms linking infection to placental trophoblast invasion defects remain unclear. In this study, we investigated pathways involved in infection-associated abnormalities in villous trophoblast invasion using early human placenta.

2015 Abstracts – Page 74
laboratory-based study used human placenta from elective first trimester (6-10 weeks, n = 20) surgical terminations of uncomplicated pregnancies. The study was performed between January 2014 and December 2014. Trophoblast cells were isolated using mild enzymatic degradation, Percoll gradient centrifugation, negative magnetic cell sorting using antibodies against CD45RB and fibroblast. The purity of isolated trophoblast cells exceeded 98% as assessed by cytokeratin-7 expression using flow cytometry. Primary trophoblast cells were treated with serially diluted lipopolysaccharide (LPS) with or without anti-Toll-like receptor 4 (TLR4) antibody. Levels of the cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-4 and IL-10, and the chemokines IL-8, macrophage inflammatory protein (MIP)-1α and CXCL12 were measured using enzyme-linked immunosorbent assay (ELISA). Cell apoptosis was determined by expression of Annexin-V and propidium iodide using flow cytometry. Trophoblast invasion was investigated using the matrigel invasion assay. In selected experiments, trophoblast cells were treated with exogenous TNF-α, IL-1β and IL-6, and trophoblast invasion was assessed. We found that LPS exposure increased first trimester trophoblast cell TNF-α, IL-1β and IL-6 production and IL-8, MIP-1α and CXCL12 secretion, decreased trophoblast invasion and induced trophoblast apoptosis. Pre-treatment of a function-blocking anti-TLR4 antibody prior to LPS exposure abrogated the effects of LPS. Exogenous TNF-α inhibited trophoblast invasion in a dose-dependent manner, while IL-1β and IL-6 had no effect on trophoblast invasion. In summary, our results indicate that LPS inhibits trophoblast invasion in vitro via mechanisms associated with increased TNF-α secretion and trophoblast apoptosis. In addition, LPS may decrease trophoblast invasion in vivo by secreting chemokines and recruiting cells of the innate immune system, such as monocytes, macrophages, natural killer cells and neutrophils, to the maternal-fetal interface. We suggest that the TLR4 signaling pathway may represent a potential target for treatments for infection-related gestational diseases such as pre-eclampsia and fetal growth restriction that are characterized by decreased trophoblast invasion and inadequate uterine spiral artery transformation. This research was supported by the National Natural Science Foundation of China (81370769 and 81200478) and Guangzhou Science and Technology Plan Project (2014Y2-00202).

189. The expression of beta-oxidation relating genes in the placenta in vivo and in vitro under hypoxic condition.
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Preeclampsia (PE) is a disorder of pregnancy characterized by high blood pressure and large amounts of protein in the urine. PE is thought in many cases to be caused by a shallowly implanted placenta that becomes hypoxic. The hypoxic condition during the pregnancy can result from a failure at any stage in the delivery of oxygen to the cells. In peripheral tissues, oxygen diffuses down a pressure gradient into cells and moves into their mitochondria, where it is used to produce energy. In our previous study, one of the beta-oxidation related gene, ACADVL was detected by gene-fishing technology using the preeclamptic placenta of human. We conducted in vitro and in vivo experiments to confirm preliminary study by inducing hypoxic stress in the human placental BeWo cells and in mouse placenta tissue. BeWo cells were cultured at 37 °C in a 20% O2, 5% CO2 as normoxic condition and in 1% O2, 5% CO2 as hypoxic condition. Pregnant mice were controlled oxygen concentrations (normoxia: 20 ± 2%, hypoxia: 10 ± 2%) from gestational day 6.5 to 17.5. And then, we conducted in triplicate for each set of experiments by using real-time PCR, western blot, PAS staining, immunohistochemistry and immunocytochemistry. The expression of genes known as biomarkers for hypoxia, HIF-1α, was increased in BeWo cells and mouse placenta which induced PE. The beta-oxidation related genes, ACADVL expression was significantly increased by hypoxic stress both BeWo cells and mouse placenta. As a result, the elevated level of HIF-1α is indicating that our experimental conditions closely mimicked PE. These results indicate that changes of beta-oxidation related genes are correlated with PE induced hypoxic condition. This work was supported by the National Research Foundation of Korea (NRF) grant of Korean government (MEST) (No. 2013-010514).

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Intrauterine growth restriction (IUGR) is a disease affecting 10% of all pregnancies and is one of the major causes of fetal morbidity and mortality. Cigarette smoke during pregnancy is known to induce IUGR, which is often characterized by hypoxia, asphyxia, and fetal demise. In severe cases, IUGR is implicated in spontaneous abortion and stillbirth. Unfortunately, studies of the effects of secondhand smoke (SHS) exposure during pregnancy are limited. Receptors for Advanced Glycation End-products (RAGE) are transmembrane receptors activated by cigarette smoke that function as a potent feed-forward inducer of inflammation. We tested the hypothesis that SHS is sufficient to induce IUGR and that RAGE modulates placental responses to SHS. C57Bl/6 mice (n=6) were exposed to SHS for 4 days from day 14 to day 17 of gestation (DGA). At the time of necropsy (18 DGA) placental and fetal weights were recorded and tissues were immediately frozen for histological and protein analysis. To address the effects of cigarette smoke in trophoblast cell invasion, first trimester trophoblast cells were treated with cigarette smoke extract (CSE) for 24 hours. Mice treated with SHS demonstrated reduced birth weight (~51%; p<0.002) and placental weight (~51%; p<0.002) when compared with controls. These reductions were associated with a decrease in mTOR activation (4.0-fold; p<0.03) and p70 (1.1-fold; p<0.02) and a decrease in trophoblast invasion. Placental RAGE protein expression was increased (1.2-fold; p<0.05) following SHS exposure. There was a profound protection from SHS-induced reductions in placental beta-oxidation related genes, ACADVL expression was significantly increased by hypoxic stress both BeWo cells and mouse placenta. As a result, the elevated level of HIF-1α is indicating that our experimental conditions closely mimicked PE. These results indicate that changes of beta-oxidation related genes are correlated with PE induced hypoxic condition. This work was supported by the National Research Foundation of Korea (NRF) grant of Korean government (MEST) (No. 2013-010514).

191. Extravillous trophoblast cells derived from induced pluripotent stem cells from preeclamptic patients identify a potential invasion defect in preeclampsia.
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2015 Abstracts – Page 75
Preeclampsia (PE) is a pregnancy-specific disease affecting ~5% of pregnancies and characterized by a shallow and poorly perfused placenta and onset of hypertension and proteinuria in the mother. The early onset, more severe form of the disease typically requires premature delivery of the infant often by caesarian section. The underpinnings of the disease remain unknown, although there appears to be an ill-defined genetic component to it. There is presently no means for diagnosing PE in early pregnancy when the dysfunctional placenta becomes established and a lack of models relevant to progression of the disease. To address this problem, we derived induced pluripotent stem cells (iPSC) from umbilical cords of infants born to mother who had experienced early onset PE and from infants born after a normal pregnancy. These two kinds of iPSC were then converted to trophoblast by exposing them to BMP4 (B, 10 ng/ml) together with the ACTIVIN signaling inhibitor A83-01 (A, 1 μM) and the FGF2 signaling inhibitor PD173074 (P, 0.1 μM) (BAP conditions). This treatment leads to unidirectional commitment to the trophoblast lineage, and provides both syncytiotrophoblast (STB) and HLA-G-expressing extravillous trophoblast (EVT). In PE, the latter cell population has been suggested to be responsible for the shallow placentaent and a failure to modify the spiral arteries, which supply maternal blood to the placenta. To compare the invasion efficiency of EVT from the PE and control pregnancies, the iPSC were plated on Matrigel-coated invasion chambers with 8.0 μm pores and exposed to BAP treatment for 6 days to induce trophoblast differentiation. EVT under 20% O2 compared to 4% O2 (p < 0.05) These results suggest that the abnormalities documented for EVT in PE pregnancies in vivo may be recapitulated in the phenotype of EVT generated from umbilical cord-derived iPSC in vitro. These experiments indicate that events of a past pregnancy can be recapitulated in this model system and suggest a means for studying the pathophysiology of the earliest events in preeclampsia in vitro. Supported by NIH grant HD067759 to RMR.

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Signals for maternal recognition of pregnancy are poorly understood in the domestic horse, and ~18% of pregnancies are lost before implantation. Markers that distinguish reproductive state and early pregnancy could enhance reproductive outcomes. Our aim was to profile the proteome of the uterine environment during the estrous cycle and early pregnancy of the horse. We characterized the protein profiles of mares during: 1) estrus (Day [d] -1; d0 = ovulation); 2) diestrus (d 12 non-pregnant); and 3) early pregnancy (d 12 pregnant). Mares (n=3) were monitored by ultrasound through cycles and uterine fluid collected by infusing ~250 ml of lactated Ringers solution followed by gravity flow recovery on day of estrus and 12 d post-ovulation in the first cycle. In a subsequent cycle, each mare was inseminated using fresh sperm and uterine fluid collected at 12 d of pregnancy confirmed by ultrasound and embryo recovery. Fluid was pooled for the same reproductive stage, and then concentrated (3 kDa centrifugation filter). For each sample, 100 μg of total protein was analyzed by mass spectrometry (iTRAQ technique) with proteins identified by comparing mass spectra against the Equus caballus non-redundant RefSeq protein (20130923 build) using Proteome Discoverer 1.4. Resulting files were loaded into Scaffold Q+ (version 4.3.2) to quantitate peptides and proteins. A total of 606 proteins was identified. The threshold for declaring differential expression of a protein between reproductive states was set at ±1.5-fold change with statistical significance (P<0.05). Fifty-six (9%; 39 up-, 17 down-regulated) proteins met threshold during diestrus compared to estrus and 71 (11%; 65 up-, 6 down-regulated) were differentially expressed in diestrus versus early pregnancy. Proteins up-regulated in diestrus versus estrus included those related to inflammation (secretory phospholipase A2 [sPLA2]), cell adhesion (desmocollin, osteopontin, angiomodulin), and immune function (properdin, dipeptidyl peptidase, complement component C6). There was higher expression of annexin A1, annexin A5, testin, and prostaglandin E synthase (PGES) in uterine fluid on d 12 of confirmed pregnancy versus non-pregnancy. Testin (cell adhesion) and PGES (prostaglandin synthesis) experienced the largest (>6.5) fold increase during pregnancy compared to non-pregnancy. Other proteins increased in pregnancy compared to estrus and diestrus were apolipoprotein A-I (cholesterol transport), apolipoprotein A-II (cholesterol metabolism), haptoglobin (inflammatory response), fibrinogen α-chain (platelet aggregation), α-1-antiproteinase 2 (immune response), and prothrombin (wound healing). Therefore, type and amount of uterine protein fluctuated depending on reproductive state, with certain changes consistent with predicted function of the identified protein. For example, down-regulation of cell adhesion and immune proteins during estrus may be related to fertilization. Up-regulation of sPLA2 during diestrus perhaps was related to luteolysis, whereas absence at pregnancy with up-regulation of annexin A1 and A5 (that inhibit sPLA2) may relate to sustaining luteal function. We predict that such assessments will contribute to understanding the complex interplay among mechanisms controlling reproductive success, including early pregnancy recognition in the horse. [Supported by Challimor Fellowship, the Siech Endowment and Cooperative Research Farms.]

193. Levels of naturally occurring regulatory T-lymphocytes in mares during estrous, luteal phase and pregnancy.
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The fetal-placental unit is an allograft that has to escape destruction by the maternal immune system. Exposure of the dam to non-maternal antigens begins already at mating and results in changes of the maternal immune system. Among the different lymphocyte subpopulations, regulatory T cells (Tregs) contribute to balance of immune responses. They are subdivided into lineages that are either derived from the thymus (naturally occurring Tregs) or generated in the periphery (inducible Tregs). The forkhead box transcription factor (FOXP3) is considered a specific marker of Tregs. Demethylation of a highly conserved region within the FOXP3 gene (Treg-specific demethylated region, TSDR) is restricted to naturally occurring Treg cells and is used for their identification and quantification. In oestrous mares, the level of naturally occurring Tregs in peripheral blood was lower in animals undergoing early pregnancy loss than in mares that lost their pregnancy at later times or carried their foal to term. Low concentrations of naturally occurring Tregs may thus explain early pregnancy loss in individual mares. In the present study, we investigated the hypothesis that levels of naturally occurring Tregs in the peripheral blood of mares are influenced by estrous cycle and pregnancy. Warmblood mares (n=30), that were either not bred (Con, n=10), bred and conceived (Preg, n=10), bred and did not conceive (Nonpreg, n=7), or were bred, conceived and lost their pregnancy < day 30 after ovulation (n=3; Loss) were included. Blood samples were collected in estrus as well as on day 7 and 14 after ovulation. Estrous samples were always collected before the first insemination. Pregnancy was confirmed by transrectal ultrasound. In mares of group Preg, additional blood samples were collected on days 14, 20, 40, 60, 100 and 250 of pregnancy. Quantitative PCR analysis for quantification of naturally occurring Tregs was done. The equine FOXP3 TSDR locus was used to quantify Treg cells. The amount of methylated and demethylated FOXP3 TSDR template DNA was calculated from calibration curves by linear regression on crossing points using the LC480 Light Cycler software applying the second-derivative maximum method. Statistical analysis was performed with the SPSS Statistics 21 software (IBM-SPSS, Armonck, NY, USA) with the General Linear Model for repeated measures. Treg levels were found to be slightly, but significantly (p<0.001) decreased from oestrus to day 14 after ovulation (e.g. oestrus: Con 1.4±0.1, Preg 1.6±0.1, Nonpreg 1.4±0.1, Loss 1.2±0.2%; day 14 after ovulation: Con 1.2±0.1, Preg 1.2±0.1, Nonpreg 1.0±0.1, Loss 1.0±0.1%), and tended to differ among groups (p=0.089). From the 3 mares of the Loss group, Treg level was below the mean, these mares had already undergone early pregnancy loss in previous pregnancies. Six mares of the Preg group lost their pregnancies between days 254 and 315 due to infection with equine herpes virus type 1 (EHV1), four carried their foals to term. Outcome and time of pregnancy did not affect Treg levels (e.g. day 100: term pregnancy: 1.0±0.1, EHV1 abortion 1.1±0.1%). In conclusion, naturally occurring Tregs slightly but significantly decrease from estrus to the late luteal phase but not during ongoing pregnancy. This decrease may be related to a change from estrogen dominance in estrus to progesterone dominance during the luteal phase and pregnancy. A decrease of lower Treg levels in individual mares with early pregnancy loss was confirmed in the present study.

194. Maternal Fructose Consumption Leads to Impaired Fetal-Placental Development: an Underlying Role of Uric Acid Induced Placental Dysfunction.
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Maternal metabolic syndrome and diabetes as well as low birth weight increase offspring risk for developing obesity, cardiovascular disease, and diabetes in adulthood. One prominent cause of maternal metabolic syndrome is the consumption of fructose. Excess fructose consumption likely contributes to offspring health risks by impairing function of the placenta, the transitory organ that mediates exchange of nutrients and wastes between the mother and fetus. However, the mechanism by which consumption of a high-fructose diet impairs placental function and the in utero milieu is unknown. Fructose is metabolized to fructose-1-phosphate, leading to a reduction in intracellular ATP levels and activation of AMP deaminase, which catalyzes the conversion of AMP to xanthine. Xanthine is then converted to uric acid by xanthine oxidase. Uric acid can act as a potent antioxidant and is beneficial to normal physiology. However, excess uric acid can lead to lipotoxicity, oxidative stress and cellular dysfunction. Thus we hypothesized that excess fructose consumption leads to adverse effects in the mother and her offspring by driving placental uric acid synthesis and transport. To test this, we placed six-week old C57BL/6J female mice on a high fructose diet (HFrD) or standard rodent chow diet (CD) for six weeks prior to mating with chow fed C57BL/6J males. HFrD-fed mice did not exhibit obesity, insulin resistance or dyslipidemia but were glucose intolerant. Maternal serum, fetal and placental data were collected on embryonic day 18.5 after a four hour fast (n=13-14 mothers in each group with siblings treated as replicates for average fetal and placental values). Serum fructose levels were significantly elevated in HFrd-fed mice as well as their fetuses (P<0.01). HFrD-fed mice had smaller litter sizes (P<0.01) and placenta collected from HFrD-fed mice were larger (P<0.001). Despite the increase in placental size, HFrD led to fetal growth restriction (P<0.01). The fetal to placental weight ratio was significantly decreased, suggesting an inefficient placental growth environment. In addition, the placentas from HFrD-fed mice had decreased ATP levels, increased xanthine oxidase activity and significantly increased uric acid accumulation (P<0.05). Allopurinol inhibits xanthine oxidase and thereby inhibits uric acid production. In HFrD-fed mice that received allopurinol (150 mg/l) in their drinking water, the placental insufficiency phenotype was reversed and normalized as assessed by two- way anova analysis. Thus we conclude that fructose drives uric acid synthesis and transport within the placenta which likely contributes to the adverse fetal and placental phenotype.

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Gestational diabetes mellitus (GDM) is defined as diabetes that begins during pregnancy, and it usually resolves after parturition. Although obesity is a risk factor for GDM, it occurs in women who are not overweight. It has both immediate and long-term impacts on metabolic and cardiovascular health of the mother and fetus. GDM is one of the most common obstetrical complications, affecting 7-18% of all pregnancies. Despite its high prevalence, there is no widely accepted animal model. Previous studies in rodents have shown that a 60% high fat diet (HFD) for 1 month before pregnancy induces obesity and type II diabetes. The heterozygous leptin receptor mutant (Lep

<sup>db</sup>) has been used as a model of GDM, but its phenotype may be dependent on diet. We therefore set out to develop a mouse model...
of GDM. For the first experiment, both wild type (Wt) C57/B6/J and Lepr<sup>db/+</sup> mice were placed on either a control or 45% kcal/fat high fat diet (HFD) from one or three weeks prior to mating and throughout pregnancy. At d17.5, glucose tolerance tests were performed. Regardless of genotype, mice placed on a HFD one week prior to mating developed glucose intolerance [Area under the curve (AUC) 12474 ± 721 (Wt CD) vs. 23921 ± 2902 (1WkHFD), 13657 ± 435 (Lepr<sup>db/+</sup> CD) vs. 23033 ± 933 (Lepr<sup>db/+</sup> 1WkHFD)] (p<0.05). Weights differed by genotype, but were not affected by the brief HFD: Wt CD (31.26±0.93g) vs. WT HFD (31.13±0.78g) and Lepr<sup>db/+</sup> CD (36.53±0.80g) vs. Lepr<sup>db/+</sup> HFD (38.22±0.80g). Glucose tolerance was not significantly impaired in mice placed on a HFD three weeks prior to mating (AUC Wt 3WkHFD 15578 ± 1429 vs. Lepr<sup>db/+</sup> 3WkHFD 17454 ± 2471), suggesting adaptation to the HFD over time. Fasting serum insulin levels were significantly decreased (p<0.05) in 1WkHFD (0.69±0.20 ng/ml) and 3WkHFD (0.55±0.08 ng/ml) Wt mice compared to Wt CD (1.36±0.18 ng/ml). At 30 minutes post glucose bolus, serum insulin was not different among Wt mice regardless of diet. Another hallmark of GDM is increased circulating leptin. Serum leptin was significantly increased (p<0.05) in 1WkHFD mice compared to 3WkHFD and CD mice of the same genotype, which did not differ from each other. For the second experiment we assessed whether the effects of HFD were specific to gestation. Fasting glucose was not different between CD and 1WkHFD prior to mating, but was again elevated in the 1WkHFD at pregnancy day 17.5. At weaning, glucose tolerance did not differ between CD and 1WkHFD mice [AUC 13191±950 (Wt CD) vs 13514±913 (Wt 1WkHFD)]. Four weeks of HFD diet alone, without pregnancy, had a modest effect on glucose tolerance [AUC 19169±945 (Wt NPCD) vs 22741±1308 (Wt NPHFD) p=0.044)]. This effect was less dramatic when mice were pregnant. Collected whole blood into heparinized tubes and granulocyte fractions were collected by a density gradient cell separation using Histopaque 1083 solution. Separated fractions were applied to total RNA extraction and they used for interferon-stimulated genes detection by quantitative RT-PCR. Simultaneously whole blood was collected into commercial tube for direct RNA extracted tubes. After extracted their RNA also used for a quantitative PCR measurement of genes. Average amounts of four interferon-stimulated genes in granulocyte fraction were relatively lower in early period of estrous cycle but the amounts in some blood material were increased in middle and later of estrous cycle. They might be not specific changes because the higher values were detected randomly without the progress of estrous cycle. These fluctuations were detected in all four genes. However fold changes of average values between gestation Day 21 and estrous cycle were different; ISG15, MX1, MX2 and OAS1 were 5.1, 2.6, 2.4 and 3.3, respectively. In direct RNA extracted method, the fluctuations were also detected and their patterns were similar even genes copy number (/GAPDH) was decreased. The average copy number of ISG15 in granulocyte was about five times higher than that in direct RNA extracted method. These data suggest that ISG15 is the most reliable indicator for the diagnosis of early gestation within three weeks after insemination. The data imply that a cow expresses less than three (copy number/GAPDH) of ISG15 gene in granulocyte is non-pregnant around Day 21 after insemination. Additionally the examination indicates a granulocyte is more suitable source for interferon-stimulated genes. The Research Program on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development from the Ministry of Agriculture, Forestry, and Fisheries of Japan has granted for this study (REP-1003).

196. **Interferon-stimulated genes expression in bovine granulocyte during estrous cycle, and exploring the reliable threshold values for the diagnosis of early pregnancy in cows.**

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The expressions of interferon-stimulated genes, interferon-stimulated protein 15 kDa (ISG15), myxovirus -resistance (MX) 1, MX2, and 2'-5'-oligoadenylate synthetase 1 (OAS1), in peripheral white blood cells, specifically during early gestation, have been examined for a suitable indicator for the diagnosis in bovine early gestation. Although this method is reliable, some reports showed the difficulty for practical application in the field. Unclear factors and methodological problems are involved in this new technique, namely; what day after insemination is suitable, which blood cell type is suitable, which gene is most reliable, how much threshold value is reliable for detection of gestation signals, and so on. The purpose of this study is to determine the threshold levels of interferon-stimulated genes using the expression of their genes during estrous cycle in cows. Peripheral bloods were collected from Holstein cows throughout estrous cycle (detection day of estrous designated as Day 0) and some early gestation cows as a gestation group to compare the expression levels in pregnant. Collected whole blood into heparinized tubes and granulocyte fractions were collected by a density gradient cell separation using Histopaque 1083 solution. Separated fractions were applied to total RNA extraction and they used for interferon-stimulated genes detection by quantitative RT-PCR. Simultaneously whole blood was collected into commercial tube for direct RNA extracted tubes. After extracted their RNA also used for a quantitative PCR measurement of genes. Average amounts of four interferon-stimulated genes in granulocyte fraction were relatively lower in early period of estrous cycle but the amounts in some blood material were increased in middle and later of estrous cycle. They might be not specific changes because the higher values were detected randomly without the progress of estrous cycle. These fluctuations were detected in all four genes. However fold changes of average values between gestation Day 21 and estrous cycle were different; ISG15, MX1, MX2 and OAS1 were 5.1, 2.6, 2.4 and 3.3, respectively. In direct RNA extracted method, the fluctuations were also detected and their patterns were similar even genes copy number (/GAPDH) was decreased. The average copy number of ISG15 in granulocyte was about five times higher than that in direct RNA extracted method. These data suggest that ISG15 is the most reliable indicator for the diagnosis of early gestation within three weeks after insemination. The data imply that a cow expresses less than three (copy number/GAPDH) of ISG15 gene in granulocyte is non-pregnant around Day 21 after insemination. Additionally the examination indicates a granulocyte is more suitable source for interferon-stimulated genes. The Research Program on Innovative Technologies for Animal Breeding, Reproduction, and Vaccine Development from the Ministry of Agriculture, Forestry, and Fisheries of Japan has granted for this study (REP-1003).

197. **Influence of Gender on the Phenotype and Immunomodulatory Properties of Mesenchymal Stromal Cells From the Amniotic Membrane of Human Term Placenta.**

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The influence of gender on the immunomodulatory properties of human amniotic mesenchymal cells (hAMSC) on PBMC. To this end, the influence of the gender of both hAMSC and PBMC donors was evaluated. We have analyzed data from 405 hAMSC samples (Female(F):227/Male (M):178) previously prepared and evaluated for their immunophenotypical characterization and immunomodulatory properties. Immunophenotype was performed by FACS analysis and immunomodulation was performed by co-culturing irradiated hAMSC with PBMC activated with antiCD3. PBMC proliferation in presence or absence of hAMSC was analyzed by H<sup>3</sup>-thymidine incorporation.
measured by microplate scintillation and a luminescence counter. A total of 10 PBMC preparations were used in this study (F:3/M:7). Student’s paired t-test, baseline-corrected unpaired t-test and ANOVA plus Tukey’s test were performed using GraphPad Prism software Version 6 (Significance P<0.05). First F and M hAMSC did not differ in their marker expressions (%CD90-F:71.6/M:70.6; %CD13-F:79.5/M:79.1; %CD166-F:10.2/M:10.0; %CD324-F:11.0/M:12.0; %CD45-F:10.9/M:11.3; %CD66b-F:1.9/M:1.7; and %SSEA-F:10.6/M:9.5). Second, we observed that hAMSC gender didn’t appear to influence PBMC proliferation. hAMSC from F and M donors were able to inhibit PBMC proliferation in a similar manner (35% inhibition for both F and M). From the total number of hAMSC preparations, 76% of F and 72% of M were able to suppress the proliferation of PBMC more than 20%, 14% of F and 16% of M samples inhibited less than 20%, and a small number of samples were not able to inhibit or stimulated PBMC proliferation (F: 10%, M:12%). Finally, we divided the samples into 4 groups in order to analyze the effects of gender of both hAMSC and PBMC donors: F or M hAMSC + F PBMC (G1 and G2, respectively), and F or M hAMSC + M PBMC (G3 and G4, respectively). No significant differences were observed between M and F hAMSC inhibition of F PBMC proliferation (G1:40.9%/G2:49.8%), and this was also true when M PBMC were used (G3:32.7%/G4:34.3%). We conclude that gender does not seem to influence the capacity of hAMSC in primary culture (P0) to suppress PBMC proliferation, neither when F and M PBMC are grouped together, or when they are considered separately. Our observations suggest that there is no need of gender selection, even though other studies regarding advantages of gender selection are needed. The authors would like to thank São Paulo Research Foundation for the Postdoctoral Internship Abroad Fellowship, Fondazione Cariplo and Fondazione Poliambulanza that supported this research.


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Preterm delivery of an underdeveloped fetus is a global problem accounting for 75% of all fetal morbidity and mortality. Babies delivered prior to full development at term have multiple medical problems that plague these individuals throughout their lifetime. There are no effective (or FDA-approved) medications that prevent contractions of the uterus in patients who enter labor preterm (PTL). What is used is ineffective at allowing the fetus to remain in the womb until term. Drugs employed to prevent PTL (tocolytics) are only evaluated for an ability to prevent labor for 48 hours, a time during which treatments can ready the fetus to breath air. PTL leads to preterm delivery (PTD) in over 50% of cases. Spontaneous PTL (sPTL, no explanation such as infection) accounts for a large share of PTL cases. While this problem undoubtedly has a number of potential causes, we have discovered that patients that enter labor spontaneously preterm, have a blunted response to the smooth muscle relaxant, nitric oxide (NO). To determine the response of preterm vs. term tissues to NO, we used tissue bath studies and found that NO cannot relax preterm tissues to the same extent as term tissues. These striking results are the first studies we are aware of that have directly measured the ability of NO to relax preterm vs. term pregnant human myometrium. Strips of myometrium from patients at term or preterm in labor were hung in tissue baths and allowed to contract spontaneously following K+ induced contraction and equilibration after washout. Strips served as their own control in the absence of NO-donor addition (cysteine-NO). Cys-NO relaxed term laboring myometrium with a K₈ of 1mM. In spontaneous preterm laboring tissues, the K₈ was 10 times higher and relaxation was significantly blunted (26% vs. 86% relaxation). In a set of tissues from the same patients, oxytocin (OT, 100 nM) was added and the ability of Cys-NO to relax agonist-induced contractions over 5 min was measured as area under the curve. In OT stimulated preterm laboring tissues Cys-NO produced only 12% relaxation (K₈ = 10-5.5 M) that did not reach significance (p =0.6) compared to control, while in term laboring tissues, Cys-NO was equally effective as when spontaneous contractions were measured. The effect of NO to relax pregnant human (term) myometrium (EC₅₀, 1 mM) together with the fact that elevations of cGMP do not mediate the relaxation effect despite phosphorylation of myometrial proteins by PKG, established that another mechanism is involved in NO-mediated relaxation in myometrium. Thus, myometrium is fundamentally different from other smooth muscles in its response to NO and cGMP. Our data suggest that the unique changes in S-nitrosations of proteins seen in myometrium following NO treatment are mechanistically associated with relaxation. Furthermore, one or more of these S-nitrosations is dysfunctional in preterm labor and may explain the failure of NO to relax the tissue. Understanding the failure of NO to relax preterm myometrium at the level of protein S-nitrosations may offer therapeutic targets unique to myometrium.

199. Stretch Induced Phosphoproteomic Signaling Networks in Pregnant Human Myometrial Cells Highlight the Importance of the Integrin Signaling Pathway. Craig C. Ulrich1, Christian Copley-Salem1, David Quilici2, Karen Schlauch1, Iain L. Buxton1, Heather R. Burkin1.

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The switch from a quiescent phenotype to a contractile state during pregnancy likely involves the integration of multiple signaling networks generated from both hormonal and mechanical signals. Identifying how these networks change and interact is crucial to understanding the biochemical processes involved in the induction of labor. We have conducted phosphoproteomic studies to identify and quantify the global phosphorylation changes that occur in telomerized pregnant human myometrial smooth muscle (hTRT-HUSM) cells in response to acute mechanical stretch. We performed 18% biaxial stretch experiments to elucidate the signaling pathways activated by mechanical distention of hTRT-HUSM cells. Protein from 3 replicates of each condition (0 minute and 5 minute stretch) was extracted and subjected to western blotting or enriched for mass spectrometry based analysis. For the mass spectrometry based analysis, samples were digested, desalted, and phospho-peptides were enriched using Ti02 affinity columns. Enriched samples were differentially TMT labeled and further enriched using hydrophilic interaction liquid chromatography. Protein and peptide identification and quantification were measured using reverse phase liquid chromatography coupled to tandem mass spectrometry. All data were analyzed in conjunction with the Nevada Center for Bioinformatics. DAVID Pathway Analysis of the phosphorylation changes induced by biaxial stretch of hTRT-HUSM cells confirmed an increase in phosphorylation of multiple peptides on proteins involved in the focal adhesion kinase (FAK)/integrin signaling pathways. This included ERK2 and the ERK scaffolding protein AHNAK. We also identified an increase in stretch induced
phosphorylation of the regulatory contractile proteins MYLK and MYL9. These are well known and integral proteins in the smooth muscle contraction pathway. The 5 minute time point gives us a static cross section of the stretch induced signal that appears to utilize the FAK/Integrin pathway, among others. Future work will utilize specific inhibitors of target integrins in order to positively identify the phosphorylation events attributed to the integrin of interest. Future work will also focus on later time points in which downstream signaling molecules, such as mTOR, are expected to be significantly altered during acute stretch. This research was supported by the National Institute of General Medical Sciences (NIH award number P20GM103440) and a Pathway to Independence Award from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NIH award number R00HD067342) to H. Burkin. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.

200. MMP2 and MMP9 Expression and Upregulation in Preterm Myometrium.
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Much evidence suggests Matrix metalloproteases (MMPs) are involved in the process of parturition and the regulation of birth timing. Secreted MMP9 has been implicated in initiating signaling events that regulate cellular contraction and recent work indicates MMP9 and MMP9 inhibitors rapidly enhance the oxytocin-induced contractile response in rat myometrium. We performed experiments to test the hypothesis that MMP2 and MMP9 are expressed in human myometrial cells during pregnancy and that their activity is altered in preterm laboring myometrium where it may exert local effects on contractility. Human myometrial tissue samples were collected under informed consent and with Institutional Review Board approval. MMP expression was assessed by semi quantitative western analysis and normalized to GAPDH expression. MMP activity was assessed by gelsatinase zymography. Statistical analyses were performed with one-way ANOVA. MMP localization in pregnant human myometrial tissue was determined by immunofluorescent confocal microscopy. Primary myometrial cells were isolated cultured for 1-3 passages and conditioned medium collected to confirm MMP expression by myometrial cells. Pro-MMP2 and MMP2 gelatinolytic activity was higher in preterm laboring myometrial tissue (p<0.05) than in nonpregnant myometrial samples and myometrial samples from other pregnant groups (preterm not in labor, term not in labor, and term in labor, n=8-13/group). Pro-MMP9 and MMP9 gelatinolytic activity was also higher in the preterm laboring myometrial tissue (p<0.001, n=8-13/group), although MMP9 expression was also increased in the term not in labor group (p<0.05). Similar expression patterns were obtained by western analysis of human uterine tissue. MMP expression and activity were detected in conditioned medium from isolated human myometrial cells and confocal microscopy detected MMP protein adjacent to myometrial cells in pregnant uterine tissue. These data indicate MMP2 and MMP9 are expressed in pregnant myometrium and that myometrial MMP activity is higher in preterm laboring myometrium. The localization of MMP9 adjacent to myometrial cells is consistent with a potential role in regulation of myometrial contractility as well as ECM degradation. Future work will determine if MMP9 can affect contractility in pregnant human myometrial tissue. This research was supported by a Mountain West Clinical Translational Research-Infrastructure Network subaward (to H. Burkin) under a grant from the National Institute of General Medical Sciences (NIH award number 1U54GM104944) and a Pathway to Independence Award from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NIH award number R00HD067342) to H. Burkin. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.

201. Postpartum uterine repair is mediated by the Notch family transcription factor, RBP-Jκ, in the mouse.
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Notch signaling is an evolutionarily conserved pathway regulating key cellular events, including proliferation. The Notch family transcription factor RBP-J kappa (RBP-Jk) coordinates signaling through all Notch receptors (Notch1-4). Both Notch1 and RBP-Jk are critical during decidualization in the mouse and human. Selective ablation of RBP-Jk in the mouse uterus (Pgrcre/+Rbpjflox/flox; Rbpjflox/flox) results in sub-fertility with reduced litters and litter sizes compared to Rbpjflox/flox mice. Rbpjflox/flox mice become infertile following two litters most likely due to failed postpartum repair, as indicated by the accumulation of hemosiderin-laden macrophages, commonly referred to as nodule cells. Nodule cells aid in uterine repair following placental detachment and are a component of postpartum nodules. RBP-Jk has been shown to control mechanisms important for promoting tissue regeneration and repair after injury in the liver. We hypothesized that RBP-Jk regulates the processes responsible for repair of the uterus following parturition. To test our hypothesis, we collected Rbpjflox/flox (control) and Rbpjflox/flox (KO) mouse uterine segments corresponding to placental detachment sites at key time points postpartum: postpartum days (PPD) 3, 5, and 10 (n=4/group/d). We evaluated uterine morphology and performed immunostaining for markers of proliferation (Ki-67), endothelial cells (CD31) and macrophages (F4/80). Specific morphological analysis included: 1) reformation of the luminal epithelium (LE) along the mesometrial pole, where the placenta detaches, 2) reformation of the myometrium along the circumference of the uterus with isolation of a distinct postpartum nodule, 3) the presence and location of macrophages, and 4) vascular abnormalities. In the control mice, the LE reformed along the mesometrial pole by PPD5. The LE of KO mice on PPD5 resembled control mice at PPD3, with a monolayer of epithelial cells or exposed stroma present at the mesometrial pole. Consistent with the morphology, Ki-67 positive cells were only present in the mesometrial LE on PPD5 while the entire LE stained positive in the KO, resembling PPD3 controls. In control mice, glanular epithelium (GE) stained strongly for Ki-67 on PPD3, and decreased by PPD5, while the KO GE stained strongly for Ki-67 on both PPD3 and 5 suggesting that epithelial proliferation is delayed or regeneration is impaired in both LE and GE following parturition. In the controls, the myometrium reformed along the circumference of the uterus by PPD10, defining the postpartum nodule within the mesometrium, while this was not evident in the KO. F4/80-positive cells were present throughout the uterus and to a greater extent in the KO mice, while in the control mice, they were confined to the mesometrium. CD31 staining at PPD3 and 5 indicated the presence of slightly larger, irregular vasculature in the KO endometrium. In summary, ablation of RBP-Jk results in impaired resolution of placental detachment injury and failed postpartum repair, suggesting Notch signaling through RBP-Jk plays a central role in this process. Potential mechanisms include abnormal epithelial proliferation, myometrial and vascular regeneration and aberrant macrophage recruitment. (NIH HD042280 to ATF)
202. **RAC1 Regulates Paracrine Mechanisms Controlling Stromal-endothelial Crosstalk During Early Pregnancy.**

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Implantation is a complex multistage process during which the embryo attaches to the uterine epithelium, invades the stroma, and establishes the placenta. During implantation, in rodents and humans, the stromal cells undergo a dramatic transformation to form a specialized tissue, known as the decidua. This process, known as decidualization, involves proliferation and differentiation of fibroblastic stromal cells into decidual cells that support embryo growth and survival until placentation ensues. Concomitant with the differentiation process, new blood vessels form in the maternal decidua from pre-existing vasculature through the process of angiogenesis. While it is clear that decidualization and angiogenesis play crucial roles during early pregnancy, the complex molecular pathways underlying these processes remain largely unknown. Our recent studies revealed that Rac1 (Ras-related C3 botulinum toxin substrate 1), a pleiotropic signaling factor and member of the Rho family of GTPases, is induced in uterine stroma during decidualization. To address its role during pregnancy, we created a conditional knockout of the Rac1 gene in the uterus by crossing mice carrying floxed alleles for Rac1 with PGR-Cre mice. A six-month breeding study indicated a severe defect in the fertility of Rac1-ablated (Rac1\(^{f/f}\)) females. These mice exhibited extensive hemorrhage in the decidual bed accompanied by embryo resorption around days 10-12 of gestation. Further analysis of the Rac1\(^{f/f}\) females revealed that, while the early phases of decidualization progress are unaffected, the latter stages of this process, particularly in the mesometrial decidua, are compromised. The loss of Rac1 expression in uterine decidual cells led to reduced expansion of the endothelial cell network and a marked reduction in the production of several paracrine regulators of angiogenesis, such as neuropilin 1, angiopoietin 2, and vascular endothelial growth factor, resulting in impaired development of maternal blood vessels during early placenta development. Collectively, our studies indicated that decidual cells, signaling via Rac1, regulate the production of critical stromal factors that impact endothelial cell function and blood vessel formation at the maternal-fetal interface. This research was supported in part by the NIH/NIEHS postdoctoral traineeship in endocrine, developmental, and reproductive toxicology (NIEHS T32ES007326 to JD).

203. **Bioactive progestin concentrations in peri-parturient mares.**

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Progesterone is required for the maintenance of pregnancy in mammals, and birth presumably necessitates withdrawal of (progesterone) which has not always been obvious in past studies in mares. The lack of progesterone in the second half of pregnancy in mares led us to examine the bioactivity of dihydroprogesterone (DHP) which was found to be equipotent to progesterone (Scholtz et al, 2014). The current study re-examined progestin withdrawal at foaling in part because multiple pregnanes that are present (some reportedly increasing in concentration) cross-react in immuno-assays for progesterone and their bioactivity is unknown. Our understanding of progestin withdrawal has been hampered by an inability to measure multiple pregnanes with adequate specificity and a lack of knowledge of their progestogenic bioactivity. Thus, we characterized changes in pregnane concentrations by liquid chromatography mass spectrometry (LC-MS/MS) and investigated the biopotency of progesterone and DHP metabolites using luciferase reporter assays in cells expressing the equine progesterone receptor (ePR).

Methods: Blood samples were collected from mares (n=4) over the 9 days before foaling (337±8 days). Serum (1ml) was extracted with methyl tert-butyl ether (5mls) and reconstituted in methanol:water (50:50) for analysis by LC-MS/MS. Standard curves ranged from 0.1 ng/ml to 100 ng/ml for pregnenolone (P5), progesterone (P4), DHP and allopregnanolone (AP). Chinese hamster ovarian (CHO) cells were transfected with a plasmid expressing the ePR and stable transfecants were selected by antibiotic resistance. The resulting cell line was transfected subsequently with an MMTV-luciferase expression plasmid responsive to steroid agonists. Cells were grown for 2 days then incubated with increasing concentrations (0-300nM) of progesterone and two metabolites known to be in high concentrations in late pregnant mares, 20αOH DHP (20αDHP) and 5α-pregn-3β,20α-diol (3β,20α-diol). Luciferase expression was measured 24 hrs later as an indicator of relative progestogenic bioactivity. Data were analyzed by linear regression and paired t tests. Results: The concentrations of the measured pregnanes began to decline from a peak reached about 3 days before foaling (P4 1.83±0.44, P5 4.28±1.18, DHP 79.0±25.7 and AP 26.0±7.04ng/ml) to low levels on the day of birth (P4 0.19±0.07, P5 0.96±0.48, DHP 11.1±2.33 and AP 7.95±2.18ng/ml). From day -3 to -1 (day before foaling), DHP decreased by 45.5±4.1% and P4 by 25.5±9.3% (P<0.05). 20αDHP and 3β,20α-diol activated ePR but both had less biopotency compared to progesterone. Concentrations achieving half-maximal stimulation (EC50) for progesterone, 20αDHP and 3β,20α-diol were 1.15, 4.50 and 612nM, respectively. Maximum ePR activation by progesterone was at 30nM and 20αDHP at 100nM, just 3 fold higher concentrations, less than half the ranges reported in late gestation. Conclusion: Like other mammals, progestogenic support of pregnancy by known bioactive progestins in horses (P4 & DHP) begins to decline from 3 days before foaling. However, other circulating pregnane metabolites such as 20αDHP may have sufficient bioactivity, and be present in sufficient concentrations, to play an additional physiological role and changes in concentration should be (and are currently being) assessed. The bioactivity of 20αDHP was surprising because prior studies suggested it did not compete with progesterone for binding to uterine and mammary cytosol. Clearly, robust bioassays are needed to determine bioactivity. Mares and women share many similarities in the endocrinology of pregnancy and parturition, and renewed studies in mares may have relevance to mechanisms regulating equine and human birth, both of which remain poorly understood. Such studies should focus on identifying relevant progestins using bioassays built on species-homologous PR activation, and subsequently measuring those progestins with specificity.

204. **Analysis of IL6 and Its Receptor Expression at the Maternal-Placental Interface during Pregnancy in Pigs.**

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Analysis of IL6 and Its Receptor Expression at the Maternal-Placental Interface during Pregnancy in Pigs.
Interleukin 6 (IL6) is a pleiotropic cytokine with multifunctional roles in inflammatory response and adaptive immunity. It has been well established that IL6 is widely expressed in the female reproductive tract and mediates embryo implantation and placental development in many species. In pigs, uterine expression of IL6 during early pregnancy has been studied, but expression and function of IL6 at the maternal-placental interface during mid- to late pregnancy has not been well understood. Thus, we examined expression of IL6 and its receptor, IL6R and GP130, in the uterine endometrium during pregnancy in pigs. We obtained uterine endometrial tissues from day (D) 12 and D15 of the estrous cycle and from D12, D15, D30, D60, D90 and D114 of pregnancy. To investigate expression of IL6 and its receptors in the placental tissues, we also obtained chorioallantoic tissues from D30, D60, D90 and D114 of pregnancy. Real-time RT-PCR analysis showed that levels of IL6, IL6R, and GP130 mRNAs in the endometrial tissues increased dramatically during mid-to late pregnancy and decreased at term. The levels of IL6, IL6R and GP130 mRNAs in the chorioallantoic tissues were low during mid- to late pregnancy and increased at term. In situ hybridization analysis showed that IL6 mRNAs were mainly expressed in the luminal epithelial and stromal cells in the endometrium during early pregnancy, and in the glandular epithelial cells during late pregnancy. IL6R and GP130 mRNAs were exclusively expressed in the glandular epithelial cells during late pregnancy. These results indicate that expression of IL6 and its receptors in the uterus endometrium and placenta is regulated in a stage-specific fashion during pregnancy, suggesting that IL6 and its receptors may play an important role in the maintenance of pregnancy at the fetomaternal interface during mid- to late pregnancy in pigs.

205. Pregnancy and Maintenance Rate of Bovine Cloned Embryos Depends on the Condition of the Surrogate and Embryo.
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SCNT cloned embryos show a limited delivery efficiency due to a high fetal loss rate after embryo transfer. In this study, we transferred 137 SCNT cloned embryos and investigated the pregnancy and maintenance rate depending on the condition of the surrogate and the embryos. Generally, we used 7d blastocysts after SCNT (108 cases) but 6d blastocysts (12 cases) had a higher rate of pregnancy after transfer while 8d blastocysts (14 cases) had the lowest rate. Even if the embryo stage were the same, the embryos with a faster developmental speed showed had a greater potential for pregnancy and the 7d blastocyst transfer of Expanding BL showed the highest pregnancy rate. Compared to the pregnancy rate after transferring 2 embryos at once(29.4%) transferring more than 4 embryos at once had the highest pregnancy rate (40.0%) but 100 days after transfer, all surrogates miscarried. We divided the cows according to species (Holstein and Han-woo) as well as their farrowing no. (heifer or suckler) and there were no differences between the species. However, Holstein suckler cows had the lowest pregnancy rate probably due to milking while in comparison heifer cows had a lower pregnancy maintenance rate probably due to their inexperience with pregnancies. With this data the pregnancy and maintenance rates are currently increasing. This work was supported by a grant of Research Program (No. 307-02) Gyeonggi-do Project, Republic of Korea.

206. Ex Vivo OB-Rb Leptin Receptor Expression in Uterine Arteries during the Follicular Phase of the Ovarian Cycle and Late Pregnancy: Role of Leptin-induced Angiogenic Reactions in Sheep Uterine Artery Endothelial Cells (UAECs).
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INTRODUCTION: Compared to the luteal phase, follicular phase and pregnancy are states of elevated estrogen and uterine blood flow (UBF) the latter being mediated by angiogenesis and vasodilatation. Leptin is an adipokine that regulates obesity and metabolism dysfunctions in reproductive processes via its receptors (OB-Ra and OB-Rb). Leptin also partly modulates blood pressure, endothelial function, and fetoplacental angiogenesis. However, virtually nothing is known regarding Leptin and its receptors in uterine artery (UA) angiogenesis. We hypothesized that: 1) In vivo UA expression of OB-Rb in endothelium (UAendo) and UA vascular smooth muscle (UAvsm) is locally elevated in pregnancy vs. nonpregnant (Luteal and Follicular) sheep and that in vitro Leptin receptors are also upregulated in nonpregnant uterine artery endothelial cells (NP-UAECs) and late pregnant ewes (P-UAECs); and 2) in vitro Leptin treatment will differentially modulate cell proliferation in ovine UAECs from NP-UAECs less than P-UAECs. METHODS: UAs were obtained from nonpregnant, (luteal, n=4; follicular, n=4), and late pregnant (120-130d, term=147d) sheep. To evaluate local UA adaptations we utilized a unilateral pregnant sheep model where pre-breeding uterine horn isolation (nongravid) restricted pregnancy to one horn (gravid). UAs were obtained from nongravid unilateral (n=7), gravid unilateral (n=7) and control bilateral (n=7) groups. Ex vivo OB-Rb protein expression was determined on UAendo and UAvsm by Western analysis; OB-Ra and OB-Rb were also evaluated in passage 4 NP-UAECs and P-UAECs. To evaluate angiogenesis in vitro, UAECs obtained from nonpregnant luteal (n=4) and follicular (n=4), as well as late pregnant (n=4) sheep were treated with vehicle (control) or 7 doses of Leptin (0.001-1000 ng/ml; 24 hr). The effect of Leptin on UAECs proliferative responses was evaluated using the 5-ethynyl-2-deoxyuridine (EdU-labeled) assay technique. RESULTS: Compared to follicular UAendo, OB-Rb was reduced (P<0.05) in UAendo from luteal (3.5-fold), nongravid (3.5-fold), gravid (9.5-fold), and control pregnant (6-fold) ewes. Compared to follicular, UAvsm OB-Rb also was lower (P<0.05) in UAvsm from luteal (3.9-fold), nongravid (5-fold), gravid (12-fold), and control pregnant (undetectable) ewes. UAendo and UAvsm OB-Rbs in nongravid were mostly reduced (P<0.05) however, interestingly less reduced than gravid and pregnant controls. Both OB-Ra and OB-Rb proteins were still expressed in passage 4 luteal, follicular, and late pregnant UAECs but unlike the ex vivo UAendo similar levels between groups were observed. Leptin treatment did not result in significant cell proliferation in luteal phase UAECs, however it significantly increased cell proliferation in UAECs from follicular phase (P<0.01; biphasic dose response) and late pregnant (P<0.01; typical dose response) sheep yielding maximum mitogenic responses at 0.1 ng/ml of Leptin; Luteal (0.80 ± 0.20 fold of control), Follicular (1.90 ± 0.24 fold) and Pregnant (1.53 ± 0.22 fold) UAECs. CONCLUSIONS: Contrary to our hypothesis, OB- Rb is not upregulated in UAendo or UAvsm in pregnancy, but rather locally downregulated possibly by placental progesterone during pregnancy. We observed higher OB-Rb in UAendo/UAvsm during the follicular phase, suggesting a role in preparation of the uterus and its vasculature for the periovulatory period. Leptin receptors in cultured passage 4 UAECs were expressed at similar levels between groups so we therefore tested if Leptin post-receptor signaling pathways for activating of angiogenesis were functional. Leptin treatment did not modulate cell proliferation in luteal phase UAECs, however it significantly elevated UAEC proliferation during the follicular phase and late pregnancy. These data suggest that
207. Effects of pregnancy and lactation on heart rate and heart rate variability in dairy cows and their fetuses.
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The combination of gestation and milk production during lactation leads to high energetic demands in dairy cows. High lactational performance requires adaptional changes of the cardiovascular system and may also constitute a stressor for the animals. During late gestation, additional metabolic stress in cows may occur due to the increasing demands of the rapidly developing fetus. Regulation of cardiovascular function by the autonomous nervous system (ANS) can be studied via analysis of heart rate (HR) and heart rate variability (HRV) in the pregnant cow as well as her fetus. Decreases of HRV variables SDRR (standard deviation of the beat-to-beat interval) and RMSSD (root mean square of successive beat-to-beat differences) are caused by a decrease in parasympathetic and/or increase in sympathetic activity and indicative of stress. To evaluate cardiovascular and ANS changes during lactation and pregnancy in cows and their fetuses, fetomaternal electrocardiograms (ECG) were recorded once weekly during the last 14 weeks before calving (a.p.) from pregnant-lactating cows (PL; n=10), pregnant-non lactating heifers (PNL; n=10). From the ECG recordings, HR and HRV variables SDRR and RMSSD were calculated. Maternal HR changed significantly over time in both groups (p<0.001). In PNL animals, HR gradually increased (week 14 and 1 a.p. 82±4 and 98±4 beats/min, respectively). In PL cows during lactation, HR was at the same level as in PNL controls but decreased markedly with drying off at 9 weeks a.p. (75±2 beats/min, p<0.05 vs group PNL). During the last 5 weeks a.p., HR increased again and did no longer differ between groups. In cows, SDRR and RMSSD did not change over time in both groups. In fetuses, HR decreased significantly from week 14 (141±2 beats/min) to week 1 a.p. (116±5 beats/min, p<0.001) without differences between groups. HRV variables SDRR and RMSSD increased with ongoing pregnancy in both groups (p<0.05), but this increase was more pronounced in fetuses from cows than from heifer leading to significantly lower HRV in heifer fetuses versus cow fetuses during the last 2 weeks before calving (e.g. SDRR week 2 a.p. heifers 9.7±0.7 and cows 13.9±1.1 msec, p<0.05; week 1 a.p. heifers 11.8±0.7 and cows 18.2±2.8 msec, p<0.01). Decreasing HR in PL cows after drying off indicates reduced physical demands after lactation. The parallel increase in HR in both groups from week 5 a.p. onwards represents adaptation of the maternal cardiovascular system to the requirements of a rapidly growing fetus. The lack of changes in HRV in pregnant animals suggests that late pregnancy per se does not represent a stressor for cows or heifers. In the fetus decreases in HR were most pronounced during the last 6 weeks of gestation and most likely caused by fetal growth as well as maturation of the autonomous nervous system. Maturational changes in the fetus are also reflected by an increase of HRV. HRV differences in fetuses of cows and heifers at the end of gestation may be due to a lower uterine capacity in heifers compared to cows. This represents a stressor for fetuses carried by heifers which is absent or occurs only much closer to calving in fetuses carried by cows. A more pronounced antepartum fetal stress response in heifers may explain why pregnancy in heifers on average is shorter than in multiparous cows.

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208. Retrospective reproductive analysis of dairy cows grazing intensive silvopastoral system calving on dry or rainy season in a Colombian tropical dry forest.
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Several factors influence the reproductive behavior of dairy cows during postpartum period. Nutrition, genetics and environmental conditions are the main causes affecting this aspect. Regarding to the tropics, important issues such climate conditions impact the reproductive performance because of the reduction of available grazing forage and the higher temperatures affecting the comfort zone of the cows, among others. Few studies report the reproductive performance of dairy cows grazing intensive silvopastoral systems. The objective of this research was to compare the reproductive behavior of crossbred dairy cows grazing intensive silvopastoral systems when they calved in dry and rainy seasons on tropical dry forest. Crossbred dairy cows (Gyr x Holstein, n=184) calving during dry (n=82) and rainy (n=102) season, were reproductively analyzed during years 2010, 2011 and 2012. Parameters such as calving to first service interval, days open, services per conception and calving interval were registered and compared among both season of each year and between the same season of the three years. Data was collected by observation at the milking parlor of the sire and cows in service and during the arrival of herd to the intensive silvopastoral system composed by Leucaena leucocephala and Cynodon plectostachyus. Regarding to 2010, 2011 and 2012 any of the variables differed significantly (p>0.05) when comparing cows calving during dry and rainy season (Mean & SEM: Calving to first service interval: 123.7±29, 93.6±18.3; 89.7±13.4, 70.1±8.6, 57.0±6.2. Days open: 138.7±14.3, 119.1±21.8; 103.2±11, 101.1±10.4; 99.7±8, 71.6±9.3. Calving interval: 424.5±14.1, 406.3±21.8; 388.3±11.1, 386.1±10.5; 351.6±8, 356.2±9.3, respectively for dry and rainy seasons of each year) but in 2012 services per conception factor was greater during the rainy season (p<0.05) (2010: 1.35±0.1, 1.2±0.2. 2011: 1.4±0.1, 1.25±0.01. 2012: 1.1±0.05, 1.5±0.13. Even though most of the variables did not differ, a pattern of shorter intervals of all the parameters was observed on cows that calved during the rainy season. When comparing this parameters among years, calving to first service interval, days open and calving interval exhibited less days on the dry than in rainy season of 2012 (p<0.05). Services per conception factor did not differ between both seasons in the three years studied (p>0.05). Under the conditions of this study, cows calving on rainy season had better reproductive performance than cows calving during the dry season even though they did not differed statistically. It could possibly be due to higher availability of forage of the intensive silvopastoral system that provided a legume containing high amounts of protein which was more degraded by the energy sources administered at the milking parlor (corn silage and commercial concentrate), and better climate conditions in terms of humidity, temperature and luminosity. In the opposite situation, longer reproductive intervals are registered on cows calving during the dry season because of low availability of forages and its poor quality of protein and high amount of fiber in the diet. The present research confirms the impact of the season of calving on the
209. Milk yield and reproductive performance association in crossbred dairy cows grazing intensive silvopastoral system at tropical dry forest of Colombia.

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It has been a goal for the entire milk industry to increase milk production of dairy herd at early lactation. However, pursuing high milk yielding, the reproductive behavior of the herd is affected most of the times. This happens due to the asynchrony of milk production peak and dry matter intake peak, that induces the cow to a negative energy balance, which causes a delay reproductive activation. Recently, to solve this problem, the intensive silvopastoral systems have been proposed to satisfy the nutritional requirements of the cows through a higher availability of forage, turning the production system profitable and sustainable along the years. There is lack of information that demonstrate the association between milk production and reproductive behavior of crossbred dairy cows grazing intensive silvopastoral systems at a tropical dry forest in Colombia. The main objective of this research is to exhibit the correlation between milk yielding and reproduction parameters of crossbred dairy cows, such as calving to first service interval, days open, calving interval and services per conception. Data of crossbred dairy cows (Gyr x Holstein) at early lactation was registered during dry season months (n=41) (June, July, August, September, December) and rainy season (n=12) (October and November) of 2011. At the same time, reproductive performance of these cows calving during the same dry and rainy months was registered. Descriptive analysis was performed to the reproductive variables and milk production of the cows evaluated. Data were analyzed using the Pearson correlation coefficient, to associate both variables. The reproductive performance for the year 2011 was (Mean + SD): Calving to first service interval: 80.7±58.5 days; days open: 94.06±60.5; services per conception: 1.45±0.6; calving interval: 378.4±61.1 days and milk production: 29.9±9.5 L/day. Regarding to the correlations, during the dry season, days open, calving interval and services per conception exhibited a positive associations with milk production, even though it was not significant (r=0.132, 0.121 and 0.025 respectively). Rainy season presented a positive correlation coefficient between days open (r= 0.54) and calving interval (r= 0.49) and milk production, which indicates a stronger association between variables. The longer reproductive intervals, the greater milk production, which could be interesting for the dairy farmer in terms of economic income per liter of milk sold; however, for reproductive aspect, this would impede the goal of one calf per year per cow. This research concludes about the important relationship between milk production and reproductive behavior under silvopastoral system, which must be adequate for both profitability.

210. Hormonal regulation of female reproductive cyclicity: a role for progesterone?

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The fundamental elements regulating the female reproductive cycle have been known for decades and include a hierarchy of control involving the hypothalamic/anterior pituitary/ovarian axis. The hypothalamus through its secretion of gonadotropin releasing hormone drives anterior pituitary production of gonadotropins (luteinizing hormone, LH and follicle stimulating hormone, FSH), which act on the ovaries to promote follicle development, ovulation, formation of the corpus luteum, and secretion of sex steroid hormones, estrogen and progesterone, with well established actions on the female reproductive tract. At the core of the female reproductive cycle is a balance of sex steroid hormone negative and positive feedback regulation of gonadotropin secretion. Both estrogen and progesterone signaling pathways have been implicated in feedback control of gonadotropins and regulation of the female reproductive cycle. Estrogen and progesterone exhibit context dependent negative and positive feedback regulation of gonadotropin secretion. These concepts have been reinforced through phenotypic examination of mice possessing null mutations at either Esr1 or Pgr loci. Esr1 and PGR encode estrogen receptor alpha and progesterone receptor, respectively. These two nuclear receptors mediate many of the actions of estrogen and progesterone on the female reproductive system. Analysis of rats with an Esr1 deficiency has further strengthened the importance of estrogen and ESR1 in regulating cyclicity (Endocrinology 155:1991-99, 2014); however, characterization of rats with a null mutation at the Pgr locus has forced a reexamination of the role of progesterone in the regulation of the female reproductive cycle. We generated two different Pgr mutant rat models. A 136 bp deletion within exon 1 of the Pgr gene (PgrΔ136E1) was produced by using zinc finger nuclease-mediated genome editing. The other possesses a 984 bp deletion including all of exon 3 of the Pgr gene (PgrΔE3; the same exon targeted in Pgr null mice) by using CRISPR/Cas9 system. In both cases, the deletion results in a truncated protein lacking the DNA binding domain and ligand binding domain due to a nonsense frame- shift and the emergence of a stop codon. Similar to Pgr null mice, Pgr null rats were infertile due to deficits in sexual behavior, ovulation, and uterine endometrial differentiation. However, in contrast to the reported phenotype of female mice with disruptions in Pgr signaling (Endocrinology 138:4147-52, 1997), Pgr null female rats exhibit robust estrous cycles. Four to five day cycles in vaginal cytology, uterine histology, serum hormone levels (LH, FSH, estradiol, and progesterone), and wheel running activity were evident in Pgr null female rats similar to wild type controls. Furthermore, exogenous progesterone treatment inhibited estrous cycles in wild type female rats but not in Pgr null female rats. This phenotypic description of Pgr null rats resembles aspects of those previously described for a patient with progesterone resistance and normal menstrual cycles (J Clin Endocrinol Metabol 48:127-32, 1979). We conclude that in the rat, and possibly other species, progesterone signaling is not required for the establishment and maintenance of the female reproductive cycle. (Supported by American Heart A association, Japanese Society for the Promotion of Science and Lalor Foundation postdoctoral fellowships and NIH grants: HD066406, OD01478)

211. Hypoxia induces differentiation of trophoblast stem cells despite the potency-maintaining FGF4.

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The majority of pregnancies are lost during the first trimester. Beside aneuploidy, the pathology behind early pregnancy loss is still unclear but likely to be “environmental”. A major limitation is the availability of human material for experimental study. Mouse trophoblast stem cell (mTSC) is a multipotent stem cell lineage capable of reconstructing placenta in vivo. Hypoxia is a common pathological condition that may be encountered in pregnancy complicated by hypotension, diabetes, and anemia or at high altitude. To understand the impact of hypoxia on earliest placental development during peri-implantation period, we use mTSC in vitro model to mimic the change caused by hypoxia. 0.5% O2 is used as it is “running” dose of oxygen stress that causes highest potency loss and differentiation gain as early as 1 day, despite the presence of FGF4. DAPI nucleus staining was used in immunofluorescence and flow cytometry to study giant nucleus formation. First placental hormone PL1 expression in normal and stress-induced differentiation was detected by immunofluorescence to show the function of giant cells. We found that 0.5% induces trophoblast giant cell (TGC) differentiation as early as 2 days and at the same time it decreases proliferation. Compared with normal differentiation, hypoxia induces giant cell morphodology earlier. By 6 days of 0.5% O2 exposure, nearly all the cells were forced into giant cell fate despite FGF4. However, hypoxia stress-induced giant cells don’t have normal giant cell function as indicated the low expression of PL1 as compared with normally differentiated giant cells. The consequence of this prioritized yet mal-functioning differentiation towards giant cells is two-fold. One is the inadequate secretion of PL1 to maintain pregnancy and the other is depletion of stem cells which are needed for other, later-lineage functions. This may account for part of reason for early pregnancy loss.

212. Gestational Exposure to Smoking Cessation Agents, Bupropion and Varenicline, in Rats Impair Fetoplacental Development and Fetal Cardiac Function.

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Approximately, 20% of all pregnant women smoke cigarettes despite their intentions to quit. Although behavioral interventions are advocated to facilitate quitting, they are only modestly effective, thus emphasizing the need for pharmacotherapy. Nicotine replacement therapy (NRT), bupropion and varenicline are recommended first-line smoking cessation drugs. However, clinical practice guidelines do not routinely recommend pharmacotherapy for smoking cessation during pregnancy because of insufficient data on the safety of existing medications. Gestational nicotine exposure is shown to adversely affect placental development and fetal health in rats, thus raising concerns about NRT’s safety during pregnancy. Conversely, the effect of bupropion and varenicline has not been reported. It is generally accepted that smoking cessation drugs mediate their therapeutic effects via specific interaction with processes related to neurotransmitter activity in the central nervous system. However, most of these drugs also interact with ion channels, which makes these drugs candidates for cardiotoxicity during embryonic development—an area that has not been previously investigated. Thus, the objective of this study was to examine the effect of maternal administration of bupropion and varenicline on placental development as well as fetal growth and cardiac function. Pregnant Sprague Dawley rats (n = 6/group) were orally gavaged twice daily with saline, bupropion (10 mg/kg) or varenicline (1 mg/kg) from day 1 to 20 of pregnancy. On gestational day 20, the dams were sacrificed, placentas and fetuses were removed. Placental labyrinth and junctional zone weight and fetal weight were assessed. Cardiac fetal gene expression (related to cardiac function - L-type calcium channel, ryanodine receptors and protein kinase c) using qRT-PCR, intracellular Ca2+ intensity, and spontaneous beating of cardiomyocytes were also assessed. Bupropion and varenicline exposed fetuses were growth restricted (bupropion: 2.35±0.01 g, Var: 2.38±0.01 g, p<0.001) compared to controls (2.47±0.01 g). Placental weights, including both labyrinth and junctional zones, were also significantly decreased following exposure to bupropion and varenicline, compared to controls (labyrinth - bupropion: 289±1.83, varenicline: 284±3.7, control: 301±5.7 mg and junctional zone -bupropion: 178±1.4, varenicline: 172±2.3, control: 187±4.1 mg). Cardiac L-type calcium channel subunits (Cav1.1) was significantly decreased by bupropion and varenicline compared to controls. In addition, varenicline exposure also significantly decreased mRNA expression of ryanodine receptors and increased protein kinase c-α, -δ and -ε, compared to controls. The magnitude of fetal cardiomyocyte Ca2+ transients was significantly higher in bupropion and varenicline exposed groups compared to controls. In addition, spontaneous beating of isolated cardiomyocytes from bupropion (230±1.15) and varenicline (219±4.3) exposed fetuses were significantly increased compared to controls (121±1.45). These findings suggest that gestational exposure to smoking cessation drug, bupropion and varenicline, may impact fetoplacental development and cardiac structure/function. It remains to be established if these offspring are at higher risk for developing adult life cardiovascular dysfunction.

213. The progesterone responsive uterine transcriptionite of the rat: impact of uterine progesterone resistance.

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Progesterone signaling in the uterus is crucial for establishment and maintenance of pregnancy. Perturbations in progesterone signaling, especially those leading to uterine progesterone resistance, are at the core of a range of uterine diseases, including recurrent pregnancy loss, endometriosis, and leiomyoma. The Brown Norway (BN) rat has been established as a model for uterine progesterone resistance. The purpose of this investigation was twofold: 1) to identify uterine progesterone responsive transcripts; 2) to determine how the uterine progesterone responsive transcriptome is affected by progesterone resistance. Eight week old Holtzman Sprague-Dawley (HSD) and BN female rats were bilaterally ovariectomized and were rested for two weeks prior to injection with vehicle (sesame oil) or progesterone (40 µg/g body weight). Uteri were collected 9 h after the injection, snap frozen in liquid nitrogen, and subsequently processed for RNA-Seq analysis. RNA-Seq libraries were sequenced with paired-end reads (100 bp) using an Illumina HiSeq2500 Sequencing System. Sequencing data was imported into the CLC Bio Genomic Workbench. The trimmed raw sequences were mapped to an annotated Ensembl rat genome reference sequence Rnor_5.0.78. Reads per kilobase per million (RPKM) was used as a measure of expression level. Transcripts with RPKM values greater than 1 with a fold change (treatment/control) greater than 2 were further analyzed by Ingenuity Pathway Analysis. A total of 334 and 199 known transcripts were upregulated by progesterone in HSD and BN uteri, respectively. Of the upregulated transcripts, 168 were common progesterone targets between the strains. A total of 328 and 143 known
transcripts were downregulated by progesterone in HSD and BN uteri, respectively. Of the downregulated transcripts, 103 were common progesterone targets between the strains. Canonical pathways modulated by progesterone in HSD versus BN uteri differed and included Wnt/Ca²⁺, Interferon, eNOS, PTEN, and p53 signaling. Several novel progesterone responsive transcripts were also identified [upregulated: 120 (HSD) and 62 (BN); downregulated: 28 (HSD) and 22 (BN)]. There were 54 novel upregulated and 9 novel downregulated progesterone responsive transcripts shared between the strains. This collection of novel transcripts includes both coding and noncoding RNAs. Quantitative RT-PCR was used to validate selected expression profiles of 45 transcripts. Among the shared progesterone responsive transcripts some exhibited similar responses between HSD and BN strains (e.g., Slc5a5, Gmcbp, Pla2g3, Gal, Sgbh1c1, Lefyl1, Mhhf2), while for others BN uteri showed diminished responses to progesterone (e.g., Igf1, Bliha15, Orm1, Sgk1). In summary, three unique features of uterine progesterone responsiveness were identified in progesterone resistant (BN) rats: 1) restricted spectrum and number of progesterone responsive transcripts; 2) attenuated progesterone responses associated with a subset of progesterone responsive transcripts; 3) unique profile of progesterone responsive canonical pathways. These new insights will provide a foundation for elucidating molecular mechanisms impacting uterine progesterone resistance. (Supported by a Lalor Foundation postdoctoral fellowships and NIH grants: HD066406, OD01478.)

214. **Dysregulation of TGF-beta Signaling Alters Uterine Fibroblast Function.**
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An increasing number of reproductive age women face pregnancy loss and infertility, some of which is associated with uterine dysfunction. A lack of understanding of mechanisms governing uterine development and function prevents effective diagnosis and treatment of such disorders. Transforming growth factor beta (TGFβ) signaling plays a pleiotropic role in fundamental cellular and developmental processes. Canonicly, TGFβ ligands signal through type 1 (TGFBR1) and type 2 (TGFBR2) receptors. Studies from the last two decades strongly indicate that TGFβ superfamily members are essential regulators of female reproduction. Our recent studies using a gain-of-function (i.e., constitutively active TGFBR1) mouse model suggest that balanced TGF-beta signaling is a key determinant of uterine development and function. We have shown that overactivated TGFβ signaling impairs uterine decidualization and promotes myofibroblast differentiation within the uterine stromal compartment. However, the timing of the endometrial lesion formation and the underlying mechanisms remain unknown. Therefore, the objectives of this study were to define molecular underpinnings of altered fibroblast properties and explore a potential contribution to the observed decidualization defects. Immunofluorescence microscopy using alpha smooth muscle actin demonstrated that overactivation of TGFβ signaling in the uterus disrupted normal differentiation and specification of the endometrial compartment at an early stage of postnatal development. Using primary cultures consisting mostly of fibroblasts derived from mouse uterus, we showed that TGFβ1 upregulated the expression of a number of genes associated with extracellular matrix (e.g., Lama1 and Ctgf) and smooth muscle cell identity (e.g., Acta2 and Des). Western blotting revealed that TGFβ1 was capable of inducing the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) within 5 min of treatment. Further studies using a highly selective inhibitor of MEK1 and MEK2, U0126, identified the involvement of ERK1/2 cascade in the regulation of several TGFβ1-induced genes (e.g. Ctgf) by uterine fibroblasts. Using Tgfb1 conditional knockout mice created by anti-Mullerian hormone receptor type 2 (Amhr2) Cre-recombinase, we provide additional circumstantial evidence that ERK1/2-positive fibroblasts might play a role in uterine longitudinal muscle layer development. In summary, increased TGFβ signaling promotes the differentiation of uterine fibroblasts to a smooth muscle cell-like phenotype. The alteration of uterine fibroblast properties may lead to functional deficiencies observed in the uterus. This research was supported by National Institutes of Health grant R21HD073756 and Ralph E. Powe Junior Faculty Enhancement Awards (to Q.L.).

215. **Administration of bovine pregnancy-associated glycoproteins to endometrial explants – evaluation of genes known to be transcribed in the bovine uterine.**
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Pregnancy-associated glycoproteins (PAGs) belong to the aspartic protease family. They are expressed exclusively by trophoblasts of even-toed ungulates. The ability to measure PAGs in maternal serum serves as a way to detect pregnancy in cattle. Furthermore, circulating amounts of PAG are associated with embryo survival and placental viability. However, little is known about the specific function of bovine PAGs. This study was designed to provide insight into some of the biological roles of bovine PAGs in ruminant pregnancy by measuring changes in transcript abundance in cultured endometrial explants treated with PAGs. We hypothesized that the application of bovine PAG to endometrium would alter transcripts in endometrial explants collected from pregnant and non-pregnant animals. PAGs for these experiments were purified from mid-gestation bovine placental extracts. Heifers were synchronized using a timing of the endometrial lesion formation and the underlying mechanisms remain unknown. Therefore, the objectives of this study were to define molecular underpinnings of altered fibroblast properties and explore a potential contribution to the observed decidualization defects. Immunofluorescence microscopy using alpha smooth muscle actin demonstrated that overactivation of TGFβ signaling in the uterus disrupted normal differentiation and specification of the endometrial compartment at an early stage of postnatal development. Using primary cultures consisting mostly of fibroblasts derived from mouse uterus, we showed that TGFβ1 upregulated the expression of a number of genes associated with extracellular matrix (e.g., Lama1 and Ctgf) and smooth muscle cell identity (e.g., Acta2 and Des). Western blotting revealed that TGFβ1 was capable of inducing the phosphorylation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) within 5 min of treatment. Further studies using a highly selective inhibitor of MEK1 and MEK2, U0126, identified the involvement of ERK1/2 cascade in the regulation of several TGFβ1-induced genes (e.g. Ctgf) by uterine fibroblasts. Using Tgfb1 conditional knockout mice created by anti-Mullerian hormone receptor type 2 (Amhr2) Cre-recombinase, we provide additional circumstantial evidence that ERK1/2-positive fibroblasts might play a role in uterine longitudinal muscle layer development. In summary, increased TGFβ signaling promotes the differentiation of uterine fibroblasts to a smooth muscle cell-like phenotype. The alteration of uterine fibroblast properties may lead to functional deficiencies observed in the uterus. This research was supported by National Institutes of Health grant R21HD073756 and Ralph E. Powe Junior Faculty Enhancement Awards (to Q.L.).
pregnant endometrium compared to the endometrium not treated with PAG. These results indicate that PAGs are capable of inducing changes in transcript abundance in bovine endometrial explants, which suggests that this model system may be useful to assess PAG function at the placenta-uterine interface.

216. Regulation of Brain-Derived Neurotrophic Factor by Estrogen, GABA, and Melatonin in Endometrial Epithelial Cells. Jocelyn M. Wessels¹, Aamer Somanî, Warren G. Foster¹.
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Approximately 10% of women of reproductive age suffer from endometriosis; a chronic gynecological and estrogen-dependent disease. Endometriotic lesions are endometrial cells implanted at inappropriate sites that result in pelvic pain, decreased quality of life, and often infertility in women. There is no clinical marker for endometriosis, and as a result there is a substantial financial burden on the healthcare system with over $1.8 billion being spent annually on endometriosis in Canada. Recently, we described the conserved expression of brain-derived neurotrophic factor (BDNF) and its high affinity receptor in the mammalian uterus, demonstrated the uterine regulation of BDNF by estrogen in vivo, and found elevated levels of circulating BDNF in women with endometriosis as compared to controls. BDNF and its high affinity receptor are able to induce nerve differentiation, growth, and maintenance, and also activate the proliferation, adhesion, and angiogenesis pathways; each central to endometriosis. Thus, we sought to investigate the in vitro regulation of BDNF output by endometrial epithelial cells in response to estrogen, gamma-aminobutyric acid (GABA), and melatonin; factors shown to regulate BDNF in the brain. Human endometrial epithelial cells (CRL-1671) were treated for 24 or 48 hours with estradiol (E2) (1nM-1µM), GABA (62.5-2000pg/mL) or melatonin (62.5-2000pg/mL) in the presence of letrozole to suppress endogenous aromatase activity (N=4/treatment). BDNF output was measured by ELISA, and for E2 treatments BDNF was normalized to milligrams of total protein. BDNF output was significantly inhibited by treatment with letrozole at 24 (P<0.003) and 48 (P=0.016) hours. At 24 hours, 10nM E2 (P=0.003), and 62.5 and 2000pg/mL GABA (P=0.003) restored BDNF output to control levels (vehicle without letrozole) while 125 and 1000pg/mL of melatonin increased BDNF output (P<0.001) above control levels. At 48 hours effect of E2 was lost; all E2 treatments failed to increase BDNF output above controls. Conversely, at 48 hours 250, 1000, and 2000pg/mL GABA treatments significantly enhanced BDNF output above controls (P<0.001) while melatonin suppressed BDNF output at higher concentrations (1000 and 2000pg/mL; P<0.001), showing an inverse dose-response curve. Here, we demonstrated that the output of BDNF is restored by E2 and GABA, and decreased by melatonin in letrozole treated endometrial epithelial cells. As endometriosis is an estrogen-dependent disease characterized by pelvic pain, we suggest that ectopic endometriosis lesions are able to release BDNF not only in response estradiol, but also in response to factors involved in pain mediation and perception (GABA). Further, because of the link between BDNF and nerve growth its expression in ectopic lesions might contribute to disease-associated pain and offer a novel therapeutic avenue to treat endometriosis.

217. A Comprehensive, Longitudinal Analysis of Steroid Hormones During Equine Pregnancy. Erin L. Legacki¹, Scott D. Stanley¹, Elizabeth L. Scholtz², Barry A. Ball³, Alan J. Conley¹.
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Introduction: Steroid hormone concentrations change dramatically during gestation in mares as the site of synthesis and metabolism shifts from the primary corpus luteum (CL), to include accessory CL, and finally to the placenta alone which utilizes substrates supplied variably as the fetal gonads grow and regress before foaling. This includes changes in both the steroid spectrum as well as their concentrations, but few studies have attempted to characterize this complex array comprehensively. Some studies have used immunomaps to measure pregnanes, androgens and estrogens but none have included all three classes. Furthermore, antibodies lack specificity and results can be misleading even when chromatography is used. However, liquid chromatography-mass spectrometry (LC-MS/MS) provides a tool to accurately quantify steroids with great specificity. The current study developed a method to determine pregnane, androgen and estrogen concentrations in a longitudinal analysis of steroids in mares to gain a better understanding of endocrinology of equine pregnancy.

Materials and Methods: Blood was collected from 8 mares throughout gestation every other day through gestation day (GD) 80 and then found elevated levels of circulating BDNF in women with endometriosis as compared to controls. BDNF and its high affinity receptor are able to induce nerve differentiation, growth, and maintenance, and also activate the proliferation, adhesion, and angiogenesis pathways; each central to endometriosis. Thus, we sought to investigate the in vitro regulation of BDNF output by endometrial epithelial cells in response to estrogen, gamma-aminobutyric acid (GABA), and melatonin; factors shown to regulate BDNF in the brain. Human endometrial epithelial cells (CRL-1671) were treated for 24 or 48 hours with estradiol (E2) (1nM-1µM), GABA (62.5-2000pg/mL) or melatonin (62.5-2000pg/mL) in the presence of letrozole to suppress endogenous aromatase activity (N=4/treatment). BDNF output was measured by ELISA, and for E2 treatments BDNF was normalized to milligrams of total protein. BDNF output was significantly inhibited by treatment with letrozole at 24 (P<0.003) and 48 (P=0.016) hours. At 24 hours, 10nM E2 (P=0.003), and 62.5 and 2000pg/mL GABA (P=0.003) restored BDNF output to control levels (vehicle without letrozole) while 125 and 1000pg/mL of melatonin increased BDNF output (P<0.001) above control levels. At 48 hours effect of E2 was lost; all E2 treatments failed to increase BDNF output above controls. Conversely, at 48 hours 250, 1000, and 2000pg/mL GABA treatments significantly enhanced BDNF output above controls (P<0.001) while melatonin suppressed BDNF output at higher concentrations (1000 and 2000pg/mL; P<0.001), showing an inverse dose-response curve. Here, we demonstrated that the output of BDNF is restored by E2 and GABA, and decreased by melatonin in letrozole treated endometrial epithelial cells. As endometriosis is an estrogen-dependent disease characterized by pelvic pain, we suggest that ectopic endometriosis lesions are able to release BDNF not only in response estradiol, but also in response to factors involved in pain mediation and perception (GABA). Further, because of the link between BDNF and nerve growth its expression in ectopic lesions might contribute to disease-associated pain and offer a novel therapeutic avenue to treat endometriosis.

218. Larger Uterine Size Is Associated With Increased Pregnancy Loss And Reduced Fertility In Heifers Receiving An In-vitro Produced Embryo.

2015 Abstracts – Page 87
Our recent research demonstrated that greater uterine volume was associated with reduced fertility after artificial insemination (AI) in lactating dairy cows. In this study, we hypothesized that size of the uterus would also reduce fertility in recipient heifers receiving an in vitro produced embryo. A total of 336 heifers had ovulation synchronized using a 5d CIDR-Synch protocol (CIDR insertion, 5 d later, CIDR removal plus prostaglandin F2aPHA (PGF) treatment, 24 h later a second PGF treatment, and 72 h later treatment with GnRH to induce ovulation). On day 5 after the final GnRH treatment that was used to synchronize ovulation, body condition score (BCS) was scored (1-5) and uterine size was determined. Diameter of each uterine horn was measured at the greatest curvature using trans-rectal ultrasound. Length of each uterine horn was measured by rectal palpation with subsequent comparison to a hand ruler. Total uterine volume of both horns was calculated using the diameter and length measurements (πr²L). On d 6-8 after GnRH treatment, a single in-vitro produced embryo was transferred on the same side as the corpus luteum, and a blood sample was taken on day 12 for measurement of progesterone (P4) concentrations. Pregnancy was diagnosed 32 and 60 d after GnRH treatment and pregnancy loss was calculated between 32 and 60 d. Average uterine volume was 92.5 ± 1.2 cm³ (n = 336). At day 32, 41.7% (140/336) of heifers were pregnant with no difference in uterine volumes between pregnant (90.9 ± 1.8 cm³, n = 140) and non-pregnant (93.7 ± 1.5 cm³, n = 196) heifers (P = 0.23). However, at day 60, only 30.6% (103/336) of the heifers remained pregnant (26.4% pregnancy loss, 37/140), and uterine volume was smaller (P = 0.04) in pregnant (89.0 ± 2.1 cm³, n = 103) compared to non-pregnant (94.1 ± 1.4 cm³, n = 233) heifers. Furthermore, heifers that lost the pregnancy between day 32 and 60 had greater (P = 0.08) uterine volume than heifers that did not have pregnancy loss. Logistic regression analyses were also performed to compare P/AI to uterine measurements (diameter, length and volume). The P/AI at d 32 was not related to uterine diameter (P = 0.38), uterine volume (P = 0.24), or uterine length (P = 0.15). In addition, P/AI was not related to P4 concentrations at day 12 (P = 0.73) but was related to BCS (P = 0.03). Interestingly, at day 60, P/AI was related to uterine diameter (P = 0.10), uterine length (P = 0.03), and uterine volume (P = 0.03), but was not related to BCS (P = 0.28) or P4 on day 12 (P = 0.40). In addition, for the pregnant heifers at day 32 (n=140), risk of pregnancy loss was related to uterine volume (P = 0.056) and uterine length (P = 0.096) using logistic regression. In a quartile analysis for uterine volume, the differences in uterine volume were not related to circulating P4 concentrations (P = 0.98). There was an effect of body condition score of the recipient heifers on P/AI either at d 32 (26.5 vs. 48.3%; ≤ 2.5 vs. ≥ 2.75; P = 0.0002) or d 60 (21.6 vs. 34.6%; P = 0.0001) pregnancy diagnosis; however this effect was independent of any of the uterine measurements (91.8 vs. 92.8 cm³ uterine volume; ≤ 2.5 vs. ≥ 2.75; P = 0.71). Thus, greater uterine size of recipient heifers can reduce fertility following transfer of an IVF embryo, primarily due to loss of the pregnancies between d 32 and d 60. The unexpected timing of this negative effect of uterine size (d 32 to d 60 of pregnancy) suggests an important signal or maternal response during this period that is related to an interaction between the quality of the embryo (IVP in this case) and the uterine size.
Npas2, a circadian gene, is associated with metabolic regulation in fetal development. Previously, we have demonstrated that a maternal high fat diet is associated with the disruption of the circadian pathway in the fetal liver. It is not known how circadian rhythms are established in fetal development with the lack of light/dark cues through the central clock nor is it known how Npas2 plays a role in adult metabolic function. We have developed a novel Npas2 conditional knock out (cKO) mouse model to investigate the role of the peripheral clock (liver) in establishing metabolic homeostasis. Our objective is to understand the role of Npas2 in maintaining metabolic homeostasis in adult life, especially under the metabolic stress of a high fat diet. Npas2 cKO mice were generated by targeting the deletion of exon 3 through the cre-lox system of conditional gene deletion. Mice with the Albumin Cre (Albcre;Fl/+) transgene and heterozygous for the loxP flanked (Fl) region of the Npas2 gene (Albcre;Fl/+;Fl/+) were mated to controls (Fl/+) to generate Npas2 cKO (Albcre;Fl/Fl;Fl/+;Fl/+) and control mice (Albcre;Fl/Fl;Fl/+;Fl/Fl;Fl/+;Fl/Fl, n=62) for this study. Mice were genotyped between postnatal day 14-20 (p14-20) by PCR amplification of tail genomic DNA then weaned at p21 onto control (CD, Harlan Teklad TD.08485, n=76) or high fat diet (HFD, Harlan Teklad TD.88137, n=83). Mice were weighed biweekly, and after 25 weeks post weaning, a glucose tolerance test (GTT, 2g/kg glucose by oral gavage) was performed. Then, animals were rested two weeks before fat and lean mass was measured by quantitative magnetic resonance (QMR) and sacrificed for tissue and serum collection. Loss of Npas2 in the liver did not alter terminal weight of the mice. As expected, both the male and female mice on a HFD gained significantly (p<0.01) more weight than mice placed on the CD. However, male cKO mice on a HFD have significantly (p<0.05) larger mass than cKO mice on the CD two weeks before the control mice on HFD. Conversely, the female cKO mice on a HFD were significantly heavier than female cKO mice on the CD after seven weeks post weaning, while the female control mice (Fl/Fl) on a HFD became significantly heavier than the female control mice on the CD after three weeks post weaning. QMR measurements showed that female cKO mice on HFD had significantly less fat mass (p<0.05, 8.62g, 27.43% fat) than female control mice (Fl/Fl, 11.94g, 33.93% fat) on a HFD. The results of the GTT study indicate that loss of hepatic Npas2 has a minimal effect on glucose homeostasis. In conclusion, we successfully generated a novel mouse model to study to role of the peripheral clock, regulated by Npas2. The initial phenotype indicates that Npas2 in the peripheral clock (liver) has a slight protective effect against initial weight gain and lipid accumulation in female mice in response to a high fat diet. Additional work must still be done to fully characterize the Npas2 cKO mouse model, including liver histology, metabolic markers and a restricted feeding diet. Research supported by NIH DP21DP20D001500-01 (K.A.), NIH 5K12HD00849 (K.A.), and T32 GM088129-01 (D.O.).

221. Super oxide dismutase 2 overexpression alleviates maternal diabetes-induced neural tube defects by suppressing oxidative stress and restoring mitochondrial function.

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Nearly three million American women and sixty million worldwide women in reproductive age have diabetes, and these numbers are expected to double in 2030. Maternal diabetes induces structural birth defects including neural tube defects (NTDs). Oxidative stress plays a central role in the induction of diabetic embryopathy. However, the source of reactive oxygen species (ROS) in diabetic embryopathy is still unclear. We hypothesize that maternal diabetes-induced mitochondrial dysfunction causes oxidative stress in the developing embryo and mitigating mitochondrial dysfunction reduces oxidative stress and diabetic embryopathy. To eliminate mitochondrial ROS, a mitochondrial specific antioxidant enzyme, super oxide dismutase 2 (SOD2), transgenic (Tg) mouse model was used. Insulin deficient type 1 diabetes was induced in female mice by streptozotocin injections. Nondiabetic (ND) and diabetic mellitus (DM) females were bred with SOD2-Tg males to produce wild-type (WT) and SOD2 overexpressing embryos. Embryonic day 8.5 (E8.5) and E10.5 embryos were harvested for molecular analyses and NTD examination, respectively. Levels of Lipid hydroperoxide in embryos, abundance of superoxide and defective mitochondria in the developing neuroepithelium, mitochondrial translocation of proapoptotic Bel-2 members, mitochondrial membrane depolarization and necroptotic cell apoptosis were significantly increased in WT embryos of DM dams compared with those in WT and SOD2 overexpressing embryos from ND dams. SOD2 overexpression abrogated maternal diabetes-induced oxidative stress and mitochondrial dysfunction in cells of the developing neuroepithelium. Embryos from 7 ND dams and 9 DM dams were examined for NTDs. 1 out of 28 WT embryos from ND dams exhibited an NTD, whereas 7 out of 28 WT embryos from DM dams had NTDs. None of the 27 SOD2 overexpressing embryos from ND dams showed manifestation of NTDs. SOD2 overexpression ameliorated NTD formation under diabetic conditions because only 1 of 25 SOD2 overexpressing embryos from DM dams had an NTD. These findings support the hypothesis that mitochondrial dysfunction in the developing neuroepithelium enhances ROS production, leading to oxidative stress and NTD formation. Restoring mitochondrial function through SOD2 overexpression blocks maternal diabetes-induced oxidative stress and reduces NTD incidence in embryos exposed to diabetes.

222. Development of viable potency factor and first differentiated lineage reporters for stress-induced differentiation of ESCs for use in high throughput toxicology assays.

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Stress induces differentiation in embryonic and placental trophoblast stem cells (ESCs and TSCs, respectively) at exposures that result in diminished population expansion and increased differentiation of cells of the first lineage - that normally differentiates when potency-maintaining growth factors are removed. Stress induces potency loss and also imbalances differentiation by increasing first lineage and decreasing later lineages, a phenomenon of prioritized differentiation. This has been shown by biochemical means but development of high throughput screens for prioritized differentiation is sought using two types of viable reporter ESCs. One type uses multimerized potency promoters for Rex1 and Oct4 that drive red and green fluorescent reporters (RFP and GFP, respectively). A second type uses GFP knocked into to Pdgfra to employ the endogenous promoter of this gene that is expressed in the first and later lineages of extraembryonic endoderm arising from ESCs. Both reporter ESCs corroborate biochemical data by FACs and microplate reader. Although the viable potency reporters show dose- dependent potency loss the sensitivity isn’t sufficient for an HTS. In contrast the Pdgfra-GFP ESCs responded to stress with an 8-fold increase in GFP at the maximal dose using the microplate reader, similar to the previous maximal biochemical induction. This was approximately 20% of all cells. Interestingly, stress-induced ESCs that were GFP-positive were all DAB2-positive, but there were a higher number of GFP positive cells with LIF removal and most of these were DAB2-negative. The identity of

2015 Abstracts – Page 89
DAB2-negative cells is being studied, but the current data suggest that stress-induced early extraembryonic endoderm that are Pdgfra- and DAB2-coexpressing despite LIF, but LIF removal induces more Pdgfra positive cells and most are later Pdgfra-negative lineages. Thus, the Pdgfra-GFP ESCs may provide a high throughput screen for prioritized differentiation of early lineages using the microplate reader and validated by fluorescence and FACS.

223. Exposure to colony stimulating factor 2 during preimplantation development increases postnatal growth in cattle.
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   The microenvironment of the preimplantation embryo can cause changes in development that affect postnatal phenotypes. Potential mediators of this effect are embryokines secreted by the maternal reproductive tract that regulate embryonic function. One of these, colony stimulating factor-2 (CSF2), can increase competence of mouse, cattle, and pig and human embryos to establish pregnancy after transfer into recipients. We hypothesized that treatment of in vitro produced embryos with CSF2 would alter birth weight and postnatal growth of the resultant calf. Embryos were produced in vitro from Holstein oocytes inseminated with X-sorted Holstein semen. Embryos were cultured with or without (control) 10 ng/ml recombinant bovine CSF2 from day 5-7 after insemination and transferred at day 7 to lactating Holstein dairy cows. Calves were weighed and withers height measured at various intervals up to 13 mo of age. In addition to calves produced by embryo transfer (ET), body weights and heights of contemporary AI calves born from a distinct set of sires were also measured. Data on growth were analyzed in two different ways. First, growth data was analysed for all Holstein female calves born following ET control (n=5), ET CSF2 (n=20) and AI (n=49). A second data set (a subset of the larger one) consisted of those ET calves in which the same sires were used for both groups (n=5 ET control and 10 ET CSF2). Birth weights were similar between all three groups but thereafter CSF2 calves grew faster than ET control calves and AI calves (treatment x month of age, P<0.0044 for full dataset and P<0.0001 for subset). For example, birth weights were 42.2 ± 3.0 kg for ET control. 40.8 ± 1.3 kg for ET CSF2 and 39.5 ± 0.8 kg for AI, weights at 6 mo of age were 149 ± 4.7 kg for ET control, 170 ± 2.6 kg for ET CSF2 and 163 ± 2.0 kg for AI, and weights at 13 mo were 340 ± 6.8 kg for ET control, 364 ± 4.0 kg for ET CSF2 and 356 ± 2.1 kg for AI. Height was not affected by treatment at any time but the weight/height ratio was affected by treatment (P=0.0405 for large data set, P=0.0888 for subset) and treatment x age (P<0.001 for large data set and P<0.0089 for subset), with greatest ratios in the ET CSF2 group. Results indicate that exposure to CSF2 during the preimplantation period can alter the trajectory of growth in the postnatal periods. Perhaps, the embryo can be programmed in culture to improve postnatal functions important for health and productivity. Support: (USDA-AFRI-2011-67015-30688).

224. TEAD4 Promotes Proliferation and Self-Renewal of Human Trophoblast Progenitors: An Implication in Placental Homeostasis.
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   In placental mammals, trophoblast cells are essential for embryo implantation and successful progress of the pregnancy. During placental development, distinct trophoblast cell types are specified from trophectoderm stem cells (TSCs) or TSC-like trophoblast progenitors. However, molecular mechanisms, which regulate self-renewal and differentiation of trophoblast stem/progenitor cells, are poorly understood. In our study, we show that transcriptional activity of TEAD4, a TEA domain containing transcription factor, plays a crucial role in promoting cell proliferation in trophoblast progenitors, isolated from both rodent and human developing placentas. In the early stage of mouse and human placentas, TEAD4 is present within the nuclei of TSC-like progenitors and directly regulates expression of TSC-specific genes. Our Global gene expression analysis (RNA-seq) in TEAD4-depleted mouse trophoblast stem cells (mTSCs) indicated that TEAD4-mediated gene regulation is important to promote proliferation and self-renewal of mTSCs. Furthermore, analyses with primary trophoblast progenitors from mouse ectoplacental cone and cytotrophoblasts (CTBs) from first-trimester human placenta confirmed that TEAD4 promotes self-renewal of trophoblast stem/progenitors cells by directly regulating expression of several Cyclins/CDKs and TSC-specific genes. In contrast to trophoblast progenitors of a developing human placenta, differentiated trophoblast cells within a matured human placenta generally lack TEAD4 transcriptional activity due to its absence in their nuclei. However, intriguingly, matured human placenta harbor a small number of CTB population, characterized by the presence of TEAD4 in their nuclei. Laser-capture micro-dissection and gene expression analyses revealed that TEAD4 expressing CTBs in matured placenta have higher expressions of TSC-specific genes and positive regulators of cell proliferation. Furthermore, a very small percentage of TEAD4 expressing CTBs in matured placenta also express Ki67, a marker for actively proliferating cells. Thus, in matured placenta, TEAD4 transcriptional activity marks a population of quiescent trophoblast progenitors that are poised for proliferation. Not surprisingly, higher expression of TEAD4 is associated with proliferating trophoblast cells of choriocarcinomas. Our study indicates that transcriptional activity of TEAD4 marks proliferating trophoblast progenitors during human placental development and TEAD4 function could be important for placental homeostasis.

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   Introduction: Uterine leiomyomas (fibroids) are the most common benign tumor in women, which greatly affects reproductive health and well-being. They are the most frequent indication for hysterectomy in the USA. It has been found that cell proliferation combined with the production of copious amounts of extracellular matrix accounts for tumor expansion. Despite their high prevalence, there are no approved agents that provide effective treatment. Silibinin is a natural polyphenolic flavanoid extracted from the fruit and seeds of milk thistle. It has been reported that silibinin possesses antioxidant, anti-proliferative, anti-inflammatory, and anti-fibrotic properties. However, the effects of silibinin on leiomyoma cell function remain unknown. The aim of this study is to investigate the effect of silibinin treatment on leiomyoma cell proliferation, apoptosis, and collagen synthesis. Methods: The experimental protocol was approved by the Institutional Review Board of Northwestern University, and a written informed consent was obtained from all the patients.
We primarily cultured leiomyoma cells, which were obtained from surgical specimens. The cells were treated with silibinin (30, 60, and 90 μM) for 24, 48, and 72 hours and then the total protein and RNA were extracted from the cells. We measured mRNA levels of cyclin D1, collagen 1A2, and collagen 3A1 by real-time PCR and detected protein levels of cyclin D1, cleaved PRAP, and collagen 1A1 by western blot. Results: Silibinin treatment significantly down regulated mRNA levels of cyclin D1, collagen 1A2, and collagen 3A1. Silibinin dose-dependently decreased the protein levels of cyclin D1 at early time points (24 h and 48 h after treatment). In contrast, it increased the protein levels of cleaved PARP dose-dependently at later time points (48 h and 72 h after treatment). Furthermore, silibinin treatment robustly reduced collagen 1A1 protein levels. Conclusion: Our results indicate that silibinin has the potential to inhibit uterine leiomyoma formation by inhibiting leiomyoma cell proliferation, inducing cell apoptosis, and decreasing collagen synthesis.

226. Cervical viral infection as a potential cause of preterm labor.
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Preterm birth (PTB) affects approximately 12% of pregnancies in the US and is the leading cause of neonatal morbidity and mortality in the developed world. Despite the severity and frequency of PTB, 50% of cases still have unknown etiology and rates have increased over the last 30 years. A lack of progress in prevention and treatment is due, in part, to a lack of understanding of the underlying causes in many cases. For example, some estimate that approximately 30% of PTBs are associated with pathogenic bacteria at the maternal-fetal interface, yet clinical trials using antibiotics have proved to be ineffective at preventing preterm labor. This shows that, while bacteria are associated with the condition, they are not always the primary cause and we should consider alternate triggers of PTB. Recently, a small number of clinical studies have demonstrated an association between cervical viral infection and PTB. Our murine models of pregnancy also confirmed that viruses infect the pregnant cervix and sensitize animals to PTB, although the mechanism remains unknown. In the present study, we hypothesized the cervical response to virus induces molecular changes resulting in cervical ripening, and therefore our objective was to identify virus-dependent molecular changes in murine and human cervical cells and determine if these changes were conducive with PTB. Labor is the result of a tightly orchestrated cascade of physiological events. Increasing estrogen concentrations increase the expression of multiple factors that can trigger robust reorganization of the uterine cervix, termed cervical ripening. The membrane rupture and cervical remodeling are due in part to increasing expression of matrix metalloproteinases (MMPs) that cleave extracellular matrix proteins, enhancing collagen degradation. Therefore, we first determined if viral infection affected the estrogen receptor and downstream targets such as MMPs. Pregnant C57BL6/J mice were infected with the gammaherpesvirus MHV68, a virus of the same family of the majority of those that infect the human reproductive tract. Cervices were collected 7d post-infection, on average. We primarily cultured leiomyoma cells, which were obtained from surgical specimens. The cells were treated with silibinin (30, 60, and 90 μM) for 24, 48, and 72 hours and then the total protein and RNA were extracted from the cells. We measured mRNA levels of cyclin D1, collagen 1A2, and collagen 3A1 by real-time PCR and detected protein levels of cyclin D1, cleaved PRAP, and collagen 1A1 by western blot. Results: Silibinin treatment significantly down regulated mRNA levels of cyclin D1, collagen 1A2, and collagen 3A1. Silibinin dose-dependently decreased the protein levels of cyclin D1 at early time points (24 h and 48 h after treatment). In contrast, it increased the protein levels of cleaved PARP dose-dependently at later time points (48 h and 72 h after treatment). Furthermore, silibinin treatment robustly reduced collagen 1A1 protein levels. Conclusion: Our results indicate that silibinin has the potential to inhibit uterine leiomyoma formation by inhibiting leiomyoma cell proliferation, inducing cell apoptosis, and decreasing collagen synthesis.

Preliminary Screening Reveals Differences in the Proteomic Profile of Neutrophils Circulating in Women with Severe Preeclampsia Compared to Normal Pregnancy.
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Preeclampsia is a hypertensive disorder of pregnancy characterized by a sudden onset of hypertension and the appearance of proteinuria occurring after 20 weeks of gestation. It is accepted that the etiology of preeclampsia is divided into two stages. In the first one, a defect in the placentation prevents a suitable vascularization of the placenta which results in the release of factors toward the maternal circulation, which in a second stage, cause the vascular dysfunction feature of this pathology: endothelial damage, vasoconstriction, activation of the coagulation system and redistribution of fluids. It has been proposed that neutrophils are actively involved in the endothelial damage observed during preeclampsia, since the number of neutrophils circulating in peripheral blood increases, they are activated, and produce high amounts of pro-inflammatory cytokines and reactive oxygen species. It has also been described that the neutrophils infiltrate the vasculature, which is associated with the increase in the expression inflammatory markers in the endothelium and vascular smooth muscle. However, it has not been described in detail whether neutrophils from women with preeclampsia express different proteins that could conditionate the inflammatory and oxidative microenvironment that leads to vascular dysfunction observed in this pathology. The aim of this work was to identify differences in the proteome of neutrophils from women with severe preeclampsia and women with healthy pregnancies. Cell lysates of peripheral neutrophils (0.1 mg of total protein) from two different study groups, pregnant women with severe preeclampsia (n=4) and healthy pregnant (n=4) were analyzed by 2D electrophoresis. After separation, proteins were stained with Comassie Brilliant Blue R-250 and analyzed with the BioSpectrum®2D imaging system to detect differences in number, relative intensity, and presence or absence of protein spots. We detected around 119 protein spots in samples of neutrophils from women with severe preeclampsia in comparison with 73 protein spots in neutrophils from healthy women. Apparent changes in spot intensity were
detected in both study groups. The neutrophils obtained from women with severe preeclampsia showed decrease in the intensity of at least 10 protein spots, compared to the control group. In contrast, lysates of neutrophils isolated from healthy controls showed a decrease in the intensity of at least 4 protein spots compared to neutrophils from women with severe preeclampsia. Although the quantitative and qualitative analysis is in progress, these preliminary observations allow us to hypothesize that the protein profile of circulating neutrophils during severe preeclampsia is different compared to healthy pregnant women. Further identification of these proteins will enable us to describe the activation profile of neutrophils during preeclampsia, and to explore the possibility that these proteins promote neutrophil infiltration in the blood vessels, inducing the inflammatory and oxidative changes that result in high blood pressure in patients with preeclampsia.

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228. Three conceptuses phenotypes of early SCNT derived pregnancies detected by ultrasonography from day 14 to 35 of gestation in bovines: preliminary results.
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High incidence of pregnancy losses and several conceptuses abnormalities are common findings in SCNT derived pregnancies in bovines (Chavatte-Palmer et al., 2012; Placenta, p. 99-104). These occurrences are primarily caused by epigenetic changes acquired during manipulation and culture of the oocytes and embryos (Smith et al., 2012; Reprod Dom Anim, p. 107–114). Routinely, pregnancy detection exams are done only on day 35 of pregnancy (day 0 = ovulation) and, consequently, the exact times of the embryo losses are unknown before this gestational date. In addition, important developmental events and changes in conceptuses occur before day 35 and can be detected by transrectal ultrasonography. Therefore, the present study aimed to detect abnormalities during early pregnancy of SCNT bovine clones from days 14 to 35 of gestation by ultrasonography. The objective was to describe the incidence as well as morphological and vascular changes on conceptuses and mothers reproductive tract, such as uterus and corpus luteum (CL). Thereunto, 202 cloned embryos were transferred to recipients cows and pregnancies were monitored from day 14 until 35 of gestation by transrectal ultrasonography in B- and Doppler-modes to evaluate conceptus (embryo proper, membranes and liquids), CL diameter, CL area, CL vascularity, CL area of vascularity, endometrium echotexture, endometrium vascularity and mesometrium vascularity. Thirty nine (39) recipients maintained CL function until day 35 and three different phenotypes of early gestation were defined: Twenty two normal gestation (NG - detection of active CL, embryo proper with heart beat, and conceptus liquids and membranes present); Six anembryonic gestation (AG – detection of active CL, conceptus liquids and membranes presents without embryo proper with heart beat); and Eleven persistent CL (PCL – detection of active CL without either signs of embryo proper or liquids or membranes and presents); Six anembryonic gestation (AG – detection of active CL, conceptus liquids and membranes presents without embryo proper with heart beat); and Eleven persistent CL (PCL – detection of active CL without either signs of embryo proper or liquids or membranes and presents). The overall evaluation of the CL showed greater CL vascularity in the NG, intermediate in the AG and lower in the PCL. The temporal evaluation of morphological and vascular dynamics of the CL showed that NG started a gradual increase of CL area of vascularity on day 23, which was not observed neither in AG nor PCL. No differences in all parameters were found between uterine horns and means between uterine horns were used for analyses. Uterine echotexture was lower in NG and AG compared with PCL. In NG was observed a gradual increase of endometrium vascularity from day 23 and of mesometrial vascularity from day 17, which were not observed in AG and PCL. This preliminary data demonstrate the occurrence of three different early clone gestation phenotypes before day 35 and respective effects on CL and uterus of the recipient cow.
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We previously reported that the rhythmic expression of clock genes in the rat uterus is attenuated during decidualization. However, this physiological significance remains to be unclear. Many circadian clock-controlled cis-regulatory elements exist in the upstream region of the prostaglandin G/H synthase 2 (Ptgs2) gene. Prostaglandin E2 (PGE2) is a critical factor for decidualization. Therefore, we raised the possibility that the Ptgs2 gene is a downstream clock-controlled gene and that attenuation of the cellular circadian clock network characterizes the regulation of Ptgs2 expression in rat endometrial stromal cells (RESCs) during decidualization. We first compared the transcript levels of clock genes and Ptgs2 in the rat uterus on pregnancy days 4-5 (D4.5) and 6.5 (D6.5). Uterine tissues from day 6.5 rats were further cut into pieces carefully at inter-implantation site (D6.5) and implantation site (D6.5e). The transcript levels of clock genes (Per2, Bmal1, Rora and Rev-erba) decreased, especially at implantation sites on day 6.5 (D6.5e) compared to those on D4.5, while the Ptgs2 transcripts increased on D6.5e. Transcripts of Prl8a2, which was used as a decidual marker, greatly increased only on D6.5e. Similar observations of REV-ERBα and PTGS2 proteins were also obtained in the endometrium on D6.5e by immunohistochemistry and Western blotting. In addition, decidualization of RESCs prepared from pregnant rats on D4.5 was induced by 100 nM medroxyprogesterone and 0.5 mM 2-O-dibutyl-cAMP. In the induced decidual cells, the rhythmic expression levels of clock genes were significantly attenuated, while the Ptgs2 transcription was increased. Increased transcript levels of Prl8a2 were confirmed in the treated cells. These results in vivo and in vitro indicate that decidualization causes the attenuation of clock genes and the induction of Ptgs2. We next investigated the regulation of clock genes in the Ptgs2 expression. In the experiment used Bmal1-siRNA, the rhythmic expression of clock genes and Ptgs2 was attenuated by the siRNA. The transcript levels of Ptgs2 and PGE2 production were increased by treatment with 10 μM SR8278, a REV-ERBα antagonist, suggesting the inhibitory role of the nuclear receptor REV-ERBα in Ptgs2 expression. Chromatin immunoprecipitation-PCR analysis revealed that REV-ERBα could directly bind to a proximal RORE site of Ptgs2. Moreover, Rev-erba knockdown enhanced the induction of Ptgs2 transcription and PGE2 production by 10 μM forskolin. Collectively, this study demonstrated that the attenuation of the circadian clock, especially its core component REV-ERBα, contributes to the induction of Ptgs2 during decidualization. This finding may contribute to our understanding of cellular clock function in the uterine physiology and the mechanism regulating the differentiation of stromal cells into decidual cells. Research supported by the Japan Society for the Promotion of Science for Young Scientists (Grant No. 6117).

231. Two Separate Populations of Macrophages Are Involved in Endometrial Repair and Remodeling in a Mouse Model of Menses.
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Background: During the menstrual cycle the human endometrium, in response to fluctuating hormones, contains a fluctuating population of immune cells, including granulocytes (neutrophils, eosinophils, mast cells), lymphocytes (uterine natural killer cells, T cells, B cells) and the differentiated monocyte, the macrophage. Previous studies in mice and humans have studied the relationship between macrophages and neutrophils during endometrial repair during menstrual cycle using markers such as GR-1 (neutrophils) or CD68 (human macrophages) or the surface marker F4/80 (mouse macrophages). These studies suggest that macrophages are the “clean-up” cell during menstruation and do not contribute to repair, whilst the neutrophil drives re-epithelialisation. Using MacGreen mice combined with a model of induced “menses” we have identified two macrophage populations which are likely to play different roles during the repair window. Methods: Artificial “menstrual” cycles were induced in MacGreen mice using the following protocol: ovariectomized mice were treated with estradiol (E2) and progesterone (P) – 3 daily injections of E2 (100ng/100μl in oil days 7-9), P-secreting pellet placed sub-cutaneously on day 13 (100mg/ml), 3 daily injections of E2 (5ng/100μl, days 13-15). Decidualization was induced on day 15 using oil. Endometrial breakdown was initiated by removing the P pellet, 90 hours after oil injection. Tissues were recovered in PBS for FACS analysis, or fixed in 4% neutral buffered formalin or 4% paraformaldehyde for immunofluorescent detection of GFP (macrophage marker), the macrophage surface marker F4/80 and Ly6G (neutrophil marker). Tissues were recovered at 0, 24 and 48 hours after the withdrawal of progesterone. Results: Fluorescing populations of F4/80⁺, GFP⁺ and Ly6G⁺ populations were observed during endometrial repair (24-48 hours after the withdrawal of progesterone). F4/80⁺ cells were localised to the basal stroma throughout the repair window, GFP⁺ cells and Ly6G⁺ cells were observed in close proximity to the exposed luminal surface, GFP⁺ cells were identified in the repairing epithelium. Preliminary FACS analysis has identified a small subset of cells that were F4/80⁺GFP⁺ (15%); however numbers of F4/80⁺GFP⁺ cells (60%) were greater than F4/80⁺GFP⁻ cells (18%). Conclusions: We hypothesise that the F4/80⁺ cells in the basal stroma are tissue resident macrophages which contribute to remodelling of the functional stroma during the repair window, whereas the GFP⁺ cells are a subset of transient macrophages that are recruited to the endometrium to aid clearance of tissue debris and support re-epithelialisation. These data highlight the importance of using multiple markers to understand immune cell dynamics in a model of scarless healing.

232. Effects Of Periovulatory Gonadotrophin Treatment On Luteal Function And Endometrial Gene Expression In Cyclic Pony Mares.
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Progesterin concentration during the early luteal phase is crucial for endometrial function and conceptus development. We recently showed that induction of ovulation with human chorionic gonadotrophin (hCG) in horses results in stimulation of luteal function and conceptus development. We thus hypothesized that periovulatory gonadotrophin treatment via support of luteal function affects endometrial gene expression. As fertility is generally lower in old than in young mares an effect of age was analysed as well. The study included Shetland mares (Kenney-Doig grade: Ila and Iib), that were grouped into young (4-8 years: n=3) and old (15-24 years, n=4) mares. All mares were assigned to the following treatments (Tx) in a crossover design; Tx1: injection of hCG (1500 IU iv) in estrus, Tx2: injection of hCG (1500 IU iv) 24h after ovulation, Tx3: injection of equine chorionic gonadotrophin (eCG;2000 IU im) in estrus, Tx4

2015 Abstracts – Page 93
injection of eCG (2000 IU im) 24h after ovulation, Tx5: no treatment (control). Blood was collected daily from day 0 (day of ovulation) until day 10 after ovulation. Progestin concentration was determined by ELISA (Enzo Life Sciences, Farmingdale, NY) which has a 100% cross-reactivity with progesterone and 5a-pregnane-3,20-dione, thus detecting the most important equine progestins. On day 10, endometrial cells were collected by cytobrush (Minibute, Tiefenbach, Germany). Expression of mRNA of cyclooxygenase-2 (COX2), progesterone receptor, estradiol receptor, acyl-CoA-dehydrogenase, uteroglobin, ueroferrin and uterocalin was analysed by RT qPCR (normalization against GAPDH and B2M, gene expression in Tx5 was set as 1). Statistical analysis was performed by GLM ANOVA (SPSS Statistics 21, IBM-SPSS, Armonck, NY). Progestin concentration was significantly (p<0.01) influenced by day of cycle, but not by treatment; however was significantly higher in old mares (p<0.05; e.g. day 2: old 12.6±0.8 ng/ml, young 9.4±1.0 ng/ml). Gene expression of uteroglobin was significantly affected by treatment (Tx1: 3.6±1.3, Tx2: 1.7±0.4, Tx2: 1.2±0.7, Tx4: 2.0±0.8, p=0.01) and age (p<0.05), while expression of COX2 and ueroferrin was affected by age only (p<0.05). Gene expression of COX2 was negatively correlated with progestin concentration on days 1 to 10 after ovulation (e.g. day 1: r=−0.348, p<0.05; day 7: r=−0.630, p<0.01). Expression of COX2 is a key factor in induction of luteolysis in non-pregnant mares. The negative correlation between progestin concentration and COX2 expression supports the hypothesis that progestin concentration in the early luteal phase is crucial for endometrial function in the horse during early pregnancy (i.e. maternal recognition). In addition, age-related differences in endometrial mRNA expression of COX2, ueroferrin and uteroglobin are in agreement with the suggestion that changes in endometrial gene expression may account for decreasing fertility in old mares. However, in old mares, progestin concentration was significantly higher than in young mares which may have masked effects of gonadotrophin treatment on luteal function. Treatment with hCG before ovulation increased endometrial uteroglobin expression which may be beneficial for conceptus development.

233. ISOLATION AND CULTURE OF EQUINE ENDOMETRIAL MESENCHYMEAL STROMAL CELLS.
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Equine mesenchymal stem/stromal cells (MSCs) are mainly harvested from bone marrow and adipose tissue requiring surgical procedures. Other less invasive sources like whole blood, Wharton’s jelly and umbilical cord blood have also been investigated. While human endometrium is known to harbor mesenchymal precursor cells, the presence of MSCs in equine endometrium, a dynamic tissue undergoing regenerative changes during estrus cycles and the puerperium, has not been investigated. This study reports for the first time the isolation, culture and characterization of MSCs from equine endometrium which may ultimately provide a convenient source for veterinary regenerative therapies. Sections of equine endometrium were collected post mortem and either frozen or analyzed by immunohistochemistry (IHC). After thawing, endometrial tissue (n=4 horses) was digested using a dissociation medium containing collagenase I and DNase type I. Red blood cells were excluded from the resultant single cell solution using a density gradient. CD324 (E-cadherin)-bound magnetic beads were utilized to separate epithelial (CD324+) from stromal (CD324-) cell fractions. The two fractions were separately cultured in DMEM/F-12 containing 10% fetal bovine serum and gene expression was analyzed later by qPCR. IHC analyses of endometrial sections for putative markers of MSCs (CD90, CD73, CD29 and CD44) and perivascular cells (CD146 and NG2) revealed the presence of CD90 throughout the stromal compartment including small vessel walls and excluding endometrial glands. CD73 was only present in the wall of larger vessels, while CD29 was present in blood vessels and around endometrial glands. CD44 was seen in small vessel walls and isolated cells throughout the connective tissue. The perivascular markers, CD146 and NG2, were restricted to blood vessel walls, whereas CD144, an endothelial marker, was located in endothelia and around endometrial glands. Both isolated cell fractions, CD324+ and CD324-, were plastic-adherent within two days after initial seeding and grew well under standard MSC culture conditions. The CD324+ fraction primarily contained epithelial cells which rapidly formed cobblestone-like clusters, whereas the CD324- fraction contained mainly fibroblast-like cells which grew fast and quickly became confluent. With time, epithelial clusters in CD324+ cell lines were overtaken by fibroblasts and eventually disappeared after passage three. Consistent with morphological differences, qPCR analyses revealed higher expression of the epithelial markers, CD29 and CD227, in the cultured CD324+ fraction at passage one (p<0.05), and a subsequent decrease in expression of both markers in the two cultured cell fractions. Both cultures of CD324+ and CD324- fractions at passage one had detectable mRNA levels of MSC and perivascular markers, with relatively higher expression of CD105 and lower expression of CD29 in CD324- cells than in CD324+ cells (p<0.05). In conclusion, we showed the presence of putative MSCs in equine endometrium. We successfully isolated and cultured these cells, which might be useful in the future to improve a valuable source for regenerative therapy. A set of surface marker antibodies was also established which will be used in further studies to characterize equine MSCs.

234. Post-translational removal of α-Dystroglycan N-terminus by PC5/6 cleavage is important for endometrial receptivity in women.
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Dystroglycan (DG) is a large cell surface glycoprotein/adhesive molecule derived from a single gene product, consisting of α- and β-subunits. The β -DG is anchored within the plasma membrane, while α-DG is non-covalently attached to the N-terminus of β-DG extracellularly. It is the highly glycosylated central mucin-like region of α-DG (amino acids 326-485) that mediates cell adhesion. However, this region is obstructed by its large N-terminus (α-DG-N, 312 amino acid long), and whose removal by proteolytic cleavage would expose the central region of α-DG to exert DG’s adhesive function. Proprotein convertase 5/6 (PC6), a member of the PC protease family, is critical for endometrial receptivity and function by post-translational activating precursor proteins into their bioactive forms. Our hypotheses are: (i) α-DG-N on the plasma membrane is a barrier for embryo implantation and endometrial receptivity requires α-DG-N removal by PC6 in women. (ii) α -DG-N that is removed from the endometrial tissue is detectable in uterine fluids as a potential biomarker.
235. Basigin Enhances Human Extravillous Trophoblast Invasion via Activation of β-Catenin Signaling in Vitro,
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Extravillous trophoblast (EVT) is a differentiated trophoblast subset that responsible for migration and invasion into the decidua
and myometrium, remodeling the maternal spiral artery, and establishing the feta-maternal exchange to support the fetus growth.
The trophoblast invasion involves degradation of decidual extracellular matrix by secreted proteases, including metalloproteinases.

Disregulation of this invasion process is associated with various pregnancy complications leading to significant maternal and fetal death.
Pre-eclampsia is one of these complications characterized by insufficient invasion of the trophoblasts. Basigin, also named CD147, is a
metalloproteinases inductor in tumor cells that highly expressed on the plasma membrane of human trophoblast in form of protein
complexes1. Its expression has been found to be elevated during the first trimester of gestation2, an active period for trophoblast invasion,
and was decreased in preeclamptic placenta when compared to that in normal placenta3. However, the role of basigin in EVT functions is
unknown. In this study, we hypothesize that basigin regulates the invasion of human EVT. A human choriocarcinoma cell line, JEG-3, and
primary human trophoblast were used in this study. Our results showed that anti-basigin functional activation antibody-treated EVTs had a
higher invasiveness. On the other hand, inhibition of basigin expression by siRNA significantly suppressed the EVTs invason, but had no
effect on cell migration and proliferation. Reduced basigin expression was associated with decreased Wnt/β-catenin signaling pathway
activation in EVTs. A significant role of Wnt/β-catenin signaling on the biological activities of basigin was supported by the use of
pharmacological activator/inhibitor of Wnt signaling (Wnt-3a and DKK-1). Furthermore, we demonstrated that integrin-β1, a previously
identified interacting partner of basigin on the plasma membrane of trophoblast, was involved in the basigin-mediated trophoblast invasion.

Our results provide evidences that basigin may be important for early placentaion in human. Continued investigation of the area
will provide considerable understanding of the regulation of placentaion that will be useful for clinical application. Reference(s): 1. Lee, C.
metalloproteinase inducer in normal placenta and preeclampsia placenta]. Zhonghua Fu Chan Ke Zhi, 41(7), 436-439.

236. Loss of SNF2L and FGL2 in Reproductive Tissues Leads to Placental and Fetal Growth Impairment.
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Introduction: Preeclampsia affects 2-8% of all pregnancies and is an important cause of maternal mortality and fetal morbidity
and mortality worldwide. Its two main clinical manifestations, hypertension and proteinuria, are relieved upon delivery of the placenta, but
no treatment or predictive marker for the disease currently exists. Preeclampsia is thought to be caused, in part, by impaired trophoblast
invasion during placental development. Crucial to this process is the establishment of an immune balance during pregnancy. Cytokines
released by T helper cells 1 and 2 (Th1 and Th2) are known to be important in immune tolerance/rejection of the fetus. There is evidence that
Fibrinogen-like protein 2 (FGL2), a protein known for its prothrombinase activity and immunosuppressive properties, is involved in the
regulation of this balance. Previous data from our lab has shown that the chromatin-remodelling protein SNF2L regulates FGL2 expression
in mouse granulosa cells. Because SNF2L is also expressed in the placenta, we hypothesized that a similar regulatory
relationship exists in the placenta, to ensure appropriate FGL2 levels during its development. Methods: Mice with or without inactivation
of SNF2L (SNF2L Ex6DEL mice) were used to evaluate the effects of the loss of SNF2L on FGL2 expression, placental development and
fetal outcome. Five mice per genotype were euthanized at each of 12.5, 15.5 and 18.5 days of pregnancy and placentas were collected for
analysis. Results: SNF2L Ex6DEL mice have a modest impairment of reproductive capacity when compared to wild-type (WT) mice.
Trophoblast invasion occurs, in WT mice, around e12.5, which coincides with an observed peak in placental FGL2 expression, suggesting
a potential role during trophoblast invasion. This is reinforced by the finding that, in both mouse and human placenta, FGL2 is more highly
expressed in the invasive cell type: glycogen cells in the mouse placenta and extravillous trophoblasts in the human placenta. In vitro,
FGL2 expression is higher in HTR-8/Svneo trophoblasts (invasive) than in BeWo trophoblasts (non-invasive). Placental FGL2 expression
at e12.5 in SNF2L Ex6DEL mice is significantly lower than in WT mice, suggesting that inactivation of SNF2L inhibits FGL2 expression
in the placenta. Whether this leads to impaired trophoblast invasion is under investigation. Significant decreases in fetal and placental
weights of SNF2L Ex6DEL mice are also seen at e12.5, but not later in pregnancy, suggesting that impaired invasion may hinder early fetal
and placental development. It is possible that the observed smaller fetuses are in the process of resorbing, as there is a higher ratio of
resorption sites to total implantations in mice euthanized at e18.5. Further protein expression and histological analysis of SNF2L Ex6DEL
placentas and fetuses is in progress to determine possible causes of resorption. There is also evidence for the involvement of FGL2 in
human disease. In the serum of women in the first trimester of pregnancy, FGL2 is found in lower amounts in women who later developed
preeclampsia. Conclusions: These data suggest that FGL2 is expressed in the invasive trophoblast cells of the placenta, that its expression is

2015 Abstracts – Page 95
regulated by SNF2L, and that loss of expression impairs placenta and fetal development. The observation that serum FGL2 is reduced in women who will subsequently develop preeclampsia renders this a potential biomarker worthy of further investigation.

237. Uterine luminal epithelium in embryo implantation. 
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Uterine luminal epithelium (LE) is the first cell layer contacted by an embryo for implantation. The importance of LE in the establishment of uterine receptivity is manifested by its physical interactions with the implanting embryos during the initial stages of embryo implantation: embryo apposition to the LE, embryo adhesion to the LE and embryo penetration through the LE. Embryo implantation initiates ~gestation day 4.0 (D4.0) in mice. Our microarray analyses reveal 245 genes significantly downregulated (e.g., Lpar3) and 382 genes significantly upregulated (e.g., Atptv0d2) (>2 fold change) in mouse D4.5 LE. Lpar3 encodes lysophosphatidic acid receptor 3 (LPA3), a G protein-coupled receptor. Lpar3-/- females have delayed embryo implantation and embryo crowding leading to impaired fertility. They have comparable serum progesterone and estrogen levels with wild type (WT) control. Progesterone receptor (PR) disappears from D4.5 WT LE but remains highly expressed in D4.5 Lpar3-/- LE shown by immunohistochemistry. PR target genes, but not estrogen receptor a (ERa) or ERb target genes, are upregulated in D4.5 Lpar3-/- LE detected by quantitative RT-PCR, suggesting increased PR/ER signaling in Lpar3-/- LE. Indeed, a low dose of PR antagonist RU486 (5 µg/mouse, ~0.2 mg/kg) or ER agonist 17β-estradiol (E2, 25 ng/mouse, ~1 µg/kg) rescues on-time implantation but not embryo crowding in Lpar3-/- females. These data demonstrate that deletion of Lpar3 disrupts fine local balance of PR/ER signaling in LE thus adversely affects embryo implantation. Atptv0d2 encodes the d2 subunit for vacuolar-type H+-ATPase (V-ATPase), which is involved in ATP-dependent proton transport to regulate intracellular or extracellular acidic environment. V-ATPase is composed of one cytoplasmic peripheral V1 domain for ATP hydrolysis and one transmembrane integral V0 domain for proton translocation. Since d subunit is involved in the assembly of V0 domain and V1 domain for a functional V-ATPase, the dramatic upregulation of Atptv0d2 in D4.5 LE implies LE acidification upon embryo implantation. Indeed, drastically increased LE acidification is detected in D4.5 LE using a fluorescent pH indicator LysoSensor Green DND-189, which accumulates in the acidic intracellular organelles to reflect their acidity. Injection of batfomycin A1 (a specific V-ATPase inhibitor) via local uterine fat pad causes a dose-dependent disruption of embryo implantation and suppression of LE acidification in pregnant mice as well as inhibits oil-induced artificial decidualization in pseudopregnant mice. These data demonstrate the involvement of LE acidification in uterine preparation for embryo implantation. However, Atptv0d2-/- females were reported to have normal fertility. Atptv0d2-/- mouse colony is being established in our lab and investigated for the mechanism of LE acidification. The above results reveal various cellular and molecular changes in the LE that are associated with uterine preparation for embryo implantation.

Understanding the cellular and molecular mechanisms in the establishment of uterine receptivity will provide knowledge for developing diagnostic and therapeutic approaches to detect and treat defective uterine receptivity. (Supported by NIH R15HD066301, and NIH R01HD065939 (co-funded by ORWH & NICHD))

238. Continuously activated Notch signaling inhibits progesterone receptor expression via hyper- methylation of its promoter. 
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Notch signaling plays a vital role in cell survival, cellular communication and differentiation throughout development in a variety of species. We previously reported that both conditional deletion of Notch1 (Prerm/Notch1F) and Notch1 silencing in Human Uterine Fibroblast (HuF) inhibits decidualization. Pgr-Cre driven overexpression of Notch1 intracellular domain (N1ICD) in the mouse (Prerm RosaN1ICD/0 N1ICDFlx) results in complete infertility due to the failure of uterine gland development, uterine receptivity, and decidualization. This is a consequence of the altered expression of both PGR and ESR1 in uterine epithelial and stromal cells coupled with an abnormal proliferation pattern. In addition, overexpression of N1ICD in HuF cells is associated with decreased PGR and inhibition of in vitro decidualization. The mechanism by which N1ICD overexpression regulates PGR has not been previously studied. The objective of our study was to determine how N1ICD induces the down-regulation of PGR resulting in decidualization failure and infertility using both the N1ICDFlx mouse model and HuF cells. We hypothesized that methylation of Pgr promoter in our N1ICDFlx mouse model is altered. CpG islands were predicted on both the mouse and human Pgr/PGR promoter region using Methprimer (http://www.uoregon.org/). Using immunohistochemistry, we observed higher expression levels of DNA methyltransferases (Dnmts) along with increased staining for 5-methylcytosine (5mC), a methylated form of the DNA base cytosine, on E3.5 in the uteri of N1ICDFlx mice compared to control animals. These results suggested a higher level of methylation in the uterus of N1ICDFlx female mice. We further tested methylation levels specifically on the Pgr promoter using a Bisulfite PCR (BSP) method. Our data showed hyper-methylation of the Pgr promoter in uterus of N1ICDFlx mice compared to control mice (26.25% vs 1.25%) on E3.5. In HuF cells overexpressing N1ICD, treatment with 5-Aza-2′-deoxycytidine, an inhibitor of DNA methyltransferases, partially reverses the down regulation of PGR and allow the cells to undergo limited decidualization as assessed by the expression of IGFBP1. These studies suggest that hyper-methylation may contribute to decreased PGR expression in the uterus of mice overexpressing N1ICD as well as transfected HuF cells. To further evaluate the mechanism by which N1ICD down regulates PGR we identified transcription factor binding sites on the Pgr/PGR promoter using MotifMap (http://motifmap.ics.uci.edu). We identified binding sites for the transcription factor binding sites on the Pgr/PGR promoter region of both mouse Pgr and human PGR, -660 and -289 bps from transcriptional start sites, respectively. PU. 1 has been reported to hyper-methylate its target genes by recruiting DNMT3b to the promoter region of target genes and PU.1 has also been reported to be regulated by Notch signaling. We also observed increased PU.1 expression in our N1ICDFlx mice at both the mRNA and protein level. In summary, our data demonstrate that overexpression of N1ICD in the endometrium can decrease PGR via expression a PU.1-mediated hyper-methylation of Pgr/PGR promoters in both mouse and HuF cells contributing to decidualization and implantation failure. These studies have translational relevance since in clinical manifestations such as endometriosis, Notch1 is overexpression in ectopic tissues and PGR is hypermethylated leading to P-resistance. (HD 042280).
239.  Defective placentaion in Lpar3 deficient mice.  
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Lpar3 encodes the third G protein-coupled receptor for lysophosphatidic acid (LPA). LPA3, Lpar3−/− female mice have delayed uterine receptivity for embryo implantation, embryo crowding, increased post-implantational embryonic death, and reduced litter size. These phenotypes are unrelated to the genotypes of the mating males, indicating maternal defects. Embryo implantation timing and embryo spacing can both affect litter size. However, restoration of on-time implantation and partial alleviation of embryo crowding using pharmacological approaches cannot fully restore the litter size from Lpar3−/− females to that from wild type (WT) females. The gestation day 18.5 (D18.5) placentas from Lpar3−/− females are heavier than those from WT females. Conjoined placentas from Lpar3−/− females are often observed due to embryo crowding from defective implantation. However, the weights of embryos up to D18.5 from Lpar3−/− females are significantly lower than those from WT females. Delayed embryo implantation is a known cause for the reduced embryo weight. The lower embryo weight may also be caused by placental defects in the Lpar3−/− females. We hypothesize that deletion of Lpar3 causes placental defects that contribute to decreased embryo weight, increased post-implantational embryonic death, and reduced litter size in Lpar3−/− females. Preliminary data using quantitative RT-PCR indicate upregulation of Lpar3 mRNA levels in WT placentas from D9.5 to D13.5. Histology of singleton or conjoined placentas from D13.5 Lpar3−/− females reveals similar abnormalities, including a very dense spongiotrophoblast layer and reduced numbers of vascular channels in the spongiotrophoblast layer compared to WT control. Spatiotemporal expression of Lpar3 mRNA in the WT placentas is being investigated using in situ hybridization. Histology of placentas at different developmental stages will be examined to determine the time course of abnormal placentation development in the Lpar3−/− females and to correlate the abnormalities with the spatiotemporal expression of Lpar3 mRNA in the placenta. It has been reported that serum LPA levels increase during human pregnancy and human placenta is a source for LPA production during pregnancy. This study will provide novel information on LPA signaling in placental development and help understand the molecular mechanisms involved in placentaion. (Supported by NIH R15HD066301 and NIH RO1HD065939 (co-funded by ORWH & NICHD))

240.  Defective embryo implantation and impaired fertility were secondary effects of neuronal defect in Olfactomedin1 deficiency female mice.  
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Olfactomedine 1 (Olfm1) is a highly conserved glycoprotein that was originally identified in the frog olfactory neuroepithelium. Olfm1−/− female mice were reported not breeding well. Embryo implantation initiates around gestation day 4.0 (D4.0) in mice. Microarray analysis revealed Olfm1 among the most highly upregulated genes in the mouse uterine luminal epithelium (LE) upon embryo implantation. In situ hybridization confirmed this upregulation and revealed LE-specific expression of Olfm1 in the D4.5 LE, suggesting potential role of Olfm1 in embryo implantation. It was hypothesized that Olfm1 deficiency led to defective embryo implantation thus impaired fertility. Indeed, Olfm1−/− females had defective embryo implantation. However, Olfm1−/− females rarely mated and those mated rarely had successful pregnancies. Ovarian histology indicated absence of corpus lutems in Olfm1−/− females, indicating defective ovulation. Superovulation using equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) rescued mating, ovulation, and pregnancy, and eCG alone rescued ovulation in Olfm1−/− females. Olfm1−/− females had reduction of hypothalamic GnRH neurons, which paralleled with their reduced body weight and brain size, but comparable basal serum luteinizing hormone (LH) levels and GnRH-induced LH levels compared to control. These results indicated no obvious local defects in female reproductive system and a functional HPG axis. Interestingly, Olfm1−/− females were unresponsive to male urine stimulation on estrous cycle and had a 41% reduction of cFos positive cells in the mitral layer of accessory olfactory bulb, suggesting defective olfactory function. Immunohistochemistry revealed that OLFM1 had spatiotemporal expression in the main and accessory olfactory systems, including main olfactory epithelium, vomeronasal organ, main olfactory bulb and accessory olfactory bulb, with the highest expression detected in the axon bundles of olfactory sensory neurons. These data demonstrate that defective embryo implantation and impaired fertility in the Olfm1−/− females were secondary effects of neuronal defect. (Supported by NIH R15HD066301 & R01HD065939)

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Inflammatory processes are critical to female reproductive cyclicity and establishment of pregnancy; however, in women with endometriosis, a reduction in the anti-inflammatory influence of progesterone (P4) is associated with both infertility and a risk of preterm birth. The relationship between systemic inflammation and altered phenotypic changes within the eutopic endometrium that disrupt fertility are not fully understood. However, using a murine model, we previously demonstrated an adult “endometriosis-like” uterine phenotype following developmental exposure to the environmental toxicant TCDD (2,3,7,8-tetrachlorodibenzo-p-dioxin). Studies using this early life TCDD exposure model identified a synergistic, negative relationship between the presence of an endometriosis-like uterine phenotype and risk for pregnancy loss following a secondary inflammatory challenge with an infection-related agent. Specifically, in mice losing their pregnancy due to an early life TCDD exposure history, an enhanced local and distal organ accumulation of macrophages was noted following a low dose lipopolysaccharide (LPS) challenge. To translate our in vivo murine findings to the human condition, in the current
study, we established an *in vitro* model of intercellular paracrine communication between immune cells and endometrial somatic cells in parallel, perfusable microchannels fabricated in polydimethylsiloxane (PDMS). This microfluidic design allows the perfusion of immune cells and conditioned media through the cell lined channels. In the first set of experiments, we co-cultured the human monocytic suspension THP-1 with endometrial stromal cells and noted a highly significant increase in monocyte adherence to stromal cells exhibiting an endometriosis-like phenotype due to treatment with TCDD (10nM). Interestingly, we noted only minimal morphological changes in the THP-1 cells adhering to stromal cells as compared to cells adhering and differentiating on plastic in response to PMA (6 ng/ml) treatment. However, exposure of THP-1 mononuclear cells to conditioned media obtained from either TCDD treated stromal cells or the endometriotic epithelial cell line (12z cells) significantly promoted both adherence and morphologic differentiation to macrophages. Lastly, we find that THP-1 macrophages exposed to TCDD for 48 hrs exhibit increased expression of TNF-α mRNA in response to an LPS challenge. Taken together, these results support our marine observations and suggest that TCDD exposure in humans may increase endometrial inflammation by altering the adhesive properties of monocytic cells as well as monocyte differentiation to macrophages. Additionally, TCDD-mediated disruption of endometrial stromal-epithelial cell communication may also impact resident macrophage populations such that chronic production of inflammatory mediators drives the development of the endometriosis phenotype. Ultimately, our goal is to create instrumented experimental “endometrium/endometriosis on a chip” models by introducing the multilayered interactions among the multiple cell types that makeup this organ system. These models should dramatically enhance our understanding of the contribution of individual cell types to the enigmatic endometriosis phenotype. Supported by: NIEHS ES14942, and Environmental Toxicology Training Grant (NIH T32 ES007028).


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In mammalian reproduction, implantation of embryos into the uterus is associated with a multifaceted series of events and which requires a spatiotemporally regulated seemingly environments resulting from various intricate processes, governed by a number of cherished factors. Earlier studies suggested that human blastocyst derived factors stimulate HOXA10 mRNA expression in Ishikawa cells. Additionally, it was documented that the expression of a number of genes were altered by conditioned medium from human blastocysts following IVF in human. Thus it is evident that the blastocyst-derived factors potentially altered the endometrial functions for uterine receptivity. However, embryo-endometrium interactions remain to be exposed during implantation. Considering the above standpoints, the present study is aimed to focus on the regulation of gene expression in the endometrium by preimplantation embryo-derived factors. To keep pace with the objectives to determine the gene expression during preimplantation, rats in delayed implantation were analyzed. Initially, RNA-sequencing analysis was performed to identify the differential expressing genes in the delayed implantation uterus compared to pseudo pregnant. Differential gene expression revealed that 10 genes (Areg, Calca, Car3, Citd1, Fxyd4, Irg1, Lamc3, S1c13a5, Scl5a8, Sulfi) were up-regulated whereas 4 genes (Aldh1a1, Krt19, Pr3c31, Prf8a2) were down-regulated in the uterus of delayed implantation. To ratify the results of the RNA-sequencing analysis, real time quantitative PCR (RT-qPCR) was initiated which revealed that the Sulfi mRNA expression was significantly increased in the delayed implantation uterus (P<0.05), while Areg, Calca, Fxyd4 and Lamc3 mRNA expressions did not differ at significant level. Then mRNA expressions in the uterus during early pregnancy at 1.5, 3.5 and 5.5 days post coitus (dpc) were analyzed by RT-qPCR and observed that mRNA expressions were significantly increased at 3.5 dpc, just before the implantation. Areg, Calca, Fxyd4 and Sulfi mRNA expressions were decreased at 5.5 dpc compared to 3.5 dpc. While the expression of Lamc3 mRNA was sustained at increasing level until 5.5 dpc. Furthermore, the uterine gene expression modulated by embryo-derived factors were examined using conditioned media of preimplantation embryos through injecting into the uterine horn of pseudopregnant rats. Preimplantation embryos at 3.5 dpc were cultured in groups (about 20 embryos in a group) in 30 μL drop of mR1ECM. After 24 hours of culture, the conditioned media were harvested. At day 3.5 of pseudopregnancy, rats were ovariectomized and treated with P4 to maintain the condition of pseudopregnancy. Conditioned media from preimplantation embryos were injected into one uterine horn and fresh media were injected into another uterine horn as a control. It was observed that expression of Areg, Calca and Fxyd4 mRNA did not differ significantly, whereas Lamc3 and Sulfi mRNA expressions were significantly increased after the injection of embryo conditioned media. Thus it is evident that Lamc3 and Sulfi mRNA expressions in the uterus were up-regulated by embryo-derived factors. In a nutshell, based on the above observations it can be suggested that the embryo derived factors might have the potential to modulate maternal gene expression and this study may contribute to elucidate the mechanism of embryo-endometrium interaction during implantation period.


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Bisphenol A (BPA) is widely used plasticizer in the manufacture of plastics and can linings. Accumulating evidence suggests that BPA has a weak estrogenic activity and BPA (≥5mg/kg/d) affect animal reproduction and development. Whether low-dose (<220nM) long-term exposure of BPA affects embryo implantation remains largely unknown. In this study, we used in vitro cell co-culture model to evaluate the effect of low-dose long-term BPA exposure on spheroids (blastocyst surrogate). Endometrial epithelial (Ishikawa) cells and choriocarcinoma (Jeg-3) cells were used to establish the in vitro co-culture model to mimic implantation process. The Ishikawa cells were exposed to low-dose BPA (1-100nM) for 1 month and 3 months. Trophoblastic spheroids (blastocyst surrogate) were produced by overnight shaking the trypsinized Jeg-3 cells. Attachment rate was measured by counting the number of spheroids attached onto Ishikawa monolayer at 1 hour to the total number of spheroids added. It was found that BPA treatment at 100nM for 1 month significantly suppressed Jeg-3 spheroids attached onto Ishikawa monolayer (66.7% VS 81.9% control, p<0.05) when compared with DMSO control. After 1 month exposure of low-dose BPA, the expression of ERα in Ishikawa cells was up-regulated; while the expression of ERβ, GPR30 (G protein–coupled receptors 30, or G protein–coupled estrogen receptor), Wnt-signaling molecules GSK3β and Axin-2 were down-regulated. However, BPA treatment for 3 months did not significantly suppress spheroid attachment onto Ishikawa cells (61.1% VS 78.3% control, p>0.05) when compared to DMSO control. Yet, the expressions of ERα and active β-catenin were significantly up-regulated; while the GSK3β and E-cadherin were down-regulated. Acute high-dose BPA treatment to 1 month, but not 3 month low-dose BPA treated
Ishikawa cells, suppressed the spheroids attachment comparable to untreated control cells. The underlying mechanism in which BPA loss its suppressive effect on spheroid attachment after 3 months low-dose expose is being investigated. In sum, the cells response to chronic low-dose BPA and alter attachment rate by modulating steroid receptor(s) and Wnt-signaling pathway. [Supported in part by a CRCG grant to KFL]

244. Niciosamide as a Potential Therapeutic Drug for Endometriosis.
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Endometriosis affects 6-10% of women of reproductive age. Although endometriosis is a benign disorder, approximately 50% of affected women experience severe chronic pelvic pain and infertility. Because endometriosis is estrogen-dependent, the most widely used medical drugs are oral contraceptives, GnRH agonists and progestins, which suppress ovarian function and subsequently reduce pelvic disease and associated pain. However, hormonal treatments are often of limited efficacy, elicit side-effects, temporarily inhibit fertility, and ultimately result in high recurrence rates of symptoms. Therefore, it is necessary to identify therapeutic targets and efficient drug(s) that improve current treatment. The progression of endometriosis is marked by an increase in proinflammatory signaling eliciting remarkably increased macrophage, cytokine and chemokine content in the peritoneal fluid. Although nonsteroidal anti-inflammatory drugs, such as ibuprofen, have also been used for the treatment of endometriosis, these drugs primarily relieve dysmenorrhea. We have recently identified a small molecule, niciosamide, which modulates the NFkB and STAT3 signaling pathways. Therefore, we hypothesize that niciosamide inhibits the endometriotic microenvironment suppressing proinflammatory mechanisms via NFkB and STAT3 signaling. To determine the inhibitory mechanisms of niciosamide in endometriosis, endometriotic lesions were induced in mice. The uteri of PMSG-primed donor mice were minced and implanted into the peritoneal cavity of estrogen primed ovariectomized wild-type recipient females. After three days recovery from the surgery, mice were given niciosamide (50, 100, or 200 mg/kg b.w./day) or vehicle orally for 3 weeks. We observed a significant difference in the pattern of progression of endometriotic lesions after 3 weeks. While the number of lesions did not differ between treatment groups, oral administration of niciosamide dose-dependently (50, 100, or 200 mg/kg b.w.) reduced the total weight of lesions 114.9 ± 31.6 mg (n=7), 76.2 ± 18.3 mg (n=9), or 44.9 ± 7.43 mg (n=20), respectively, compared with controls (154.0 ± 29.3 mg). Niciosamide treated animals had fewer proliferating Ki67 positive cells in their lesions. The genes related to cytokine signaling and inflammatory responses were examined by quantitative RT-PCR. Treatment of niciosamide decreased cytokine and chemokine levels including: Il1b, Cxcr2, Csf3r, Il1r1, Tnf, Il10, and Osm, but increased Ccl17 in the endometriotic lesions. Immunohistochemical analysis revealed a reduction in phospho-STAT3 and iNOS following treatment of niciosamide. Current treatments for endometriosis temporarily inhibit fertility. However, niciosamide treatment did not disturb normal reproductive function in mice. Thus, these results suggest that niciosamide could be a potential therapeutic drug for the treatment of endometriosis by targeting inflammatory mechanisms while preserving normal fertility.

245. Effect of 17β-estradiol, human chorionic gonadotropin, and interleukin-1β on plasminogen activators activity and mRNA expression in porcine endometrial cells.
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During the estrous cycle in pigs, the uterus undergoes physiological, morphological and functional changes. 17β-estradiol (E2), human chorionic gonadotropin (hCG), and interleukin-1β (IL-1β) are related to change of morphological and physiological function of uterus through stimulation of prostaglandin secretion. However, the effects of these factors on plasminogen activators (PAs) activity and mRNA expression have not been reported. Therefore, we investigated whether E2, hCG and IL-1β regulate PAs activity and mRNA expression in endometrial cells from porcine uterus. Endometrial cells were isolated from porcine uterus on stage of the follicular phase. The isolated cells were stimulated with different concentrations of E2 (0.2, 2, 20, and 200 ng/ml), hCG (0.5, 1.0, 1.5, and 2.0 IU/ml), and IL-1β (0.1, 1, 10, and 100 ng/ml) for 24 hours, and determined PAs activity and mRNA expression. In results, the level of urukinase-type PA (uPA) and tissue-type PA (tPA) mRNA expression in endometrial cells was not changed by E2, hCG, and IL-1β. In contrast, PAs activity in endometrial cells was increased in high E2 (200 ng/ml) and hCG (2.0 IU/ml) concentrations (P < 0.05), whereas IL-1β-treated cells stimulated PAs activation in low concentrations (0.1 and 1 ng/ml, P < 0.05), but PAs activity was inhibited by high concentrations (10 and 100 ng/ml, P < 0.05). Together these results show that pregnancy-regulated hormones and cytokines are potential factors for inducing PAs activation in porcine endometrial cells, providing a new important link in the mechanism of PAs translation or post-translation process in endometrial cells. This work was supported by Korean Research Foundation Grant funded by the Korean Government (MOEHRD) (2010-0021580).

246. WITHDRAWN.

247. Estrogen Receptor α in Uterine Stromal Cells is Required for Epithelial Cell Proliferation and Pregnancy.
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Estrogens mediate their action mostly through the estrogen receptor α (ERα), which is expressed in female reproductive organs, especially in the uterus. Uterine tissue consists of an outer muscle layer, a mesenchymal layer (including stromal cells), and a lumen lined by epithelial cells. ERα is crucial for female reproduction as global loss of ERα (Esr1−/−) leads to a complete infertility. However, the Esr1−/− model does not allow assessment of ERα function during early pregnancy due to a hormonal imbalance and lack of mating behavior. Thus we bred Amhr2−/−, which is expressed in the stromal cells of the uterine-embryo implantation site (anti-mesometrium), with Esr1−/− animals to generate knockout of ERα selectively in the stromal cells of the uterus (Amhr2−/−;Esr1−/−), while preserving ERα expression of other uterine cells. In the control uteri, we found that estrogen (E) induced the proliferation of epithelial cells throughout the whole luminal
breeding with WT males for 6 months. At 5.5 (days post coitus) dpc, Djana Harp1, Adel Driss2, Sharifeh Mehrabi1, Indrajit Chowdhury1, Samantha Jefferson3, Neil Sidell4, Robert Taylor5, Winston 249. The Potential Role Of Exosomes In The Pathogenesis Of Endometriosis.

of its target gene FKBP4 in stromal fibroblasts from the eutopic endometrium of baboons and women with endometriosis.

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Introduction: Endometriosis is characterized by severe chronic pelvic pain and infertility. The presence of the disease results in aberrant gene expression in the eutopic endometrium and the development of Progesterone (P) resistance. Our miR microarray and qRT-PCR data generated using baboon model of induced endometriosis revealed that induction of the disease significantly increased the expression of miR-29c. This increase in miR-29c results in the decreased expression of one of its targets FKBP4 (gene coding for FKBP52; an immunophilin co-chaperone protein required for the action of progesterone receptor) expression and might contribute to observed P resistance in subset of women with endometriosis. Decorin, a proteoglycan is a P induced gene and its expression is up regulated during decidualization. Objectives: In the present study, we have characterized; 1) the expression of Decorin in baboon and women with endometriosis; 2) the effect of over expression of miR-29c on in vitro decidualization and Decorin expression using primary culture of human uterine fibroblast (HuF) cells and 3) evaluated the effect of progesterone (P) resistance in subset of women with endometriosis. Decorin expression, HuF cells were transfected with either miR-29c mimic or non-targeting negative controls and treated with MPA or vehicle treatment for 48hours. Results: Our qRT-PCR analysis revealed that expression of Decorin is significantly decreased in eutopic endometrium of baboons (n=4) following induction of disease at 3 and 15 months compared to controls (n=3). Decorin expression was also significantly decreased in the endometrium of women with endometriosis (n=18) compared to the women without endometriosis (n=6). Interestingly, Decorin expression together with FKBP4 was significantly decreased in the HuF transfected with miR-29c mimic following the decidualization stimulus compared to HuF cells transfected with non targeting negative control. In addition decidualization was also compromised in these cells as evidenced by the decreased expression of PRL and IGFBP1 transcripts. To confirm that Decorin is directly regulated by progesterone and FKBP4, HuF cells were transfected with either miR-29c mimic or non-targeting negative controls and treated with MPA or vehicle for 48h. The negative control cells had a six fold increase in Decorin expression in response to MPA compared to vehicle treated cells. In contrast, HuF cells transfected with miR-29c and treated with MPA (48h) showed significant decrease in Decorin expression compared to MPA treated non targeting negative control transfected cells suggesting that Decorin expression is directly induced by progesterone Conclusion: As a consequence of endometriosis decidualization is compromised and the expression of Decorin is decreased in HuF cells. We suggest that the compromised progesterone response is mediated via the increased expression of miR-29c and the decrease of its target gene FKBP4 in stromal fibroblasts from the eutopic endometrium of baboons and women with endometriosis.

249. The Potential Role Of Exosomes In The Pathogenesis Of Endometriosis.

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Endometriosis is a disease involving the deposition of endometrial tissue outside of the uterine cavity into the peritoneal cavity of the abdomen or pelvis. The pathogenesis of endometriosis is complex but the disease microenvironment induces the proliferation of new blood vessels around the lesions and fundamentally contributes to their invasion and inflammatory responses. Exosomes are nanoparticles released from cells, which can transfer proteins, mRNAs, small RNAs and microRNAs (miRNAs) via the extracellular environment to cells at distant sites. We hypothesized that exosomes released from endometriotic stromal cells might contribute to the pathogenesis by packaging and delivering miRNAs. To determine if there is a differential gene profile of miRNAs in endometriosis exosomes, exosome pellets were prepared by differential ultracentrifugation or using the Total Exosome Isolation method from culture media of stromal cells grown from endometriosis lesions as well as from phase- matched eutopic endometrial cells from diseased patients and controls. Exosomes were positively identified in all preparations by transmission electron microscopy and the NanoSight LM10 instrument. Particle size analysis confirmed the predominance of exosomes ranging from 35-150 nm in diameter. miRNAs were extracted from exosomes and deep sequenced. We observed differential expression of pro-angiogenic factor modulating miRNAs, several of which were increased in exosomes derived from lesion cells and diseased eutopic stromal cells compared to exosomes derived from phase- matched control eutopic
endometrial stromal cells. To determine whether exosomes have the ability to be endocytosed and work in an autocrine/paracrine fashion, we labeled exosomes with a fluorescent protein, BODIPY TR ceramide and incubated these with cultured endometrial stromal cells grown in chamber slides. Cells were then fixed after 24h of culture and confocal microscopy was used to verify internalization of labeled exosomes by the host cells. To assess if the exosomes could directly influence endothelial cell function, we next selected differentially expressed angiogenic miRNAs and transfected them into HUVEC cells to determine their pro-angiogenic effects on the endothelial tube formation assay. Pro-angiogenic miRNAs led to increased endothelial tube branching. This is the first report characterizing endometrial stromal cell-derived exosomes from normal and diseased subjects, with demonstrated cellular uptake and functional angiogenic effects of transfected candidate miRNAs. This study supports the hypothesis that exosomes derived from endometriotic stromal cells play autocrine/paracrine roles in the peritoneal microenvironment, modulating angiogenesis. The broader clinical implications build on Sampson’s theory of retrograde menstruation, but extend this concept to the idea that exosomes might work as intercellular communication modulators, with cells from diseased endometrium stimulating pro-angiogenic propensity of nascent lesions in the peritoneum to adopt a new vasculature and cause inflammatory changes that lead to pelvic pain and infertility.

250. **Disruption of Endometrial NELF Transcriptional Pausing Complex Impairs Early Pregnancy.**


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The ovarian hormones estrogen (E2) and progesterone (P4) play key roles in establishing and maintaining mammalian pregnancy via their cognate nuclear receptors, estrogen receptor (ER) and progesterone receptor (PR). These receptors serve as hormone-induced transcriptional mediators by assembling and interacting with regulators of chromatin accessibility and modulators of RNA polymerase II (Pol II) transcriptional rates. Recent studies have linked gene regulation through Pol II pausing and nuclear receptors. Pausing of Pol II during early transcriptional elongation is an important rate limiting step where an engaged transcription complex pauses near a transcription start site (TSS). Cells are able to coordinate and appropriately respond to signals by modulating the rate of pause release through recruitment of pTEF-b. Previously we performed Pol II ChIP-seq on mouse uterine tissue and analyzed Pol II accumulation near TSSs. This data revealed promoter proximal enrichment of Pol II at a number of genes relevant to reproductive biology, thus we hypothesized that pausing might impact endometrial response to hormonal signals. Here we used a conditional knock-out approach to disrupt the transcriptional pausing complex NELF (Negative Elongation Factor) in uterine cells by deleting the NELF -B subunit using PRcre (NELF-B cKO). Estrous cycles and ovulation rates of NELF-B cKO mice were comparable to WT; nonetheless, NELF-B cKO female mice were infertile, with no live births after 10 weeks of continuous breeding. Normal, healthy implantation sites were observed at 6.5 dpc, however pregnancies were subsequently lost. To ascertain causes for failed pregnancies, uteri were examined between 6.5 and 13.5 dpc, and by 8.5 dpc implantation sites of NELF-B cKO mice were smaller than WT sites. Closer examination indicated impaired decidualization of implantation sites by 7.5-8.5 dpc. Comparable levels of deciduoma markers Wnt4 and Pigs2, two genes with low promoter-proximal enrichment of Pol II in the uterus, are present in WT and NELF-B cKO 7.5 dpc implantation sites. Bmp2, a gene with high promoter-proximal Pol II enrichment that could be affected by disruption of pausing, is lower in 7.5 dpc NELF-B cKO uteri than in WT. These finding are consistent with inadequate decidualization. After artificial decidualization, WT controls responded as expected, however, NELF-B cKO mice exhibited minimal uterine decidualization. Overall, these findings indicate that Pol II pausing is not necessary to initiate but is required to maintain pregnancy. Disruption of the NELF complex results in pregnancy loss at a time relevant to decidualization, indicating a crucial role for NELF-mediated transcriptional pausing in uterine response to PR and embryonic initiated signals needed to support full-term pregnancy.

251. **Constitutive Expression of the Progesterone Receptor B Isoform Results in Severe Subfertility and the Development of Poorly Differentiated Ovarian Neoplasms.**

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The healthy ovary is responsible for the production of the steroid hormones, estrogen and progesterone, which function via their cognate receptors, the estrogen receptor and progesterone receptor (PGR). The appropriate balance of these steroids, hormone receptors, and hormone receptor isoforms is absolutely critical for reproductive function. Correct levels of the PGR-A isoform are necessary for ovulation and a successful pregnancy, while the PGR-B isoform is important for mammary gland development. Dysregulation of these PGR isoforms or a pre-dominance of a single PGR isoform is not only detrimental to reproduction but also underpins endometrial and ovarian cancer susceptibility. To further understand the role of PGR isoform dysregulation in hormone-responsive tissues, a conditional mouse model was generated to constitutively express Pgr-B, using a Prcre driven lox-STOP-lox system. Through the constitutive expression of the PGR-B isoform in PGR positive tissues, mice exhibited severe subfertility (n=8) due to the inability of embryos to implant (n=5) and the failure of uterine decidualization (n=6). At 34 weeks of age, mice developed large ovarian tumors identified as poorly differentiated neoplasms (n=9) with a tumor penetrance of 64%. To determine whether the neoplasms developed from the uterus or the ovary, ovaries were removed from young mice and allowed to age (n=13). Ovariectomized PGR-B expressing mice failed to develop tumors, confirming that tumorigenesis originates from the ovary or oviduct. Preliminary studies indicate that the PGR antagonist, mifepristone, successfully reduces tumor volume in PGR-B expressing mice (n=3). An RNA microarray was performed on ovarian tumor tissue (n=5) compared to wildtype age-matched healthy ovaries (n=3). Upon filtration by fold change and a p-value of ≤0.05, 1199 and 1653 genes were found to be upregulated and downregulated respectively. The major upregulated gene pathways represent cell proliferation and cell motility pathways such as MAPK, p53, and AKT pathways. Major downregulated pathways include organ morphology specification and development including reproductive system development. Currently, studies are being conducted to assess

2015 Abstracts - Page 101
the PGR-B specific cistrome within these ovarian tumors. Human ovarian cancer is the most lethal reproductive disease. Furthermore, the PGR-B isoform is commonly found upregulated in human ovarian cancer, yet our understanding concerning the role of the individual PGR isoforms in the progression of ovarian cancer is currently limited. Therefore, these data will increase our understanding of this disease, furthering the discovery and development of new biomarkers and effective treatment options. This work was supported by NIH Grants: R01HD042311, 5U54HD070495 (to FJD) and R01CA77530 (to JPL) and NURSA grant: U19DK62434 (to MTJ, SYT & FJD).

252. Conditional deletion of Pgrmc1 and Pgrmc2 results in aberrant endometrial gene expression, histoarchitecture and estrogen signaling.
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Progesterone receptor membrane component (PGRMC) 1 and PGRMC2 are purported non-classical progesterone (P4) receptors implicated in mitosis, apoptosis and chemoresistance. Our previous studies have shown that Pgrmc1 and/or Pgrmc2 conditional knockout mice have fewer pups per litter and fewer litters overall due to a post-implantation uterine defect. The objective of this study was to extend these initial findings by determining how PGRMC1/2 deficiency alters the functional capacity of the uterus. To achieve this goal, RNA-seq was initially used to analyze endometrial gene expression in control (Pgr+/+;Pgrmc1+/+;Pgrmc2+/+) and double conditional knockout (dcKO: Pgr+/+;Pgrmc1−/−;Pgrmc2−/−) mice (n=3). This study revealed that ablation of Pgrmc1 and Pgrmc2 leads to changes in expression of lipid metabolic proteins (e.g., Scd2), chemokine signaling proteins and transcription factors (e.g., Pparg). Further, transmission electron microscopy demonstrated that the uterus of dcKO mice displayed cysitic hyperplasia, abnormal accumulation of plasma membrane, break down of the epithelial/stromal interface and buildup of cellular debris. Because of the hyperplastic phenotype, the rate of epithelial and stromal cell proliferation in response to steroid hormone treatment was monitored by Ki67 immunostaining using two different models. First, neonatal control and dcKO mice were treated with vehicle or P4 (n=4) to determine if PGRMC1/2 mediated the anti-proliferative effects of P4 in this model of cytokine-induced epithelial proliferation. Control and dcKO mice responded similarly to P4 showing a clear reduction in epithelial cell proliferation in response to P4 treatment. Next, ovariectomized sexually mature control and dcKO mice were treated with an estrogen (E2) series or an E2+P4 series (n=3–4) in order to analyze regulation of E2-induced epithelial proliferation. While no difference was observed between control and dcKO mice in their response to E2+P4 treatment, E2 treatment induced 5-fold more (p=0.00044) luminal epithelial proliferation in uteri from control mice (58.77±4.74% Ki67+ cells) than in uteri from dcKO mice (11.55±1.60% Ki67+ cells). Similar data were observed in glandular epithelium. Interestingly, other E2 responses such as water imbibition and expression of lactoferrin remained intact in uteri from dcKO mice. Thus, while PGRMC1 and PGRMC2 are not necessary for P4 to suppress epithelial proliferation, they are essential for E2 to induce epithelial cell proliferation. Quantitative PCR revealed that expression of Ifg1, a stromal-derived cytokine that drives E2-induced epithelial cell proliferation, was reduced by 4.4-fold (p=0.02) in E2-treated Pgrmc1/2-ablated uteri compared with uteri obtained from control mice (n=3). Thus, the inability of PGRMC1/2-deficient stromal cells to express Ifg1 likely accounts for the failure of E2 to induce epithelial cell proliferation. Taken together, the results from this study reveal that PGRMC1 and 2 are involved in multiple regulatory pathways including transcriptional regulation of genes required for structural and functional maintenance and epithelial-stromal cross-talk within the uterus. Supported by NIH RR030264 and HD066297.

253. Pro-Inflammatory Proteins, S100A8, S100A9, and S100A12, and Their Receptors, TLR4 and AGER: Expression and Regulation at the Material-Conceptus Interface in Pigs.
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S100A8, S100A9, and S100A12, EF-hand type calcium binding proteins, are known as immunogenic proteins that induce inflammatory response in the damaged tissues. S100A8/S100A9 heterodimer (called calgranulin), and S100A12 bind to their shared receptors, Toll-like receptor 4 (TLR4) and receptor for advanced glycation endproducts (AGER), and subsequently, trigger secretion of pro-inflammatory cytokines, such as interleukin-1beta (IL1B), IL6, IL10, interferon gamma (IFNG), and transforming growth factor-beta (TGFβ). It has been determined that calgranulin and S100A12 regulate the inflammatory process in the uterus during pregnancy in mice and humans. However, expression, regulation, and function of S100A8, S100A9, and S100A12 in the porcine uterine endometrium during pregnancy are not well understood. Thus, the present study determined expression and regulation of pro-inflammatory S100A proteins and their receptors, TLR4 and AGER, in the uterine endometrium during the estrous cycle and pregnancy in pigs. We obtained the endometrial tissues from gilts on Days 12 and 15 of the estrous cycle and Days 12, 15, 30, 60, 90, and 114 of pregnancy, conceptuses on Days 12 and 15 of pregnancy, and the chorioallantoic tissues on Days 30, 60, 90, and 114 of pregnancy. Real-time RT-PCR analysis showed that S100A8, S100A9, and S100A12 were expressed in the uterine endometrium during the estrous cycle and pregnancy with the highest levels on Day 12 of pregnancy and showed a pregnancy-specific expression pattern, and TLR4 and AGER were also expressed in the uterine endometrium during the estrous cycle and pregnancy in a pregnancy status- and stage-specific manner. In situ hybridization analysis showed that S100A8, S100A9, and S100A12 mRNAs were localized exclusively to luminal epithelial cells on Day 12 of pregnancy, and immunohistochemical analysis showed that S100A9 and S100A12 proteins were localized to luminal epithelial cells during pregnancy. AGER mRNA was detected in luminal and glandular epithelial and stromal cells, and chorionic membrane during pregnancy. Levels of S100A8, S100A9, and S100A12 mRNAs were increased by estrogen, and abundance of S100A8 mRNA was also increased by IL1B in endometrial explant cultures. S100A8, S100A9, and S100A12 and their receptors were expressed in conceptuses during early pregnancy, and in the chorioallantoic tissues during mid- to late pregnancy. These results indicate that pro-inflammatory S100A proteins, S100A8, S100A9, and S100A12, and their receptors were expressed in the uterine endometrium in a pregnancy status- and stage-specific manner, and expression of S100A proteins were affected by estrogen and/or IL1B during the implantation period. These suggest that S100A proteins may play an important role in the establishment and maintenance of pregnancy in pigs.

254. Analysis of Type I Interferon Receptor, IFNAR1 and IFNAR2, in the Uterine Endometrium during Pregnancy in Pigs.

2015 Abstracts - Page 102
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A type I interferon (IFN), IFN-delta (IFND), is secreted by the conceptus during the implantation period in pigs. It binds to its own heterodimeric receptor, IFN-alpha/beta receptor (IFNAR1 and 2), to transmit its signal into the cell. However, expression and regulation of IFNAR1 and IFNAR2 in the porcine uterine endometrium during pregnancy are not fully understood. Thus, we analyzed expression and regulation of IFNAR1 and IFNAR2 in the uterine endometrium during the estrous cycle and pregnancy to understand the roles of conceptus-derived IFND at the maternal-conceptus interface during the implantation period in pigs. We performed real-time RT-PCR and in situ hybridization analyses using the uterine endometrial tissues collected from gilts on day (D) 12 and D15 of the estrous cycle and D12, D15, D30, D60, D90, and D114 of pregnancy, and utilized endometrial explant culture to determine the effects of steroid hormones, estrogen and progesterone, interleukin 1-beta (IL1B), and IFNG. Real-time RT-PCR analysis showed that IFNAR1 and IFNAR2 were expressed in the uterine endometrium during the estrous cycle and pregnancy, and their levels on D12 of pregnancy were higher than those on D12 of the estrous cycle and highest during pregnancy. In situ hybridization analysis showed that IFNAR1 and IFNAR2 were mainly localized to luminal epithelial cells during early pregnancy, and detected in glandular epithelial cells and chorionic membrane during mid- to late pregnancy. Abundance of IFNAR1 and IFNAR2 mRNAs was increased by IL1B and IFNG in endometrial explant cultures. RT-PCR analysis showed that IFNAR1 and IFNAR2 were expressed in conceptuses on D12 and D15 of pregnancy. Real-time RT-PCR analysis showed that IFNAR1 and IFNAR2 were also expressed in the chorioallantoic tissues during mid -to term pregnancy. These results exhibited that IFNAR1 and IFNAR2 were expressed in the uterine endometrium during the estrous cycle and pregnancy in a pregnancy status- and stage-specific manner, and their expression was affected by IL1B and IFNG. These suggest that IFNAR1 and IFNAR2 play an important role in the establishment and maintenance of pregnancy by mediating the action of conceptus- derived IFND during the implantation period in pigs.

255. Early interactions between embryo-epithelium in \textit{in vitro} mouse and human implantation sites.

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Establishment of pregnancy is limited by the time-specific window of uterine receptivity to the implanting embryo. Direct observation of mouse or human embryos attaching to human uterine epithelial cell monolayers can improve knowledge of implantation. Here we have used this model to test our hypotheses that osteopontin (OPN)-activated pathways are important at the early stage of embryo attachment and an embryo-mediated apoptotic signal allows embryo invasion into the endometrial cell layer. Artificially hatched mouse or human embryos were co-cultured with confluent receptive epithelial endometrial (Ishikawa) cells. Embryo attachments were tracked using phase contrast microscopy and graded using a scale of stability at times up to 48h. After fixation, attachment sites were observed by DAPI (DNA), rhodamine-phalloidin (actin) fluorescence staining and analysed by Z-stack optical sectioning in conjunction with immunofluorescence for components of the OPN pathway and markers of cell lineage and apoptosis. Most mouse embryos had attached weakly to the Ishikawa cell monolayer after 24h of co-culture, and progressed to stable attachment by 36h. After 48h of co-culture, few embryos remained unattached. Embryo attachments were associated with thinning of the subjacent endometrial monolayer. OPN and its receptors were immuno-detected in Ishikawa cells and also at the embryo attachment site. Cleaved caspase-3 staining at early embryonic attachment stages revealed epithelial apoptosis at the embryo-endometrial interface. In advanced attachment, beta-actin staining showed formation of prominent stress fibres in outgrowing trophoderm; the embryonic origin of these cells was confirmed using cell tracker and GATA3 immunostaining. Further advanced embryos developed outgrowth, “invading” into the endometrial monolayer. Attachment assays using human blastocysts demonstrated differences with mouse. Every hatched human embryo (n=23) was attached to the Ishikawa cell monolayer at 48h. Staining of cleaved caspase 3 showed the presence of apoptotic cells beneath and adjacent to the attached embryo. Combining E-cadherin, actin and nuclear staining, multiple early syncytiotrophoblast masses were observed per site. Syncytium formed from trophoderm that is in contact with the epithelium. Our data provide a better understanding of the progressive steps in embryo attachment in human and mouse. Embryo adhesion leads to thinning of the epithelial endometrial layer. Later, depending on attachment/invagination stages, subjacent epithelial cells may be apoptotic or displaced, with outgrowth of trophoderm. These experiments suggest an important role for the microfilament cytoskeleton in implantation and set the scene for functional investigation of the role of OPN and its receptors in triggering cytoskeletal reorganisation.

256. Analysis of CXC Chemokines, CXCL9, CXCL10, and CXCL11, and Their Receptor, CXCR3, in the Uterine Endometrium during Pregnancy in Pigs.

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CXC chemokines, CXCL9, CXCL10, and CXCL11, recruit nearby responsive cells by binding to their shared receptor, CXCR3. It has been suggested that these CXC chemokines contribute to induce the proper uterine immunological condition during pregnancy in humans and rodents. However, expression and function of CXCL9, CXCL10, and CXCL11 and their receptor, CXCR3, in the porcine uterine endometrium during pregnancy are not well understood. To determine expression and regulation of CXCL9, CXCL10, CXCL11, and CXCR3 in the porcine uterine endometrium during the estrous cycle and pregnancy, we obtained uterine endometrial tissues from gilts on day (D) 12 and D15 of the estrous cycle and D12, D15, D30, D60, D90, and D114 of pregnancy, conceptuses on D12 and D15 of pregnancy, and chorioallantoic tissues from gilts on D30, D60, D90, and D114 of pregnancy, and evaluated the effect of interferon-gamma (IFNG) on expression of these molecules in the uterine endometrium using explant tissue cultures. Real-time RT-PCR analysis showed that expression of CXCL9 mRNA showed a biphasic pattern during pregnancy with high levels on D15 and D60 of pregnancy. Levels of CXCL10 mRNA were higher on D15 of pregnancy than those on D15 of the estrous cycle. Levels of CXCL11 mRNA increased on D15 of pregnancy and maintained to term pregnancy. Levels of CXCR3 mRNA on D15 of pregnancy were also significantly higher than those on D15 of the estrous cycle. In situ hybridization analysis showed that CXCL9 mRNA was localized to stromal and vascular endothelial cells
on D15 of pregnancy, and CXCL10 mRNA was localized exclusively to subepithelial stromal cells on D15 of pregnancy. Abundance of CXCL9, CXCL10, CXCL11, and CXCR3 mRNAs was up-regulated by IFNG in endometrial explant culture. RT-PCR analysis showed that CXCL9, CXCL10, CXCL11, and CXCR3 mRNAs were not detectable or barely detectable in conceptuses on D12 and D15 of pregnancy. Real-time RT-PCR analysis showed that CXCL9, CXCL10, and CXCL11, but not CXCR3, were expressed in the chorioallantoic tissues during mid- to term pregnancy. These results indicate that CXC chemokine ligands, CXCL9, CXCL10, and CXCL11, and their receptor, CXCR3 were expressed at the maternal-conceptus interface, and their endometrial expression was increased by IFNG. These suggest that CXC chemokines and their receptor may play an important role in the establishment and maintenance of pregnancy by regulating the endometrial immune environment in pigs.

257. Expression and Regulation of the TNF and TNFR Superfamilies in the Uterine Endometrium during Pregnancy in Pigs.
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The tumor necrosis factor (TNF) superfamily is an important mediator for inducing apoptosis through its death receptors. During pregnancy, apoptosis has been observed in endometrial tissues and plays an important role in the establishment and maintenance of pregnancy in many species. However, expression and function of the TNF and TNF receptor (TNFR) superfamilies in the uterine endometrium during pregnancy has not been well studied in pigs. Thus, we determined expression of the TNF superfamily members, TNF, TNFSF10, and FASLG, and the TNFR superfamily members, TNFRSF1A, TNFRSF1B, TNFRSF10A, TNFRSF10B and FAS in the uterine endometrium during the estrus cycle and pregnancy in pigs. We performed real-time RT-PCR and in situ hybridization analyses using uterine endometrial sections from gilts on day (D) 12 and D15 of the estrus cycle and on D12, D15, D30, D60, D90, and D114 of pregnancy and chorioallantoic tissues on D30, D60, D90, and D114 of pregnancy. Using endometrial explant cultures, effects of steroid hormones and interferon-gamma (IFNG) on expression of the TNF and TNFR superfamilies were determined. Real-time RT-PCR analysis showed that mRNAs of the TNF and TNFR superfamilies were expressed in the uterine endometrium during the estrus cycle and pregnancy in a pregnancy status- and stage-specific manner. Levels of TNF, FASLG, TNFRSF10A, and TNFRSF10B mRNAs on D15 of pregnancy were significantly higher than those on D15 of the estrus cycle. In situ hybridization analysis showed that TNF mRNA was expressed to endometrial epithelial cells and markedly to chorionic epithelial cells, and TNFSF10 mRNA was also localized to endometrial epithelial cells during mid- to late pregnancy. Abundance of TNF, FASLG, TNFRSF10, FAS, and TNFRSF10A in the uterine endometrium was up-regulated by IFNG in the presence of estrogen and progesterone. TNF, TNFSF10, FASLG, TNFRSF1A, TNFRSF1B, TNFRSF10A, TNFRSF10B, and FAS were expressed in chorioallantoic tissues during mid- to late pregnancy. These results showed that the TNF and TNFR superfamilies were expressed in the uterine endometrium and chorioallantoic tissues during the estrous cycle and pregnancy, and expression of some members of the TNF and TNFR superfamily was increased by INFG during pregnancy in pigs. These indicate that the TNF and TNFR superfamilies may play an important role in the establishment and maintenance of pregnancy by regulating apoptosis at the maternal-conceptus interface in pigs.

258. WITHDRAWN.

259. Type I interferon regulates Matrix Metalloproteinases (MMPs) expression of the bovine endometrial cells in vitro.
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Interferon tau (IFNT) is a Type I IFN produced by the elongating conceptus in ruminants, which is known as the maternal recognition of pregnancy signal. Available evidence supports the idea that IFNT acts in a paracrine manner to modulate gene expression in the endometrium, which promotes implantation accompanying with the tissue remodeling. However, there are only few reports examined the consequences of INFT in remodeling of endometrium. In the present study, we investigated the effect of type I IFN on the expression of Matrix Metalloproteinases (MMPs), which is supposed to have a potential role in the endometrial tissue remodeling. The study was initiated using an in vitro culture system utilizing the spheroid, as a 3-dimensional cell mass composed of endometrial cells and ECs. Bovine endometrial epithelial cells (BEE) and bovine endometrial stromal cells (BES) were separated and purified from the caruncles and cultured in Dulbecco’s modified Eagle’s medium and Ham’s F-12, 1:1 (v/v) (DMEM/Ham’s F-12) containing 10% fetal bovine serum (FBS). Homo-spheroids (evolved from only BES) and Hetero-spheroids (evolved from both BES and BEE) were generated according to Yamauchi et al. (2003, Placenta 24:258) using ascorbate. Initially, the expression of MMP2 and MMP9 were examined utilizing the supernatant of BES, BEE, Homo- and Hetero-spheroids by gelatin zymography. The results showed that BEE predominantly expressed MMP9, whereas MMP2 was expressed in BES and Hetero-spheroids. While MMPs expression were not detected in the supernatant of the Hetero-spheroids. Since the expression of MMPs without treatments disturbs the analysis of the effect of IFNT, we used Hetero-spheroids for the further experiments. Thereafter the effect of P4, IFNalpha (Type I IFN) and the conditioned medium (CM) of the bovine elongating conceptus (day 18 of pregnancy) on MMPs expression were examined for the Hetero-spheroids. Real-time quantitative PCR (RT-qPCR) revealed that P4 and IFNalpha suppressed the gene expression of MMP2 and MMP9, respectively. On the other hand, gelatin zymography analysis of the supernatant from the cultured Hetero-spheroids showed that IFNalpha and CM strongly promote the expression of MMPs. While zymograms of the MMPs in the intact Hetero-spheroids were significantly reduced by the IFNalpha. These results suggest that the Type I IFN promotes the clearance of MMPs accumulated in the spheroid without increasing the gene expression. Since it was assumed that proteases were related to the clearance of MMPs, several protease inhibitors were used for the clearance of analysis. Zymograms of the supernatant revealed that phenylmethanesulfonyl fluoride (PMSF) and Leupeptin (both are serine proteinases) significantly repressed the clearance of MMP2 and MMP9 induced by Type I IFN. Additionally, to examine the state of ECM in the Hetero-spheroids, aniline blue staining was performed using the sections of the Hetero-spheroids. Dense collagen fibers were detected in the Hetero-spheroids of the control group. However, after the treatment with IFNalpha, intensity of collagen fibers were significantly decreased. These results indicate that Type I IFN induce the clearance of MMPs accumulated in the tissue through serine proteases. Our results suggest the novel function of Type I IFN in the uterus, the possibility to participate in the tissue remodeling of the endometrium. Supported by JSPS 25660214 and JSPS26292141.
260. **Does local regulation of intra-uterine androgens play a critical role in preparation for implantation in women?**


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During the establishment of pregnancy, the endometrium undergoes dynamic remodeling in order to establish a ‘receptive’ microenvironment able to support and sustain pregnancy. Decidualization is characterized by differentiation of endometrial stromal fibroblasts which secrete growth factors and cytokines that regulate vascular remodeling and immune cell influx, processes that are essential for implantation and placental development. Recent studies in our laboratory have revealed that decidualization of endometrial stromal cells results in significant changes in the expression of enzymes that regulate biosynthesis and metabolism of estrogens. We believe that intra-tissue steroid production may play an important regulatory role in the endometrium during the establishment of pregnancy. In the present study, we tested the hypothesis that changes in the availabilty of bioactive androgens influence endometrial function during decidualization. Primary human endometrial stromal cells were isolated from endometrial biopsies collected from women during the proliferative phase of the cycle using a method approved by the local institutional ethics committee (n=20). None of the women were receiving hormonal therapy or suffering from endometriosis. In vitro decidualization was induced by incubating cells with progesterone and cAMP. The expression of androgen biosynthetic enzymes was assessed by qPCR, Western Blot and immunocytochemistry. Concentrations of the decidualization marker IGFBP-1 and the androgens testosterone (T) and dihydrotestosterone (DHT) were determined by ELISA.

We found that decidualization was associated with biosynthesis of androgens. Time-dependent increases in the androgen biosynthetic enzymes *AKR1C3* and *SRD5A1* (5α reductase) were detected by qPCR (n=16 patients, p<0.001) and Western blot. Increased enzyme expression was associated with secretion of the potent androgen receptor agonists T and DHT (n=8, p<0.001). Androgen action was inhibited by co-treatment with the antiandrogen flutamide which significantly reduced secretion of the decidualization marker IGFBP-1 (n=8, p<0.01) and altered the expression of AR-regulated genes such as *SPP1* (osteopontin; n=8, p<0.001). Co-treatment with the adrenal androgen precursor dehydroepiandrosterone (DHEA) increased biosynthesis of T and DHT and increased secretion of IGFBP-1 (p<0.05). These data suggest intra-uterine androgens may be critical for decidualization and thus impact on establishment and maintenance of pregnancy. We speculate that decidualization is associated with creation of a steroid microenvironment that may play a critical role by ‘fine tuning’ the endometrium in preparation for pregnancy.

**261. Endometrial vascularization during the ovarian follicular growth in llamas.**

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In llamas and alpacas more than 95% of gestations are carried out in the left uterine horn which is slightly larger than its right counterpart. It is suggested that this anatomical asymmetry is the result of differences on the vascular irrigation and drainage between both uterine horns, originating in the presence of a prominent cross-over arterial branch extending from the right uterine artery to the left uterine horn. The aim of this study was to evaluate changes on endometrial irrigation between the left and right uterine horn during the ovarian follicular growth in llamas. Adult non-pregnant and non-lactating llamas (n=5) were submitted to ultrasound guided follicle ablation of every follicle ≥4 mm in diameter to induce the synchronous emergence of a new follicular wave (Day 0 = day of follicle ablation). Females were examined, every other day from Day 1 to Day 15, using B mode transrectal ultrasonography to evaluate the interval from ablation to new wave emergence, the diameter profile of the dominant follicle, the day on which the new dominant follicle reached the middle segment of each uterine horn was conducted using Color-Power Doppler mode. Vascular perfusion of the endometrium was assessed by off-line measurement of the number of colored pixels as an indicator of blood flow area. The average of three still images of each horn was used in the analyses. Doppler Power images were recorded, edited, and analyzed using the ImageJ software. Blood samples were taken from all females by jugular venipuncture every other day from Day 1 to Day 15. After blood centrifugation at 1800 rpm for 10 min plasma was stored at -20°C. Plasma estradiol concentration was determined using a commercial, double-antibody radioimmunoassay kit. Serial data was analyzed by one way ANOVA for repeated measures using the MIXED Procedure in SAS. If significant (P<0.05) main effects or interactions were detected, Tukey’s post-hoc test for multiple comparisons was used to locate differences. The interval from ablation to follicular wave emergence was 3.2 ± 0.8 days. There was a significant (P<0.01) effect of day on the growing diameter profile (from 4.5 ± 0.2 to 14.4 ± 2.6 mm in diameter) of the induced dominant follicle from the day of follicular wave emergence to Day 15 that correlated to a significant (P<0.05) increase of plasma estradiol concentration, from 41.0 ± 5.7 to 64.9 ± 6.8 pg/mL, and to an increase (P<0.05) in endometrial vascularization area of left uterine horn, from 0.6 ± 0.09 to 1.36 ± 0.3 cm².

On the contrary, endometrial vascularization area of the right uterine horn ranged from 0.56 ± 0.13 to 1.05 ± 0.22 cm² and it was not affected during the follicular growth(P=0.01). In conclusion, endometrial vascularization in the left uterine horn is increased during the follicular growth under high concentrations of systemic estradiol in llamas. If this feature relates to the high incidence of embryo implantation in this uterine horn warrants further investigations. Research supported by Project Fondecyt de iniciación 11140396, CONICYT, Government of Chile.

262. **WITHDRAWN.**

263. **Adrenomedullin Promotes Organization and Function of Junctional Proteins in the Uterus.**

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Defects in blastocyst implantation can prevent pregnancy or set the stage for clinical complications of pregnancy that present at advanced gestational ages. A critical variable of the implantation equation is uterine receptivity, which determines whether a blastocyst can
attach to and invade the endometrium. However, the cellular and molecular mechanisms underlying uterine receptivity are not well understood. Our lab has identified adrenomedullin (Adm, AM) as a maternal- and fetal-derived endocrine factor that is important for the establishment and maintenance of a healthy pregnancy. Using genetic mouse models, we have uncovered a subfertility phenotype in Adm<sup>−/−</sup> females, which birth smaller litters that are abnormally spaced and overcrowded in utero. Importantly, we have shown that the maternal genotype is causative of this phenotype, underscoring the importance of maternal-derived AM dosage in the uterus during implantation. However, further investigation has been limited by the embryonic lethality of Adm<sup>−/−</sup> pups, so the subfertility phenotype of Adm<sup>−/−</sup> females remains unexplained. Previous studies by our group demonstrated that AM contributes to cellular organization and structure in the lymphatic endothelium by affecting the expression, localization, and function of several junctional proteins that are also highly expressed in the peri-implantation uterus. Intriguingly, misregulation of proteins involved in junctional integrity and cellular polarization in the uterine epithelium has been associated with infertility due to dysfunctional uterine receptivity. Therefore, we hypothesized that AM promotes uterine receptivity and supports implantation and early embryonic development by providing organizational cues to uterine epithelial and stromal cells. Here, we demonstrate that AM stimulates linearization of the tight junction proteins ZO-1 and claudin 1 (CLDN1) at uterine epithelial cell contacts in vitro. We also present evidence of basolateral collapse of CLDN1 in the epithelium of Adm<sup>−/−</sup> inter-implantation sites in vivo. Furthermore, we demonstrate that AM enhances gap junction-mediated communication between primary human endometrial stromal cells in a scrape loading assay. Together, these results demonstrate that AM dosage in the uterus has implications not only for the expression patterns of junctional proteins but also for their functionality. Ultimately, data from these and future studies will advance our understanding of uterine receptivity and inform efforts to develop therapeutics for infertility; preventative strategies for complications of pregnancy; and novel contraception methods.

264. Effects of Low Peripheral Progesterone Concentrations on the Equine Endometrial Transcriptome. Alejandro Esteller-Vico<sup>1</sup>, James N. MacLeod<sup>1</sup>, Daniel G. Graugnard<sup>2</sup>, Kirsten E. Scoggin<sup>1</sup>, Edward L. Squires<sup>1</sup>, Mats H. Troedsson<sup>1</sup>, Barry A. Ball<sup>1</sup>.

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Progestational-induced changes in endometrial gene expression that are essential for maintenance of pregnancy have been reported in a number of species. In the horse, serone progestosterone (P4) concentrations are routinely measured to assess progestational support of pregnancy and low P4 is implicated as a cause of embryo loss; however, there is little information available concerning changes in the endometrial transcriptome associated with low peripheral P4 concentrations. Therefore, the objectives of this study were to evaluate changes in the endometrial mRNA transcriptome at Day 12 of the estrous cycle between mares with normal versus low P4 concentrations. Mares (n=6) were randomly assigned to control (0.5 mL of saline as placebo) or treatment cycles (125 mg of cloprostenol IM on Days 0 through 3 postovulation), and subsequently the same mares underwent the opposite treatment after a rest cycle in a switchback experimental design. Blood samples were collected daily from the jugular vein, and the reproductive tract was examined by transrectal palpation and ultrasound on a daily basis. P4 concentrations were measured via ELISA and expressed as area under the curve (AUC).

Endometrial biopsies were collected at Day 12 postovulation, RNA was isolated and the purity and integrity were assessed (NanoDrop ND-1000; Bioanalyzer, Agilent, 2100) prior to RNA sequencing. Library preparation and subsequent nucleotide sequencing were performed according to Illumina's standard mRNA-seq kit protocols (TruSeq Stranded RNAseq Sample Prep kit). The libraries were quantified by qPCR and sequenced on two lanes for 101 cycles from 1 end of the fragments on a HiSeq2000 using a TruSeq SBS sequencing kit version 3, generating an average of 2.73x10<sup>6</sup> stranded 100-bp paired-end reads per sample. The raw sequences (fastq) were analyzed using CLC Genomics Workbench software (version 6.0). After filtering and trimming for low quality, the reads were mapped to the equine reference genome (EquCab2.0) and quantified for nucleotide coordinates corresponding to Ensembl's consensus gene models (www.ensembl.org). Gene expression analyses were performed using an empirical analysis of DGE and a false discovery rate (FDR) correction of the P-values (significance set at P-value<0.05). Gene functions and pathways were analyzed using Ingenuity® Pathway Analysis (IPA®) on the differentially expressed genes setting pathway significance at p-value<0.05 with a z-score<-2 or >2 to indicate inhibition or activation, respectively. For the control estrous cycle, AUC for P4 was 94.7±4.3 ng mL<sup>-1</sup> day<sup>-1</sup>±SEM versus 19.6±1.0 in treated cycles (p<0.0001). Differential expression of 623 gene loci (p<0.05 after adjusting for FDR) was identified. Of these, 381 were up-regulated and 242 were down-regulated in the low P4 samples. Some of the functions and canonical pathways significantly altered included cholesterol biosynthesis and lipid metabolism and activation of estrogen mediated S-phase entry, cyclins and cell cycle: G1/S checkpoint regulation, oxidative phosphorylation, and apoptosis signaling. In conclusion, lower concentrations of progesterone during the early and mid luteal phase resulted in substantial levels of differential gene expression and significant changes for several cell signaling pathways in the equine endometrium.

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265. Impact of Uterine Glands and Uterine Luminal Fluid on Uterine Receptivity in Mice. Andrew M. Kelleher<sup>1</sup>, Thomas E. Spencer<sup>1</sup>.

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Uterine glands and, by inference, their secretions are essential for blastocyst implantation and stromal cell decidualization in mice and likely humans. In mice, the uterus becomes receptive to implantation on day 4 post-mating and, by the afternoon of day 5, becomes refractory to blastocyst implantation. Glandular epithelia (GE) synthesize and secrete or transport bioactive substances fundamental for the attainment of uterine receptivity and blastocyst implantation. In example, leukemia inhibitory factor (LIF) is expressed in the GE on day 4 in response to ovarian estrogen, and LIF null mice are infertile due to defects in blastocyst implantation. Treatment of newborn C57BL/6 mice with progesterone ablates postnatal differentiation of GE, resulting in uterine gland knockout (UGKO) phenotype. UGKO mice are infertile and exhibit defects in blastocyst implantation and uterine stromal cell decidualization. In Study One, adult control wild type (WT) (n=4) and progesterone-induced UGKO (PUGKO) mice (n=4) were mated to intact fertile males at estrus and flushed at 1600 h on day 5 post-mating. No blastocysts were recovered from WT females, whereas an average of 4 blastocysts were recovered from PUGKO uteri. In Study Two, WT and PUGKO mice were mated to vasectomized males, and uteri and uterine luminal fluid (ULF) obtained on days 3, 4 and...
5 of pseudopregnancy (DOPP; n=4 mice/day). Total RNA was isolated from the uteri and sequenced. From DOPP 3 to 4 in WT uteri, 243 genes were increased (P<0.05, >2-fold) and 148 genes decreased, whereas only 20 genes increased and 111 genes decreased from DOPP 4 to 5. As compared to WT uter, 254 genes were decreased and 117 genes were absent in the PUGKO uteri. Proteomic profiling of the ULF was conducted using liquid chromatography-tandem mass spectrometry. This approach identified 1,111 proteins in WT ULF, but Lif was not among them. In the PUGKO ULF, 19 proteins are more abundant (P<0.05, >2-fold) on DOPP 3, 48 on DOPP 4, and 258 on DOPP 5. Study Three tested the hypothesis that apical secretions of the GE into the uterine lumen regulate uterine receptivity. First, ULF was collected from WT and PUGKO uteri on DOPP 4. Next, WT mice (n=4) received injections of mouse serum albumin (MSA) into the lumen of one uterine horn at 0800 h on DOPP 4. Similarly, DOPP 4 PUGKO mice (n=4/treatment) received injections of MSA, WT DOPP 4 ULF, PUGKO DOPP 4 ULF, recombinant mouse Lif (100 ng), or PUGKO DOPP 4 ULF and Lif. Uteri were obtained on DOPP 5. Expression of four uterine receptivity genes (Hdc1, Msx1, Ptgs2, Wnt7a) was increased (P<0.05) and one gene (Abpl) was decreased in the PUGKO compared to WT uterus. However, none of the intraluminal treatments altered expression of uterine receptivity genes (Abpl, Hdc1, Hegf1, Hlh, Lif, Msx1, Mx2, Mac1, Noggin, Sfrp4, Ptgs2, Wnt7a) in the PUGKO uterus. Collectively, these results support the working hypothesis that blastocyst implantation defects in the PUGKO uterus stem from a lack of uterine receptivity and/or blastocyst competency for attachment due to alterations in gene expression and ULF homeostasis. Results also support the idea that uterine glands secrete factors, such as Lif, in a basolateral direction and that paracrine crosstalk between the GE and other cell types (stroma and luminal epithelium) is required for establishment of uterine receptivity. Supported by NIH R01 HD076347.

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Infertility and subfertility represent major problems in domestic animals and humans. The majority of embryonic loss in those species occurs during the first month of gestation when pregnancy recognition and conceptus (embryo and associated extraembryonic membranes) implantation are obligatory. The receptive endometrium in ruminants represents a temporary, but unique physiological state of the uterus when conceptus elongation and implantation is possible. Thus, inadequate uterine receptivity may compromise conceptus growth, signaling for pregnancy recognition, and pregnancy establishment. However, the critical genes and physiological pathways that mediate uterine receptivity and pregnancy success are not well understood. Predominantly Angus heifers (n=270) were classified based on fertility using serial ET (n=4 rounds) to select animals with intrinsic differences in pregnancy loss. In each round, a single in vitro-produced embryo of high quality was transferred into synchronized heifers (n=228), and pregnancy determined on day 28 by ultrasound. Heifers were then classified as high fertile (HF; 100% pregnancy success) or subfertile (SF; ≤25% pregnancy success). Following fertility classification, a single in vivo-produced embryo was transferred into HF (n=29) and SF (n=32) heifers on day 7 post estrus. Uteri of all recipient heifers were flushed on day 14 to recover the conceptus. If present, the recovered conceptus (n=35) was imaged on a Zeiss Discovery V8 stereomicroscope with an AxioCam I cc 1 and AxioVision version 4.6 software. Conceptus length and area were then determined using ImageJ (NIH, version 1.48). The effect of recipient fertility classification on pregnancy rate and conceptus length and area were determined using embryo donor as a covariate. Pregnancy rate was not different (P=0.93) in HF (64%) and SF (56%) heifers. Conceptus length was not different (P>0.10) between HF (3.0 ± 1.7 mm) and SF (5.0 ± 1.62 mm) heifers. Area was not different (P>0.10) between HF (3.68 ± 1.74 mm²) and SF (4.09 ± 1.81 mm²) heifers. The HF (n=29) and SF (n=31) heifers were genotyped using blood sample DNA and Illumina’s 778K SNP BovineHD Genotyping BeadChip. A genome-wide association study (GWAS) was then conducted using the EMMAx mixed- model association test. Highly significant associations (n=20; P<5X10^-7) were detected on several chromosomes (BTA 2, 3, 4, 5, 6, 8, 13, 15, 16, X) and queried against the cattle Quantitative Trait Loci (QTL) database which revealed 8 SNPs located in loci with at least one known fertility trait. Results of these studies indicate that pre-implantation conceptus growth is not compromised in SF heifers that are fertility-classified by ET. Further, they support the idea that the observed difference in capacity for pregnancy success is manifested between days 14 and 28 when pregnancy recognition signaling and conceptus implantation must succeed for the establishment of pregnancy. The genetic markers identified in this study may be useful to select animals for fertility and enhance our understanding of the physiological pathways governing pregnancy success and loss in mammals. Supported by 1 R01 HD072808 from the Eunice Kennedy Shriver National Institute of Child Health and Human Development.

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Basigin (BSG) is a transmembrane glycoprotein that belongs to the immunoglobulin superfamily. BSG is known to regulate tissue remodeling and cellular differentiation and is critical for male and female reproductive processes including spermatogenesis and embryo implantation. BSG is expressed by trophoblast cells beginning with the trophectoderm layer of the blastocyst. During the first trimester of pregnancy these trophoblast cells invade into the maternal endometrium and form the functional placenta at the implantation site. Pre-eclampsia, which is associated with shallow invasion of placental trophoblast cells, has been linked to reduced BSG expression in trophoblast. We hypothesize that BSG is an important regulator of trophoblast migration and that knockdown of BSG expression in HTR-SVNeo trophoblast cells would decrease their migration capacity. In order to knockdown BSG expression HTR-SVNeo cells were treated with 5nm of siRNA for BSG and compared to a negative control group treated with the same concentration of a scrambled siRNA. Cell lysates were collected using RIPA extraction buffer at 24, 48, 72 and 96 hours after treatment with siRNA to evaluate the knockdown efficacy. Immunoblotting revealed that siRNA treatment successfully knocked-down BSG protein expression in the HTR-SVNeo cells after 72 and 96 hours of treatment. Scratch migration assays were used to evaluate cell migration post knockdown. Migration was assessed at 24, 48, 72 and 96 hours after treatment with siRNA or scrambled siRNA control. Migration assays showed that BSG knockdown after 72-96
hours of siRNA treatment impaired cell migration compared to HTR cells treated with the scrambled siRNA negative control. We also carried out immunoblotting for BSG protein in cell lysates, conditioned medium and isolated microvesicles from HTR cells. We found that HTR cells consistently secrete quantities of BSG protein into the medium. Isolation of microvesicles in the conditioned medium was carried out by ultracentrifugation and confirmed that BSG protein was in the microvesicle fraction. These results provide support for a local paracrine and/or autocrine mechanism by which BSG shed in microvesicles from trophoblast cells can act on neighboring cells to regulate invasive behavior. BSG has been reported to interact with cortactin in formation of podosomes. Cortactin is a cytoplasmic protein that promotes polymerization of cytoskeletal elements and is considered a marker for podosomes. We hypothesize that cortactin should localize along the leading edge of the plasma membrane of migrating HTR-SVNeo trophoblast cells. In order to evaluate cortactin localization during migration, HTR-SVNeo cells were treated with epidermal growth factor for 48 hours to stimulate cell migration. Cortactin was visualized using immunofluorescence microscopy. Cortactin was found to be localized near the cytoplasmic membrane of EGF-treated cells whereas it was located near the nucleus in the untreated control cells. These results show that cortactin protein is drawn toward the cell membrane during cell migration and may play an essential role along with BSG in this process. We are currently evaluating the effects of BSG knockdown in HTR cells on MMP-2, MMP-9 and cortactin expression. We are also investigating the signaling pathways that may be involved in regulating trophoblast cell migration through these two proteins. Funding: NIH P01HD057877 to RAN

268. **Curcumin attenuates pro-angiogenic factors and pro-inflammatory cytokines and chemokines in human eutopic endometrial stromal cells from patients with endometriosis.**


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Endometriosis is a chronic gynecological inflammatory disorder in which immune system deregulation may play a role in its initiation and progression. Due to reduced receptor concentrations and other signaling defects, eutopic endometriotic tissues have an attenuated response to progesterone and are considered to be progesterone-resistant, which contributes to lesion proliferation and survival. Current hormonal therapies, including synthetic progestins, GnRH-agonists, and danazol are often of limited efficacy and counterproductive to fertility, and can cause systemic side effects because of suppression of endogenous steroid hormone levels. Therefore, in our current study, we compared normal endometrial stromal cells (NESC) to cells derived from eutopic endometrium of endometriosis subjects (EESC). Basal levels of pro-inflammatory chemokines and cytokines, and angiogenic factors were measured in the supernatants and the effects of curcumin were assessed at different doses over a time course. Curcumin, a naturally occurring polyphenolic compound from Curcuma longa, has long been used as an anti-inflammatory folk medicine in Asian countries. Our results confirmed that basal secretion of pro-inflammatory chemokines, cytokines, angiogenic factors and survival factors, including tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1), IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1) and vascular endothelial growth factor (VEGF) is higher in EESC compared to NESC. Furthermore, our studies showed that treatment of EESC and NESC with curcumin significantly reduced expression of those chemokines, cytokines angiogenic and survival factors in a dose-dependent and time-dependent manner. These findings demonstrate cytokine production differences in eutopic stromal cells derived from normal versus endometriosis patients and suggest that curcumin may have potential therapeutic uses in the prevention and treatment of this disease.

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269. **The Role of Early Postnatal Nutrition and Estrogen Administration in Uterine Gland Development in Holstein Dairy Calves.**

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Adenogenesis, or the development of uterine glands, is initiated in the early postnatal period. It involves rapid endometrial epithelial cell proliferation, germinal bud formation, invasion into the stroma, and extensive branching and coiling. Little is known about how nutrition impacts adenogenesis in ungulates. The overall objective was to determine if two different milk replacer diets impact early reproductive tract development, and specifically adenogenesis in cattle. In the first study, Holstein heifer calves (n=11) were assigned to a accelerated milk replacer (ACC) diet containing 28% crude protein and 25% fat that was fed at 1,077 g of dry matter per day. In week 8, heifers were restricted to one-half of the daily milk replacer allowances. Starter grain (25% crude protein; 4% fat [w/w]) was pair-wise in week 8. Diet did not affect weights of the entire reproductive tract, cervix, ovaries or uterus. Estradiol treatment increased (P < 0.05) reproductive tract and cervix weights but not uterus and ovary weights. Diet by estradiol interactions (P < 0.05) were detected on total reproductive tract and uterus weight. In both cases, weights were increased (P < 0.05) in estradiol-treated ACC-fed calves but not in estradiol-treated CON-fed calves. Follicle numbers were increased (P < 0.001) by estradiol treatment but not by diet, and no interaction...
was detected. Uterine gland number, average gland size, and total gland area were not affected by diet, estrogen, or diet by estradiol interactions. Collectively, these observations suggest that plane of nutrition impacts adenogenesis. However, compensatory mechanisms likely exist to allow uterine glands to develop as plane of nutrition improves. Prepubertal estradiol exposure impacts overall reproductive tract weights but does not have a pronounced effect on adenogenesis when it is provided after a majority of uterine gland development has occurred.

270. Prolactin Signalling In Reactivation From Embryonic Diapause In The Mink.
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Embryonic diapause is a period of developmental arrest in which the embryo is maintained in a dormant state for an extended period of time. Over 130 species of mammals undergo embryonic diapause but the molecular mechanisms that control it remain unknown. In many species with embryonic diapause, the main factor responsible for reactivation from diapause is an increase in either progesterone and/or estrogen signalling. In contrast, in the mustelid carnivores, neither hormone is able to reactivate the embryo, despite a rapid increase in circulating progesterone preceding implantation. In the mink, prolactin is the factor essential for embryo implantation. Treating mink with prolactin during diapause results in precocious termination of diapause, whereas treatment with dopamine agonists prevents implantation. However, prolactin cannot reactivate embryos in culture and it is unknown how prolactin acts at the uterine level to induce implantation.

In order to further understand the endocrine requirements permissive to reactivation of the mink embryo, we first characterised the expression of the prolactin, progesterone and estrogen receptors in the mink uterus. These results confirmed that although both the progesterone and estrogen receptor were highly expressed before diapause, levels of both were reduced at diapause and remained low throughout all reactivation stages examined. Hence, steroid signalling does not appear to be required for the initial reactivation of the mink embryo from diapause, consistent with the inability of either hormone to terminate embryonic diapause in the mink. In contrast the prolactin receptor was strongly expressed in the glandular and luminal epithelium before, during and at reactivation from diapause with significant increases in mRNA around the time of implantation.

Therefore, for the second part of this study, we sought to determine whether prolactin has direct effects on uterine processes associated with termination of embryonic diapause. Immunofluorescence analysis revealed phosphorylated STAT1 protein expression in the mink endometrium was low during diapause and increased at reactivation. In immortalized mink endometrial epithelial cells phosphorylated STAT1 protein expression increased significantly after 30 min of prolactin treatment indicating stimulatory effects of this hormone. We then examined whether prolactin mediates its effects on the mink uterus via upregulation of factors previously implicated in embryonic reactivation in either the mink and/or other species, including steroid receptors, growth factors, cytokines and factors related to the polyamine pathway. To date, we have discovered no differences in abundance of the prolactin receptor, progesterone receptor, LIF, HBEGF or ODC1 after prolactin treatment at the time points examined. Previous studies have shown that prolactin has direct effects on the mink ovary, this is the first indication of its potential direct effects on stimulating uterine signals to reactivate the embryo from diapause.

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271. UTERINE-EXPRESSED FOXL2 REGULATES THE EXPRESSION OF GENES INVOLVED IN UTERUS RECEPTIVITY AND EMBRYO IMPLANTATION.
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Forkhead transcription factor L2 (FOXL2) is a forkhead family transcription factor with important roles in reproduction. Recent publications have demonstrated that FOXL2 is expressed in human and bovine endometrium and its expression varies during the cycle and pregnancy. We aimed to explore FOXL2 contribution for embryo implantation. We investigated the role of FOXL2 in endometrial cells by comparing the expression levels of important components in high-FOXL2 expressing human endometrial cells, AN3-CA, to levels in low-FOXL2 expressing Ishikawa endometrial cells or FOXL2-depleted AN3-CA cells as well as in FOXL2-overexpressing Ishikawa cells. All experiments were performed at least three times. We determined that human endometrial cells and mouse uteri express FOXL2, and that FOXL2 expression in mouse uterus is hormone- and pregnancy-dependent (n=4 mice per each time point). FOXL2 in the mouse uterus is localized to the endometrium and myometrium. We demonstrate that FOXL2 depletion upregulates the expression of wnt/fzd family members including fzd6 (2-fold increase) and wnt11 (6-fold increase), while it reduces by 70% the expression of Kremen2, a wnt/fzd antagonist. FOXL2 depletion also induces the expression of some apoptosis-regulated genes, TNFIP3 and AFT3 (each by 7-fold) but inhibits the expression of others, such as IER3 by 75%. Apoptosis has been shown to play an important role in uterine function and embryo implantation. We show that FOXL2-depleted cells express higher levels of MSX2 (2-fold increase) and cytokines such as CXCL1 (15-fold increase) that are involved in uterus receptivity and embryo implantation. Moreover, the expression of RGS2, a GTPase-accelerating protein expressed only at implantation sites, is 6-fold higher in FOXL2-depleted uterus and endometrial cells compared to control. Conversely, overexpression of FOXL2 in low-FOXL2 expressing Ishikawa cells causes a significant but opposite trend in the expression of the same genes outlined above. These results suggest that FOXL2 has a role in regulating uterus receptivity and embryo implantation. Better understanding of the contribution of FOXL2 for embryo implantation would potentially lead to the establishment of novel strategies for improving reproduction.

272. Human Chorionic Gonadotropin (hCG) modulates Transforming growth factor-β (TGF-β) signaling decreasing the activation of ERK 1/2 and SMAD2/3 in a human endometrial stromal cell line.
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One of the key processes in embryo implantation and placenta development is a correct endometrial stromal decidualization and an adequate extravillous trophoblast (EVT) invasion into maternal decidua. hCG is one of the earliest hormones mediating embryo maternal communication which signals through the LH/hCG G-protein coupled receptor [expressed in human endometrial stromal cells (ESC)] and acts in the endometrium as a growth and differentiation factor during pregnancy. We have shown that hCG activates ERK1/2 signal transduction pathway in ESC, modulating gene expression and inducing extracellular matrix remodeling that increases EVT invasion in vitro. TGF-β is a multifunctional cytokine whose expression is regulated in human endometrial tissue. ESC expresses both the TGF-β receptors and its signal transduction machinery. TGF-β1 is required for ESC decidualization but paradoxically it restrains the invasiveness of EVT in vitro through molecules secreted by ESC. Hence TGF-β1 and hCG seems to have opposite effects in the endometrium, suggesting that hCG modulates the effects of TGF-β1 in ESC. The objective of the present study was to evaluate the regulation of hCG on TGF-β1-induced activation of ERK1/2 and Smad2/3 signal transduction pathways in human ESC. For that we used the telomerase-immortalized human endometrial stromal cell line, Si-Tib which was cultured in 60mm petri dishes. Prior to the assays, the cells were serum-starved for 8-12 hour and then stimulated with increasing doses of TGF-β1 (1, 2.5, 5 and 10 ng/ml) in presence of hCG (10 IU/ml) or vehicle for 2.5, 5, 10, 20, 40, 80 and 160 min. All experiments were performed in duplicate at least in 3 times. The results showed that ERK phosphorylation occurs with all doses of TGF-β1 assayed with a peak at 40 min. TGF-β1-induced ERK1/2 activation was significantly reduced (~40%) in presence of hCG in all time points evaluated with TGF-β1 10 ng/ml. Phosphorylated Smad2/3 increased with all doses of TGF-β1 with a maximum at 40 to 80 min. hCG significantly decreased TGF-β1-induced Stat3/2/3 activation in the doses of 2.5, 5 and 10 ng/ml with a 50% to 100% reduction. In summary our results suggest that hCG modulates TGF-β signalling in endometrial stromal cells by decreasing the activation of transduction pathways mediated by ERK1/2 and Smad2/3. These observations are important because they suggest that in vivo a similar regulation might take place during embryo implantation thus allowing correct EVT invasion through the maternal uterus in humans. Research supported by FONDECYT 1140614 to A. T- P.

273. **Over-activation of stromal beta-catenin results in sub-fertility due in part to decidualization defects.**

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Two of the most crucial events in early pregnancy in species such as humans, non-human primates and rodents are embryo implantation and uterine decidualization. Embryo implantation is a tightly regulated series of events, beginning with apposition of the embryo to the uterine luminal epithelium, followed by physical attachment to the epithelium, and culminating with penetration beyond the basement membrane into the underlying stroma. Decidualization, which is initiated during embryo attachment, is the process by which stromal cells terminally differentiate into decidual cells and is required for early pregnancy support. Perturbation at any stage of implantation or decidualization can result in pregnancy loss. Wnt ligand signaling through the canonical beta-catenin (β-cat) pathway plays a pivotal role in embryonic Müllerian duct development, postnatal uterine maturation, and establishment of early pregnancy. Previous studies in our lab and others revealed that over-activation of the transcriptional role of β-cat (OA- β-cat) in the Müllerian duct mesenchyme (MDM) of mice resulted in sub-fertility primarily due to malformed oviducts. To investigate the role of β-cat in the uterus during early pregnancy, we used mice with OA- β-cat in the MDM, which includes the stroma and myometrium, to test the hypothesis that dysregulation of stromal β-cat during early pregnancy results in implantation and/or uterine decidualization failure. To bypass the malformed oviducts and determine implantation efficiency, WT embryos were transferred into uteri of pseudopregnant control and mutant mice. Although 3/4 mutants had at least one implantation site, significantly fewer embryos implanted in mutants compared to controls (p<0.001). We next evaluated whether reduced implantation was due to attachment or decidualization defects. Attachment was indirectly assessed by expression of epithelial Esr1 and Muc1 and epithelial and stromal proliferation by Ki-67 on days 4 and 4.5 of pseudopregnancy (DOPP) during uterine receptivity. Qualitatively, 3/4 mutants showed decreased epithelial Esr1, Muc1 and Ki-67 and increased stromal Ki-67 expression comparable to controls. Additionally, both mutants and controls displayed proper uterine closure, which is important for embryo apposition and attachment. These data suggest that mutant uteri are receptive to embryo attachment. To determine whether subfertility might be attributable to, decidualization was induced by both luminal scratch and oil injection methods in one horn of pseudopregnant mice and evaluated on DOPP 7.5 and 9, respectively. Although some mutants had regional decidualization, both methods resulted in no significant difference between control and induced horns as measured by uterine wet weights in mutants (p=0.9) but did in controls (p<0.01), indicating a reduced decidualization response in mutant uteri. Bmp2, Wnt4 and Cox2, which are progesterone regulated factors critical for decidualization, were used to compare decidual tissue in mutants and controls by IHC on DOPP 5.5 and DOPP 9. In mutant uteri with implantation sites (DOP 5.5) or in those in which decidualization did occur (DOPP 9), expression patterns were similar to that of controls, suggesting complete decidualization. However, regions of mutant uteri where embryos did not implant (DOP 5.5) or decidualization did not occur (DOP 9) show different patterns of expression compared to inter-implantation sites of controls. These results suggest that although embryos may be able to attach to the luminal epithelium of mutant uteri, OA- β-cat in the stroma may alter signaling pathways that are critical for initiation of decidualization. Interestingly, RNA sequencing of estrus mutant and control uteri revealed 1075 differentially expressed genes including Ptg2 (Cox2) and Ihh implying that OA- β-cat may impact priming of the uterus for proper signaling during early pregnancy. Future studies are designed to delineate the pathways affected by OA- β-cat signaling that impact decidualization.

274. **The guinea pig vaginal closure membrane as a model of epithelial membrane fusion.**

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Introduction: Epithelial membrane fusion is a fundamental process in embryogenesis, but is a rare event post-natally under normal physiological circumstances. A notable exception is the vaginal closure membrane (VCM) of the guinea pig (and other hystricomorph rodents), which exhibits recurrent opening and closing. Closure involves the fusion of opposing stratified squamous epithelia without disruption of basal membranes and mesenchymal invasion, occurring over a period of days. This novel biological phenomenon may be related to the epithelial fusion that takes place during formation of the eyelids or buccal palate during fetal
development, tympanic membrane repair after birth and possibly even wound healing which has been shown to involve changes in trans-epithelial bioelectric currents. The accessibility of the VCM, predictively recurrent fusion and co-ordination with reproductive cycles makes for a unique model of membrane fusion and physiology. The current studies were conducted to begin to investigate physiological events involved in formation and disruption of the VCM in cyclic guinea pigs.

Methods: Peripubertal female guinea pigs (n = 8) housed in pairs were observed daily. VCM status was noted until vaginal opening and for several cycles thereafter to estimate cycle length. Bioelectric currents were recorded 3-5 times for each animal using a highly sensitive, vibrating probe at both open and closed states under gaseous isofluothane anesthesia. Following the final measurement, animals were euthanized and blood, urine, gonadal, and vaginal tissue samples were taken for analysis. Nonlinear regression analysis of bioelectric currents and day of the cycle was carried out in R using function nls(), and competing models were assessed using AIC(). Results: Cycle periods were established setting the first day of opening as cycle day 1. The estrous period extended until closure was observed (days 1-3), and early luteal (days 4-7), mid luteal (days 8-10), and late luteal (days 11-14) periods were defined accordingly. Animals established regular cycles of average length 15.08 ± 0.57 days, as estimated day 1 to next day of opening of the VCM. Bioelectric currents (µA/cm2) across the VCM of cyclic females during estrus varied somewhat, and a robust direction of current was not observed (0.154 ± 0.82). However, early and late luteal periods were characterized by a robust inward currents (early -1.914 ± 0.71, late -2.941 ± 0.45), similar to that determined previously in pregnant female closed VCM. The mid luteal period, interestingly, exhibited a distinct reversal to outward current (2.114 ± 0.65). Sinusoidal (model=a1sin(x)+a2) and polynomial (power=3) models were found to fit at significance level p=0.005, and AIC was lower for the sinusoidal model (sinusoidal 142.2, polynomial 155.1). Luteal period measurements were similar in magnitude to those observed previously in VCM of pregnant females and in epithelia undergoing repair, including human skin and cornea. The lack of a clear outward current across the vaginal membrane during the open conformation differed from previous measurements in pregnant adult females where a stronger outward current was observed during transient VCM opening around day 30 of pregnancy.

Conclusion: We thus conclude that bioelectric currents are correlated with cycle periods in guinea pigs, and further hypothesize that sex steroids may be a causative factor in the sinusoid current pattern observed.

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Introduction: Estrogens potently dilate various vascular beds throughout the body with the greatest responses in the uterus. Endogenous circulating estrogens increase during the follicular phase of the menstrual cycle and during pregnancy in women. The physiological responses to estrogens occur primarily via signaling through tissue/cell-specific expression of estrogen receptors, ERα and ERβ. We have previously shown the expression of these receptors in the ovine uterine vasculature; however, the expression of these receptors in human uterine vasculature has yet to be determined. Hypothesis: ERα and ERβ are expressed in uterine vasculature and are associated with endogenous estrogens during the menstrual cycle and pregnancy. Methods: Intact uterine artery (UA) and myometrium tissues were obtained from hysterectomies from women with different endogenous estrogens, including late pregnant women (very high estrogens, n=3), premenopausal women in the proliferative (high estrogens, n=8) and secretory (low estrogens, n=8) phases of the menstrual cycle, and postmenopausal (very low estrogens, n=5). ERα and ERβ mRNA was assayed by RT-PCR and protein was assessed by immunoblotting with specific antibodies. Cellular specific expression of ERα and ERβ was determined by immunohistochemistry with ER antibodies co-stained with markers of endothelial and smooth muscle cells in sections of UA and myometrium. Results: ERα and ERβ mRNA and proteins were detected in all human nonpregnant and pregnant UA and myometrium, and both levels of ERα and ERβ proteins are associated with the physiological status with endogenous estrogen levels. Immunohistochemical analysis also confirmed that ERα and ERβ were highly expressed in nonpregnant and pregnant UA and myometrium. In UA, ERα and ERβ were expressed in endothelial and smooth muscles cells and were highly localized to the nucleus. Both ER isoforms were also detected in the stroma and microvessels of myometrium. Conclusions: ERα and ERβ are highly expressed in the human uterine vasculature and myometrium in association with endogenous levels of circulating estrogens (funded by ROI HL70562 and AHA PRE18570033).

276. Lin28B Regulation of Trophoblast Cell Differentiation.
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LIN28A and LIN28B are RNA-binding proteins that serve an essential role in the maintenance of embryonic stem cells. Both proteins directly inhibit the let-7 miRNA family and prevent stem cell differentiation. As cells begin to differentiate, LIN28A and LIN28B decrease, allowing mature let-7 levels to increase. LIN28A has been detected in mouse trophoblast stem cells and human first trimester ACH3P cells as trophoblast stem cells differentiate, Lin28a decreases whereas let-7 miRNA levels rise. Subsequently, shRNA mediated knockdown of LIN28A in ACH3P cells drove cells toward a more differentiated state as made evident by increased levels of differentiation markers. These results suggested a role for Lin28A in the regulation of trophoblast differentiation. Interestingly, our studies show that Lin28b is very low in mouse trophoblast cells, however, in human first trimester and term placental tissue LIN28B is more abundant than LIN28A. Moreover, analyzing LIN28 levels in sheep conceptuses also displayed more abundant expression of LIN28B compared to LIN28A. This suggests that LIN28B may have an important role in the regulation of human and sheep placenta.

We hypothesize that LIN28B acts as an important regulator of trophoblast cell proliferation in human and ovine placenta. LIN28B was detected by immunofluorescence in human first trimester placenta at 8 and 11 weeks and was localized to cytrophoblast cells. LIN28B was also detected in ovine trophoblast cell lines and in Bewo and ACH3P human trophoblast cell lines, and higher levels of LIN28B than LIN28A was confirmed. To investigate the role of LIN28B in the development of the human placenta, LIN28b knockdown ACH3P cell lines were generated using a lentiviral delivered LIN28b shRNA targeting construct. Knockdown was confirmed using quantitative PCR and western blot. It was discovered that proliferation was significantly decreased in LIN28b knockdown compared to scramble control ACH3P cells. Furthermore, hCG, a marker of differentiated syncytiotrophoblast cells, was increased in media from the knockdown cells compared to scramble controls. Analysis of let-7 miRNA levels showed that the let-7s were increased in the LIN28B knockdown compared...
to controls. These findings suggest that in the absence of LIN28B, let-7 levels increase and drive trophoblast cells to a more differentiated state. These results indicate an important role for LIN28B in human placentation.

277. INFERTILITY IN WOMEN WITH DORMANT GENITAL TUBERCULOSIS MAY BE DUE TO ABBERRANT GROWTH FACTOR SIGNALING CAUSED BY THE MYCOBACTERIAL HEAT SHOCK PROTEIN HSP65.
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The dormant genital tuberculosis (GTB) is associated with infertility due to recurrent implantation failure in women. Heat shock protein, HSP65 is a mycobacterial antigen, which plays a role in the pathogenesis of tuberculosis. However, mechanisms of implantation failure in women with dormant GTB are not completely understood. We explored molecular and morphologic phenotype of endometria of dormant GTB and control women at the expected time of implantation. Women with dormant GTB had lower endometrial thickness, indicative of lower embryo receptivity, despite normal concentrations of circulating steroid hormones. There were lower protein levels of endometrial receptivity markers and reduced formation of endometrial pinopodes in dormant GTB than control endometria. Compared to control subjects, endometrium of dormant GTB women had a lower protein abundance of LIF and phosphorylated-STAT3 indicative of lower growth-factor signaling. As stromal cells decidualization is critical for endometrial receptivity, we used human endometrial stromal cell line (hESC) to study the effect of mycobacterial HSP65 on decidualization. Pretreatment with HSP65 reduced decidualization, while PBS challenged hESCs underwent normal decidualization. Aberrant decidualization due to HSP65 was associated with lower transcript abundance of PRL, LIFR, IL6 and CXCL8 along with reduced phosphorylation of STAT3 in hESC. In summary, these data demonstrate that endometria of dormant GTB women had reduced receptivity as indicated by lower endometrial thickness, receptivity markers and cytokine signalling proteins. Treatment of hESC with mycobacterial HSP65 compromised endometrial decidualization due to reduced cytokine and STAT3 signalling. Therefore, we conclude that implantation failure in women with dormant GTB may be due low cytokine-driven decidualization caused by mycobacterial HSP65 leading to decreased endometrial receptivity.

278. Estrogen up-regulated Sprr2g in the uterine luminal epithelium during mouse periimplantation.
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Embryo implantation involves the involves synchronized preparation of a competitive embryo and a receptive uterus. However, it remains largely unknown. Sprr2g is is the one of Spr family that can cornified cell envelop in the epithelium cell. Their functions in the simple epithelium such as the uterine epithelium are not clear during the periimplantation. We have examined the expression and regulation of Sprr2g in WT and Lpar3-/- mouse uterus by microarray assay, realtime PCR and In situ Hybridization. Sprr2g mRNA appeared more abundant in Lpar3-/- than in WT on day 4.5 uterine LE by microarray analysis and these differential expressions were confirmed by realtime PCR. Realtime PCR indicates that Sprr2g mRNA is highly expressed in the day 0.5 uterus LE; the expression level drops dramatically afterward and reaches the lowest level in day 5.5 uterus. It is comparable expression level of Sprr2g mRNA to that of WT are detected in the day 0.5 and 2.5 Lpar3-/- uterus using realtime PCR and in situ hybridization. However, significantly lower Sprr2g mRNA levels than that in the WT uterus are detected in the day 3.5 and higher in the day 4.5 and 5.5 lpar3-/- uterus. Hormonal regulation in ovarioctomized uterus indicated up-regulation of Lpar3 mRNA by estrodial (E2) in the luminal epithelium. Intriguingly, Sprr2g highly expressed on the decidualization in day 7.5 uterus and in vitro induced decidual the uterine stromal cell. Sprr2g expression in uterine luminal epithelium is stimulated by E2 and in decidualization suggest its essential role in embryo implantation. However, the function of Sprr2g in the LE and decidualization has not been established.

279. Analysis of PLAC1 in mouse placental formation by a lentiviral vector.
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In mammalian reproductive systems, the placenta is the first organ to be formed during embryogenesis, and is required for nutrient supply and gas exchange to the fetus. The investigation of the functions of placental genes is important to understand the development of pregnancy related diseases. The present study shows that the X-linked Plac1 (placental specific protein 1) gene is involved in trophoblast cell invasion and migration. We analyzed the biological functions of PLAC1 in mouse placental formation. Plac1 mutant mice were generated using embryonic stem cells. To evaluate the fertility of Plac1 mutant mice, we first measured the average litter size produced from natural mating. Whereas the intercrossing of wild type (WT) females with mutant male mice showed normal litter size, Plac1 mutant females showed decreased litter size with reduced numbers of the maternally mutated embryos. These results indicated that the maternally-expressed PLAC1 protein plays important roles for the embryonic development. The placental weight of Plac1 mutant mice was drastically increased (around 2-fold) mid-gestation compared with WT placenta. Histological analysis showed Plac1 mutant placentas had morphological abnormalities of the adjacent maternal and fetal blood vessels within the labyrinth layer. We next tried to rescue Plac1 mutant mice by lentiviral vector transduction of blastocysts, as this would result in placenta-specific gene expression. This transduction improved the morphology of blood vessels in the labyrinth layer and the birth rate of Plac1 mutant mice. Unexpectedly, LV-Plac1 transduced mutant placentas still demonstrated hyperplasia. These results indicated that PLAC1 derived from embryonic tissues but not placental tissues may be important for determination of placental size. Our findings suggest that PLAC1 is necessary for placental size regulatory mechanisms and correct functioning of the labyrinth trophoblast cells. In the future, we will examine the relationship of this model other mouse models which have been shown to have similar phenotypes to further investigate the molecular mechanisms of mammalian placentation.
280. Prostaglandin E$_2$ (PGE$_2$) Induces Human Endometrial Fibroblast Cell Decidualization In Vitro via PGE$_2$ Receptor 2 (PTGER2).
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Prostaglandins, especially of the E series, play an important role in endometrial fibroblast differentiation into decidual cells (decidualization) during embryo implantation in many species. To study the regulation of human decidualization by PGE$_2$, we utilized the human uterine fibroblast (HuF) in vitro cell model. Treatment of HuF cells with PGE$_2$ (10 microM) alone for 1-3 days caused significant (P<0.01) increases in the expression of the decidual markers IGFBP1, PRL and FOXO1A compared to cells treated with vehicle. This increase was significantly (P<0.01) greater in the presence of the steroids, progesterone and estradiol. Since induction of decidualization can be also stimulated by 8Br-cAMP, we hypothesized that PTGER2 and/or PTGER4 were, at least in part, responsible for mediating PGE$_2$-induced decidualization. To test this, we incubated the cells with 17-PT-PGE$_2$ (10 microM), butaprost (10 microM) or Cay10580 (1 microM) which are PTGER1, 2 and 4 agonists, respectively. In the presence of steroids, exposure to 17-PT-PGE$_2$ or Cay10580 for 2 days induced a small but significant (P<0.05) increased expression of IGFBP1 and PRL. However, the PTGER2 agonist butaprost dramatically increased (P<0.001) the expression of these genes. To confirm these findings, we incubated the cells with the PTGER 1.2 and 4 competitive antagonists SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220 (1 microM), butaprost (10 microM) or Cay10580 (1 microM) which are PTGER1, 2 and 4 agonists, respectively. In the presence of steroids, exposure to 17-PT-PGE$_2$ or Cay10580 for 2 days induced a small but significant (P<0.05) increased expression of IGFBP1 and PRL. However, the PTGER2 agonist butaprost dramatically increased (P<0.001) the expression of these genes. To confirm these findings, we incubated the cells with the PTGER 1.2 and 4 competitive antagonists SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively. In the presence of steroids, SC19220, PF04418948 or L-161,982 (10 microM), respectively.

281. Endometrial Expression of Genes Involved in Growth Factor, Cytokine, Hormone, and WNT Signaling During the Early Estrous Cycle of the Cow.
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Knowledge of the molecules used by the maternal reproductive tract to regulate development of the preimplantation embryo is largely incomplete. To identify possible candidates for this function, an experiment was conducted to assess expression patterns during the first seven days after ovulation for 93 genes that could potentially be involved in control of development. Included were genes for 29 growth factors, 11 cytokines, 22 interleukins, 3 hormones, 19 WNT ligands and 9 WNT regulatory molecules. Ovulation was synchronized in 15 cows and cows were slaughtered at days 0, 3, 5 and 7 relative to predicted ovulation. Reproductive tracts were obtained and intercaruncular regions of endometrial tissue were harvested for gene expression analysis from uterine horns ipsi- and contralateral to the corpus luteum. Abundance of specific mRNA molecules was determined using the the NanoString nCounter analysis system. Data were normalized against 6 housekeeping genes (ACTB, ERK1, GAPDH, RPL19, SLC30A6, SUZ12) and internal positive controls. Genes were considered expressed if the number of reads was greater than two standard deviations above the mean of negative controls. Data were analyzed by analysis of variance using the GLM procedure of SAS with day, side, and day*side as fixed effects, and cow as random effect. Nineteen genes were significantly affected by day with values highest at Day 5 (FGF12, HGF, VEGFA, HDGF, PTGER1, 2, 4 receptors in HuF cells to determine whether they change during 8Br-cAMP induced decidualization. Interestingly, HuF cells express all three receptors initially but expression of PTGER1 and PTGER4 dramatically decreased during decidualization while that of PTGER2 significantly increased. In conclusion, the results of this study suggest that PGE$_2$-induced decidualization of HuF cells is mediated mainly via the PTGER2 receptor. (This work was supported by Research Seed Grant from SIU School of Medicine to BB, SIU CURCA Undergraduate Student Assistantship to DD, and R15HD073868 to DT).
These results demonstrated that pig SCNT fetus showed abnormal protein expression in the extraembryonic tissue, and extensive apoptosis occurred in the extraembryonic tissue of the SCNT fetus due to an increase in apoptotic protein expression or a decrease in antioxidant control fetus. Moreover, a marked increase in the frequency of TUNEL-positive cells was observed in the extraembryonic tissue in SCNT fetus. An immunohistochemical analysis showed that the expression of 33 proteins was significantly increased or decreased in the extraembryonic tissue of SCNT fetus compared to control fetus. The differentially expressed proteins in the extraembryonic tissue of SCNT fetus included ATP or lipid binding proteins, antioxidant proteins, translation elongation factors, and transcription factors. Western blotting analysis indicated that antioxidant enzymes and anti-apoptotic proteins were down-regulated; however, the expression levels of apoptotic proteins, Bax and Hsp27, were increased in the extraembryonic tissue of SCNT fetus. Moreover, immunohistochemical analysis also showed that the expression of the catalase or GPX genes was decreased in the extraembryonic tissue with SCNT fetus compared to those with control fetus. In addition, we observed a significant decrease in DNA methyltransferase1 (Dnmt1) expression in SCNT extraembryonic tissue, and the expression levels of Dnmt3a and Dnmt3b were abnormally higher in SCNT fetus compared to control fetus. Moreover, a marked increase in the frequency of TUNEL-positive cells was observed in the extraembryonic tissue in SCNT fetus. These results demonstrated that pig SCNT fetus showed abnormal protein expression in the extraembryonic tissue, and extensive apoptosis occurred in the extraembryonic tissue of the SCNT fetus due to an increase in apoptotic protein expression or a decrease in antioxidant protein expression.

**283. Aberrant expression of DNA methyltransferase (Dnmt) family in the cloned porcine extraembryonic tissue.**

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Somatic cell cloning by nuclear transfer (SCNT) in pig is clearly of great benefit for basic research and biomedical applications. Even though cloned offspring have been successfully produced in pig, SCNT is struggling with the low efficiency. In the present study, we investigated differentially expressed proteins of the extraembryonic tissue from pig SCNT fetus compared to control (normal) fetus. We obtained the extraembryonic tissue from embryos at day 35 of pregnancy and examined the protein expression profiles using two-dimensional electrophoreses (2-D) and Western blotting. The extraembryonic tissue of fetus in control pregnancy was compared to the extraembryonic tissue of SCNT fetus, which showed an abnormally small size and shape as well as exhibited abnormal placental morphology compared to control fetus. A proteomic analysis showed that the expression of 33 proteins was significantly increased or decreased in the extraembryonic tissue of SCNT fetus compared to control fetus. The differentially expressed proteins in the extraembryonic tissue of SCNT fetus included ATP or lipid binding proteins, antioxidant proteins, translation elongation factors, and transcription factors. Western blotting analysis indicated that antioxidant enzymes and anti-apoptotic proteins were down-regulated; however, the expression levels of apoptotic proteins, Bax and Hsp27, were increased in the extraembryonic tissue of SCNT fetus. Moreover, immunohistochemical analysis also showed that the expression of the catalase or GPX genes was decreased in the extraembryonic tissue with SCNT fetus compared to those with control fetus. In addition, we observed a significant decrease in DNA methyltransferase1 (Dnmt1) expression in SCNT extraembryonic tissue, and the expression levels of Dnmt3a and Dnmt3b were abnormally higher in SCNT fetus compared to control fetus. Moreover, a marked increase in the frequency of TUNEL-positive cells was observed in the extraembryonic tissue in SCNT fetus. These results demonstrated that pig SCNT fetus showed abnormal protein expression in the extraembryonic tissue, and extensive apoptosis occurred in the extraembryonic tissue of the SCNT fetus due to an increase in apoptotic protein expression or a decrease in antioxidant protein expression.
in the catalytic activity of UCH-L3 and the chymotrypsin-like activity of the proteasome which result in an increase in Lysine 48-linkage specific polyubiquitylated protein. The related mechanisms may be referred to the increased level of phosphorylated histone h3Y41. (This project was supported by grants of the National Natural Science Foundation of China, Nos. 30871316, 31071053).

285. **Testicular sterols lanosterol and meiosis-activating sterol differentially regulate Sertoli cell gene expression.**

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Introduction: Early precursors and intermediates of cholesterol synthesis include the enzymatic catalytic formation of lanosterol (LAN) from squalene leading to the core structure of steroids. In the gonads, lanosterol is a precursor of meiosis-activating sterols (MAS), a factor suggested to have role in the regulation of meiosis during early spermatogenesis (T-MAS) and oocyte maturation (FF-MAS). To identify potential transcriptional effects on Sertoli cell gene expression by several sterols, including LAN and MAS, we employed a primary rat Sertoli cell (SC) model using cholesterol (CHOL) and 22-hydroxycholesterol (22-CHOL) for comparison. Our initial findings indicated that testicular sterols may temporally coordinate, and thereby integrate, nuclear activities of certain transcription factors resulting in changes in SC function. This current study was designed to further characterize effects of steroid exposure on steady-state expression of functionally-relevant steroid receptors and lipid handling transfer START-domain proteins (StARs) as well as monitoring several SC homeostasis regulators including cytokines, peroxiredoxins (PRDXs) and prostaglandin-endoperoxide synthase (PTGS). Methods: Sertoli cells (SC) were isolated from the testes of 18-day-old SD rats and purified as previously reported. Primary SC cultures (≥95% pure; Leydig- and macrophage-negative) were maintained in serum-free, supplemented DMEM/F12 medium at a density of 1×10⁷ cells/100mm. On day 2–3 ex vivo, sterols were added in parallel and their pharmacokinetics evaluated by dose (nano-micro M) and time (20min-24h) compared to vehicle-matched controls. Triplicate or duplicate samples were analyzed for each experiment. High quality RNA was prepared and Quantitative Real-time RT-PCR used for analyses with normalization to respective 18S level. Here we report findings at 24h following sterol addition, with statistical significance at p<0.05. Results: Steroid receptors Neither LAN nor MAS altered the expression of the androgen (AR) or progesterone (PR) receptors compared to matched controls; however AR and PR mRNA levels were significantly decreased by CHOL or 22-CHOL. MAS decreased ESR1 mRNA levels; ESR2 expression was significantly and differentially regulated by both LAN and MAS. START-domain proteins Dose-dependent effects of LAN and MAS on StARD1 and D5 expression were similar and in the absence of any changes in D4 mRNA. In contrast, CHOL and 22-CHOL decreased D4 mRNA. Cytokines MAS decreased the expression of all the cytokines evaluated: interleukins 1alpha, 1beta, 6, interferon-gamma and tumour necrosis factor, findings similar to CHOL and 22-CHOL responses were cytokine-specific and dose-dependent. Homeostasis Compared to matched controls, MAS decreased the expression of PRDX-2 and -5 mRNAs, effects also seen with CHOL and 22-CHOL. In contrast, only LAN resulted in dose-related increases in PRDX-1, -3, -4 mRNAs. All sterols increased PGTS-1 expression; CHOL and 22-CHOL decreased PGTS-2 mRNA levels. Summary: LAN and MAS can regulate ESR2 but do not affect AR and PR expression in SC. All sterols decreased expression of several known regulatory cytokines. LAN and MAS dose-dependently regulate StARD1, which is involved in mitochondrial CHOL transfer and StARD5, which redistributes free cholesterol in the cell. While increases in StARD4 can indicate a response to cell stress, D4 expression was unchanged with either LAN or MAS. Furthermore, CHOL and 22-CHOL decreased StARD4. All sterols increased PGTS-1 expression, which is involved in cell signalling maintaining homeostasis. LAN and MAS regulated specific PRXs that can mediate signal transduction by regulating cytokine-induced peroxide levels. Conclusion: Taken together, our findings suggest that particular intra-testicular sterols can regulate Sertoli cell signalling pathways and gene expression, influence intracellular lipid binding and transfer and thereby modulate SC function. Support: Studies funded by NICHD, The F.M. Kirby Foundation and Population Council.

286. **Reprogramming from Differentiated Cells into Pluripotent Cells by using Non-Integrating Vector System with activation-induced cytidine deaminase (AICDA).**

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Stem cells can provide a useful tool for studying on mechanisms of embryonic development and production of transgenic animal. Although somatic cells are able to convert into reprogrammed cells by introduction of exogenous reprogramming factors, however, iPS cells are not safely fully because of the use of retrovirus and inhibition of inserted exogenous reprogramming factors to differentiate from stem cells into beneficial cells (Choi et al., 2014). According to recent reports (Bhutani et al., 2010 and Popp et al., 2010), immune system protein called activation-induced cytidine deaminase (AICDA) plays an important role on DNA demethylation in promoter regions of two key pluripotent genes (Oct4 and Nanog ). Here we attempted whether to convert bovine ear cells into pluripotent cells by non-integrating vector with AICDA. AICDA genes were inserted to pCMV6-AC-IRES-GFP-Puro expression vector ( OriGene Technologies, USA) and transfected into the bovine ear cells. They were passaged on inactivated mouse embryonic fibroblasts after transfection. Transfected cells were cultured in DMEM/F-12 supplemented with 20% Knockout Serum Replacement and two cytokines (2000 U/ml LIF and 20 ng/ml bFGF) for a week at 37°C. As results, Oct4, Nanog and AICDA were expressed in the transfected cells but not expressed in control cells. The patterns of DNA demethylation in the promoter regions of Oct4 and Nanog were significantly increased when compared to the control cells using bisulfite sequencing and real time PCR. These results showed that AICDA can help epigenetic reprogramming by induction of DNA demethylation in the promoter sites of two key pluripotent genes (Oct4 and Nanog). * This work was partly carried out with the support of "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ009418022015)" Rural Development Administration, Republic of Korea.

287. **Histone H4K12ac in sperm chromatin represents a potential factor for epigenetic mediated subfertility.**
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During spermiogenesis, DNA-binding histones are replaced by protamines as nucleoprotamine package DNA is approximately ten times more efficient than nucleohistones. In men, histone to protamine exchange is incomplete and the remaining histones are located in the annular region and are highly acetylated. Although histone acetylation is a characteristic feature of transcriptionally active genes, it is known that spermatozoa are transcriptionally inactive, however, histone acetylation represents an epigenetic mark that is transmitted from sperm to oocyte and involved in the regulation of gene expression in the early embryo. Acetylation of histone H4 at lysine 12 (H4K12ac) was observed prior to full decondensation of sperm chromatin after fertilization suggesting an important role for the regulation of gene expression in early embryogenesis. Similarly, DNA-methylation may contribute to gene silencing of several developmentally important genes. Following the identification of H4K12ac-binding promoters in sperm of fertile and subfertile patients, we aimed to investigate whether the depletion of histone binding is associated with aberrant DNA-methylation in sperm of subfertile men. Furthermore, we monitored the transmission of H4K12ac, 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC) from the paternal chromatin to the embryo applying mouse in-vitro fertilization and immunofluorescence. Using a murine model, immunofluorescence revealed that H4K12ac co-localize with 5mC in the sperm nucleus. During fertilization, when the pronuclei are formed, the paternal pronucleus exhibits a strong acetylation signal on H4K12, while in the maternal pronucleus, there is a permanent increase of H4K12ac until pronuclei fusion. Simultaneously, there is an increase of the 5mC signal and a decrease of the 5mC signal. Chromatin immunoprecipitation (ChIP) with anti-H4K12ac antibody was performed with chromatin isolated from spermatozoa of subfertile patients with impaired sperm chromatin condensation assessed by aniline blue staining. Fertile donors were used as control. DNA-methylation analysis of selected H4K12ac-interacting promoters in spermatozoa was performed by pyrosequencing. Depletion of binding sites for H4K12ac was observed within the following developmentally important promoters: AFF4, EP300, LRPP5, RUVBL1, USP9X, NCOA6, NSDI1, POUP2F1. We found 5-10% hypomethylation within CpG islands of selected promoters in the sperm of fertile donors and it was not significantly altered in the subfertile group. Our results demonstrate that the H4K12ac depletion in selected developmentally important promoters of subfertile patients was not accompanied by a change of DNA-methylation. We suggest that aberrant histone acetylation within developmentally important gene promoters in subfertile men, but not DNA methylation, may reflect insufficient sperm chromatin compaction affecting the transfer of epigenetic marks to the oocyte. This research was supported by the Grant Agency of the Czech Republic GACR No. 14-05547S.


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Endometriosis is a common gynecological disease that affects many women of reproductive age. It involves growth of the endometrial cells outside the uterus on peritoneal surfaces, primarily due to retrograde menstruation. Although the estrogen (E2) dependence of endometriosis is well characterized, the role of progesterone (P4) in establishment and progression of this disease remains less understood. In the present study, we developed and validated an immunocompetent mouse model of endometriosis, in which minced uterine tissue fragments collected from donor females with intact immune system were introduced into the peritoneal cavities of the recipients. These fragments were cultured for approximately 10 days to allow the lesion to develop. The ectopic lesions were monitored by immunofluorescence, histology and hemodynamic analysis. The lesions were comprised primarily of stromal cells with cystic filled with turbid fluid. In contrast, 1-3 small, white colored, nonvascular lesions, with either small cysts or compact content, were seen in the E2 plus P4-treated group. Immunohistochemical analysis revealed that there was a significant reduction in the expression levels of Ki67, F4/80, CYR61, NF-κB p65, and p-ERK1/2 as well as in the diameters of supporting blood vessels at the interface between lesion and peritoneum in E2 plus P4-treated lesions, when compared to those in E2-treated group. These results indicate that P4 alleviates E2-dependent establishment and growth of ectopic lesions by inhibition of endometrial cell proliferation, inflammatory responses and angiogenesis. Moreover, alteration in the numbers of pro-inflammatory T helper 17 (RORγ) cells and anti-inflammatory T-regulatory (FoxP3) cells, attenuation in the expression levels of estrogen receptor alpha and progesterone receptors, and aberrant myofibroblast activation were observed in the E-treated, but not in the E plus P-treated cystic lesions, when compared to the corresponding eutopic endometrium. Suppression of DNA methylation by administration of 5-aza- 2'-deoxycytidine to E2-treated recipients inhibited lesion expansion and restored the aberrant expression of target genes and fibrotic reaction. Collectively, this immunocompetent mouse model of endometriosis provides opportunities to study the impact of endometriosis on physiological functions of the uterus, as well as progression and molecular pathogenesis of this disease that may be associated with aberrant steroid actions, genetic mutation, epigenetic modification, chronic inflammation, or exposure to environmental toxicants. Supported by R21 ES024198 to QLI, U54 HD 055787 to MKB and ICB, and U54 HD40093 to RAN.

289. Studying DNA methylation in animal reproduction and development.

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Aberrant DNA methylation patterns have been associated with the failures during animal reproduction and development. Using cattle as a model, we are studying one of the major epigenetic components: DNA methylation in various tissues, gamete production, early embryo development. We have systematically characterized the impacts of tissue types, ages and generations on the DNA methylation status in cattle. We detected over 34 million potential methylated sites of ten tissues from three cattle using RRBS method. An average of 1.5% sites were detected with methylation level when we use >7 reads as a threshold. These results will be used for detecting the relationship between methylation level and genome structures, the relationship between methylation level and gene expression when combined with the published RNA expression data, and the methylation differences among different tissues. To estimate the impacts of DNA methylation on animal fertility, we are in the process of generating genome-wide DNA methylation maps at a single-base resolution with bull sperm of high and low fertility. These results will provide insights into the roles of DNA methylation in animal reproduction and development.

290. **Nutritional Programming of Accelerated Puberty in Heifers: Alterations in DNA Methylation in the Arcuate Nucleus.**

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High rates of body weight gain during the juvenile period appear to program molecular events within the hypothalamus, leading to advancement of puberty. The hypothalamic arcuate nucleus (ARC) integrates nutritional inputs through intermediate neuronal or glial circuits that regulate GnRH release, and differential gene transcription within the ARC appears to be involved with nutritional programming leading to accelerated puberty in heifers. This is supported by our previous studies reporting altered mRNA abundance of key genes associated with the control of GnRH release, neuronal plasticity, and signaling pathways in the ARC, and accompanying increases in adiposity and secretion of leptin and insulin-like growth factor 1 (IGF-1). Methylation of DNA, an epigenetic mechanism that controls gene expression, is associated with metabolic programming events and proposed to play a role in the pubertal process. Herein, we hypothesized that an altered pattern of DNA methylation in key genes within the ARC occurs in response to an elevated rate of body weight gain during the juvenile period. We assessed DNA methylation in the ARC of juvenile heifers fed to gain body weight at relatively high (1 kg/day; High- gain, n = 4) or low (0.5 kg/day; Low-gain, n = 4) rates from 4.5 to 8.5 mo of age. At the completion of the experiment, heifers (as anticipated) remained prepubertal, with earliest puberty expected around 9 mo of age in High-gain heifers. A block of tissue containing the hypothalamus was dissected, snap frozen, cut in 20-μm coronal sections, mounted on slides, and stored at -80°C. Genomic DNA was isolated from bilateral tissue scrapes of a 1-mm diameter area within the ARC and a methyl-cytosine enrichment assay was used to capture methylated regions of the genome. Using a custom-designed oligonucleotide array targeted to imprinted genes and genes associated with nutritional inputs and the control of puberty, a comparative-genomic-hybridization array was used to identify differentially methylated regions between High- and Low-gain heifers. Treatment effects on DNA methylation patterns were assessed by t-test and by Fisher’s exact test (comparing the proportion of heifers of each treatment identified as exhibiting hypermethylation of a given sequence). Differential methylation of genomic regions was observed at genes involved in the modulation of growth and metabolism, including those encoding the receptors of growth hormone (GHR), IGF-1 (IGF1R) and leptin (LEPR), as well as the imprinted IGF2 and PEG3 genes. Importantly, differential methylation was observed at LIN28B and HMGA2 genes recently proposed to be central components of a key inhibitory network that controls onset of puberty. Hence, an elevated rate of body weight gain during the juvenile period alters DNA methylation patterns in the ARC and these changes may be critical for programming the age at puberty in heifers. Collectively, these findings show dietary dependent epigenetic modulation of metabolic pathway genes within the ARC. Supported by USDA-NIFA-AFRI 2009-65203-05678.

291. **Karyotyping analysis and epigenetic characteristics of porcine ESCs.**


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Pluripotent stem cells can be categorized according to their pluripotent state. The distinct biphasic states, naïve and primed, represent cells of the preimplantation embryo and later epiblast cells, respectively. However, naïve stage in pigs has been difficult to capture in vitro. We already reported that primed embryonic stem cell (ESC) lines were derived from porcine embryos of various origins, including in vitro fertilized (IVF), parthenogenetic activation (PA) and, in particular, nuclear transfer (iPS-NT) from a donor cell with iPSC. Karyotyping analysis revealed that the representative four cell lines contained a normal number of 38 XX chromosomes at 10-25 passages. The pericentric inversion of chromosome 8, which is considered a normal variation, has been detected in IVF 0214 and PA 0531B lines. The expression of Xist has only been able to observe in female porcine ESC lines by RT-PCR, except for the male cell line TG-NT1, which was established recently. Additionally, immunofluorescence staining with H3K27me3 as a marker of the state of XCI has been performed in porcine ESC lines. Interestingly, only iPS-NT lines showed multiple aberrant patterns of nuclear foci, whereas other female cell lines had a single-positive focus and male cell lines did not have a focus in the nucleus. These observations suggest that iPS-NT lines have epigenetic instability compared with other porcine ESC lines.

This work was supported, in part, by a grant from the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ011288), Rural Development Administration, and the National Research Foundation of Korea Grant funded by the Korean Government (NRF- 2012R1A1A4A01004885, NRF-2013R1A2A2A04008751), Republic of Korea.

292. **Tissue-specific expression of estrogen receptor 1 is regulated by DNA methylation in a T-DMR (tissue-dependent and differentially methylated region).**

RYO MAEKAWA, MAKI OKADA, HIROSHI TAMURA, NORIHIRO SUGINO.

2015 Abstracts – Page 117
respectively) than that with 1.5mM VPA or that for the controls (7.94±1.24 and 7.28±0.72, respectively). The expression level of (45.7±7% and 337.8±33.3). However, the apoptotic index was lower (P<0.05) with 3.0 and 4.5 mM VPA (2.3±0.39 and 3.2±0.43, and number of blastocysts (297.6±33.8, 360.6±40.4 and 373.3±32.7, respectively) were not significantly different from that of controls DNMT3a

VPA treatment (3.0 and 4.5 mM) altered the cell morphology, increased (P<0.05) the population doubling time and decreased (P<0.05) the cell viability. VPA treatment increased the global level of H3K9/14ac, H4K5ac and H3K18ac but not that of H3K27me3 in the donor cells than that in untreated controls. The treatment decreased (P<0.05) the expression level of ESR1/1 promoter, suggesting that the region includes the T-DMR for tissue-specific ESR1 expression. It is known that ESR1 has several transcription starts sites (TSS) and corresponding upstream exons (upstream Exon-A to -E1). The transcription of ESR1 starts from any of these upstream exons, and the upstream exons are used in a tissue-dependent manner. Three upstream exons, upstream Exon-A, -B and -C, are often used in the tissues with high ESR1 expression. In the present study, we investigated whether human ESR1 has a T-DMR and whether DNA methylation of the T-DMR regulates its expression. We also investigated whether T-DMR is present in each upstream exon of ESR1.

Materials and methods: We obtained informed consent from patients and approval by Yamaguchi University. DNA methylation profiles and mRNA expression profiles of ESR1 were analyzed in the endometrium, mammary gland, placenta, skin, and breast cancer tissues by sodium bisulfite sequencing. T-DMR-methylated reporter assay was performed to examine whether DNA methylation at the T-DMR actually suppresses transcription of ESR1. Results: ESR1 expression was tissue-specific, being high in the endometrium and mammary gland and low/nill in the placenta and skin. In all of the tissues, the proximal promoter regions were unmethylated. On the other hand, the distal regions were unmethylated in the endometrium and mammary gland, but were hypermethylated in the placenta and skin, indicating this region is a T-DMR. T-DMR-methylated reporter assay revealed that DNA methylation of the T-DMR suppressed ESR1 transcription. DNA methylation analysis around upstream Exon-A, -B and -C showed each upstream exon has its own T-DMR and DNA methylation of the region is associated with transcriptional regulation in each upstream exon. In some breast cancer cases, T-DMRs regulate ESR1 transcription via DNA methylation in a manner similar to normal tissues, while in other cases, ESR1 transcriptional regulation deviates from the regulation seen in normal tissues. Conclusions: This is the first report to demonstrate that ESR1 has T-DMRs, and that the T-DMRs regulate tissue-specific ESR1 expression via DNA methylation in normal tissues. We also found that each upstream exon has a corresponding T-DMR, of which DNA methylation status is involved in regulating transcription of the upstream exon. Furthermore, our results show some breast cancer cases deviate from the normal regulatory mechanism of the transcription regulation of ESR1.

293. Valproic acid-induced histone hyperacetylation of donor cells does not improve the developmental competence and epigenetic status of cloned buffalo (Bubalus Bubalis) embryos.

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Abnormal epigenetic nuclear reprogramming after somatic cell nucleus transfer (NT) is considered to be the primary reason responsible for very low cloning efficiency. This study was aimed at examining the effects of treatment of donor cells with valproic acid (VPA), a histone deacetylase-inhibitor, on histone acetylation, preimplantation development and gene expression in buffalo embryos produced by Hand- made cloning (HMC). HMC, examination of total cell number (TCN), level of apoptosis, immunofluorescence staining and qPCR were performed as described earlier (Selokar et al., 2014, PLoS One,10;9(3):e90755). The donor cells were treated with VPA (0, 1.5, 3.0 and 4.5 mM) for 24 h following which reconstructed embryos (n = 72, 84, 87 and 84, respectively) were generated from these cells. VPA treatment (3.0 and 4.5 mM) altered the cell morphology, increased (P<0.05) the population doubling time and decreased (P<0.05) the cell viability. VPA treatment increased the global level of H3K9/14ac, H4K5ac and H3K18ac but not that of H3K27me3 in the donor cells than that in untreated controls. The treatment decreased (P<0.05) the expression level of HDAC1, DNMT1, DNMT3a and P53, and increased (P>0.05) that of CASPASE3 compared to that in the untreated control cells. Following use of treated cells for HMC, the blastocyst rate of donor cells treated with 1.5, 3.0 and 4.5 mM VPA (49.4±4.6, 48.5±5.0 and 52.4±8.4%, respectively) and the total cell number of blastocysts (297.6±33.8, 360.6±40.4 and 373.3±32.7, respectively) were not significantly different from that of controls (45.7±7% and 337.8±33.3). However, the apoptotic index was lower (P<0.05) with 3.0 and 4.5 mM VPA (2.3±0.39 and 3.2±0.43, respectively) than that with 1.5mM VPA or that for the controls (7.94±1.24 and 7.28±0.72, respectively). The expression level of HDAC1 and CASPASE3 was lower (P<0.05) in the blastocysts produced from VPA-treated cells than that in controls whereas that of DNMT1, DNMT3a and P53, and the global level of H3K9/14ac were not significantly different between the two groups. In conclusion, our results provide evidence that DNA-induced hyperacetylation of histones in the donor cell does not affect the developmental competence and global level of H3K9/14ac although the expression level of some genes is altered.

294. Rat blastocysts resulting from somatic cell nuclear injection and time-lagged enucleation.

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Introduction: Estrogen receptor 1 (ESR1), which codes estrogen receptor alpha, shows tissue-specific expression. The mechanism controlling tissue-specific expression of ESR1 is still unclear. It has been reported that DNA methylation of a specific region of the gene has an important role in determining tissue- and cell-specific gene expression. This region is called the T-DMR (tissue-dependent and differentially methylated region). We previously found a possible link between the mRNA expression of ESR1 and the DNA methylation status of ESR1 promoter, suggesting that the region includes the T-DMR for tissue-specific ESR1 expression. It is known that ESR1 has several transcription starts sites (TSS) and corresponding upstream exons (upstream Exon-A to -E1). The transcription of ESR1 starts from any of these upstream exons, and the upstream exons are used in a tissue-dependent manner. Three upstream exons, upstream Exon-A, -B and -C, are often used in the tissues with high ESR1 expression. In the present study, we investigated whether human ESR1 has a T-DMR and whether DNA methylation of the T-DMR regulates its expression. We also investigated whether T-DMR is present in each upstream exon of ESR1.

Materials and methods: We obtained informed consent from patients and approval by Yamaguchi University. DNA methylation profiles and mRNA expression profiles of ESR1 were analyzed in the endometrium, mammary gland, placenta, skin, and breast cancer tissues by sodium bisulfite sequencing. T-DMR-methylated reporter assay was performed to examine whether DNA methylation at the T-DMR actually suppresses transcription of ESR1. Results: ESR1 expression was tissue-specific, being high in the endometrium and mammary gland and low/nill in the placenta and skin. In all of the tissues, the proximal promoter regions were unmethylated. On the other hand, the distal regions were unmethylated in the endometrium and mammary gland, but were hypermethylated in the placenta and skin, indicating this region is a T-DMR. T-DMR-methylated reporter assay revealed that DNA methylation of the T-DMR suppressed ESR1 transcription. DNA methylation analysis around upstream Exon-A, -B and -C showed each upstream exon has its own T-DMR and DNA methylation of the region is associated with transcriptional regulation in each upstream exon. In some breast cancer cases, T-DMRs regulate ESR1 transcription via DNA methylation in a manner similar to normal tissues, while in other cases, ESR1 transcriptional regulation deviates from the regulation seen in normal tissues. Conclusions: This is the first report to demonstrate that ESR1 has T-DMRs, and that the T-DMRs regulate tissue-specific ESR1 expression via DNA methylation in normal tissues. We also found that each upstream exon has a corresponding T-DMR, of which DNA methylation status is involved in regulating transcription of the upstream exon. Furthermore, our results show some breast cancer cases deviate from the normal regulatory mechanism of the transcription regulation of ESR1.

2015 Abstracts – Page 118
Effects are transmitted transgenerationally. Regulation, cell cycle and metabolism, acting in a step-wise fashion from zygote to morula occurred. This study provides the first comprehensive analysis of pig lincRNAs and gives a revealing insight into the gene regulatory mechanism responsible for early embryonic development. Genome-wide transcriptome studies have identified thousands of long intergenic noncoding RNAs (lincRNAs), some of which play important roles in early embryonic development. Pig is an ideal model for reproduction, however, due to the limited sequence annotation, porcine lincRNA have not yet been characterized and it is unknown if they are associated with early embryonic development. Here, we identified 4776 lincRNA genes derived from RNA sequence (RNA-seq) data sets of 30 samples through Coding-Non-Coding Index (CNCI) and Coding Potential Assessment Tool (CPAT), which are two tools for lincRNA identification independent of known annotations. These lincRNAs show short length, low exon number and low expression level, which is consistent with previous studies in other species. Weighted co-expression network analysis suggested that each developmental stage can be delineated concisely by a small number of functional modules of co-expressed genes. A sequential order of transcriptional changes in pathways of protein assembly, gene regulation, cell cycle and metabolism, acting in a step-wise fashion from zygote to morula occurred. This study provides the first comprehensive analysis of pig lincRNAs and gives a revealing insight into the gene regulatory mechanism responsible for early embryonic development in pig. Research supported by National Basic Research Program of China (973) (2011CBA01006).

Identification of Long Intergenic Noncoding RNAs and Their Genetic Programs Analysis in Pig Early Embryos. Jingyu Li, Zhonghua Liu.

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This study is important for understanding the role of lincRNAs in early embryonic development. Pig is an ideal model for reproduction, and the study provides the first comprehensive analysis of pig lincRNAs. This provides insight into the gene regulatory mechanism responsible for early embryonic development in pig.


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In somatic cell nuclear transfer (SCNT), nucleosomal environment of differentiated cells seems to impair the genomic reprogramming for its proper development. We have shown that histone H2A.Z, which is essential for the cell survival but is not expressed in the early embryos, was carried from the donor cell into the SCNT egg and suggested a possibility that such aberrant histone conformation impairs the reprogramming. Recently, it was shown that a histone chaperone ANP32E, a member of the p400/Tip60 complexes, is responsible for the removal and deposition of H2A.Z in the nucleosome via the acetylation of histone H3 on lysine 56 (Ac-H3K56). In this study, we examined the effect of HDACi on the modulation of H2A.Z in SCNT embryos and analyzed the subcellular localization of Ac-H3K56 and histone chaperones, Tip60 and ANP32E, in IVF and SCNT embryos by immunocytochemistry. In IVF embryos, histone H2A.Z was absent from the PN to 4-cell stages and appeared thereafter, whereas it existed from the 1-cell to blastocyst stages in SCNT eggs and the most of SCNT embryos arrested at the 2-cell stage. Either trichostatin A (TSA), oxamflatin or suberoylanilide hydroxamic acid (SAHA) treatment to SCNT eggs led to the elimination of H2A.Z from the 1- to 4-cell stages and then it emerged thereafter similar to IVF embryos and improved their embryonic development. Unlike those HDACi, histone H2A.Z in the valproic acid (VPA)-treated SCNT eggs remained in the PN and vanished thereafter. The disappearance of histone H2A.Z was prolonged until the morula stage and some embryos were arrested at the morula-blastocyst transition (MBT). As for the histone H3, Ac-H3K56 was localized in nucleus from the 1- to 4-cell stages of IVF and SCNT embryos, although acetylation level of histone H3 in HDACi-treated SCNT embryos was apparently lower at the pronuclear stage. Both Tip60 and ANP32E showed no difference in its localization among IVF and SCNT embryos regardless of HDACi treatments. These results indicated that HDACi induces an acetylation of histone H3 on lysine 56, which initiates a removal of donor cell-derived histone H2A.Z in SCNT eggs by means of p400/Tip60 and ANP32E complexes. Regulating the histone H2A.Z may affect adversely ZGA and/or MBT and will provide novel chromatin remodeling technology to improve the developmental ability of SCNT eggs as well as the stem cell engineering.

Overexpression of the histone H3 lysine specific demethylase 1 A (KDM1A) in oocytes alters embryo development and the effects are transmitted transgenerationally. Christine Lafleur, Keith Siklenka, George Chountalos, Maren Godmann, Hugh Clarke, Sarah Kimmins.

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297. Overexpression of the histone H3 lysine specific demethylase 1 A (KDM1A) in oocytes alters embryo development and the effects are transmitted transgenerationally.
The gamete epigenome carries a molecular memory of a parent’s lifetime experiences to influence the health and development of offspring, yet little is known of the molecular mechanisms underlying epigenetic inheritance. The epigenome refers to the chromatin content including histones that function to control gene expression. The objective of this study was to alter the oocyte epigenome to determine its role in oocyte gene expression and the consequences for offspring. The lysine specific demethylase 1A, KDM1A, removes demethylation on histone H3 at lysine 4 and is highly expressed in the oocyte and ovary. To investigate the role of KDM1A in transgenerational epigenetic inheritance we designed transgenic mice to over-express KDM1A in the oocyte only. Oocytes from transgenics showed altered expression of 71 genes including those linked to embryo development. Analysis of pregnancies and offspring of transgenics revealed increased pre-implantation loss, smaller litters and fetuses with skeletal abnormalities. Intriguingly, descendants of transgenics that did not carry the transgene themselves, nontransgenics also had abnormal reproductive outcomes, suggesting KDM1A targets regions of the oocyte epigenome that escape epigenetic reprogramming. Transgenerational inheritance is thought to occur via alterations in DNA methylation in the germline. In sharp contrast, this study demonstrates that the histone targeting demethylase KDM1A alters embryo development with lasting maternal effects that span generations. This research was funded by the Canadian Institutes of Health Research, Réseau Québécois en Reproduction, Fonds Québécois de Recherches Nature et Technologies.

298. Therapeutic targeting of the Hippo pathway in a canine model of mammary gland cancer.
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Breast cancer is the second most common form of cancer worldwide and the second leading cause of cancer death in women. The mouse is the most widely used engineered model in breast cancer research. Murine induced tumor models have contributed significantly to our understanding of fundamental aspects of breast cancer biology, but don’t always provide sufficient similarity with the human disease for effective preclinical drug development. The bitch develops spontaneous mammary gland tumors with an incidence approximately three times as great as in women. About one-third of these are carcinomas that resemble human breast carcinomas. The prognosis following mastectomy for invasive tubular carcinoma and solid carcinoma is poor in the bitch, and these histological subtypes make the best models for translational breast cancer research. Studies of the hippo pathway, and more specifically of its two main effectors YAP and TAZ, have demonstrated that it is involved in mammary gland differentiation, growth and tumorigenesis. Furthermore, pharmacological inhibitors of the Hippo pathway such as Verteporfin (an inhibitor of YAP/TAZ-TEAD transcriptional activity) hinder tumor growth. Our hypothesis is that a misregulation of the Hippo pathway contributes to the development of mammary gland cancer in dogs, and that this pathway represents a pharmacological target that can be used clinically, with results that could translate to human medicine. Immunohistochemistry for YAP and TAZ was done on formalin fixed, paraffin embedded blocks of three normal canine mammary glands, six canine mammary gland adenomas and thirteen canine mammary gland carcinomas. Preliminary results show that, as in human, TAZ is expressed in reserve cells in the normal mammary gland and in benign tumors. This expression increases and broadens in malignant epithelial cells in a subset of canine mammary gland carcinomas. To assess the effect of Verteporfin on canine mammary cancer, we exposed five different canine mammary cancer cell lines (CMT-12, CMT-47, CMT84, CMT9 and CMT-28) to six different concentrations of Verteporfin (0 uM, 0.03 uM, 0.1 uM, 0.3 uM, 1 uM and 3 uM) and assessed cell viability after 48 hrs. Verteporfin killed cancer cells, with effective dosages (ED) for the five lines varying between 0.1uM and 0.4uM. These concentrations are well below the effective tumor and plasma drug levels obtainable in vivo in mouse xenograft models. These preliminary results suggest that the Hippo pathway plays an important role in canine mammary gland tumorigenesis in dogs and that it is possible to target this pathway as therapeutic avenue for certain types of mammary gland cancers. This work represents a first step towards the development of a targeted molecular therapy for inoperable mammary gland cancers with results that could translate to human medicine.

299. Changes in histone modification, DNA methylation and C/EBPb binding of the Cyp11a1 promoter region in rat granulosa cells undergoing luteinization during ovulation.
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Introduction: The ovulatory LH surge rapidly alters the expression of steroidogenesis-related genes such as StAR, Cyp19a1 and Cyp11a1 in granulosa cells (GCs). Cyp11a1 codes P450scc and plays an important role in production of progesterone. The ovulatory LH surge induces rapid up-regulation of Cyp11a1 in granulosa cells (GCs) undergoing luteinization during ovulation, but its mechanism was poorly understood. Recent evidence has shown that epigenetic mechanisms such as histone modification and DNA methylation are essentially involved in the regulation of gene expression. In this study, we investigated whether epigenetic mechanisms including histone modifications and DNA methylation are involved in the rapid change of Cyp11a1 expression after LH surge and also investigated whether transcription factor is associated with the change.

Methods and Results: 21-day-old immature rats were injected with eCG (15 IU) followed by hCG (15 IU) injection 48 h later. The ovaries were removed and luteinized GCs were collected before hCG (0 h), and 4 h, 8 h, and 12 h after hCG injection. 1) In RT-PCR analysis, Cyp11a1 mRNA levels rapidly increased after hCG injection, reached the peak at 4 h, and remained high level until 12 h. 2) Histone modification status in the Cyp11a1 promoter region (-206 bp to -23 bp) was analyzed by chromatin immunoprecipitation (ChIP) assay. The level of trimethylation of histone-H3 lysine-4 (H3K4me3), which is an active chromatin marker, was increased and significantly higher at 12 h than 0 h. The level of trimethylation of histone-H3 lysine-9 (H3K9me3) and trimethylation of histone-H3 lysine-27 (H3K27me3), which is a suppressive chromatin marker, was decreased and significantly lower at 4 h and 12 h than 0 h. 3) DNA methylation status was analyzed from -1427 bp to -73 bp around Cyp11a1 promoter region by sodium bisulfite sequencing. 5 CpGs (-403 bp to -73 bp) were demethylated while the other 3 CpGs (-1427 bp to -1095 bp), which locate at relatively distal region, were methylated. This DNA methylation profile did not change during luteinization induced by hCG injection. 4) Chromatin accessibility assay showed that the chromatin condensation of the Cyp11a1 promoter region decreased after hCG injection, suggesting that the chromatin structure of the Cyp11a1 promoter becomes loose after hCG injection. 5) Since we previously found C/EBPb, which is transcription factor, is involved in the expression of other steroidogenesis-associated genes with accompanying histone modification, we examined the binding of C/EBPb to the promoter region of Cyp11a1. ChIP assay showed that the binding of C/EBPb was significantly higher at 12 h than 0 h.
Conclusion: Changes of histone modification status and chromatin structure in the Cyp11a1 promoter region in addition to DNA hypomethylation status of the promoter are closely associated with the rapid increase of Cyp11a1 mRNA expression in GCs undergoing luteinization during ovulation. In addition, it is suggested that the binding of C/EBPβ together with the epigenetic change is involved in the increase of Cyp11a1 mRNA expression.

300. Donor cell metabolism decides the production efficiency of transgenic pigs by somatic cell nuclear transfer.
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The incidence of metabolic syndrome is increasing globally, as the prevalence of obesity continues to rise. However, the basic mechanisms of metabolic syndrome are not completely known yet. Therefore, animal disease models are required for the study of metabolic syndrome. However, long transgenic (TG) efficiency has been an obstacle to the production of TG pigs. For successful animal cloning by somatic cell nuclear transfer (SCNT), not only the quality of oocytes or handling techniques but the quality of donor cell is also an important factor. A SCNT method in which somatic cells derived from TG pig are used as the nuclear donor (re-cloning method) is an effective technique for TG pig production. Although there have been various studies on the effects of donor cell quality cloning success rate, the selection criteria of donor cell is still deficient. Hence, we aimed to investigate the distinct characteristics of donor cells with different developmental competency. Two TG cell lines (Normal TG cell line for Line 1 and re-cloning TG cell line for Line 2) derived from the fetal cells of Yucatan pig were analyzed. In the evaluation of the development following SCNT, a formation rate of blastocyst derived from Line 2 was significantly higher compared to Line 1 (30.8% vs 20.0%). By contrast with the full-term pregnancy, following SCNT embryo transfer, the rates were rather the opposite (3.7% vs 13.6%). This interesting finding demonstrated that high in vitro developmental competence cannot always ensure high production efficiency of animal cloning. To investigate genetic factors affecting successful cloning, the differential expression genes (DEGs) were identified by the comparison of mRNA of two cell lines using next generation sequencing (NGS), and DNA methylation pattern was characterized by bisulfite sequencing. By combining the two information, several genes were identified that showed significant difference in their expression profile. Additionally, the oxygen consumption of the respective cell lines were measured to determine mitochondrial function by XF cell mito stress test. We confirmed that the results of oxygen consumption rate were consistent with efficiency of full-term development. The present results suggest that the success of animal cloning is critically dictated by the quality of donor cell, and some differential expression genes and oxygen consumption have can serve as a novel marker of donor cell which has high cloning efficiency.

301. Angelman syndrome imprinting center encodes a transcriptional promoter.
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Prader-Willi Syndrome (PWS) and Angelman Syndrome (AS) are two genetic imprinting disorders that arise from disruption of either paternal or maternal gene expression at 15q11-q13. Allele specific gene expression in this region is regulated by a bipartite imprinting center (IC) composed of the PWS-IC and AS-IC. The PWS-IC is a positive acting element that promotes the expression of genes on the paternal allele, while the AS-IC functions to epigenetically inactivate the PWS-IC in the oocyte, such that the PWS-IC does not function on the future maternal allele. Thus the AS-IC sets the imprint at the locus by establishing a maternal specific mark at the PWS-IC. The PWS-IC and AS-IC elements have been identified by shared deletion overlaps in PWS and AS individuals in which parental allele identity was not correctly assigned. The PWS-IC spans SNRPN exon 1 in both human and mouse. The human AS-IC is located 35 kb upstream of SNRPN but lacks sequence conservation to the mouse, hindering efforts to understand its molecular activities. Using BAC transgenesis approach, our lab recently demonstrated that several mouse oocyte specific promoters that drive transcripts across the PWS-IC are necessary for maternal allele identity and thus constitute the murine AS-IC activity. However analogous transcripts transiting the PWS-IC in human have not been previously identified. To explore the molecular function of the human AS-IC, we characterized SNRPN transcripts in both human and bovine oocytes. This study has identified several classes of transcripts that initiate within or nearby the human AS-IC, indicating that the human AS-IC is an oocyte specific promoter. A transcript with similar origin is also present at the conserved bovine AS-IC. The results strongly suggest that some cases of Angelman syndrome imprinting defects arise from the absence of these imprint-setting transcripts during epigenomic programming in the growing oocyte.
conformation. Acetylation of lysine 5 on histone 4 (H4K5ac) is associated with a relaxed chromatin structure and a state of active transcription. In contrast, di-methylation of lysine 9 on histone 3 (H3K9me2) is associated with areas of heterochromatin and a less permissive state of transcription. We hypothesized that increased maternal age or exposure to superovulation will alter the levels of these epigenetic modifications and modifiers and contribute to the decline in fertility associated with these events. To test this, fully grown oocytes (≥70µm) were retrieved from ovaries of aged (69-70 weeks) and young (10-13 weeks) CF1 mice and immunofluorescent staining was performed for DNAm, H4K5ac, H3K9me2, or MeCP2. Fluorescence was visualized by confocal microscopy. All image acquisition settings were maintained within the same within each trial. We used MetaMorph software to compare the fluorescence intensity of these epigenetic marks between age groups by analyzing the average pixel intensity of the germinal vesicle (GV) excluding the nucleolus. The number of oocytes within a group analyzed for each epigenetic modification was: H3K9me2= 62 young and 30 aged, H4K5ac= 42 and 9, MeCP2= 27 and 10, and DNAm=91 and 29. The number of oocytes within a group analyzed for each epigenetic modification was: H3K9me2= 6 young and 12 aged, H4K5ac= 4 and 3, MeCP2= 3 and 6, and DNAm=9 and 13. There was an age related decrease in average intensity level (units=pixel intensity value) for MeCP2 (mean ± SEM for young and aged, respectively; 8.51 ± 0.77 and 4.89 ± 1.48; P<0.05), H3K9me2 (20.48 ± 1.04 and 11.56 ± 1.69; P<0.001), and H4K5ac (23.77 ± 1.86 and 13.74 ± 4.63; P<0.05). No significant age related difference for global DNAm was observed between groups. These preliminary results suggest that maternal age affects the oocyte epigenome. Effects of superovulation of the abovementioned epigenetic marks are currently under investigation.

303. The gestational cytokine Leukemia Inhibitory Factor (LIF) and derivatives of para-aminobenzoic acid regulate the activation of pro-inflammatory pathways in murine macrophages.

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The immune system of the uterine endometrium is exceptional in its ability to protect the mucosa from a variety of pathogens while being supportive to a developing semi-allogeneic embryo. However, aberrant activation of inflammatory pathways in macrophages and uterine epithelial cells at the maternal-fetal interface, leading to the production of nitric oxide (NO), can affect trophoblast survival and function and potentially induce pregnancy complications such as early embryo loss in humans. Our data with human macrophages demonstrates that the gestational cytokine LIF and a new class of synthetic molecules derivative of para-aminobenzoic acid (AL-361 and AL-549) have the ability to modulate activation of the nuclear factor kappa B (NFκB) pathways and the transcription factor signal transducer and activator of transcription family Stat1, both key signaling pathways involved in the inducible nitric oxide synthase (iNOS) transcription and NO production. This study aims at investigating the effect of those molecules in a murine model. Murine macrophages were pre-treated with either AL-549 or recombinant LIF, and then activated by the pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ), alone or in combination with bacterial lipopolysaccharide (LPS). Those studies demonstrate that the molecule AL-549 diminish the level of activation of signaling pathways IFNγ/Stat1, TNFα/NFκB and LPS/NFκB and reduce the production of nitric oxide (NO). Preliminary acute toxicity studies show that AL-549 molecule has no effect on normal development and viability in mice. Moreover, no difference in hematocrit, weight of organs and mice total weight was detected after 3 weeks of treatment with the molecule AL-549 (8 µg per group). Meanwhile, preliminary data suggests that LIF also reduced NO production in peritoneal macrophages activated by LPS. These results indicate that the inflammatory signaling pathways associated with pregnancy complications or gynaecological diseases may be targeted effectively with natural and/or synthetic molecules. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada (NSERC), the Fonds Québécois de la Recherche sur la Nature et les Technologies (FQRNT), and the Réseau Québécois en Recherche sur la Nature et les Technologies (RQRNT), and the Réseau Québécois en Recherche sur la Nature et les Technologies (RQRNT).

304. The Mammalian Embryo as a Toxicogenomic Sentinel to Evaluate Water Contaminants.

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Drinking water is considered safe. Nevertheless, the disinfection processes such as chlorination produce by-products that could potentially affect humans on sanitary, reproductive and developmental terms. It is well known that the mammalian embryos cultured in vitro are very sensitive to water quality. It is also known that the first week of embryonic development is crucial considering that the substantial erasure of the parental DNA methylation patterns followed by the establishment of a new one that will drive tissues differentiation and may last for the entire life of the person. During these processes, the embryo becomes highly vulnerable to environmental disruptions. Thus, our goal is to develop a tool to analyse the toxicity of a variety of products as well as their genomics targets, beginning with water and using the pig embryo as a model for human metabolic and reprogramming processes. Chlorination by-products (CBP) have been measured for a whole year in Quebec City drinking water and the average concentration (1X) of 4 of the CBPs – bromodichloromethane (10 µg/L), chloropicrine (1,5 µg/L), dichloroacetonitrile (1,5 µg/L) and dichloroacetic acid (15 µg/L) – has been tested separately on embryos. The phenotype results show that both 1X and 10X CBPs concentrations produce no significant phenotypical effect on the blastocysts rates or quality. Using EmbryoGENE porcine transcriptomic and epigenetic microarrays, we have begun the analysis of the genomic signature of the CBPs. These platforms can measure simultaneously RNA and DNA in samples as small as 10 blastocysts with 40K genes (RNA) and 180K (DNA) analyzed in a automatic pipeline. Such data will then form a foundation to determine the genes and the signalisation networks specifically altered by the exposition to the molecule, thus ensuring a better understanding of the toxicity mechanisms. More broadly, we expect our method to become a powerful tool to determine the toxicity of a variety of molecules as well as the signalisation pathway altered by the exposition to them.

305. The Impact of GATA6 on Steroidogenic Enzymes in Endometriosis.

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Endometriosis is a chronic disease defined by the presence of estrogen-sensitive and progesterone-resistant endometrial stromal cells and glands growing outside of the uterus. The persistence of this ectopic endometrial tissue leads to infertility and chronic pelvic pain in up to 10% of reproductive aged women. As factors in the pathogenesis of the disease have not been completely elucidated, it has been challenging to develop new diagnostic methods as well as novel treatments. We suggest that endometriosis arises as a consequence of abnormal DNA methylation, making it an epigenetic disease. In profiling the DNA methylome of endometriosis, we uncovered an epigenetic switch governing the expression of GATA transcription factors in endometriosis, which express GATA6 at the expense of GATA2. Following this we examined the expression of GATA6 in healthy eutopic cells, which strikingly altered their gene expression patterns, often mirroring the phenotype of endometriotic cells. Notably, GATA6 increased the expression of steroidogenic factor 1 (NR5A1). In endometriotic cells the NR5A1 locus is methylated in a pattern similar to steroidogenic tissues, and is responsible for mediating the expression of genes important for estradiol synthesis; however, the mechanism by which its expression is activated is unknown. Because GATA6 and NR5A1 are known regulators of the steroidogenic gene pathways in healthy steroidogenic tissues, we hypothesize that the expression of GATA6 alters the cell fate and steroid sensitivity of endometrial stromal cells. To test this we first examined how exogenous GATA6 affected the methylation of the NR5A1 promoter in healthy eutopic endometrial stromal cells. We observed a loss of methylation at this locus in approximately 16% of our cells that were expressing GATA6. This was similar to the effect of treating cells with the DNA methyltransferase inhibitor decitabine. We next examined the most highly affected genes and pathways altered by GATA6 using RNAseq on cells transduced with wither GFP or GATA6. Pathway analysis revealed that many genes affecting tissue morphogenesis and differentiation potential were repressed following GATA6 expression. In contrast, pathways contributing to inflammatory response and steroid biosynthesis were significantly upregulated. This prompted us to ask if GATA6 was able to transform endometrial stromal cells into sterioigenically competent cells. To test this we compared how steroidogenic gene expression is influenced by GATA6 and NR5A1 each alone as well as in combination. Healthy endometrial stromal cells were again transduced with null virus, GATA6, NR5A1, or both GATA6 and NR5A1. Expression of steriodogenic genes critical to the development of endometriosis were examined. Both NR5A1 and GATA6 expression at the mRNA and protein level were increased when NR5A1 and GATA6 were infected together compared with infection by either individually. The mRNA and protein expression of cholesterol side chain cleavage enzyme (CYP11A1), 17α-hydroxylase (CYP17A1), and steroidogenic acute regulatory protein (STAR) were all also higher with the addition of both NR5A1 and GATA6. Expression of CYP19A1 at the mRNA level was higher with the combination of NR5A1 and GATA6 as well. These findings suggest that GATA6 works synergistically with NR5A1 to increase expression of steroidogenic enzymes critical in the development of endometriosis. It is our hope that by more thoroughly understanding the impact of GATA6 expression and the pathways it modulates, improved diagnostic and treatment strategies to aid women with endometriosis will be developed. Research supported by NIH R37HD038691-12S1 and a grant from the Friends of Prentice at Northwestern Memorial Hospital.

306. Sperm Histone-Mediated Epigenetic Inheritance in Mice.
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During mammalian spermiogenesis, most, but not all, histones are replaced by protamines and the DNA is highly condensed. The few histones that remain bound in the sperm genome are selectively located in distinct nuclear domains including gene coding regions. It has therefore been proposed that sperm histones could play an important role in regulating gene expression in the early embryo. Supporting evidence of this idea was recently provided by the demonstration of a link between histone association with genes in sperm and their subsequent expression in 2-cell embryos when poly(ADP-ribose) (PAR) metabolism was perturbed during spermatid development in mice. PAR metabolism is mediated by PARP and PARG enzymes with specific functions in epigenetic gene regulation, chromatin reorganization and the regulation of topoisomerase II beta activity. Inhibition of PARP activity, using either a genetic mouse model or pharmacological approach, led to deviation retention of histones in sperm. Consequently, genome-wide histone mapping in mature sperm showed that defined genes were affected by abnormal histone positioning due to PAR inhibition during spermiogenesis. Transcript frequencies of the affected genes were either elevated or reduced in 2-cell embryos generated with such sperm, depending on the individual genes affected by abnormal histone occupancy in sperm. These results provoke the hypothesis that the presence or absence of nucleosomes and the activating or silencing nature of their histone modifications provide a connection between their expression profile in early embryos and histone modification status in the sperm.

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Introduction. Certain prostaglandin-inhibiting analgesics are used in pregnancy and are generally regarded as harmless to both mother and child. However, prostaglandins up-regulate DNA methyltransferases (DNMTs) which mediate methylation in germ cells during gametogenesis. Increasingly, in utero exposures are recognised as moderators of long-term health in offspring, mediated via epigenetic change. Thus, it was hypothesised that maternal exposure to prostaglandin-inhibiting analgesics would inhibit the production of DNMTs in the fetal gonad, which may impact upon the development of the gametes. Methods. Pregnant Wistar rat dams were randomly allocated to three treatment groups from e15.5-e21.5 – indomethacin (0.8mg/kg/day), paracetamol (350mg/kg/day) or low dose paracetamol (60mg/kg/day) (n=3-7 from a minimum of two litters). DNMT3a or DNMT3b expression in fetal germ cells was investigated using fluorescence immunohistochemistry followed by semi-quantitative analysis of intensity of immunostaining. Statistical comparisons were carried out using two-way ANOVA and Sidak’s multiple comparison tests. Results. Reductions in DNMT3a and DNMT3b immunostaining were seen in indomethacin- and paracetamol-exposed fetuses in the rat fetal gonad. DNMT3a immunostaining was particularly reduced in fetal ovaries. Immunonegative germ cells were significantly increased following maternal indomethacin or paracetamol exposure (p<0.0001 and p< 0.001, respectively, versus controls). Indomethacin dosing was within a therapeutic range, but initial paracetamol dosing was much higher than in humans. Following maternal exposure to therapeutic paracetamol dosing, DNMT3a immunostaining was not significantly reduced in fetal ovaries, but mRNA analysis showed a reduction in DNMT3a. Conclusion. Maternal
exposure to prostaglandin-inhibiting analgesics results in a reduction in DNMT3a and DNMT3b immunostaining in rat fetal gonads. This could result in aberrant germ cell methylation, which may impact the long-term health of the developing gametes, with the potential for intergenerational inheritance of epigenetic defects. This study provides a basis for determining if similar effects are observed in the human fetal gonad. This research was funded by the Medical Research Council (MRC), United Kingdom.

308. The methyltransferase Setdb1 controls meiosis and mitosis in mouse oocytes and early embryos.

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Oocytes acquire meiotic and embryonic developmental competence during oocyte growth and maturation. Setdb1 is a histone H3 lysine 9 methyltransferase implicated in silencing of genes and endogenous retroviral elements in embryonic stem cells, during somatic differentiation and in disease. Setdb1 is required for early embryonic development since loss of zygotic expression results in lethality shortly after implantation. Setdb1 is also expressed during oogenesis and is maternally provided to the early embryo up to the blastocyst stage at which paternal expression starts. To address the function of Setdb1 for oogenesis and early embryogenesis, we conditionally deleted Setdb1 in growing oocytes. We observe that Setdb1 is required for meiotic resumption and chromosome segregation. In mutant oocytes, chromatin condensation dynamics, kinetochore-spindle interactions and bipolar spindle organization are disturbed. These phenotypes relate to specific changes in gene expression in mutant oocytes. Setdb1 maternally deficient embryos arrest early during pre-implantation development and show comparable defects during cell cycle progression and in chromosome segregation. Our study reveals a novel key regulatory function for the lysine methyltransferase Setdb1 for meiosis and mitosis, thereby safeguarding genome integrity at the onset of life.

309. Histone deacetylases (HDACs) regulate histone H4 lysine 12 acetylation and histone H3 serine 10 phosphorylation during mouse oocyte meiosis.

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In mammalian oocytes, most studies of histone modifications have been focused on meiotic stage-specific changes of individual modification, whereas little is known about mutual regulations and molecular pathways among distinct histone modifications. Recently, growing evidence shows that cross-regulations of different histone modifications are important for both mitosis and meiosis, but related mechanisms remain largely unknown. Here we present the interactions between histone acetylation and histone phosphorylation in mouse oocytes, both of which have been found to be crucial for oocyte maturation and embryo development. GV and MII oocytes were treated with broad-spectrum HDAC inhibitors and class-specific HDAC inhibitors, and the corresponding changes of histone acetylation and phosphorylation were then assessed to confirm the role of histone acetylation in regulation of histone phosphorylation and determine the class of HDACs responsible for histone phosphorylation. Besides, related pathway of histone phosphorylation was explored by detection of the changes of mRNAs and proteins in histone phosphorylation-related kinases. After that, the pathway was further confirmed by inhibition of individual HDAC using small interfering RNA (siRNA). The effects of HDACs on oocyte maturation and embryo development were also evaluated thereafter. Results indicate that histone phosphorylation is regulated by acetylation during meiotic resumption of oocytes in mice, and Class I HDACs have a function in regulation of histone H4 lysine 12 (H4K12) acetylation and histone H3 serine 10 (H3S10) phosphorylation in GV oocytes. Furthermore, HDAC1/3 can accelerate H3S10 phosphorylation by increase of expression of Aurora kinase C. In MII oocytes, however, class II HDACs play the main role and Ia class HDAC accelerate H3S10 phosphorylation by increase of autophosphorylation of Aurora kinase family (Aurora A/B/C). Although oocytes maturation and spindle morphology were not affected after inhibition of class I or class II HDACs during oocyte maturation, the developmental capacities of parthenogenetic and in vitro fertilized embryos were indeed impaired after inhibition of HDAC1/3 during embryo activation. Taken together, the present results show that histone phosphorylation is regulated by acetylation via Aurora kinases pathway during meiotic resumption in oocytes and these would shed light on the molecular mechanisms of cross-regulations among different histone modifications. This research was supported by National Natural Science Foundation of China [31101715] and Jiangsu Agriculture Science and Technology Innovation Fund [CX(12)S012].

310. The histone H3K9 methyltransferase Suv39h2 participates in the maintenance of the trophoblast stem cell state.

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Hemochorial placentation involves lineage specific development of trophoblast cell types, which orchestrate the efficient redirection of blood flow to the placenta and delivery of nutrients to the fetus. As pregnancy progresses, specialized trophoblast cell lineages arise through the precise expansion and differentiation of trophoblast stem (TS) cells. Failure of proper trophoblast lineage development is associated with pathological conditions such as preeclampsia, intrauterine growth restriction, and preterm birth. Thus we can gain insights into these disorders through elucidating regulatory mechanisms controlling trophoblast cell fate decisions. Epigenetic mechanisms, including histone modifications, have been demonstrated to possess key roles in controlling cell differentiation but little is known about their involvement in trophoblast development. In this investigation, we examined the contributions of a histone H3K9 methyltransferase, Suv39h2, in the regulation of TS cell differentiation. Suv39h2 catalyzes the dimethylation and trimethylation of histone H3K9, histone marks generally associated with transcriptional repression. Among the H3K9 methyltransferases, we targeted Suv39h2 because it exhibited a robust difference in its expression in stem versus differentiation states unlike other histone H3K9 methyltransferases (Suv39h1, Setdb1, Ehmt2), which show modest expression differences during differentiation. Suv39h2 transcript and protein expression is significantly elevated in the TS cell stem state and declines as TS cells differentiate. Disruption of Suv39h2 expression had striking effects on TS cells. Suv39h2-specific shRNA delivery inhibited TS cell proliferation and promoted trophoblast cell differentiation. Initially, using a candidate approach we observed a significant upregulation of transcripts for markers of differentiation (Adm, Hand1, Prl3b1) in the Suv39h2-shRNA expressing TS cells. These results suggested that Suv39h2 participates in the maintenance of the TS cell stem state and
311. Transgenerational Effects of a Maternal High-fat Diet on Offspring Hepatic Steatosis.
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Background: It is well established that alterations in the early life environment result in an increased risk for obesity and metabolic disorders in offspring. We have also shown that postnatal exposure of offspring to an obesogenic diet following maternal obesity exacerbates these outcomes, potentially contributing to transgenerational effects. In the present study, we sought to determine the impact of a maternal high fat (HF) diet on offspring risk of hepatic nonalcoholic fatty liver disease (NAFLD) over 3 generations. We hypothesized that maternal HF intake would impair offspring hepatic beta-oxidation and promote de novo lipogenesis, through increased hepatic endoplasmic reticulum (ER) stress, and that these effects would persist in subsequent generations. Methods: Pregnant Wistar rats (F0) received either a HF diet (45% kcal as fat, n=8) or a control diet (5% kcal as fat, n=8) throughout pregnancy and lactation. F1 offspring were randomized to receive a HF or control diet from weaning. Two further generations of rats (F2 and F3) were fed the control diet. Hepatic tissue was collected at postnatal day 120, and analyzed either histologically for steatosis or for changes in gene and protein expression levels in key signaling pathways. The severity of steatosis was evaluated by grading histological sections based on visualization of lipid vacuoles: <5% = 0, 5-33% = 1, >33-66% = 2, >66% = 3. Results: Maternal HF diet, in combination with post-weaning HF diet, was associated with increased hepatic lipid deposition in adult offspring, which persisted into F2 and F3 offspring. Beta-oxidation enzyme Cpt1a mRNA levels were decreased in F1 males, but not females, born to HF-fed mothers (p<0.001), as well as those fed a post-weaning high fat diet (p=0.001). These changes were not associated with differences in the phosphorylation of key enzymes regulating lipogenesis including ACC or AMPK. In F1 females, but not males, levels of a lipogenic transcription factor Pparγ2 mRNA were decreased in HF exposed offspring (p<0.031), with a modest reduction due to post-weaning HF intake (p=0.087). In F1 females only, we observed increased mRNA levels of ER stress related factor EIF2AK3 due to postnatal HF intake (p<0.001), and a significant interaction between maternal and postnatal diets (p=0.001). However, this effect did not persist in F2 or F3. Conclusions: Our results indicate that early life exposure to maternal HF affects a sex specific response - with the level of post-weaning nutrition on hepatic lipid deposition in adult offspring. These changes appear to be a result of decreased hepatic beta-oxidation, but not de novo lipogenesis, and may be mediated by ER stress.

312. Specific deletion of LKB1/Stk11 in the Müllerian duct mesenchyme drives hyperplasia of the prostatic stroma and tumorigenesis.
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Benign prostatic hyperplasia (BPH) is a highly prevalent non-malignant overgrowth of the peri-urethral prostate stroma in older men with unknown etiology. To assess the role of these tumor suppressor genes in male reproductive tracts, we generated conditional deletion of Pten/Lkb1CreER mice by using the Amhr2-Cre to drive expression of Cre recombinase. We show that Müllerian duct mesenchyme (MDM) cells contribute to the postnatal stromal cells at base of the seminal vesicles, the ampullary glands on the dorsocranial aspect of the prostatic urethra, and the peri-urethral and ductal space by lineage tracing with Amhr2-Cre-driven β-galactosidase activity. The Pten/Lkb1CreER mice develop prostatic hyperplasia with bladder obstruction with nearly 100% penetrance within a few months postnatally, always due to stromal expansion and must be euthanized. The stromal areas from prostates of Pten/Lkb1CreER mice, were estrogen receptor (ESR1) positive, which is consistent with both Müllerian duct mesenchyme-derived cells and its purported importance in BPH development and/or progression in humans. Promoter CpG hypermethylation and transcriptional inactivation of tumor suppressor genes have been found in a variety of human malignancies. To determine whether stromal hyperplasia in Pten/Lkb1CreER mice could be a model for human BPH, we decided to investigate the frequency of epigenetic inactivation of STK11 in prostate samples of BPH patients. Analysis of the UCSC genome browser revealed a number of CpG islands present both upstream and downstream of the STK11 promoter. Based on sequence conservation and its location, bisulfite-sequencing analysis was done on CpG island “2” using a cohort of five patient samples. Preliminary bisulfite analysis displays marginally higher methylation levels compared to control, indicating that hypermethylation of CpG island “2” may not be a significant mechanism for STK11 silencing. Currently, we are investigating additional CpG islands in a larger number of patient samples.

313. Epigenetic reprogramming during spermatogenesis.
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Studies suggest that the paternal epigenetic contribution in sperm may serve as a route to altered offspring phenotypes. We recently determined that paternal folate deficiency (FD), in mice, is associated with increased birth defects in offspring. Suggesting a potential epigenetic association, in sperm from FD sires there was altered DNA methylation at genes implicated in development and disease. Spermatogenesis is a highly complex cell differentiation process fueled by spermatogonial stem cells (SSC). The progression from

2015 Abstracts – Page 125
SSC to spermatozoa is characterized by an intense epigenetic reprogramming leading to important changes in gene expression. However, nothing is known about the sensitivity of the SSC to environmental exposures, or if epimutations imposed to these cells can escape reprogramming and persist through meiosis and spermiogenesis. The objective is to determine if an epigenetic memory is retained at specific gene regulatory regions from the SSC to the spermatozoa. To address this question we isolated an SSC-enriched germ cell population and compared its epigenome with that of mature spermatozoa. Using Oct4-GFP mice, we were able to isolate about 100 000 germ cells per animal at post-natal day 6. RNA-sequencing experiments confirmed that these cells were free of any somatic contamination, and expressed high levels of the recently identified SSC marker Id4 and undifferentiated spermatagonia markers such as Puf5, Lin28a, Sal1a, Oct4, and Gfra1. Using ChIP-sequencing, we are comparing the epigenome of these SSC-enriched population to that of sperm with a focus on histone H3 trimethylation at lysine 4 (H3K4me3) and lysine 27 (H3K27me3). These histone methyl marks, in sperm, localize to genes involved in embryonic development. Furthermore, SSC-enriched cell isolates express high levels of several epigenetic modifiers and in particular Kdm2b/Mll2, an H3K4me3 methyltransferase and Kdm6b/Mbd3, an H3K27me3 demethylase, suggesting an important role of these two marks in SSC biology.

This study will provide important new information on the level of epigenetic reprogramming that occurs during spermiogenesis and has important clinical ramifications, as it will reveal how much of the epigenome is determined in the testis stem cell population and how much it can affect the health of the next generation.

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314. Over-expression of KDM1A in spermatogenesis alters the sperm epigenome and has dire consequences for development of the embryo.
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Parental effects can influence health and development of generations to come through a process called epigenetic inheritance. The mechanisms underlying epigenetic inheritance are largely unknown but may involve transmission via the sperm epigenome. Recent studies have shown that sperm histones are retained at genomic regions high in CpG, and activating histone marks such as histone H3 lysine 4 dimethylation (H3K4me2) are retained at gene promoters implicated in embryonic development. Thus, we hypothesized that the epigenetic marks on sperm histones play a major role in the development of offspring, and serve to influence the inheritance of non-genetic information in future generations. To test this hypothesis we designed a transgenic inbred mouse model with a disturbed sperm-epigenome induced by over-expression of the human histone demethylase KDM1A in the testes. Offspring sired by transgenic males have a range of gross abnormalities and increased frequency of death. Importantly, the offspring sired by the wild type littermates (nonTG) also exhibited the same phenotype, which persisted for three generations. To elucidate a potential mechanism for epigenetic inheritance we employed genomic and epigenomic approaches to profile histone marks, DNA methylation and the RNA population in sperm from TG and nonTG males. We also analyzed gene expression two-cell embryos to associate changes in gene expression with a altered sperm epigenome. Our data reveals a possible complex collaboration between sperm chromatin and RNA to regulate not only offspring health, but a persistent epigenetic memory.
of DB and WT did not differ in serum insulin levels, but on a high fat diet, offspring of DB had lower insulin. In the females, offspring of DB had lower insulin than offspring of WT on a standard diet, but this disappeared on a high fat diet. At 30 weeks, glucose tolerance was not significantly different across treatment groups, but in offspring of DB dams, glucose tolerance was numerically improved compared to offspring of WT (p= 0.06, n=23WT, 19DB). Preliminary results show that pancreatic beta cell count does not differ across treatment groups. Leptin mRNA was reduced in visceral adipose tissue of offspring from DB dams, compared to that in offspring of WT (p= 0.03, n=7WT, 4DB), despite similar body composition, suggesting greater leptin sensitivity in offspring exposed to hyperleptinemia in utero. These results show that maternal hyperleptinemia alone does not cause metabolic disease in offspring, and may be protective of insulin and leptin sensitivity. Supported by American Diabetes Association 1-14-BS-181.

316. Effect of progesterone on beating rate of differentiation of mouse embryonic stem cells into cardiomyocytes.
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Introduction: Mouse embryonic stem cells (mESCs) have the ability to form aggregates, called mouse embryonic bodies (mEBs), which are required for early development of mESCs. These mEBs differentiated spontaneously into cardiomyocytes. Sex steroid hormones work in the early developing embryo of mice. We researched the influence of progesterone (P₄) among the sex steroid hormones during early differentiation of mESCs. Methods: mESCs were performed hanging-drops for four days, which were then suspended in differentiation medium without LIF for three additional days. Seven-day-old mESCs-derived mEBs were attached onto 6-well culture plates and differentiated into cardiomyocytes. Differentiated mESCs were harvested every four days for 20 days. We analyzed expression of cardiomyogenesis-related genes, and sex steroid hormone receptors, and observed beating-rate during differentiation of mEBs. We replaced FBS with charcoal-dextran treated FBS (CD-FBS) to investigate the effects of sex steroid hormones during differentiation of mESCs. mEBs were treated with P₄ or mifepristone (progesterone receptor antagonist). Results: The highest beating-rate (92.64%) of mESCs (E14) was reached at differentiation 6d. We observed time-dependent increased expression in mRNA levels of various cardiac markers, including Tbx20, Isl1, Foxh1, cTnt1, and Ryr2. In addition, we identified expression of cardiac markers, including alpha-actinin, troponin I, and atrial natriuretic peptide (ANP) via immunocytochemistry method. Thus, our mESCs (E14) were differentiated into cardiomyocytes. To examine effects of sex steroid hormone, we measured mRNA expression of steroid hormone receptors. Expression in mRNA level of ERα, ERβ, and AR showed a time-dependent increase. However, expression of mRNA of PR showed an opposite pattern of beating-rate during differentiation. In CD-FBS treated mEBs, the highest beating-rate (95.08%) was reached at differentiation 12d. CD-FBS treated mEBs was remarkably delayed compared with mEBs cultured in CD-untreated FBS, meaning that steroid hormones have an influence on differentiation of cardiomyocytes. Beating rate (67.56%) of P₄ treated mEBs was more decreased. Also, mRNA expression of PR of P₄ -treated mEBs increased more than P₄-untreated mEBs. Conclusions: In our study, we confirmed that sex steroid hormones affected the differentiation of mESCs into cardiomyocytes. The expression profile of PR gene suggests that progesterone might repress cardiac beating, and other sex steroid hormones might have an effect on increase of beating-rate. This study was supported by a grant (15182K2FDA460) from the Korea Food and Drug Administration.

317. Abnormal Development of Distal Paramesonephric Ducts and Mesonephric Ducts in Lhfpl2-mutant Mice.
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Defective development of distal reproductive tracts could lead to congenital abnormalities in humans, such as vaginal imperforation in females and distal vas deferens blockage in males. These abnormalities count for 1% female infertility and 1-5% male infertility in humans. Studies using genetic and tissue recombination approaches have demonstrated the origins of vagina and vas deferens from paramesonephric ducts (Müllerian duct) and mesonephric ducts (Wolffian duct), respectively. Despite these significant progresses, the mechanisms involved in distal reproductive tract development are still largely unknown. A spontaneous point mutation in exon 2 of mouse lipoma HMGIC fusion partner-like 2 (Lhfpl2) gene leads to distal vaginal blockage and infertility in 100% females. The mutant females have normal ovarian development and ovulation, normal oviductal and uterine development, and normal uterine responses to exogenous estrogen stimulation. Histology of female reproductive tracts from early postnatal days to adult reveals double lumens in the upper vagina but no lumen in the position of lower vagina. The reproductive tracts of the adult mutant females are bloated with liquid due to vaginal closure, which is the cause for infertility. Interestingly, infertility is also observed in ~70% mutant males, which have normal mating behavior and sperm counts, while the rest ~30% mutant males have normal fertility. The absence of sperm in the reproductive tracts of wild type (WT) females positively mated with infertile Lhfpl2 mutant males indicates blockage of male reproductive tracts. Indeed, distal vas deferens atresia is observed in these infertile males. Blue dye injection via vas deferens also shows distal vas deferens abnormalities in the fertile mutant males. Therefore, this Lhfpl2 point mutation leads to defective development of distal reproductive tracts in both female and male mice. LHFPL2 belongs to LHFP family and encodes a tetra-transmembrane protein with unknown functions. In situ hybridization and immunohistochemistry reveal its specific expression in the epithelia of vagina and vas deferens in postnatal WT mice. The spatiotemporal expression of Lhfpl2 in the developing paramesonephric ducts and mesonephric ducts is being investigated. Whole-mount immunofluorescence of PAX2 (paired box 2, a marker of developing reproductive tracts) and histology reveals that the developing ducts fail to merge with the urogenital sinus in the embryonic day 15.5 mutant embryos. The above data demonstrate that LHFPL2 plays an essential role in the merging process of developing paramesonephric and mesonephric ducts with urogenital sinus. The functions of LHFPL2 as well as the underlying cellular and molecular mechanisms involved in distal reproductive tract development will be investigated. These studies will give insight into the related congenital abnormalities in humans. (Supported by NIH R15HD066301 & R01HD065939)

318. Timing of colostrum consumption and duration of nursing from birth affect neonatal porcine uterine and cervical MMP9 and TIMP1 expression.

2015 Abstracts - Page 127
In the neonatal porcine uterus, research indicates effects of age at first nursing and duration of nursing on uterine expression of morphoregulatory proteins, including matrix metalloproteinase (MMP) 9, at postnatal day (PND) 2. In these studies uterine MMP9 was reduced in gilts when nursing was delayed by feeding milk replacer for up to 12 h from birth (PND 0) or when nursing duration was restricted. MMPs are a family of proteolytic enzymes that facilitate growth, development and connective tissue remodeling of the female reproductive tract and other tissues. MMP activity is tightly regulated by specific tissue inhibitors of metalloproteinases (TIMPs) which complex with active and latent MMPs to control their proteolytic activity. Whether MMP9 expression is affected by the timing or duration of nursing in female reproductive tract tissues other than the uterus is unknown. Further, whether lactocrine-associated changes in neonatal uterine MMP9 expression are accompanied by changes in expression of its inhibitor, TIMP1, or MMP9 activity is undetermined. Here, objectives were to determine effects of: (1) age at first nursing from birth (0 h, 30 min, 12 h); and (2) nursing duration (30 min, 12 h, 48 h) on cervical MMP9 and uterine TIMP1 proteins at PND 2. Treatment effects of timing and duration of nursing on uterine MMP9 activity were also studied. Gilts (n=4-6/group) were assigned at birth to one of six treatment groups: (1) nursed for 30 min from birth and gavage-fed milk replacer for 47.5 h; (2) nursed for 12 h and gavage-fed milk replacer for 36 h; (3) nursed ad libitum for 48 h; (4) gavage-fed milk replacer for 30 min from birth and nursed for 47.5 h; (5) gavage-fed milk replacer for 12 h from birth and nursed for 36 h; or (6) gavage-fed milk replacer for 48 h. Uteri and cervices were collected at PND 2 (50 h). MMP9 and TIMP1 proteins in tissues were evaluated by immunoblotting, using actin as a loading control, and quantified by densitometry. Zymography was used to detect uterine MMP9 activity. Immunoreactive bands at 84 kDa for MMP9 and 92 kDa for proMMP9 were consistently detected in uterine and cervical tissues on PND 2 when nursing was initiated at 0 h or 30 min of age. Likewise, in the uterus, TIMP1 (26 kDa) was expressed at these times. However, as observed for gilts fed replacer for 48 h, these targeted proteins were undetectable in tissues on PND 2 when gilts were nursed starting at 12 h after birth. Increasing the duration of nursing from 30 min to 12 h increased uterine (p < 0.05) and cervical (p < 0.05) MMP9 protein levels to those observed in gilts nursed for 48 h. Similarly, uterine TIMP1 expression increased (p<0.05) with duration of nursing. Uterine MMP9 activity, detected by zymography, paralleled immunoblotting data. This confirmed that uterine MMP9 proteins were enzymatically active. These studies illustrate the importance of age at first nursing and duration of nursing from birth on MMP9 and TIMP1 expression in the neonatal porcine female reproductive tract. Results also support and extend previous studies by confirming that the window for lactocrine signaling in both the uterus and cervix is open within 12 h of birth. [Support: USDA-NIFA 2013-67016-20523; NSF-EPS-158862].

319. Localization of Candidate Proteasomal Interactors ADAM5 and NEDL2 in Porcine Spermatids and Spermatozoa.
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It is well established that the ubiquitin proteasome system (UPS) has a role in the acquisition of the spermatoozoa’s ability to fertilize oocytes, a propriety conveyed by the process of sperm capacitation within the female reproductive system. The UPS controls the turnover and activity of sperm head and tail proteins by protein ubiquitination. Catalyzed by the ubiquitin activating enzyme E1/UBA1, E2-type ubiquitin-conjugating enzymes and E3-type ubiquitin ligases, ubiquitination makes substrate proteins recognizable to the 26S proteasome, a multi-subunit ubiquitin-specific proteolytic holoenzyme highly enriched in the mammalian sperm acrosome, but present in all parts of sperm head and flagellum. Here, we investigate two candidate proteosome-interacting sperms proteins, identified by our and others’ published studies as possibly involved in sperm capacitation and/or acrosomal function during fertilization. The WW-domain containing NEDD4-like ubiquitin ligase 2 (NEDL2) catalyzes covalent ligation of ubiquitin to internal Lys-residues of substrate proteins and may be involved both in gametogenesis and fertilization through its interaction with WW-domain binding sperm/oocyte proteins such as PAWP. A-disintegrin-and-metalloproteinase domain proteins ADAM 1a, 2, and 3 genes result in considerably decreased fertility. Our recent co-purification study found that sperm proteasomes co-purified with ADAM5, which plays a role in one of the aforementioned ADAM complexes. The objectives of this ongoing study are to confirm the presence of ADAM5, NEDL2 and several other candidate proteasomal substrates and interactors in boar spermatozoa, and to determine the nature of their interaction with sperm proteasomes. We detected both ADAM5 and NEDL2 proteins by Western blotting and localized them in boar spermatids and spermatozoa by immunofluorescence. Western blot analysis confirmed the presence of ADAM5 in porcine testes and spermatozoa, and immunofluorescence microscopy localized it mainly in the nucleus of round and elongating spermatids, and in the post-acrosomal sheath of fully differentiated spermatozoa. The NEDL2 protein was localized to the pro-acrosomic granule of round spermatids, caudal manchette of elongated spermatids. Mature spermatozoa showed NEDL2 in the sperm head-acrosomal equatorial segment and post-acrosomal sheath (only ~10% spermatozoa), as well as in the connecting piece/centriolar region and, to a lesser extent, whole flagellum of fully differentiated spermatozoa. All of the above NEDL2 also showed the presence of ubiquitinated proteins detected by polyclonal anti-ubiquitin antibodies. Further research is being pursued to identify additional proteasome-interacting sperm proteins, to characterize the developmental dynamics of proteasomal subunit localization and activity during sperm accessory structure biogenesis on isolated and in situ spermatids, and to assess changes in localization and activity of the above and other proteasome interactors during sperm capacitation altered by pharmacological interventions with UPS. This research was supported by grant R2011-67015-20025 from the USDA-NIFA and by MU F21C Program funding to P.S.

320. Metabolomics reveals specific metabolic adaptations in mid and late gestation sheep fetuses in response to maternal overnutrition/obesity during pregnancy.
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Maternal Obesity (MO) during gestation has marked effects on fetal growth and development including programming of increased offspring risk of adult metabolic disease. We have shown that MO throughout gestation in sheep increases fetal blood glucose and insulin, and fetal adiposity at 0.5 gestation (G) and 0.9G. F1 and F2 lambs born to MO mothers or grandmothers are predisposed to similar metabolic alterations as their mothers, suggesting a multigenerational programming effect (International Journal of Obesity 2014:1–7). We utilized targeted electrospray ionization liquid chromatography-mass spectrometry-based metabolomics (West Coast Metabolomics Center, Davis, CA) to compare the effects of maternal nutrition in gestation on fetal plasma metabolite (carbohydrates, lipids, amino acids and nucleic acids) profile at mid (n=6) and late gestation (n=5) in our well characterized MO sheep model. Sixty days prior to conception ewes were assigned to control [CON, 100% of National Research Council (NRC) recommendations], or obese [MO, 150% NRC] diets until necropsy at 0.5G and 0.9G. Immediately before necropsy, ewes were weighed, and a 10 ml blood sample was collected via jugular venipuncture into a chilled heparinized tube and plasma frozen at -80°C. Analysis of 150 metabolites was done utilizing the univariate and multivariate methods of the web-based metabolomic data processing software MetaboAnalyst 2.0. Body weights of CON, and MO ewes were similar at the start of the experimental diets. By 0.5 and 0.9G, weights of MO ewes were markedly greater (P < 0.01) than CON. At 0.5G, 24 plasma metabolites differed (P < 0.05) between fetuses of MO and control dams. Partial least squares discriminant analysis (PLS-DA) indicated that during mid gestation, MO resulted in a greater (P < 0.01) accumulation of carbohydrate and lipid metabolites in fetal plasma compared to amino acid and nucleosides. Further, greater concentrations of metabolites of carbohydrates and lipids were observed in MO fetuses compared to CON fetuses (64.5 ± 3.2 vs. 35.4 ± 3.6 %, respectively). Concentrations of amino acid and nucleic acid metabolites did not differ between CON and MO groups. At 0.9G, 17 metabolites differed (P < 0.05) in fetal plasma from MO versus CON dams. PLS-DA indicated that during late gestation, MO resulted in greater (P < 0.01) accumulation of amino acid metabolites compared to carbohydrate, lipid metabolites and nucleosides in fetal plasma. Further, higher concentrations of amino acid metabolites were observed in MO vs. CON fetuses in late gestation (60.5 ± 5.2 vs. 40.2 ± 4.7 %, respectively). The other 3 metabolite classes remained similar. The pattern observed in the fetal plasma metabolome was consistent with that observed in the maternal plasma, and suggest a direct link between maternal and fetal metabolic changes in response to maternal obesity. These data are consistent with the concept that MO/overnutrition in pregnancy produces in utero metabolic adaptations with potential long-term effects on the risk of adult-onset diseases such as obesity and metabolic syndrome. NIH INBRE 1P20RR16474 and NIH HD070096-01A1.

321. Delineating the Functional Significance of mTOR Signaling in Human Leiomyoma Cells.
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Uterine fibroids, the most common benign tumors of the female reproductive tract, are typically treated by suppression of endogenous ovarian sex steroids and/or surgery. Although several alternative pharmacological approaches have been proposed recently, efficacy has been limited due to an incomplete understanding of the cell signaling pathways involved in the pathology of uterine fibroids. A role for atypical activation of the mammalian target of rapamycin (mTOR) pathway in the pathogenesis of leiomyoma has been proposed. Since there is evidence that Rapalogs targeting Torc1 signaling may reduce proliferation and/or viability of leiomyoma cells, we hypothesized a dual inhibition of both Torc1 and Torc2 signaling may demonstrate a more significant contribution of this complex signaling pathway to the pathology of leiomyoma cells. To this end, the objective of this study was to compare the in vitro impact of a Torc1 inhibitor (MK-8669) and dual Torc1/Torc2 inhibitor (INK128) on immortalized human leiomyoma and patient matched myometrial cells. Leiomyoma and myometrial cells were treated with either vehicle, MK-8669 (0, 62.5, 1.25, 2.5 and 5 nM) or INK128 (0, 1, 5, 10, 20 and 50 nM) and a MTT assay was performed as a surrogate for cell viability at 48 hrs post treatment. All experiments were done in triplicate. To assess on target effects, immunoblotting for phosphorylated S6 was performed at 6, 12 and 24 hrs following treatment with vehicle, MK8669 (5 nM) or INK128 (5nM). Significant reduction (p < 0.05) in viability was observed following INK128 treatment at all concentrations (1, 5, 10, 20 and 50 nM) compared to control. A significant reduction (p < 0.05) in viability was only observed at the higher concentrations of MK8669 (2.5 and 5 nM) compared to vehicle control. The decrease in viability observed in myometrial cells was tempered when compared to the response observed in the leiomyoma cells. Downstream target effects were observed in response to both MK-8669 and INK-128 as evidenced by a significant reduction in phosphorylated S6. In summary, treatment with a Torc1 specific inhibitor and dual Torc1/Torc2 inhibitor induced a decrease in phosphorylated S6 yet only the dual inhibitor could induce meaningful changes in cell viability. Indirectly these data suggest that both Torc1 and Torc2 signaling may contribute to the pathobiology of leiomyoma cells.

322. Impact of intrauterine growth restriction on long-term fertility in male pig model.
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Recent studies have shown that reproductive capacity of offspring is programmed by fetal development. We have shown that intrauterine growth restriction (IUGR) neonates have a reduced growth performance and a weakened antioxidant defense system. Based on these findings, we hypothesized that IUGR would alter the adult male reproductive function in the domestic pig, an excellent biomedical model for studying human development and fertility. We used 7 pairs of male piglets including 7 normal birth weight (NBW) piglets (1.57 kg) and 7 IUGR piglets (1.14 kg) on 0.9G. Analysis of 150 metabolites was done utilizing the univariate and multivariate methods of the web-based metabolomic data processing software MetaboAnalyst 2.0. Body weights of CON, and MO ewes were similar at the start of the experimental diets. By 0.5 and 0.9G, weights of MO ewes were markedly greater (P < 0.01) than CON. At 0.5G, 24 plasma metabolites differed (P < 0.05) between fetuses of MO and control dams. Partial least squares discriminant analysis (PLS-DA) indicated that during mid gestation, MO resulted in a greater (P < 0.01) accumulation of carbohydrate and lipid metabolites in fetal plasma compared to amino acid and nucleosides. Further, greater concentrations of metabolites of carbohydrates and lipids were observed in MO fetuses compared to CON fetuses (64.5 ± 3.2 vs. 35.4 ± 3.6 %, respectively). Concentrations of amino acid and nucleic acid metabolites did not differ between CON and MO groups. At 0.9G, 17 metabolites differed (P < 0.05) in fetal plasma from MO versus CON dams. PLS-DA indicated that during late gestation, MO resulted in greater (P < 0.01) accumulation of amino acid metabolites compared to carbohydrate, lipid metabolites and nucleosides in fetal plasma. Further, higher concentrations of amino acid metabolites were observed in MO vs. CON fetuses in late gestation (60.5 ± 5.2 vs. 40.2 ± 4.7 %, respectively). The other 3 metabolite classes remained similar. The pattern observed in the fetal plasma metabolome was consistent with that observed in the maternal plasma, and suggest a direct link between maternal and fetal metabolic changes in response to maternal obesity. These data are consistent with the concept that MO/overnutrition in pregnancy produces in utero metabolic adaptations with potential long-term effects on the risk of adult-onset diseases such as obesity and metabolic syndrome. NIH INBRE 1P20RR16474 and NIH HD070096-01A1.
expression of DNA methyltransferase 1 (Dnmt1), Dnmt3a, SUV39h2 and lysine (K)-specific demethylase 4A (Kdm4a, previously known as Jumonji Domain Containing 2A) genes were up-regulated in IUGR boars' testis. Such a transcriptional de-regulation could alter downstream-regulated gene expressions, leading to a decline in sperm production. These observations provide evidence that male offspring reproduction could be programmed by growth restriction during prenatal development, hinting at possible mechanism involved in infertility in human and animals. This research was supported Sichuan Agricultural University Innovation Fund. PS was supported by grant #2011-67015-20025 from the USDA-NIFA and by MU F21C Program funding to P.S.

323. The Effects Of A High Fat Diet On The Morphology Of The Small Intestine In The Ossabaw Mini-Pig Model.
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Obesity, metabolic syndrome, and type 2 diabetes are serious health challenges facing the United States and other western countries. Recently, there has been increased interest in the possible role of the gut microbiome and entero-endocrine system in the exacerbation of obesity and its sequelae. Our laboratory is studying the Ossabaw mini-pig as a novel animal model for obesity. The Ossabaw mini-pig has a mutation in the Val199Alle region of the PRKAG3 gene (the ~/isofrom of AMPK) that causes increased intramuscular fat and contributes to their thrifty genotype. This pig breed, when fed an excess calorie high fat/cholesterol/fructose diet, naturally develops features of metabolic syndrome including visceral obesity, glucose intolerance, and dyslipidemia. Our research goal was to determine whether feeding an excess calorie high fat diet and the consequent development of obesity would lead to alterations in the morphology of the small intestine and distribution of enteroendocrine cells. Nineteen Ossabaw gilts were fed either an excess calorie high fat diet/cholesterol/fructose diet (n=10) or control diet (n=9) for eight months, after which the animals were euthanized and samples of the ileum were collected for histology. Ossabaw gilts on the high fat diet showed marked increases in overall body weight, heart girth and abdominal girth and also developed metabolic syndrome with elevated androgen levels. We used immunohistochemistry methods to measure intestinal villus height and assess expression of specific cell types in the ileum. An anti-chromagranin antibody (AB) was used to identify enteroendocrine cells, anti-glucagon-like peptide-1 (GLP-1) AB to detect L cells, and Periodic Acid-Schiff stain to highlight goblet cells. Histological tissue sections were scanned using NanoZoomer Digital Pathology System (Hamamatsu Photonics) and images obtained were analyzed using NDP.view2 software. Normal distribution of data was conducted using PROC UNIVARIATE and non-normal data were log transformed prior to analysis with an ANOVA using PROC MIXED (SAS, Cary, NC). Statistical significance was set at p<0.05. The ileal epithelium of obese Ossabaw gilts had significantly greater villi length than that of lean Ossabaw gilts (obese: 488.9 mm ± 18.8 mm; lean: 389.4 mm ± 17.1 mm.; p<0.0001). Obese Ossabaw gilts also had significantly more goblet cells per villus than lean Ossabaw gilts (obese: 31.3 ± 2.0 goblet cells/villus; lean: 26.0 ± 2.0 goblet cells/villus; p<0.05). We also identified numerous L cells that secrete GLP-1 and other enteroendocrine cells within the ileum. The L cells were located primarily in the intestinal submucosa with only a few in the villi. Lean Ossabaw gilts showed a trend towards fewer L cells than the obese group. We hypothesize that the differences in villi height and goblet cells may be due to increased proliferation of stem cells in the crypts of the intestinal lining in obese animals. We are currently investigating potential differences in intestinal epithelial barrier integrity by evaluating expression of the tight junction protein claudin-4. Recent studies have reported gender differences in intestinal villus height and epithelial cell integrity in response to injury in rodent models and have reported alterations in gut enteroendocrine cell secretion in obese women. The Ossabaw pig may be a useful model to investigate links between gut function and reproductive hormones. Funding: NIH R21HD060105 to RK and USDA ILLU-538-319 to RAN.

324. Effect of Trolox supplementation on sperm characteristics during semen cryopreservation in Korean Native Pig.
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Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a cell permeable, water soluble derivative of Vitamin E with potent of antioxidant properties to prevent mediate oxidative stress and apoptosis. The objective of this study was to evaluate the effect of Trolox supplementation to freezing extender on sperm motility, viability and acrosome integrity during semen cryopreservation in Korean Native Pig. Boars were kept under uniform feeding and handling conditions in the National Institute of Animal Science, RDA, Korea. In this experiment, whole semen was collected from four mature Korean Native boars by a gloved-hand technique and frozen twice weekly for total of four freezing operation per animal. Semen samples were diluted into a freezing extender (LEY) containing lactose hen-egg yolk with glycerol and Orvus Es Paste (OEP) and loaded in 0.5ml plastic straws. In this experiment, each ejaculated semen sample was split into four aliquots and extended withLEY that supplemented with 0, 50, 100 or 200 μM Trolox. For freezing the semen samples were cooled from 5°C to -5°C at 6°C/min, held at -5°C for 30s while ice crystal formation was induced, then further cooled from -5 to -80°C at 40°C /min, and thereafter from -80°C to -150°C at 60°C /min using programmable semen freezer. To evaluate the post-thaw sperm quality, semen was thawed at 38°C for 20sec. Sperm quality in terms of motility, viability and acrosome integrity was improved by addition of Trolox to freezing extender during semen cryopreservation. In particular, the sperm motility and viability at 1 and 2 h of incubation was higher (P<0.05) in the group treated with 200 μM Trolox during freezing procedure when compared to the control group. The result of this study shows that the addition of Trolox to the freezing extender (LEY) improves motility and viability of frozen-thawed sperm in Korean Native Pig. This work was carried out with the support pf "Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ00948101)" Rural Development Administration, Republic of Korea.

325. WITHDRAWN.
326. FOLLICULAR FLUID INFLUENCE IN THE OOCYTE COMPETENCE: IDENTIFICATION OF FACTORS INVOLVED IN OOCYTE QUALITY AND EMBRYONIC DEVELOPMENT.
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2015 Abstracts - Page 130
In the process of *in vitro* production of embryos (IVP), one of the most important stages for embryo development is *in vitro* maturation (IVM), since essential events that influence the quality of the future embryo occur during this phase. The oocyte removal from the follicular environment results in the spontaneous resumption of meiosis, interfering in the oocyte capacitation period *in vitro*. Thus, follicular conditions can result in differences that can later impact the embryo phenotype. The objective of this study was the investigation of fundamental molecules present in follicular fluid (FF) that are possibly involved in oocyte capacitation, embryo viability and competence. For this analysis, bovine ovaries were obtained in commercial slaughterhouse. Follicles of 7–8mm were individually aspirated and Cumulus-oocyte complexes (COCs) with their respective FF (5 follicles/ovary) were separated. *In vitro* maturation of COCs from the same ovary were made in 50µl drops of culture medium using Well of the Well system for 22-23 hours in an incubator at 38.5°C and 5% CO2 and high humidity. Oocyte fertilization was made at the same atmospheric conditions of the IVM for 18 hours, followed by *in vitro* culture (IVC) until D7 in SOFaa medium containing 5% of FCS, 20 L / mL of essential amino acids and 10 uL / mL of nonessential amino acids. Cleavage and blastocyst rates were evaluated at 40hpi and 168hpi respectively. Glucose, cholesterol and pyruvate molecules present in the FF were quantified by fluorimetric assays using commercial kits and analyzed according to the cleavage and blastocyst rates. The data obtained was then analyzed using the Wilcoxon-Mann-Whitney test (n = 6 replicates) on GraphPad Prism 5.0 software. The results showed a higher glucose level in the FF of cleaved embryos than of non-cleaved ones (CI = 0.234 ± 1.327µM; NCl = 0.554 ± 0.108 uM). Likewise, oocytes that were able to develop into blastocysts were obtained from FF with higher pyruvate and cholesterol concentration (cholesterol - BI = 33.14µM ±1.98; NBl= 28.86µM ± 1.32), (pyruvate - BI= 35.83µM ± 2.67; NBl= 28.42µM ± 2.30). These results indicate that glucose can be an important substrate for embryo cleavage and that the presence of cholesterol and pyruvate in the FF is essential for the development to blastocyst stage, thus resulting in higher oocyte quality, which is an essential factor for a better embryo development. Financial support: UFABC and FAPESP.

327. **Global O-GlcNAc modified proteins in the human placenta: a mass spectrometry analysis of pregnancies complicated by maternal diabetes.**

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The nutrient sensitive hexosamine biosynthetic pathway (HBP) utilises cellular glucose to produce uridine diphosphate N-acetylglucosamine (UDP-GlcNAc) for the post-translational addition of N-acetyl-glucosamine (GlcNAc) to Ser/Thr residues of intracellular proteins. GlcNAcylation alters protein function by influencing location, protein-protein interaction and phosphorylation status. Placental function is altered in pregnancies complicated by maternal diabetes. We hypothesise this altered placental function is mediated through glucose-induced changes in protein GlcNAcylation. Our aim was to discover which proteins are GlcNAc-modified in normal placenta, and then compare the GlcNAc proteome to that of placentas from pregnancies exposed to high blood sugar (e.g. maternal diabetes); GlcNAc-proteins that differed in abundance were used to determine the pathways that might contribute to altered placental function in diabetes. Placentas were obtained at term from mothers with pre-existing type 2 diabetes mellitus (n=6: T2DM) and BMI-matched women with uncomplicated pregnancies (n=6: T2CON). GlcNAc-modified proteins were isolated from pooled tissue lysates using succinylated wheat germ agglutinin lectin-bound beads and separated by SDS-PAGE. Gel slices were processed for mass spectrometry (MS) analysis and the resulting data were submitted to MASCOT to enable protein identification. Proteins with a change (≥2fold) in abundance between biosamples were further studied using Ingenuity pathway analysis (IPA) to identify parent canonical signalling pathways. Proteins identified by MS were validated by Western blot. MS analysis identified 943 proteins, of which 716 were present in both T2DM and T2CON. The abundance of 170 proteins was significantly different between biosamples with 85 proteins more GlcNAcylated and 85 proteins less GlcNAcylated in T2DM compared to control. IPA revealed a group of proteins that contribute to clathrin-mediated endocytosis. Species involved in early endosomal formation (Rab5B and Rab5C, -2.09 fold and -4.67 fold respectively) and in receptor recycling via late endosomes (Rab11A, -5.45fold) were less-GlcNAc-modified in T2DM compared to control. Western blot analysis of GlcNAc-modified proteins in lysates from T2DM and T2CON placentas confirmed their GlcNAc status. Such analysis revealed that other components of this pathway, e.g. the clathrin heavy chain are also GlcNAcylated. This study has revealed the placental GlcNAc proteome, thereby aiding understanding of the molecular mechanisms governing placental function. Our data suggest that proteins involved in clathrin-mediated endocytosis are differentially GlcNAcylated in placentas from women with T2DM, which may result in altered activity of this pathway, contributing to altered placental function and perhaps fetal macrosonia. Ultimately, placental endocytosis may be a therapeutic target in pregnancy complicated by maternal diabetes.

328. **Colostrum, Oral Insulin-like Growth Factor 1, and Pregnant Sow Serum Support Neonatal Porcine Uterine Cell Proliferation.**

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Milk-borne bioactive factors delivered from mother to offspring via nursing are important for neonatal development as proposed by the lactocrine hypothesis. Lactocrine effects on uterine development are evident as early as postnatal day (PND) 2, suggesting that effects are induced shortly after ingestion of colostrum (first milk). Insulin-like growth factor (IGF) 1, present in high concentrations in porcine colostrum, enhanced cervical cell proliferation at 12 h postnatal when supplemented orally at birth (PND 0). Whether oral IGF1 affects neonatal uterine development is unknown. Whether maternal serum constitutes a potential source of lactocrine-active factors is also of interest. Here, objectives were to determine effects of feeding a single dose of the following: (a) colostrum or milk replacer, with or without orally supplemented IGF1; and (b) serum obtained from late pregnant sows vs a non-pregnant gilt on uterine cell proliferation and IGF1 signaling at 12 h postnatal. Gilts at birth (n=3-6/group) were gavage- fed a single dose (15 ml/kg BW) of either: 1) colostrum collected at birth; 2) colostrum supplemented with IGF1 (1 μg/ml); 3) milk replacer; 4) milk replacer supplemented with IGF1; 5)
pregnancy day 109 sow serum; or 6) non-pregnant gilt serum. After the initial feeding, all neonates were gavage-fed milk replacer until 12 h postnatal when uterine tissues were collected. Uterine sections were immunostained for proliferating cell nuclear antigen (PCNA) and labeling indices (LI) were determined for epithelial and stromal cell compartments. Additionally, proteins involved in the IGF1 signaling cascade, including anti-apoptotic B-cell lymphoma 2 (BCL2) and phosphorylated (p)AKT uterine protein were evaluated by immunoblotting using total AKT as the loading control. Uterine stromal and epithelial PCNA LI were greater (P<0.05) in gilts fed a single dose of colostrum, colostrum with IGF1, or replacer with IGF1 compared to those fed replacer alone from birth. Both stromal (P<0.005) and epithelial (P<0.0005) PCNA LI were higher in neonatal gilts fed a single dose of serum from late pregnancy as compared to those fed serum from a non-pregnant adult gilt. Immunoreactive uterine BCL2 (26 kDa) and pAKT (60 kDa) proteins were detectable in immunoblots at 12 h however, no effects of treatment were observed. Results indicate that: 1) a single feeding of colostrum stimulates uterine endometrial cell proliferation by 12 h postnatal; 2) oral IGF1 supplementation supports uterine cell proliferation and can rescue the lactocrine-null phenotype as defined by patterns of endometrial cell proliferation; and 3) orally active factors in serum from late pregnancy are uterotrophic as reflected by increased endometrial cell proliferation at 12 h postnatal. In addition to providing the basis for a bioassay system which can be used to identify uterotropic lactocrine activity by 12 h postnatal, results also indicate that maternal serum is a potential source of lactocrine-active factors in colostrum. [Support: USDA-NIFA 2013-67016-20523; NSF-EPS-158862].

329. Insufficient Methionine and B-vitamin Levels in Zebrafish Feed Increases the Liver Lipid Accumulation and Affects Redox Regulation and One Carbon Metabolism in the Next Generation.

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In aquaculture, the feed raw materials have changed from containing high levels of fishmeal and fish oil to high levels of plant based alternatives. This changes the delivery of essential nutrients from the feed ingredients. Several amino acids and B- vitamins, such as methionine, folate and vitamin B12 are low in the plant based diets. These are important as methyl donors (1-C donors) and for the regulation of the methylation potential of the cells, vital for many biological processes including gene regulation through DNA methylation. Negative effects of plant based ingredients on growth performance and flesh quality for fish have been observed, but how the fish feed affects the gene transcription for the following generations remains an important unexplored field. To study this we made one feed that was just below the requirements for methyl donors (low 1-C feed) and one feed that was enriched with methyl donors (high 1-C feed). These feeds were given to the F0 generation (6 separate tanks of 20 fish for each diet) of zebrafish from 27 days post fertilization until maturity, while both groups of the next generation (F1) were given the same type of feed enriched with methyl donors. Mature livers from F1 generation were sampled for transcriptome analyses. Genes related to redox regulation and lipid metabolism, but also genes related to 1-C metabolism, were significantly differentially regulated in the liver samples from F1. We are currently investigating if these changes in gene expressions are caused by changes in DNA methylation. The alterations in lipid metabolism were confirmed with a higher inclusion of lipids in the hepatocytes of F1 mature livers of the low 1-C feed group. This research was supported by The Research Council of Norway (EpiFeedFish 225250/E40).

330. Defining the Age-Sensitive Neonatal Porcine Uterine Transcriptome.

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Uterine development in the pig begins prenatally and is completed postnatally. Maternal support of development in mammals continues after birth by communication of signals in milk via a lactocrine mechanism. At birth (postnatal day = PND 0), the porcine endometrium consists of a simple, corrugated luminal epithelium (LE) supported by stroma that extends to the myometrial interface. Glandular epithelium (GE), absent at birth, differentiates from LE shortly thereafter. Onset of uterine glandogenesis is marked by estrogen receptor-alpha (ESR1) expression, and immunostaining for proliferating cell nuclear antigen (PCNA) in nascent GE is evident by PND 2. The fact that PCNA labeling indices were reduced for LE and nascent GE by PND 2 in lactocrine-null gilts, fed porcine milk replacer in lieu of nurse, indicates that morphogenetically critical events supportive of porcine uterine wall development occur within 48 h of birth. Therefore, it is important to define the uterine developmental transcriptome during this period of early neonatal life. The objective of this study was to determine effects of age on the porcine uterine transcriptome between birth and PND 2 using RNA Sequencing (RNAseq). Newborn gilts (n = 4/group) were assigned to be: 1) euthanized at birth; or 2) nursed ad libitum for 48 h. Uteri were collected either at birth or at 50 h postnatal. Total RNA was extracted from each uterus and RNA quantity and integrity were determined. For RNAseq, RNA (500 ng/μg) was used to create cDNA libraries for each uterus. These were uniquely indexed and sequenced at > 90 million reads per sample. Raw reads were mapped to the most recent Suscrofa 10.2 build. Results of RNAseq analyses were validated using qPCR. Gene enrichment and functional analyses were determined using a) Database for Annotation, Visualization and Integrated Discovery; b) Kyoto Encyclopedia of Genes and Genomes; c) Panther; d) Reactome; and e) Ingenuity Pathway Analysis. Results of RNAseq revealed 3283 genes for which expression changed from birth to PND 2 (P ≤ 0.05). Of these, 737 gene transcripts were up-regulated while 2546 transcripts were down-regulated at least 2-fold or more in uteri from PND 2 as compared to PND 0 gilts. Gene enrichment and functional analysis tools revealed that age-sensitive, up- and down-regulated transcripts were linked to alterations affecting signaling by chemokines, Wnt β-catenin, transforming growth factor-β, matrix metalloproteinases, and ESR1. Results extend previous findings of age-sensitive events associated with neonatal porcine uterine development and indicate that developmentally sensitive gene expression events associated with this process are extensive. [Support: USDA-NIFA 2013-67016-20523; NSF-EPS-158862]
331. In utero exposure to inorganic arsenic results in metabolic and reproductive defects in male CD-1 mice.
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Exposure to harmful chemicals or endocrine-disrupting agents during fetal life disrupts organ formation and increases the risk of diseases when the affected fetuses reach adulthood. One such agent is inorganic arsenic, a naturally occurring metalloid found in soil, groundwater, and plants such as rice. Arsenic contamination in groundwater and rice has led to widespread arsenic poisoning in many countries. In animal models such as mice, gestational exposure to high doses of inorganic arsenic at ppm level causes tumors in tissues such as the ovary, adrenal, and liver. In this study, we evaluated the effect of in utero exposure to human relevant and low dose of arsenic at 10 ppb, the maximum contaminant level set by the EPA, on reproductive and metabolic parameters in male mice when they reach adulthood. Pregnant CD-1 mice were exposed to sodium arsenite in the drinking water at 0 (control), 10 ppb, and 42.5 ppb (tumor-inducing dose in mice) from embryonic day 10 to 18, the window when the reproductive system develops. In utero exposure to arsenic did not affect maternal weight gain, number of pups per litter, sex ratio, and anogenital distance of the pups. After birth, pups were fostered to unexposed females to avoid maternal effects of arsenic exposure on lactation. The exposed animals were allowed to develop to adulthood and evaluated for body fat content, glucose tolerance test, and fertility. We found that male mice exposed to 10 ppb in utero exhibited significant increase in body weight at birth when compared to controls (p<0.05) while males exposed to 42.5 ppb showed a tendency for increased body weight compared to control males (p=0.08). This increased body weight in arsenic-exposed pups continued to 11 weeks of age. Furthermore, at 5 months of age, males exposed to both 10 ppb and 42.5 ppb were obese with significant fat/lean body mass ratio (p<0.001). The body weight gain was accompanied with defects in glucose metabolism. To assess the effect of in utero arsenic exposure on male reproductive function, exposed males were paired with unexposed females for 1 year of continued breeding. Regardless of the dose, total numbers of litters sired by the arsenic exposed male were reduced significantly (p<0.05). Male exposed to 42.5 ppb in utero also exhibited a significant decrease in the number of pups per litter (p<0.05). These results indicate that in utero exposure to arsenic, including the 10 ppb EPA maximum contaminant level, results in obesity as well as fertility defects in male mice. This work was supported by the National Institute of Health Intramural Research Fund.

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Development of replacement beef heifers on dietary energy levels to achieve 55 vs. 65% of mature body weight at 14 mo of age did not affect their ovarian follicular development or subsequent fertility (Eborn et al., 2013, J. Anim. Sci. 91:4168). Because bovine ovarian gametogenesis and folliculogenesis begin early in fetal development, the objective was to determine the impact of maternal nutrition during gestation on ovarian development and fertility of their female progeny. In a 2x2 factorial, prepubertal beef heifers (8-month-old) were fed either a high (HE) or low (LE) energy diet for 6 mo before breeding (prebreeding) plus the first 22 d of a 47-d breeding period to achieve 55 or 65% of mature body weight, respectively, at breeding. Pregnancy was diagnosed at about 75 d of gestation. The pregnant heifers were then housed and managed together on grass pasture until onset of the third trimester of gestation, at which time they were randomly and equally reassigned to two precalving (PC) dietary energy levels to achieve a gain of either 0.70 (PCHE) or 0.45 (PCLE) kg/d until parturition. After weaning, all female progeny (n = 114) were housed together and managed by a standard heifer protocol. Number of antral follicles (AFC) and ovarian size were measured for their ovaries by transrectal ultrasonography at 14 mo of age and just prior to a 29-d natural breeding period with multiple fertile bulls. Data were analyzed by analysis of variance with diet, year, and diet by year as fixed effects. Progeny birth weight (34.0 ± 0.4 kg) was not affected by prebreeding dietary level but calves of PCHE-treated dams were heavier (P ≤ 0.02) at birth than PCLE calves (34.9 vs. 33.2 ± 0.6 kg, respectively). Ovaries of progeny born to HE-treated dams contained more (P ≤ 0.02) small (2 to 5 mm) follicles (21.6 ± 17.9 ± 0.9; HE vs. LE), and total AFC (23.7 ± 19.8 ± 1.0; HE vs. LE), whereas total AFC did not differ between precalving treatments (21.8 ± 21.9 ± 1.0; PCHE vs. PCLE). Ovarian length (28.9 ± 0.3 mm) and height (15.1 ± 0.2 mm) were not affected by prebreeding or precalving diets. Proportion of daughters pregnant to the 29-d breeding period did not differ between prebreeding diets (71.0 vs. 71.3 ± 0.5%; HE vs. LE) but was greater (P = 0.08) for progeny of PCHE- vs. PCLE-treated dams (79.7 vs. 66.0 ± 0.6%, respectively). Treatment differences in progeny ovarian AFC indicate that maternal dietary energy levels influenced ovarian gametogenesis and folliculogenesis during early gestation, whereas nutritional influence on fetal growth and fertility occurred later in gestation. USDA is an equal opportunity provider and employer.

333. STUDIES ON THE IMPACT OF INTRAUTERINE EXPOSURE TO MATERNAL OBESITY AND ENDOCRINE DISRUPTORS ON THE OFFSPRING METABOLISM IN MICE: ROLE OF LIVER ER STRESS.
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Intrauterine exposure to adverse environmental conditions can affect adult offspring health, through yet still mostly unexplained mechanisms known as "fetal programming". The endoplasmic reticulum (ER) stress response is an adaptive process that restores the normal function of cells after exposure to diverse stressors. Prolonged ER stress can alter cell signaling, affect cell function and in some cases, trigger apoptosis. ER stress is activated in various tissues, i.e. liver, muscle and adipose tissue, under pathological conditions such as obesity and type 2 diabetes. Hepatic ER stress contributes to the development of liver steatosis and insulin resistance, and some components of the unfolded protein response have been shown to regulate liver lipid metabolism. Endocrine disruptors (ED), pollutants commonly used to synthesize plastics that exhibit hormonal activity, are also able to disrupt ER function. Our hypothesis is that fetal programming of metabolic disease given by maternal obesity and exposure to ED is mediated by liver ER stress. Objectives: a) To evaluate
if intrauterine exposure to maternal obesity, alone or combined to a mixture of ED, affects the adult offspring metabolism. b) To evaluate liver ER stress response as a possible mechanism mediating those metabolic disturbances in adult offspring. Methods: C57BL/6J pregnant mice with normal weight or obese (~25% overweight by high fat diet 45% kcal from fat feeding) were injected intraperitoneally (i.p.) daily from gestational day 7 (E7) to E15 with vehicle (sesame oil) alone or containing a mixture of endocrine disruptors (mED) [DEHP + DPP + BBP (300mg/Kg/day) + 4-nonylphenol + 4-ter-Octylphenol (50mg/Kg/day)] in a complete dose (1X) and in 1/2X and 1/10X dilutions. Male and female adult (90 and 120 day-old) offspring were analyzed for body weight, glucose tolerance (i.p. glucose tolerance tests (GTTs)), insulin and cholesterol levels. Classic ER stress sensors (phospho-IRE1α, phospho-eIF2α and ATF6) were detected in liver protein extracts by western blot. Results: Intrauterine exposure to the mED at 1X and 1/2X doses was associated to gestational shortening, smaller litters and death of neonates. Litter sizes and viability were unaffected by the 1/10 dose, so this was used for subsequent experiments. Male offspring exposed to mED 1/10 or to maternal obesity exhibited altered glucose tolerance (area under curve p<0.05, n=7 per group), not associated to overweight or abnormal total plasma cholesterol levels. Interestingly, no metabolic disturbances were detected in females. Male, and not female, livers exposed to maternal obesity exhibited an activated ER- stress response, as shown by a significant increase in phosphorylation of proteins IRE1α and eIF2α (p>0.05). Animals from both genders exposed to mDE did not exhibit a significant increase in ER stress markers. Strikingly, the combined exposure to mED and maternal obesity did not produce detectable additive effects in the offspring metabolism. Conclusions: Overall, these results suggest that intrauterine exposure to maternal obesity or ED can induce fetal programming of metabolic abnormalities in a gender-specific manner and shows reticulum endoplasmic stress as a possible programming mechanism induced by maternal obesity. Acknowledgements: This research was supported by Fondecyt grants. #1121145 (M.F.), #1110712 (A.R.), #1110778 (R.M.), #1141236 (D.B.). CONICYT SCHOLARSHIP #21120903 and NUT14-02 grant.

334. Impact of a Total Western Diet on oocyte quality in swine.

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Maternal nutrition will obviously have a direct impact on the health and well-being of the mother but it can also have profound consequences for embryonic/fetal fitness. It has been suggested that in humans, maternal preconception diet plays an important role in dictating reproductive success possibly by affecting oocyte quality. The periods of final oocyte maturation and early embryo development have been shown to be very sensitive to changes in the maternal environment. Our hypothesis for this study is that the total western diet (TWD), which is macro- and micro-nutrient unbalanced, will have profound effects on the molecular components of developing oocytes. The TWD for pigs was formulated using the principle of nutrient density (mass of nutrient/kcal/day). The 50th percentile daily intake levels for macro and micronutrients were selected from the NHANES survey. To determine effects of the newly formulated pig TWD on metabolic and reproductive parameters, ten female pigs were assigned to either the TWD (n=5) or a control (CON) commercial pig diet (n=5). Pigs were fed for 12 weeks and allowed ad libitum access to diets. Pigs fed the TWD gained significantly more weight than CON sows, 92.8 ± 9.5 vs. 54.2 ± 11.2 kg (p < 0.001). A gene expression analysis was done to quantify and compare relative expression levels of 60 selected genes in 10 individual oocytes from CON pigs versus 12 individual oocytes from the TWD pigs using quantitative PCR. Expression levels of 72 genes were also evaluated in the pooled cumulus cell samples from each pig. qPCR data was analyzed via ΔΔCt method. Fold change values were analyzed via ANOVA (P<0.05) and the Benjamini-Hochberg multiple testing correction was applied. Preliminary analysis showed that 11 genes were significantly differentially expressed in the oocytes (AHCY, AKT2, ATM, BAD, CRP, ENO1, G6PD, HYOU1, LDLR, PPAR-D, PRDX6), while none of the genes tested were differentially expressed in the cumulus cells. Biological pathways that were affected by diet were related to apoptosis, carbohydrate metabolism, glycolysis, one carbon metabolism, cholesterol synthesis, fatty acid oxidation, protein metabolism, and epigenetic chromatin modification. In addition to the gene expression analysis, cytochemistry experiments were carried out to quantify and compare levels of glutathione and reactive oxygen species in oocytes

335. Impact of HDL Cholesterol Metabolism on the Oocyte’s Cholesterol Content.

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INTRODUCTION: Cholesterol is a key molecule for mammalian reproduction due to its involvement as a substrate for gonadal steroid hormone synthesis. Cholesterol is transported in circulation by different lipoproteins, classified based on their size and density in: chylomicrons, very low density lipoproteins, low density lipoproteins and high density lipoproteins (HDL). The Scavenger Receptor Class B Type I (SR-BI) in the liver participates in cholesterol homeostasis by taking up cholesterol from circulating HDLs, in a process known as reverse cholesterol transport. SR-BI is also expressed in the ovary; it is up-regulated in the periovulatory period, when it is involved in cholesterol uptake as substrate for progesterone synthesis. In most mammalian species analyzed, HDLs are the main lipoproteins composing follicular fluid, probably due to the fact that bigger lipoproteins cannot permeate through the follicle’s basal membrane pores. SR-BI KO mice, which have abnormally large, cholesterol rich HDL particles due to their defective reverse cholesterol transport produce labile oocytes which die shortly after ovulation and are unable to develop into viable embryos. The major cause of infertility in SR-BI KO females is not the lack of SR-BI in the ovary itself but the presence of abnormal circulating HDLs, as different strategies used to normalize the HDL metabolism, i.e. dietary administration of the cholesterol- lowering drug probucol or liver expression of SR-BI by adenoviral infection, are able to restore their fertility. We recently showed that SR-BI KO mice exhibit high levels of cholesterol in ovulated eggs, as
assessed by staining with the cholesterol fluorescent dye filipin. Cholesterol-loading of wild type eggs in vitro phenocopies the defective MII arrest observed in SR-BI KO eggs. OBJECTIVES: The aims of this work were: 1) To describe the dynamics of cholesterol accumulation in SR-BI KO oocytes during ovarian folliculogenesis, and analyze if restoration of fertility in SR-BI KO females after HDL normalization is mediated by a prevention of cholesterol accumulation in oocytes, 2) To analyze the presence and localization of different components required for HDL cholesterol metabolism in the ovary. RESULTS AND CONCLUSIONS: Ovarian cryosections from WT and SR-BI KO mice were stained with filipin and fluorescence was quantified in oocytes from follicles of different stages of folliculogenesis. Whereas the cholesterol fluorescence was similar in oocytes from primary and secondary preantral stage follicles, a significantly higher filipin fluorescence was found in SR-BI KO oocytes from antral follicles (*p<0.01 for early antral follicles and *p<0.005 for late antral follicles). Thus, our results show that cholesterol accumulation in oocytes from SR-BI KO mice starts at the antral stage of folliculogenesis, suggesting that cholesterol excess in SR-BI KO oocytes could be due to exposure of these cells to abnormal HDLs in follicular fluid. Additionally, both SR-BI KO mice treated with probucol as well as double SR-BI/ApoAI KO mice, which have very low plasma cholesterol, exhibit reduced cholesterol content in oocytes from antral follicles and in ovulated eggs in comparison to SR-BI KO mice (*p<0.005), indicating that reducing cholesterol accumulation in oocytes may restore fertility. HDLs acquire its cholesterol content from cells expressing cholesterol transporters such as ABCA1, ABCG1. The cholesterol transporter ABCA1, and the main proteins that compose the HDLs, ApoAI and ApoE, were detected in the ovary using different techniques such as RT-PCR, Western Blot and immunofluorescence. ABCA1 was shown to be expressed in the oocyte and granulosa cells, whereas apoAI and apoE were found to be present in follicular fluid, suggesting the existence of molecules capable of exchanging cholesterol between HDLs, oocytes and granulosa cells in the ovarian follicles. ACKNOWLEDGMENTS: FONDECYT grants 1141236 to DB and 1110712 to AR.

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Milk composition has varies with stage of lactation, age, breed, nutrition, energy balance and health status of the udder. Colostrum differs considerably in the concentration of milk protein that may be more than double in colostrum compared to later in lactation (Ontsouka et al., J Dairy Sci, 2005). Bovine milk contains low concentrations of serum proteins, including albumin, immunoglobulins, complement proteins, members of the IGF family and lactoferrin, compared with human colostrum (Korhonena et al., Brit J Nutr, 2000). This study was performed to identify differential proteins between bovine and human colostrum. Also, we tried to sort out different proteins between bovine colostrum and mature milk proteins. Whey milk proteins of 3 sets of bovine and human colostrum, which were collected 12h after parturition, were analyzed separately using 2-dimensional electrophoresis (2-DE) within the isoelectric point ranges of 3.0 to 10.0 and then differential proteins were identified using MALDI-TOF analysis. Firstly, in comparison of bovine colostrum and mature milk, a total of 29 protein spots on 2-DE gels were differentially expressed and identified as polymeric immunoglobulin receptor, zinc-alpha-2-glycoprotein, vitamin D-binding protein, IGHM protein in bovine milk, etc. Majority of bovine colostrum specific proteins were related with immunity functions. Secondly, we identified 33 proteins incomparision of human colostrum and bovine colostrum among which 8 spots were up-regulated proteins in human colostrum samples, 6 spots were up-regulated proteins in bovine samples, 12 spots were human colostrum-specific proteins and 7 spots were bovine colostrum-specific proteins. A number of identified proteins were included in multiple functional categories. Of these, two human colostrum proteins, IP6K2 and ATP6V1C1, which have very low plasma cholesterol, exhibit reduced cholesterol content in oocytes from antral follicles and in ovulated eggs in comparison to SR-BI KO mice (*p<0.005), indicating that reducing cholesterol accumulation in oocytes may restore fertility. HDLs acquire its cholesterol content from cells expressing cholesterol transporters such as ABCA1, ABCG1. The cholesterol transporter ABCA1, and the main proteins that compose the HDLs, ApoAI and ApoE, were detected in the ovary using different techniques such as RT-PCR, Western Blot and immunofluorescence. ABCA1 was shown to be expressed in the oocyte and granulosa cells, whereas apoAI and apoE were found to be present in follicular fluid, suggesting the existence of molecules capable of exchanging cholesterol between HDLs, oocytes and granulosa cells in the ovarian follicles. ACKNOWLEDGMENTS: FONDECYT grants 1141236 to DB and 1110712 to AR.

337. Mono(2-ethylhexyl) Phthalate Inhibits the Synthesis of Sex Steroid Hormones in Cultured Mouse Antral Follicles.
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Di(2-ethylhexyl) phthalate (DEHP) is a ubiquitous environmental toxicant used as a plasticizer in common consumer, medical, and building products. Humans are exposed to DEHP on a daily basis, and this is concerning because DEHP is an endocrine disruptor with toxicity likely mediated by its bioactive metabolite, mono(2-ethylhexyl) phthalate (MEHP). Previous studies indicate that MEHP may have a direct effect on inhibiting ovarian steroidogenesis in cultured mouse antral follicles by decreasing aromatase and estradiol levels following 96hr of exposure. However, the temporal effects of MEHP upstream of estradiol and aromatase and the mechanism by which MEHP inhibits steroidogenesis are unknown. Thus, we tested the hypothesis that MEHP directly inhibits the synthesis of sex steroid hormones upstream and prior to the decrease in estradiol levels. To test this hypothesis, antral follicles from adult CD-1 mice were cultured with vehicle control (dimethylsulfoxide [DMSO]) or MEHP (0.1-10 µg/ml) for 24-96hr (n=3-9 separate experiments). Following each 24hr time-point, media were subjected to enzyme-linked immunosorbent assays for the measurements of sex steroid hormone levels, and follicles were subjected to quantitative polymerase chain reaction to measure the mRNA levels of the steroidogenic enzymes. Following 24hr of exposure, MEHP decreased androstenedione and testosterone levels (androstenedione: DMSO=2.4±0.8; 10µg/ml=0.7±0.1 ng/ml; testosterone: DMSO=2.4±0.6; 10µg/ml=0.8±0.1 ng/ml, n=3-9, p<0.05). Following 48hr of exposure, MEHP decreased the mRNA levels of Star and Cyp11a1, which are rate-limiting enzymes responsible for initiating the conversion of cholesterol to pregnenolone (Star: DMSO=1.0±0.07; 1µg/ml=0.5±0.04; 10µg/ml=0.5±0.2 fold change; Cyp11a1: DMSO=1.0±0.09; 1µg/ml=0.7±0.05; 10µg/ml=0.5±0.1 fold

EFFECT OF ENVIRONMENT ON OVARY: Program Numbers 337–365
change, n=3-9, p≤0.05). Following 72h of exposure, MEHP increased the mRNA levels of Hsd17b1, which is responsible for generating testosterone, and Cyp19a1, which is responsible for generating estradiol (Hsd17b1: DMSO=1.0±0.1; 0.1µg/ml=1.8±0.3; 1µg/ml=2.6±0.2; 10µg/ml=2.8±0.4 fold change; Cyp19a1: DMSO=1.1±0.2; 0.1µg/ml=2.5±0.2; 1µg/ml=2.5±0.3; 10µg/ml=3.4±0.3 fold change, n=3-9, p≤0.05). However, following 96h of exposure, MEHP decreased the mRNA levels of Hsd17b1 and Cyp19a1 (Hsd17b1: DMSO=1.0±0.06; 0.1µg/ml=0.4±0.08; 1µg/ml=0.5±0.1; 10µg/ml=0.3±0.09 fold change; Cyp19a1: DMSO=1.0±0.06; 0.1µg/ml=0.4±0.1; 1µg/ml=0.4±0.2, 10µg/ml=0.3±0.2 fold change, n=3-9, p≤0.05). Further, MEHP decreased testosterone and estradiol levels (testosterone: DMSO=30.0±2.7; 0.1µg/ml=10.2±2.6; 1µg/ml=17.5±4.1; 10µg/ml=9.7±2.2 ng/ml; estradiol: DMSO=3929.9±408.1; 0.1µg/ml=1327.6±150.1; 1µg/ml=2302.8±419.2; 10µg/ml=2009.1±267.7 pg/ml, n=3-9, p≤0.05). Collectively, these data suggest that MEHP has a direct effect on inhibiting steroidogenesis in the antral follicle. The mechanism by which MEHP inhibits steroidogenesis may involve initial toxicity of MEHP, then a failed compensatory response to restore steroidogenesis, then ultimately an inhibition in the synthesis of testosterone and estradiol. Supported by R01ES019178 (JAF) and an Environmental Toxicology Fellowship (PRH).

338. The Effects of In Utero Bisphenol A Exposure on Oxidative Stress-Related Genes in Multiple Generations of Mice. Amelia Berger1, Wei Wang1, Changqing Zhou1, Jodi A. Flaws1, Ayelet Ziv-Gal1.

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Bisphenol A (BPA) is a chemical that is commonly used as a plasticizer in the production of polycarbonate plastics and epoxy resins. It is also a known endocrine disrupting chemical. Previously published data indicate that in utero BPA exposure can affect proper ovarian development and function of the F1 progeny in mice; however the mechanism by which it does so is unknown. Interestingly, in other tissues including muscle and testes, embryonic BPA exposure results in abnormal development by causing oxidative stress. Oxidative stress occurs when there is alteration in the clearance of reactive oxygen species by anti-oxidant enzymes. In the current study, we examined if in utero BPA exposure alters oxidative stress-related genes in the ovaries of mice from the F1 and F3 generations. For this study, pregnant dams (FVB mice; F0) were orally dosed with vehicle control (tocopherol-stripped corn oil) or BPA (0.5, 20, or 50 µg/kg/day) daily from gestation day 11 until birth. After birth, F1 females were bred to reach to the F3 generation. Pooled ovaries from F1 and F3 generations (postnatal day 4) were examined for gene expression analyses. We focused on superoxide dismutase 1 (Sod1), catalase (Cat), and glutathione peroxidase (Gpx), which are known to reduce the amount of reactive oxygen species. Our results indicate that compared to control, BPA (0.5 µg/kg/day) increases the levels of Sod1 (1.3 fold change (FC), p≤0.05) and Gpx (1.3 FC, p≤0.05) in the F1 generation, but decreases the levels of Gpx (0.5 FC, p≤0.05) in the F3 generation. BPA (20 µg/kg/day) increases the levels of Sod1 (1.5 FC, p≤0.05) and Gpx (1.4 FC, p≤0.05) in the F1 generation, but decreases the levels of Gpx (0.4 FC, p≤0.05) in the F3 generation.

Lastly, BPA (50 µg/kg/day) increases the levels of Sod1 (1.7 FC, p≤0.05), Gpx (1.7 FC, p≤0.05), and Cat (1.4 FC, p≤0.05) in the F1 generation, whereas it increases the levels of Sod1 (1.3 FC, p≤0.05) and decreases the levels of Gpx (0.6 FC, p≤0.05) in the F3 generation. These data suggest that in utero BPA exposure significantly alters the expression of anti-oxidant related genes in postnatal day 4 mouse ovaries, but the effects are different in the F1 and F3 generations. Supported by: NIH P01 ES022848 and EPA RD-83459301.


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In mice, early stages of ovarian development begin around gestation day 10.5 and continue after birth until about postnatal day (PND) 8. During this developmental period, primordial germ cells populate the female gonads and start forming the ovarian follicular pool by a process termed folliculogenesis. During the early stages of folliculogenesis, clusters (nests) of germ cells break apart and develop into primordial and then primary follicles. Toxic effects on the female mouse reproductive system and folliculogenesis have been observed following exposure to the endocrine disrupting chemical bisphenol A (BPA). For example, a previous study indicates that in utero exposure to BPA (GD11 until birth) inhibits germ cell nest breakdown in the female offspring (F1; PND 4) when compared to the vehicle control group. In the current study, we hypothesized that the observed effects on the F1 generation can carry over to subsequent generations (F2, F3). To test our hypothesis, pregnant dams (FVB mice; F0) were orally dosed daily with tocopherol-stripped corn oil (vehicle), diethylstilbestrol (DES; 0.05 µg/kg, positive control), or BPA (0.5, 20, or 50 µg/kg/day) from gestation day 11 until birth. Sexually mature F1 females and their offspring were bred with untreated males to form the F2 and F3 generations. F2 and F3 ovaries were collected on PND 4 and further processed for histological evaluation. Specifically, the ovaries were serially sectioned and every 10th section was used to count the numbers of germ cells, primordial, and primary follicles. The percentages of germ cells, primordial, and primary follicles in each treatment group were statistically compared to the control group. The results indicate that in the F2 generation, the percentages of germ cells, primordial follicles, and primary follicles in BPA treatment groups were similar to those of the control group (p>0.05; n=3-4).

Similarly, in the F3 generation, the percentages of germ cells, primordial follicles, and primary follicles in BPA treatment groups were similar to the control group (p>0.05; n=3-5). Together, these data suggest that the effects of in utero BPA exposure on folliculogenesis on the F1 generation at PND 4, are not likely to persist in the F2 or F3 generations. Supported by NIH P01 ES022848 and EPA RD 83459301.

340. High-throughput (HTP) toxicology test system in 3-dimensional (3D) murine ovarian follicle culture. Hong Zhou1, Malika A. Malik2, Aarthi Arab1, Matthew Hill1, Ariella Shikanov1.

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Multiple toxicants, drugs and their metabolites are damaging to the functional unit of the ovary – the follicle. Ovarian follicles are susceptible to this damage at all stages of their development, yet assays to study reproductive toxicity are limited. Historically follicle growth and development was studied in 2D cultures. However 3-dimensional (3D) in vitro culture preserves the 3D architecture of the ovarian tissue and physiological structure-function relationship and allows detailed mechanistic studies of reproductive toxicology. Here we applied the novel 3D high-throughput (HTP) in vitro ovarian follicle culture system, based on fibrin alginate interpenetrating network (FA-IPN) to study the ovotoxic effects of an anti-cancer drug, Doxorubicin (DXR). The fibrin component in the system is degraded by plasmin and it appears as a clear circle around the encapsulated follicle. The follicle health strongly correlated with the degradation area.
around the follicle. For the purpose of the high throughput image analysis we wrote a MATLAB® custom code. We did not observe any significant difference between manual images processing using ImageJ to the MATLAB® methods, confirming that the automated program is suitable to measure fibrin degradation in attempt to evaluate follicle health. The cultured follicles were treated with DXR at concentrations ranging from 0.005 nM to 200 nM, corresponding to the therapeutic plasma levels of DXR in patients. Follicles treated with DXR demonstrated decreased survival rate in greater DXR concentrations. We observed partial follicle survival of 35.4% (n=80) in 0.01nM treatment and 47.6% (n=92) in 0.005nM, which we identified as the IC50 for secondary follicles. In summary, we established a 3D in vitro ovarian follicle culture system that could be used in a HTT approach to measure toxic effects on female reproductive system.

341. Genistein exposure inhibits growth and alters hormone levels through dysregulation of steroidogenic enzymes in cultured adult mouse antral follicles.
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Genistein is a naturally occurring isoflavone phytoestrogen commonly found in a number of plant products, such as soybeans, lentils, chickpeas, and sunflower seeds. Genistein, like other phytoestrogens, has the potential to mimic, enhance, or impair the estradiol biosynthesis pathway, thereby potentially altering ovarian follicle growth. Previous studies have inconsistently indicated that genistein exposure affects granulosa cell proliferation and hormone production, but no studies have examined the effects of genistein on intact antral follicles. Thus, this study was designed to test the hypothesis that genistein exposure inhibits follicle growth and steroidogenesis in mouse antral follicles. To test this hypothesis, antral follicles were mechanically isolated from adult CD-1 mice and cultured in supplemented α-minimum essential media treated with either a vehicle control (dimethylsulfoxide; DMSO) or genistein (6.0 and 36µM) for 96 hours (h). Individual follicle diameters were measured every 24 h as a measurement of follicle growth. At the end of the culture period, media were collected and subjected to enzyme-linked immunosorbent assays for measurements of progesterone, androstenedione, testosterone, and estradiol levels. Additionally, the follicles were collected and subjected to quantitative real time polymerase chain reaction (qPCR) assays to measure the gene expression of key steroidogenic enzymes. The results indicate that genistein (36µM) significantly inhibits antral follicle growth beginning at 24h when compared to control (n=3, p≤0.05). Exposure to genistein (6.0 and 36µM) significantly decreases estradiol levels at 72h and 96h when compared to control (n=3, p≤0.05). In contrast, genistein (6.0 and 36µM) increases progesterone levels at 48h, 72h, and 96h (36 µM only) when compared to control (n=3, p≤0.05). Genistein exposure (6.0 and 36µM) also increases testosterone levels at 48h and 72h when compared to control (n=3, p≤0.05). Androstenedione levels remained unaffected at every time point. The results also indicate that genistein exposure increases the expression of steroidogenic acute regulatory protein (StAR) at 72h (DMSO: 1.01 ± 0.10, genistein 36µM: 3.57 ± 0.96 relative fold change) and 96h (DMSO: 1.23 ± 0.49, genistein 36µM: 8.46 ± 3.25 relative fold change) and increases expression of cytochrome P450 cholesterol side-chain cleavage (Cyp11a1) at 96h (DMSO: 1.06 ± 0.24, genistein 36µM: 2.74 ± 0.56 relative fold change) when compared to control (n=3, p≤0.05). Additionally, genistein decreases the expression of 3β-hydroxysteroid dehydrogenase (Hsd3β1) at 24h (DMSO: 1.02 ± 0.10, genistein 36µM: 0.42 ± 0.01 relative fold change) and the expression of cytochrome P450 17α-hydroxylase 1 (Cyp17a1) at 24h (DMSO: 1.76 ± 0.65, genistein 36 µM: 0.42 ± 0.11 relative fold change) and 96h (DMSO: 1.05 ± 0.20, genistein 36µM: 0.16 ± 0.04 relative fold change) when compared to control (n=3-5, p≤0.05). Genistein exposure did not significantly affect the expression of 17β-hydroxysteroid dehydrogenase 1 (Hsd17β1) or cytochrome P450 aromatase (aromatase (Cyp19a1)). Collectively, these data indicate that genistein exposure inhibits antral follicle growth through dysregulation of the estradiol biosynthesis pathway. Supported by NIH ES019178.

342. Prenatal exposure to di-(2-ethylhexyl) phthalate adversely affects selected female reproductive outcomes in the F2 generation of mice.
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Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer commonly used in materials such as vinyl flooring, medical devices, baby toys, automotive parts, and food containers. DEHP can leach from products and expose the general population through ingestion, inhalation, and dermal contact. Prenatal exposure to DEHP has been shown to adversely affect the development and function of the male reproductive system in multiple generations, but little is known about the multi-generational effect of DEHP on the female reproductive system. Thus, the objective of this study was to test the hypothesis that prenatal DEHP exposure affects female reproductive outcomes in the F2 generation of mice. To test this hypothesis, pregnant CD-1 mice (7-10 dams per treatment group) were orally dosed with tocopherol- stripped corn oil (vehicle control) or DEHP (20µg/kg/day, 200µg/kg/day, 500mg/kg/day, or 750 mg/kg/day) daily from gestation day 10.5 until birth of the pups. The subsequent generation (F1) offspring were mated with proven male breeders to produce the F2 generation, without further DEHP treatment. On postnatal days (PNDs) 8, 21, and 60 of the F2 offspring, at least one female pup from each litter was euthanized, and the ovaries and uteri were collected and weighed. At PNDs 21-60, the pups were weaned and subjected to measurements of puberty onset and estrous cyclicity. At PND 90, the mice were mated with untreated, proven male breeders to produce a set of offspring. The results show that prenatal exposure to DEHP did not affect the litter size or the male to female ratio of the F2 mice compared to controls (n=3-10 dams per treatment; p≤0.05). However, prenatal DEHP exposure (750mg/kg/day) significantly altered estrous cyclicity so that prenatally DEHP exposed mice spent less time in proestrus compared to controls (n=3-10 dams per treatment; p≤0.05). Further, DEHP exposure (500mg/kg/day) significantly influenced the weight at the time of weaning so that prenatally DEHP exposed mice weighed more when compared to controls (n=3-10 dams per treatment; p≤0.05). In contrast, DEHP exposure did not affect the mating ability of the dams (n=3-10 dams per treatment; p≤0.05). Further, DEHP exposure did not affect the ability of the dams to become pregnant, litter size, or the male to female ratio of the pups produced by the F2 generation (n=3-10 dams per treatment; p≤0.05). These data suggest that prenatal DEHP exposure may have two-generational effects on some female reproductive outcomes such as amount of time spent in proestrus and weight at the time of weaning. Supported by NIH P01 ES022848 and EPA RD-83459301.

343. Glutamate cysteine ligase modifier subunit (Gclm) null mice have increased apoptotic follicle loss in the ovary.
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Glutathione (GSH) plays an important role in regulating the redox balance of the cells and provides cytoprotection against oxidative stress. Mice lacking the modifier subunit of glutamate cysteine ligase (Gclm), the rate-limiting enzyme in GSH synthesis, have decreased tissue concentrations of GSH. It has been reported that apoptosis accounts for ovarian follicle loss via follicle atresia during postnatal life. Our previous in vitro studies have shown that GSH deficiency escalates apoptosis in ovarian follicles and that apoptotic death of ovarian follicles is related to an early and sustained increase in reactive oxygen species. We have also previously reported that Gclm-/- female mice have oxidized GSH redox ratio at 2 months and 6 months of age and increased oxidative damage at 21 days and 9 months of age, along with accelerated age-related decline in ovarian follicles in Gclm-/- females compared to wild type littermates. The differences in primordial follicle numbers between Gclm-/- and Gclm+/+ mice were statistically significant beginning at 3 months of age. We have also previously observed evidence for accelerated recruitment of primordial follicles into the growing pool in Gclm-/- ovaries. We hypothesized that oxidative stress in Gclm-/- mice would increase apoptosis in vivo in ovaries at 2 months of age, which leads to accelerated depletion of ovarian follicles beginning in 3 month old Gclm-/- mice. In this study, we found marginally significant Gclm genotype-related differences in the numbers of primordial and primary follicles in ovaries from 2 month Gclm-/- compared to Gclm+/+ mice (P=0.086 and P=0.085, respectively). For in situ detection of apoptosis, we used immunostaining with antibodies directed against cleaved caspase-3, a critical executioner of apoptosis, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), a hallmark of apoptosis, in ovaries of 2 month old Gclm-/- and Gclm+/+ mice. We found significantly increased immunostaining for cleaved caspase-3 in granulosa cells of histologically healthy-appearing and histologically healthy plus atretic antral follicles in 2 month old Gclm-/- ovaries compared to Gclm+/+ ovaries (P=0.024 and P=0.021, respectively). We also found that the percentage of histologically healthy-appearing antral follicles with TUNEL positive granulosa cells was higher at 2 months of age in Gclm-/- ovaries than in Gclm+/+ ovaries (P=0.008). There was a nonsignificant trend towards a greater percentage of TUNEL positive secondary follicles in Gclm-/- compared to Gclm+/+ ovaries (P=0.051). No significant Gclm genotype-related differences in cleaved caspase-3 and TUNEL were observed in primordial or primary follicles of 2 month old mice. These data suggest that an increase in apoptotic antral and secondary follicles at 2 months of age in Gclm-/- mice, together with our previously observed accelerated recruitment of primordial follicles into the growing follicle pool, results in statistically significantly lower numbers of total healthy and healthy primordial follicles in Gclm-/- ovaries at 3 months of age. Together the current and our prior findings indicate that oxidative stress induced by GSH deficiency increases oxidative damage, recruitment of primordial follicles, and apoptosis in ovaries of Gclm-/- mice, driving the accelerated age-related decline in ovarian follicles that we observe in Gclm-/- mice. Supported by NIH ES020454.

344. Effects of prenatal di-(2-ethylhexyl) phthalate exposure on ovarian follicle numbers in mice.
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2Di-(2-ethylhexyl) phthalate (DEHP) is a plasticizer used in medical tubing, building materials, car seats, and children’s toys. Humans are exposed to DEHP on a daily basis by ingestion, inhalation, and dermal contact. It is known that prenatal exposure to DEHP can adversely affect the reproductive organs and functions of the male reproductive system, but limited information exists about the effects of prenatal DEHP exposure on female reproductive organs, especially the ovary. Thus, we tested the hypothesis that prenatal DEHP exposure affects the numbers and histologic appearance of ovarian follicles in the F1 generation of mice. To test this hypothesis, pregnant CD-1 mice (7-14 dams per treatment group) were orally dosed daily with tocopherol-stripped corn oil (vehicle control) or DEHP (20 µg/kg/day, 200 µg/kg/day, 200 mg/kg/day, 500 mg/kg/day, or 750 mg/kg/day) from gestation day 10.5 until the birth of pups. On postnatal days (PNDs) 8, 21 and 60, at least one female pup from each litter was euthanized, the ovaries were collected, and fixed in Dietrich’s solution. The fixed ovaries were embedded in paraffin, sectioned at 8 µm, and stained with Weigert’s hematoxylin and methyl blue. Every tenth ovarian section then was used to count the numbers of primordial, primary, pre-antral, and antral follicles. The results show that prenatal exposure to DEHP does not affect primordial, primary, or antral follicles in F1 ovaries collected at PND 8 or 60 (n=3-11 dams/treatment group, except n=2 for the 20 µg/kg/day group; p<0.05). In contrast, DEHP (200 µg/kg/day and 500 mg/kg/day) exposure significantly increased the number of pre-antral follicles compared to controls on PND 21. Specifically, numbers of pre-antral follicles in control ovaries were 38.3±3.4, but were 63.4±11.0 in ovaries treated with 200 µg/kg/day DEHP and were 78.5±18.6 in ovaries treated with 500 mg/kg/day (n=3-11 dams/treatment group; p<0.05). These data suggest that prenatal DEHP exposure may not affect follicle numbers in F1 ovaries at PND 8 or 60, but it may increase pre-antral follicle numbers at PND 21. Supported by NIH P01 ES022848 and EPA RD-83459301.

345. 17β-Estradiol Sensitizes Ovarian Surface Epithelium To Transformation By Suppressing Dab2 Expression.
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Ovarian cancer is the leading cause of death from gynecological malignancies in North America with a five-year survival rate of 42%. The high mortality rate is because not much is known about the initiating events, which therefore limits the development of methods for early detection and prevention. Epidemiological studies have shown a positive correlation between estrogen exposure and ovarian cancer incidence such that women using estrogen therapy for more than 10 years are twice as likely to develop the disease. Using a mouse model of ovarian cancer, our lab has established that prolonged exposure to high levels of estradiol (E2) accelerates the timing of tumor onset and increases the incidence of morphologically dysplastic ovarian surface epithelium (OSE), lesions that could be sites of cancer initiation. To further investigate these findings, the goal of this project is to recapitulate the effects of E2 on mouse OSE (mOSE) with an in vitro model to elucidate the mechanisms by which E2 sensitizes OSE cells to transformation. OSE proliferation and morphology are tightly regulated by the asymmetrical distribution of cell polarity proteins. Maintenance of polarity provides positional cues for surface localization and growth inhibition. We hypothesize that the ability of E2 to cause OSE dysplasia is by inhibition of a tumor suppressor gene called Disabled-2 (Dab2). DAB2 plays a critical role in maintaining polarity by mediating the polarized distribution of cell surface proteins and it is highly expressed in ovarian tissue but is lost in the majority of ovarian carcinomas. Mice treated with exogenous E2 for 30-60 days showed loss of DAB2 and reduced E-cadherin in the dysplastic OSE cells, suggesting that loss of DAB2 may be an early event.
in ovarian tumorigenesis. To reproduce the E2-induced OSE dysplasia seen in vitro, an in vitro model system was established using primary cultures of mOSE cells and the effects of E2 in this model were tested in growth curve experiments, and by immunofluorescence staining, quantitative RT-PCR, and western blot analysis. In response to long term (15 days) treatment with E2, mOSE formed multiple foci of stacked cells reminiscent of the areas of dysplasia seen in vivo and displayed evidence of loss of contact inhibition and polarity. DAB2 and other polarity proteins known to be dysregulated in ovarian cancers, such as E-cadherin, Cytookeratin-19, Beta-catenin, and Smad, were all inappropriately expressed or mislocalized in mOSE displaying dysplasia due to prolonged E2 exposure. Dab2 mRNA levels were reduced within 3 hours of E2 treatment and protein levels fell by 48 hours, before morphological changes were observed, confirming that the suppression of DAB2 is an up-stream event of OSE dysplasia. mOSE cells from Esr1 knockout mice did not suppress Dab2 expression in response to E2, indicating that E2 down-regulates Dab2 through the ESR1 pathway. Chromatin immunoprecipitation showed that ESR1 does not bind the estrogen response element site found in the Dab2 gene but several microRNAs are regulated by E2 and have sequences consistent with regulation of Dab2 transcripts and are therefore primary targets for current investigation. Taken together, the results demonstrate that E2 is capable of suppressing Dab2, leading to mislocalization of E-cadherin and increased OSE cell proliferation. Prolonged exposure results in morphologically dysplastic OSE which have lost polarity and contact inhibition, rendering them more susceptible to transformation.

### 346. Phthalate Metabolites Are Associated with Hot Flashes in Midlife Women.

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Hot flashes are the most common symptom that perimenopausal women report to their health care providers and the primary reason that perimenopausal women seek medical care. Despite the high prevalence of hot flashes and their negative impact on quality of life, little is known about the risk factors for hot flashes. Thus, the objective of this study was to test the hypothesis that an environmental chemical exposure (phthalates) is associated with the occurrence, frequency, and severity of hot flashes in midlife women. Phthalates are synthetic chemicals commonly used as plasticizers in consumer goods such as children’s toys, deodorants, lotions, and cosmetics. Phthalates are also used in adhesives, pesticides, solvents, wood finishes, lubricants, and in medical devices including surgical gloves, tubing, blood bags, and dialysis equipment. With 18 billion pounds of phthalates used worldwide each year and exposure occurring through inhalation, ingestion, and dermal absorption, human exposure to phthalates is ubiquitous. To test whether phthalate exposure is associated with hot flashes, data were obtained from 195 women aged 45 to 54 years enrolled in the Midlife Women’s Health Study. Each woman completed a self-administered questionnaire that provided detailed information on hot flash history and potential confounders such as race, body mass index, and reproductive history. Additionally, each woman provided urine samples that were subjected to measurements of phthalate metabolites by isotope dilution high-performance liquid chromatography negative-ion electrospray ionization-tandem mass spectrometry. Specifically, the following phthalate metabolites were measured: monomethyl phthalate (MMP), mono-(5-carboxy-2-ethylpentyl) phthalate (MCCEPP), mono-(3-carboxypropyl) phthalate (MCPP), monobutyl phthalate (MBP), monoethyl phthalate (MEP), mono-(2-ethyl-5-oxo)hexyl) phthalate (MOEHP), monobenzyl phthalate (MBzP), monoisobutyl phthalate (MiBP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), and monoethylhexyl phthalate (MEHP). Further, serum samples were subjected to measurements of estradiol using enzyme-linked immunosorbant assays. The data indicate that higher mean MCEPP levels were positively associated with ever having hot flashes (p < 0.04), severe hot flashes (p < 0.02), and trended towards an association with daily hot flashes (p < 0.09). Additionally, higher MEHP levels were positively associated with ever having hot flashes (p < 0.04), severe hot flashes (p < 0.03), and trended towards a positive association with daily hot flashes (p < 0.06). MEP levels were also positively associated with ever having hot flashes (p < 0.05), having hot flashes in the past 30 days (p < 0.01), daily hot flashes (p < 0.008), and trended towards a positive association with severe hot flashes (p < 0.06). There were no statistically significant associations between the other metabolites and hot flashes outcomes. The data also indicate that MEP levels are negatively correlated with estradiol levels (p < 0.05) and that MBP and MBzP levels trended towards a negative correlation with estradiol levels (p < 0.06). Collectively, these data suggest that some, but not all, phthalate metabolites are associated with an increased risk of hot flashes in perimenopausal women. These data also suggest that some phthalates may be associated with hot flashes through a mechanism that includes low estradiol levels. Supported by NIH AG18400.

### 347. Effect of Bisphenol A on HSP70 Expression During Bovine Oocyte Maturation.

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Bisphenol A (BPA) is an endocrine disrupting chemical that can have detrimental effects on fertility and reproductive health. BPA is one of the highest volume chemicals produced worldwide and can be found in a wide range of everyday products including plastic containers and cans. BPA has been detected in over 90% of individuals in North America in different samples, including blood, urine, tissue and follicular fluid. BPA has been linked to infertility and reproductive failure, such as reduced in vitro fertilization (IVF) success in humans and other mammals. This study evaluated the effects of BPA exposure during in vitro bovine oocyte maturation, on embryo cleavage and blastocyst development rates. Environmental toxins such as BPA can induce apoptosis in cumulus cells, oocytes and embryos. Since, the expression levels of specific survival, stress, and epigenetic markers are determinants of oocytes maturation, their evaluation following BPA exposure at different concentrations is important. To investigate the stress correlated with BPA exposure we selected heat shock protein 70 (HSP70), an upstream inhibitor of apoptosis, which is up regulated during toxic stress. We hypothesized that BPA affects in vitro developmental rates and HSP70 expression during oocyte maturation. To test our hypothesis, oocytes were in vitro matured following exposure to BPA concentrations of 0.5, 0.05 and 0.005 mg/mL and controls (no treatment and vehicle). These concentrations represent environmentally significant levels of BPA; particularly 0.5 mg/ml which is equivalent to the Lowest Observed Adverse Effect dose (LOAEL) as for FDA guidelines. Oocytes were fertilized and cultured up to day 8 to determine developmental rates. To measure HSP70 expression levels, proteins were extracted from pools of 40 oocytes from each treatment group. Cleavage rate in the 0.5 and 0.05 mg/mL BPA treatment group was significantly lower compared to the no treatment and vehicle controls, while no difference in
the number of cleaved embryos was detected if oocytes were treated with 0.005mg/mL. The blastocyst rate was significantly lower in all the three treatment groups compared to controls, with no embryos reaching the blastocyst stage in the LOAEL treated group. Western Blot analysis of HSP70 expression in oocytes showed a marked increase in HSP70 levels in those matured in 0.05 and 0.005 mg/mL BPA doses, suggesting that BPA affects the in vitro production system, through changes in protein levels involved in stress and apoptotic pathways. These preliminary results suggest that oocyte exposure to BPA compromise early embryo development affecting key stress proteins, such as HSP70, thereby negatively influencing in vitro produced bovine embryos. This research is supported by Canada Research Chairs program and an Ontario Veterinary College scholarship (NK).

348. Aberrant SIX1 Expression May Contribute to the Development of Uterine Adenocarcinoma Following Neonatal Xenoestrogen Exposure.
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In a mouse model of neonatal xenoestrogen exposure, 95% of animals treated with pharmacologic doses of the synthetic estrogen, diethylstilbestrol (DES, 1 mg/kg/day), and 35% of animals treated with physiologic doses of the phytoestrogen, genistein (50 mg/kg/day), develop uterine adenocarcinoma by 18 months of age. This phenotype is prevented by prepubertal ovariectomy, suggesting that it depends on endogenous hormone exposure. We aim to understand the mechanistic underpinnings by which early xenoestrogen exposure in combination with endogenous hormone exposure leads to uterine cancer development later in life. Sine oculis-related homeobox 1 (SIX1), a cancer-associated transcription factor, becomes permanently ectopically expressed in the uterus following neonatal xenoestrogen exposure. Aberrant SIX1 expression is localized to a population of abnormal uterine basal cells that develop following exposure and proliferate with age. We hypothesize that carcinogenesis occurs when neonatal xenoestrogen exposure directs permanent changes in uterine SIX1 expression, resulting in aberrant cellular reprogramming that is exacerbated by endogenous hormone exposure. In this study we compared uterine SIX1 mRNA expression, SIX1 expression patterns, and histopathology in control and neonatal DES- or genistein-treated mice at 6, 12, and 18 months of age and tested whether pre-pubertal ovariectomy impacted aberrant SIX1 mRNA expression. At 6 months of age, DES and genistein treatment resulted in 13- and 7-fold increases in SIX1 mRNA expression, respectively (n=30-33 mice per group). Furthermore, SIX1 protein localized to all cells in hyperplastic and neoplastic lesions. Regardless of the timing of ovariectomy, neonatal DES treatment resulted in increased SIX1 mRNA expression. However, SIX1 mRNA expression was 3-fold lower in DES-treated prepubertally ovariectomized mice compared to DES-treated intact or adult ovariectomized mice (n=4-10 mice per group). This indicates that prepubertal ovariectomy prevents further increases in SIX1 mRNA expression. These findings suggest that uterine SIX1 expression is initiated by neonatal DES treatment and perpetuated by endogenous hormones beginning at puberty. As the mice age, SIX1 expression patterns are associated with proliferative histopathologic changes, suggesting that it could play a role in carcinogenesis. To determine if SIX1 plays a causative role in cancer formation, we are currently investigating if conditional deletion of SIX1 in the uterus prevents the development of both abnormal uterine basal cells and uterine adenocarcinoma following neonatal DES exposure.

349. WITHDRAWN.

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The impact of nutritional restriction during gestation on the fetus varies depending on the species, timing, severity and type of the restriction. In this study, sheep were placed on a maintenance diet, based on the metabolisable energy intake, or a diet equivalent to 60% of maintenance for the first 55 days of gestation. Maternal blood samples were collected at gestational days 55 (n=36) and 75 (n=15). These samples were assayed for leptin and progesterone. Fetuses from these animals were also collected at gestational days 55 (n=34) and 75 (n=25). Birth weight and weaning weight of male and female lambs were recorded (n=65) from animals brought to term within each diet group. Age at puberty was determined through weekly monitoring of marking by a harnessed vasectomized ram. The ovaries of female offspring (n=30) were assessed at 6-7 months of age by laparoscopy and transrectal ultrasound scanning to determine ovulation rates and antral follicle counts; two indicators of fertility. During the first 55 days of gestation, maintenance ewes increased body weight by 4.2% while body weight decreased 6.4% in those on the restricted diet. Differences in body weights were maintained between the groups until at least day 90 of gestation. At both days 55 and 75 of gestation, no nutrition induced differences in fetal weight or fetal gonad weight were observed in either male or female fetuses. At cessation of nutrition restriction (day 55), maternal plasma leptin levels were significantly lower in those animals exposed to reduced nutrition (maintenance 2.9 ng/ml vs restricted 2.2 ng/ml, P=0.048). By gestational day 75 maternal leptin levels in restricted animals had returned to levels comparable to those found in maintenance animals (maintenance 3.4 ng/ml vs restricted 2.9 ng/ml). Progesterone concentrations were increased in restricted animals at gestational day 55 (maintenance 6.68 ng/ml vs restricted 7.78 ng/ml, P=0.021) while levels at day 75 were similar (maintenance 9.51 ng/ml vs restricted 9.03 ng/ml). At birth, there were no differences between the groups in the number of lambs born per ewe (maintenance = 1.77, restricted =1.73) or in the weights of those lambs born (maintenance = 5.4 kg vs restricted =5.7 kg). Growth of the lambs to weaning was also unaffected. Weights of female lambs remained similar between the groups until at least 7 months of age. The gestational nutrition restriction did not affect the onset of puberty in female offspring with the average time taken to attain puberty being 225 days for maintenance animals vs 226 days for those from the restricted group. At 6-7 months of age, no significant differences were noted between the groups in either ovulation rate (maintenance 1.40 vs restricted 1.48) or antral follicle counts (maintenance 14.3 vs restricted 15.5). While further studies are ongoing with this cohort of sheep, the nutritional regime used here, 60% of maintenance for the first 55 days of gestation, did not appear to affect birth rates, size or growth of offspring or peripubertal fertility of female offspring.
351. RNA transcript profiles for porcine oocyte-cumulus complexes treated with follistatin at germinal vesicle breakdown and metaphase 2.

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During the latter stages of folliculogenesis, the cumulus-oocyte complex(COC) is bathed in follicular fluid (FF), which provide an important and specialized micro-environment for in vivo oocyte maturation. Characterisation of FF and information about FF constituents have been performed to overcome limited laboratory environment outside of a body and to provide efficiency gains of oocyte competence in vitro. Among many factors in FF, this study was conducted to examine effect of Follistatin(FSH) on porcine oocyte, which was isolated from porcine FF (pFF) In mammal, It is because FST is emerging evidence that it has a role in modulating the biology of other members of TGF-β superfamily. Firstly, in oocytes culutred with or without FST at GVBD stage, we checked maternal mRNA expression level but FST did not make a difference of cumulus-related genes; Has2, Agre,Ptggs2, Ptx3, and Tnfaip6 and oocyte- related genes; BMP15 and GDF9. Secondly, effect of FST in oocyte maturation in vitro was investigated. FSH-induced cumulus oocyte complex exposed to exogenous FST for 44h IMV had significantly increased the maturation percentage of oocytes compared to controls. Also, mRNA expression level of target genes, presented above, were analysed and we found that FST and BMP15 expression level increased meaningfully while GDF9 level did not differ significantly at metaphase 2 stage. Lastly, to define FST-induced cytoplasmic maturation, we checked maternal mRNA expression; Cdx2, CyclinB1 and c-mos, expression level for futher analysis. Real-time PCR experiments revealed greater abundance of mRNA for c-mos which is an upstream activator of mitogen-activated protein kinase (MAPK), responsible for metaphase 2 arrest. In conclusion, these results demonstrate that positive association of exogenous FST, as separate action.

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352. Microfluidic platform supports mouse ovarian follicle development and recapitulates human 28 days menstrual cycle.

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The main female reproductive organs include ovary, fallopian tubes, uterus, cervix, and vagina. These organs function in relation to one another to provide hormonal support and the anatomical structure through which gametes travel for the embryo to undergo development, transport, implantation and placentation. Our objective is to develop an ex vivo female reproductive tract that can be used for reproductive toxicology and therapeutic discovery based on the ovarian hormone production and their effects on the downstream gynecologic tissues. The ex vivo female reproductive tract consists of three-dimensional cultures of ovarian follicles, fallopian tube epithelium, endometrial and myometrial co-cultures, and endo and ectocervix connected by the unidirectional flow of culture media in a microfluidic culture system. The microfluidic platform designed for ovarian follicle culture includes a media donor, culture module, and media collection compartments. Computer control of media flow through the culture modules was achieved by a series of pneumatically actuated valves and pumps. The flow rate (40 μl/h) and culture volume (700 μl) were determined by computational models of follicle estradiol production based on empirical data. Mouse primary follicles were isolated from day 12 CD-1 mice and multiply encapsulated in 0.5% alginate hydrogels. To mimic human 28 days menstrual cycle, follicles were cultured for 14 days with follicle-stimulating hormone (FSH) to phenocopy the follicular phase, which was followed by human chorionic gonadotropin (hCG) administration on day 14 to trigger the luteal phase and an additional 14 days of culture without FSH. Media was collected every 24 h from the collection compartments, and the flow rate and ovarian hormone production were monitored. Results indicated that encapsulated in vitro follicle growth (eJFVG) was supported from primary to antral stage in the microfluidic system. After hCG treatment, follicles produced metaphase II oocytes with barrel-shaped bipolar spindles and tightly aligned chromosomes on the metaphase plate. During the follicular phase, estradiol production increased and peaked on day 14 when follicles reached maturity. During the luteal phase, the progesterone levels increased significantly and peaked 2 days after the hCG treatment; and, the histologic analysis indicated that follicles initiated luteinization at the cellular level based upon granulosa cell hypertrophy. Compared to follicles in the static culture group, follicles cultured in the microfluidic platform produced significantly higher levels of estradiol and progesterone. Taken together, our results demonstrate that the microfluidic platform provides a dynamic environment in which hormone production is maintained over a 28 day in vitro hormone cycle. This tool provides great potential to monitor rapid ovarian hormone changes and their effect on downstream reproductive tissues in vitro, and to provide a model to study the reproductive toxicology and therapeutic discovery (This work is supported by NIEHS/OWHR/UTHES022920 and NCATS/NIEHS/NICHD/OWHR/UTH3TR001207 and the NIH Common Fund).

353. Where are they now? Determining the mechanisms regulating cold-induced granule formation in oocytes.

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Ribonucleoprotein complexes (RNPs) are aggregates of RNA and proteins within membrane-less particles. By bringing together RNA molecules and RNA-binding and -processing proteins, RNPs create micro-environments that can regulate translation (stress granules), enhance RNA degradation (e.g. P-bodies), transport and localize messenger RNAs (e.g. sponge bodies) or store them (e.g. subcortical aggregates in murine oocytes). Tightly regulating the modifications and the expression of maternal transcripts is particularly important for oocytes because of the absence of transcription during meiotic maturation and early embryogenesis. Translation of mRNAs must be precisely regulated so that development can occur normally. P-bodies and P-body-like RNPs have been described in murine oocytes and have been suggested to play roles in silencing and storing maternal mRNAs. Here we report that growing and fully grown oocytes at the germinal vesicle (GV)-stage oocytes develop granules that contain the canonical P-body marker, GW182, in response to...
cold-shock. These granules disappear following return to 37°C, indicating that their formation is reversible. Surprisingly, the GW182 foci did not co-localize with other canonical stress granule markers such as TIA-1 and G3BP. To investigate the nature of the GW182 cold-induced granules, we examined their behaviour under various conditions. Translational inhibitors induced a slight but significant increase in the number of GW182-foci. Microtubule-disruptors on the other hand, prevented their formation. Mature metaphase II eggs were not able to form GW182 granules when cold-shocked. This loss of granule-forming activity was not due to the decrease in PKA activity at the onset of maturation or to germinal vesicle breakdown. However, inhibition of MAP kinase activity in mature oocytes restores the ability to form GW182 granules in response to cold-shock. Determining the regulators of the GW182 cold-induced granule formation will allow us to manipulate their formation and assess their role in mediating the stress response of oocytes.

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The multidrug resistance-associated protein (MRP1/ABCC1), a member of the ATP-binding cassette (ABC) transporters, serves as a drug transporter for cellular excretion of hydrophobic compounds, organic anionic drugs and chemical conjugates. Cyclophosphamide (CPA) is a prodruk which must undergo hepatic biotransformation to the anti-neoplastic metabolite, phosphoramide mustard (PM). PM depletes the finite pool of ovarian primordial follicles leading to infertility and premature ovarian failure. We had previously reported PM-induced increased mRNA encoding Mrp1 in an ex vivo ovarian culture system and hypothesized that MRP1 is involved in the ovarian response to PM exposure as a cellular defense mechanism to export the ovotoxicant from the ovary. Spontaneous immortalized granulosa cells (SIGC) were cultured in control media or media containing PM (6 mM) for 48h until 80% confluent. In addition, postnatal day 4 ovaries were cultured for 4 days in the presence or absence of PM (60 µM). The conditions used in the SIGC and PND4 ovary culture were those at which cell death and follicle loss, respectively, had been previously characterized. Protein isolation, followed by western blotting, was performed to investigate MRP1 presence and activation following PM exposure. In contrast to our expectations, no impact of PM exposure were observed on MRP1 protein abundance in either the SIGC or PND4 ovary culture models, raising the possibility that the MRP1-PM conjugate could be recycled or degraded to prevent detection of an increase in total MRP1 protein in ovaries exposed to PM. These data add to our knowledge on ovarian mechanisms of Phase III chemical biotransformation.

355. A Murine Model Of Platinum And Taxane-Induced Ovarian Toxicity To Evaluate Gonadoprotective Agents.  
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Platinum agents are frequently used to treat solid tumors, such as ovarian, cervical, endometrial, head and neck, bladder, anal, gallbladder, gastric, esophageal, anaplastic thyroid, osteosarcoma, colorectal, and pancreatic. However, platinum-based CT is gonadotoxic and can result in a reduction in number of ovarian follicles, leading to premature ovarian insufficiency, amenorrhea, early menopause, and decreased fertility. This has an important impact on the post-cancer quality of life in these women. In a previous study we reported that granulocyte colony stimulating factor (G-CSF) protected PMFs and ovarian microvasculature from the damaging effects of cyclophosphamide and busulfan combination chemotherapy. Our objective was to establish a mouse model of platinum chemotherapy-induced diminished ovarian reserve and evaluate the gonadoprotective properties of vascular stabilizing agents, including G-CSF. C57Bl/6 mice were divided into 6 groups (n=18 per group) and treated with vehicle (control), CT (carboplatin 80mg/kg IP and paclitaxel 20mg/kg IP), or CT concurrent with one of 3 agents that inhibit vascular remodeling (fingolimod, bevacizumab, G-CSF) or lupon. Immunohistochemistry (IHC) was performed to evaluate and quantify microvessel density (MVD, PECAM/CD31) and apoptosis (caspase-3). Outcome measures for ovarian reserve included differential follicle counts, serum anti-mullerian hormone (AMH) levels and breeding. Groups were compared using analysis of variance with pairwise comparisons. Compared to controls (3.3±0.55 microvessels/µm²), platinum CT caused a 58.8% reduction in MVD (1.96±0.94 microvessels/µm²). With the exception of lupon, all the protective agents rescued MVD. CT treated mice had a 93.5% reduction in PMF (53±133.6) compared to vehicle-treated controls (830±387.5; p<0.0001) and lower AMH levels (58.5±21.4 vs. 89.3±36.1 ng/ml; p=0.008). While none of the protective agents improved mean PMF counts compared to CT alone, 27% of the mice (4/15) treated with CT + G-CSF retained ≥20% of PMFs compared to only 7% of CT mice (1/15). CT treated mice had more apoptotic oocytes (65% compared to 0%, p<0.0001), but none of the protective agents reduced the number of apoptotic oocytes. Granulosa cell apoptosis did not differ between groups. Fingolimod, bevacizumab and G-CSF improved MVD in animals treated with platinum chemotherapy, but none of these agents preserved PMF. These results are in contrast to our previous observations in animals treated with cyclophosphamide and busulfan, indicating that the mechanisms of gonadotoxicity from platinum agents may be different than alkylating agents.  
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356. Effects of Extended Exposure to the Antibacterial Triclosan in the Adult Female Rat.  
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Triclosan (TCS), an antibacterial, has been shown to have endocrine disrupting activity in the rat. We reported previously that TCS advanced puberty in the female rat in the female pubertal assay and potentiated the estrogenic effect of ethynyl estradiol (EE) on uterine growth in weanling rats acutely exposed to EE and TCS in the weanling uterotrophic assay. In the female pubertal study, we observed a decrease in serum thyroxine (T4) without concomitant changes in T3, TSH or thyroid histopathology following a 21 day
exposure. Therefore, the purpose of our current study was two-fold: 1) to assess changes in adult female reproductive function, including evaluating pituitary and serum hormone concentrations, reproductive tissues and estrous cyclicity and 2) to assess thyroid hormones, thyroid weight and histopathology following an extended exposure to TCS. Adult female Wistar rats were exposed to vehicle control, positive control EE (1.0 µg/kg), or TCS (2.3, 4.7, 9.4, and 37.5 mg/ kg) daily by oral gavage for eight months. Extended dosing with an estrogen or compound which enhances estrogens might lead to early cessation of estrous cyclicity. Therefore, estrous cyclicity was assessed daily by vaginal cytology throughout the dosing period. Although there was no difference between the controls and the EE females in the age of estrous cycle cessation (reproductive senescence), there was a difference in the pattern of irregularity with controls exhibiting mostly persistent diestra and the EE females exhibiting predominately persistent estrus. Extended exposures to TCS (ranging from 2.3 to 37.5 mg/kg) did not cause significant changes in estrous cyclicity, either in timing or pattern of reproductive senescence. In addition, there was no difference in reproductive hormone levels, or uterine epithelial cell height as compared to controls. When thyroid status was assessed at the end of the study, serum T4 was significantly decreased in TCS 9.4 and 37.5 mg/kg groups with no effect on serum T3, TSH, thyroid tissue weight or histology. Therefore, this study showed that there was no concomitant increase in TSH or change in thyroid histopathology following an extended oral exposure to TCS. Importantly, extended exposure to TCS does not significantly alter the timing of reproductive senescence, even though triclosan has been shown to enhance estrogen activity in shorter term studies. This abstract does not necessarily reflect EPA policy.

357. Evaluation of cytotoxicity in isolated antral follicles treated with mono-butyl phthalate and hydroxyurea.
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Ovarian antral follicles are essential for female fertility because they are the main source of ovarian sex steroid hormones and the only follicle type capable of ovulation. Various in-vitro follicle culture systems have revolutionized the field of follicle biology and toxicology by allowing a controlled environment to study the impacts of chemical insults on follicular function. Mono-butyl phthalate (MBP) is the metabolite of di-n-butyl phthalate (DBP), an endocrine disruptor present in plastics, cosmetics and some oral medications. We have previously shown that in vitro treatment with DBP causes follicular cell cycle arrest and apoptosis in isolated antral follicles. However, no studies have evaluated the effects of MBP in isolated antral follicle cultures. Hydroxyurea (HU) is an antineoplastic drug commonly used in the treatment of sickle cell anemia and myeloid leukemias. HU is a well-characterized DNA replication inhibitor and inducer of cell cycle arrest; however, its interactions with the ovarian follicle have not been studied. Therefore, we designed this study to test the hypothesis that in vitro treatment with MBP and HU decreases cell survival in isolated antral follicles. To test our hypothesis, we mechanically isolated antral follicles from the ovaries of adult CD-1 mice (35-39 days old) and individually exposed them (n=5-8 cultures with 4-10 follicles per treatment) to supplemented media alone (NT, control for HU), dimethylsulfoxide (DMSO, vehicle for MBP), MBP (0.001-100 µg/ml), and HU (100 mM) for 24-72 h. Follicle growth and survival were monitored by measuring follicle diameter and cytotoxicity (compromised membrane integrity; CellTox Green) every 24 h, while number of metabolically active cells (ATP concentration; Promega CellTiterGlo) were determined at 72 h. MBP did not affect the ability of antral follicles to grow in vitro, but HU-treated follicles failed to increase their diameter overtime (% change at 72 h; NT: 119.6 ± 4.5%, HU: 99.8 ± 1.6%; n=8 cultures, p<0.05). MBP treatment did not alter ATP production by isolated antral follicles, but HU-treated follicles produced less ATP and, thus, contained less viable cells at 72 h (luminescent units; NT: 4500 ± 599.5, HU: 1179 ± 46.3, n=3 cultures, p<0.05). In agreement with follicle diameter and ATP levels data, MBP treatment did not affect follicle cell membrane integrity; however, HU-treatment resulted in cytotoxicity at all time points (% change at 72h, NT: 360 ± 101.9%, HU: 738 ± 186.1%; n=4, p<0.05). These results show that MBP did not cause alterations in follicle function under the conditions tested. This suggests that MBP may not be responsible for the effects of DBP in isolated ovarian follicles. These results also show for the first time that in vitro exposure to HU disrupts growth, viability and membrane integrity of antral follicles in vitro. Thus, future experiments will further characterize the effects of HU treatment on ovarian function as they are of concern to women undergoing treatment with HU. Finally, our results support the use of cell-based viability assays to evaluate potential ovarian toxicities using isolated ovarian follicle cultures. This work was supported by NIH grant R00ES021467 (ZRC).

358. Effects of in vitro exposure to the phthalate substitute acetyl tributyl citrate on isolated antral follicle viability.
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Phthalates are endocrine-disrupting chemicals (EDCs) that cause reproductive toxicity in both males and females. Several chemicals already approved by the Food and Drug Administration have been proposed as phthalate substitutes. One example is acetyl tributyl citrate (ATBC). Studies have reported that no reproductive toxicity occurs when ATBC is given to rodents at doses up to 1000 mg/kg/day. However, no studies have tested whether ATBC causes direct toxicity to the ovary. Ovarian antral follicles are essential for female fertility because they are the major producers of ovarian steroids and are the only follicle type that can ovulate in response to gonadotropin stimulation. Previous studies have used in-vitro ovarian follicle culture as a screening tool to demonstrate that EDCs can cause direct ovarian toxicity. Therefore, we designed this study to test the hypothesis that in vitro treatment with ATBC decreases cell survival in isolated antral follicles. To test our hypothesis, we mechanically isolated antral follicles from the ovaries of adult CD-1 mice (35-39 days old) and individually exposed them (n=3 cultures with 4-5 follicles per treatment) to supplemented media alone (NT), dimethylsulfoxide (DMSO, vehicle for ATBC), and ATBC (0.001-100 µg/ml) for 24-72 h. Follicle growth and survival were monitored by measuring follicle diameter, cytotoxicity (compromised membrane integrity; CellTox Green), and number of metabolically active cells (ATP concentration; Promega CellTiterGlo) every 24 h. The DNA synthesis inhibitor hydroxyurea (HU, 100 mM) was used as a positive control for the viability assays. Exposure to ATBC did not affect the ability of antral follicles to increase their diameter over time at most concentrations. Interestingly, there was a transient (at 48 h only) increase in diameter in follicles treated with ATBC at 1µg/ml when compared to vehicle control-treated follicles (48 h % change, DMSO: 110.4 ± 2.9; 1ATBC: 114.1 ± 3.7, n=3 cultures, p<0.05). ATBC treatment did not cause compromised membrane integrity and did not inhibit ATP production at any of the concentrations tested. HU inhibited follicle growth (48-72 h), decreased follicle cell membrane integrity (24-72 h), and inhibited ATP production (72 h). These
experiments show that ATBC does not cause antral follicle toxicity and, thus, may be a safer alternative to phthalates. This work was supported by NIH grant R00ES021467 (ZRC).

359. The Marmoset Monkey as a Model for Doxorubicin Chemotherapy Ovarian Toxicity.
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Pediatric cancer chemotherapy induces ovarian damage leading to follicular demise and decrease fertility. Drug-based approaches such as pretreatment with dexrazoxane and bortezomib were shown to prevent doxorubicin (DXR)-induced ovarian toxicity in the mice. Prior to translation of drug-based ovarian protection to humans, safety and efficacy of putative ovarian shielding agents need to be confirmed in a nonhuman primate model. Marmosets are ideal nonhuman primate model for fertility preservation given their rapid generation time (~2 years) and short gestation (5 months). A pilot study has been initiated to assess DXR chemotherapy in adolescent female marmoset monkeys as a model for ovarian chemotherapy toxicity and drug-based protection in children. To test the hypothesis that adolescent marmosets exhibit DXR-induced ovarian toxicity, three 1-year-old adolescent female marmosets were injected intravenously with either normal saline for vehicle control, DXR at 1 mg/kg (a human equivalent dose of 6mg/m²), or DXR at 10mg/kg (a human equivalent dose of 60mg/m²). Each female underwent unilateral oophorectomy at 12 h following DXR or vehicle injection to assess acute DXR ovarian damage. Interim analysis revealed increase in cells undergoing DNA double strand breaks as demonstrated by increased γ-H2AX phosphorylation in the high dose DXR-treated animal when compared with the control animal. The second ovary of high dose DXR-treated animal was harvested 9 days post DXR-treatment. Analysis confirmed the persistence of a small size ovary and the progression of ovarian toxicity with follicle degeneration 9 days post DXR treatment. This was evident by moderate multifocal degeneration of the oocytes. Hyper-eosinophilia of the ooplasm, Pyknosis, karyorrhexis, and karyolysis of multiple oocyte nuclei and granulosa cells were noted throughout most follicles. We conclude that the near maximum human equivalent dose of DXR causes persistent ovarian damage in the marmoset. The marmoset model requires DXR dose adjustment and proactive supportive therapy similar to standard human chemotherapy protocols to withstand DXR chemotherapy administration. This research was supported by the Research and Development funds (MSN132883) and NIH training grant K12 HD0558941 to SMS.

360. Maternal obesity induces a loss of fetal oocytes and results in ovarian dysfunction in offspring.
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Maternal obesity predisposes offspring to non-communicable disease and reproductive dysfunction. We have previously shown that female rat offspring born to mothers fed a high fat (HF) diet throughout pregnancy and lactation enter puberty early and display aberrant reproductive cyclicity characterized by prolonged or persistent estrus. It is currently unknown what mechanisms are driving this reproductive phenotype. This study set out to investigate key ovarian mediators of follicle development. Methods: Pregnant Wistar rats were fed either a control (n=8) or a HF diet [(n=7) 45% kcal as fat] throughout pregnancy and lactation and offspring were fed a control diet post weaning. Ovarian tissues were collected from offspring at 4 different time points: embryonic day 20 (E20), postnatal day (P)4, P27, and P120 during diestrus (fetal, neonatal, prepubertal, and adult, respectively). Ovarian histological analysis was conducted using fixed H&E stained ovarian sections (4-10um) to determine oocyte and follicle counts. Key reproductive mediators of folliculogenesis including antimullerian hormone (AMH) and its receptor (AMHRI) were localized using immunohistochemistry and semi quantified using computerized image analysis. Atresia was evaluated using the apoptotic marker caspase-3 in histological sections. Circulating levels of FSH, LH and estradiol were investigated using ELISA, and key genes measured using qPCR. Results: Fetuses of HF fed mothers had a 13% loss of oocytes at E20 compared to control. At P4, HF neonates had increased numbers of primordial follicles and transitioning follicles, and demonstrated decreased immunopositive granulosa cell localization of AMH and AMHRII. These changes were accompanied by an increased proportion of apoptosis in P4 follicular cells. At P27, primordial follicle number in HF offspring was similar to control, but HF offspring had smaller secondary follicles, increased type II atretic follicles, and increased apoptosis compared to control offspring. Prepubertal HF offspring also had decreased plasma E2 concentrations despite comparable circulating FSH and LH concentrations. At P120, adult HF offspring displayed increased number of type II atretic follicles, increased follicular apoptosis and increased circulating FSH:E2 ratio. These changes were also accompanied by elevated immunopositive AMH protein in antral follicles compared to control. Adult HF offspring demonstrated decreased mRNA levels of the oocyte-secreted factor, GDF9, oocyte-specific RNA binding protein, DAZL, and estrogen receptor α. Other key genes mediating folliculogenesis (steroidogenic enzymes, FSH receptor, proinflammatory cytokines) were similar between groups. Conclusion: The present study demonstrates that in this rodent model of maternal obesity, ovarian dysfunction in offspring likely stems from impaired ovarian development as early as fetal life. We speculate that maternal obesity may impair fetal germ cell development, and accelerate primordial follicle recruitment rates but that these follicles are then lost to atresia. Given the high rates of obesity in female populations worldwide, future studies are needed to fully delineate the impact of maternal obesity on germ cell development, long term ovarian function, and whether maternal obesity epigenetically alters the germine of female offspring, potentially conferring increased disease risk for future generations.

361. The Role of Follicular Fluid on MAPK Activity in Heat-Shocked Oocytes.
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The bovine oocyte is surrounded by follicular fluid (FF) and layers of cumulus cells that play a major role on oocyte maturation. Oocyte maturation requires a series of orchestrated events leading to increased MAPK activity (Reprod Dom Anim 45:1074–1081, 2010) and meiotic progression to metaphase II stage. There is strong evidence that heat shock compromises oocyte maturation and function.
Indeed, exposure of bovine cumulus-oocyte complexes (COCs) to elevated temperature reduced oocyte nuclear maturation, mitochondrial activity, developmental competence, induced apoptosis and cytoskeleton disorganization. Therefore, the objective of this study was to determine the role of FF on MAPK activity in heat-shocked bovine oocytes. Slaughterhouse COCs were distributed in the following groups: positive control (38.5°C for 22 h, 10% fetal bovine serum), control (38.5°C for 22 h, 0% FF) and heat shock (41°C for 14 h followed by 38.5°C for 8 h) in the presence of 0, 5, 10, 15 and 25% FF during in vitro maturation (IVM). MAPK activity (ratio between phosphorylated and total MAPK) was determined by western blotting. This experiment was replicated 5 times using 140-180 oocytes/treatment. MAPK activity did not differ between positive control (1.14 ± 0.20 a.u.) and 0% FF control (0.84 ± 0.20 a.u.) as well as controls versus 0% FF heat shock group (0.76 ± 0.22 a.u.). Addition of 5, 10 and 25% FF to maturation medium reduced MAPK activity (0.57 ± 0.22, 0.59 ± 0.2 and 0.33 ± 0.22 a.u., respectively) as compared to positive control. On the other hand, MAPK activity of heat shocked oocytes matured in the presence of 15% FF (0.79 ± 0.22 a.u.) was similar to both control groups. In conclusion, heat shock for the first 14 hours IVM did not alter oocyte MAPK activity. The effect of FF supplementation on MAPK activity was dose-dependent. While FF doses such as 5, 10 and 25% exerted an inhibitory effect, the dose of 15% FF maintained MAPK activity in heat-shocked oocytes.

**362. The Immediate Effects of Gonadotoxic Agents on the Viability of the Mammalian Ovary.**

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Background: Annually 790,740 women in the United States will be diagnosed with some form of invasive cancer. Approximately 8% of these women are under the age of 40. Current treatment regimens utilize aggressive gonadotoxic chemotherapy such as cyclophosphamide aimed at cure. This has lead to chemotherapy-induced gonadotoxicity becoming a leading cause of ovarian failure. Objective: Determine the immediate effects of gonadotoxic chemotherapy on the viability of granulosa cells, oocytes, and ovary both in situ and in vivo. Design: Experimental laboratory study. Animals: C57Bl/6 6 week old unstimulated female mice that have never been mated. Main Outcome measure(s): Cell viability in the ovarian cortex using multiphoton microscopy of whole ovaries incubated with calcein-am and ethidium homodimer-1. Results: 75 mg/kg of cyclophosphamide intraperitoneal (IP) injection resulted in a mosaic pattern of cell death in somatic cells likely granulosa cells 24 hours after treatment, at 48 hours there is activation of follicles within the islands of nonviable granulosa cells adjacent to oocytes, and at 7 days the ovarian cortex appears devoid of follicles and has markedly more cell death compared to saline-treated control mice at 7 days and cyclophosphamide treated mice at 24 and 48 hours. Conclusions: Multiphoton microscopy using the Live/Dead Cytotoxicity Assay provides a baseline method to image whole live ovaries. Cyclophosphamide causes a non-uniform pattern of somatic cell death. Activation of quiescent follicles may occur as early as 48 hours after cyclophosphamide and activation is complete by 7 days after administration of IP cyclophosphamide. Somatic cell death is widely spread throughout the ovary one week after treatment. Future Directions: Continue experiments using cisplatin and doxorubicin for comparison and determination of sensitivities to various chemotherapies. Determine if effects of gonadotoxic chemotherapy is different in aged mice and primary ovarian insufficiency phenotypes.

**363. Effect of Melatonin on Estrus and Ovulation Induction in Pubertal Does during the Anestrous Season.**

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Temperate goats are seasonal breeders and photoperiod changes cue estrus cycles. When does are exposed to short photoperiods, the pineal gland secretes the hormone melatonin to stimulate the hypothalamus to produce gonadotropin releasing hormone. This hormone is responsible for the initiation of a cascade of hormonal events that lead to the estrous cycle. When ambient photoperiod is prolonged, it inhibits melatonin secretion and the reproductive cycle of the doe enters a dormant state of the non-breeding season. The first estrus cycle is initiated at puberty. Previous research studies at Fort Valley State University has demonstrated that pubertal does are able to show estrus and ovulation when synchronized with a Controlled Internal Drug Release-progesterone and gonadotropic hormone combination during the non-breeding season, but were not able to become pregnant during the anestrous season. This finding insinuates that pre-pubertal does have not reached their full reproductive capacity, despite their advanced age. This may be due to the inactive state of the corpus luteum (CL) of does in deep anestrus. Melatonin has been shown to increase the integrity of the CL by reducing the harmful effects of reactive oxygen species (ROS). Melatonin could also be used to induce multiple cycles and ovulations in does in anestrous. Thus, the objective of this study was to find out if exogenous melatonin could increase the integrity of the CL so that the pre-pubertal doe would sustain pregnancy and determine if multiple estrous cycles would increase the conception rate of pre-pubertal does. The study was conducted from April to August in 2014 at the Fort Valley State University, Fort Valley, GA. Twelve pre-pubertal does were subjected to long photoperiod regimen (18 hr Light: 6 hr Dark) in two similar controlled rooms, over a three week period simulating a controlled anestrous condition. They were then given daily 0.17 mg per kg of body weight of melatonin injections, intramuscularly. The first group of does was bred at the detection of first estrus; each doe in the second group was allowed to skip the first estrus and bred at their respective second estrus. Ultrasoundography was performed to detect the presence of corpora lutea on the ovaries. Blood samples were drawn and the serum was stored to determine peripheral progesterone concentrations. Results showed that estrus and ovulation were induced in all the does in both treatment groups. None of corpora lutea detected in any of the treated does were able to secrete progesterone. Thus, melatonin was not able to improve the conception rate of the pre-pubertal does. The expected improvement in the CL integrity of the does bred on their second cycle also did not result in any conception. It is postulated that reactive oxygen species may be the cause of the poor integrity of the CL during luteinization in pubertal does resulting in zero levels of progesterone secretion and poor conception rate.

**364. The Effect of Photoperiod on Estrus and Ovulation of Nulliparous Alpine Does induced During the Non-Breeding Season.**
The seasonality of goats in temperate regions causes estrous cycles and ovulation to be limited to fall and winter months. About a third of pregnancies occur in late spring and the kids are dropped late causing them to miss the subsequent breeding season because of sexual immaturity. These kids mature during the non-breeding season when they are in deep anestrus and may not breed until the next breeding season, a year later, thus, wasting a reproduction period and productivity. An earlier study at our facility has shown that using exogenous progesterone and gonadotropin hormones to induce estrus and ovulation during the non-breeding season was not able to sustain pregnancy in pubertal does. It was postulated that this could be due to infertility, probably caused by exposure to less fertile bucks or the lack in integrity of corpora lutea (CL) capable of secreting adequate progesterone in pubertal does induced from deep anestrus. By using photoperiodic rooms, artificial lighting can be manipulated to control reproductive efficiency with multiple inductions of estrus and ovulation in nulliparous does during the non-breeding season. Exposure of the induced does for breeding in their first or second cycles may enable us to compare the fertility and integrity of their CL in sustaining pregnancy. Twelve, one-year old Alpine nulliparous does were allocated to two groups in two photoperiod controlled, air conditioned rooms, between April, 2014 and August, 2014. The intensity of illumination inside the rooms due to the leakage of outside light ranged from 0.02 to 0.04 lux. Six fluorescent light bulbs illuminated each room with light intensity ranging from 130-220 lux depending on the position of the measurement on the floor, and was approximately 185 lux at the height of 60 cm over most of the floor area. The first group of does (n=6) were bred when their first estrus were detected, while the second group (n=6) skipped the first cycle and was bred when they repeated estrus/ovulation on their second estrus. The does in both treatment groups were exposed initially to long photoperiod (16 hr light: 8 hr dark) for three weeks. Photoperiod regimen was drastically decreased (8 hr light: 16 hr dark) after the three week period, on day-21, to simulate breeding season conditions. Each doe was hand bred with a buck induced with melatonin treatment to enhance libido and ensure copulation. Blood samples (5mL per doe) were collected, twice weekly, and stored for assay to monitor progesterone levels, in order to determine CL integrity. An ultrasound was used to search for corpora hemorrhagica (CH) or CL on the ovaries after the does showed overt signs of estrus and to determine pregnancy two months after hand breeding the does. The results showed that the does were growing on average 0.13kg – 0.16kg per day. All does subjected to the long photoperiod and subsequent short photoperiod regimen showed estrus and ovulation but no conception was recorded. Estrus was successfully demonstrated by estrous physical signs without mucus and does’ receptive behavior for copulation by the buck; while successful ovulation was confirmed by presence of CH/CLs on the ovaries using ultrasound sonography. Progesterone concentrations in blood serum collected from both treatment groups showed 0ng/mL, which suggested lack of corpora lutea integrity, possibly due to persistent dormancy and infertility of the nulliparous does deep in anestrus state of the non-breeding season.

**365. Effect of Ovarian Tissue Transportation in *Morus nigra* Extract on the Morphology and Apoptosis of Ovine Preantral Follicles.**

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Studies have demonstrated that *Morus nigra*, known as black mulberry, has antibacterial, antifungal, antinociceptive, anti-inflammatory effects, in addition to its antioxidant activity. However, there were no reports of the use of *M. nigra* extract as an in vitro preservation medium in the transportation of preantral follicles enclosed in ovine ovarian tissue. The aim of this study was to evaluate the effect of *Morus nigra* extract during ovine ovarian tissue transportation on the survival and apoptosis of preantral follicles in vitro. Ovine ovaries (n=8) were divided into fragments. One fragment was fixed and processed for histology, caspase-3 expression and TUNEL analysis (fresh control). The other fragments were placed in Minimal Essential Medium (MEM) or *M. nigra* extract (0.025; 0.05 or 0.1 mg/mL) and stored (simulating transport) at 4°C for 6, 12 or 24 h. Preserved fragments were also destined to histological, caspase-3 and TUNEL analysis. Preantral follicles were individually classified as morphologically normal or atretic. In addition, caspase-3 expression and TUNEL analysis were performed in all treatments preserved for 6 h. Immunostaining for caspase-3 was classified as absent, weak, moderate or strong in the ovarian cells. The rates of follicular survival after preservation were analyzed through ANOVA and Tukey’s test. TUNEL positive cells rate were compared using qui-square test (P<0.05). There was a decrease (P<0.05) in the percent of morphologically normal preantral follicles after preservation in all treatments compared to the fresh control. Moreover, in all treatments, follicular survival decreased (P>0.05) from 6 to 12 h, except for MEM, which showed a reduction in the percentage of normal follicles only after 24 h. The percentage of normal follicles was higher (P<0.05) after preservation of ovarian tissue in 0.05 *M. nigra* for 6 h than in MEM or 0.025 mg/mL *M. nigra* and similar to 0.1 mg/mL *M. nigra*. Apoptosis increased (P<0.05) after conservation for 6 h in all treatments compared to the fresh control. Moreover, TUNEL positive cells decreased (P<0.05) after preservation in 0.05 or 0.1 mg/mL *M. nigra* compared to MEM or 0.025 mg/mL *M. nigra*. Immunostaining for caspase-3 in fresh control and after 6 h of preservation in 0.025 or 0.05 mg/mL *M. nigra* was weak in the oocytes and absent in granulosa cells. A moderate and weak staining for caspase-3 was observed in oocytes and granulosa cells, respectively, after preservation in 0.1 mg/mL *M. nigra*. In addition, a moderate reaction for caspase-3 was observed in both oocytes and granulosa cells preserved in MEM. In conclusion, 0.05 mg/ml *M. nigra* can be used for the preservation of ovine ovarian tissue at 4°C for up to 6 h, providing lower preantral follicle apoptosis. The use of *M. nigra* is recommended as an alternative media due to the higher cost of MEM.

**Poster Topic Area 3: Assisted reproductive technology & Fertilization and preimplantation development**
**366. Production of Somatic Cell Nuclear Transfer Equine from Donor Fibroblasts in G2 and Oocytes in Telophase II.**

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Somatic cell nuclear transfer (SCNT) has been successful in producing animals of commercial interest, especially cattle and horses. The birth of Brazil's first 3 cloned foals in 2011 resulted from our current bovine protocol which utilizes donor cells in G2 phase of the cell cycle and oocytes in Telophase II of meiosis(G2/TII). However, embryo development and birth rates of normal offspring still remain low. To improve production of SCNT equine embryos, a series of experiments were conducted to modify the bovine G2/TII protocol. Experiment 1 evaluated the effect of maturation media supplemented with either 10% fetal bovine serum (FBS) or 10% Invitrogen Knockout serum (KO) on oocyte maturation and subsequent development of parthenogenetically activated embryos. Experiment 2 evaluated the effects of culturing SCNT embryos in media without dextrose for either 3 or 5 days post-activation on blastocyst development and initial pregnancy rates and at 150 days of gestation. Data was analyzed by T-test and considered significant if P<0.05. In experiment 1, oocytes were matured with FBS or KO, respectively. No differences in genetic maturation (FBS 62% vs KO 67%) or blastocyst development (FBS 41% vs KO 44%) was detected. A significant difference (P= 0.03) was observed in hatching rates (FBS 13% vs 38%). In experiment 2, embryos were produced using donor cells in G2 phase of the cell cycle and oocytes in Telophase II. No significant differences were observed in NT blast development, initial pregnancy or d 150 pregnancy rates (10% vs 15%, 25% vs 23% and 14% vs 15%) for embryos cultured in the absence of dextrose for the first 3 or 5 days, respectively. A comparison between the initial protocol from 2011 and currents protocols demonstrate and increase in efficiency of obtaining pregnancies based on the number of embryos transferred (2% in 2011 to 20% in 2013). These results demonstrate that oocyte maturation conditions greatly influence parthenogenetic blastocyst hatching rates as well as pregnancy rates of transferred cloned embryos produced under the same conditions.

**367. Mouse offspring from oocytes produced by meiotic spindle transfer.**

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Meiotic spindle transfer (ST) between metaphase II oocytes offers a strategy for mitochondrial genome (mtDNA) replacement and prevents transmission of mtDNA-based disease from mothers to children. ST also allows complete replacement of defective cytoplasm in patient oocytes due to advanced age with the expectation of improved developmental potential. We previously pioneered ST in a nonhuman primate model and demonstrated live birth and normal postnatal development of monkey ST offspring. Here, we investigated feasibility and efficacy of ST in hybrid (B6D2F1; C57BL/6N x DBA/2N) and inbred C57BL/6N (B6N) mouse strains. Initially, standard ST approach using oocyte spindle isolation under spindle imaging system (Cri Oosight) and Sendai virus-assisted fusion (HJV-SeV) allowed production of live hybrid BDF1 offspring but not inbred B6N.WE then further modified micromanipulations along with in vitro fertilization (IVF), embryo culture and embryo transfer steps to optimize ST for B6N oocytes. Modified protocols improved development of ST embryos from B6N oocytes and supported normal development to live pups. Furthermore, ST offspring were healthy and developed to fertile adults.

These results suggest that ST allows complete cytoplasmic replacement in both hybrid and inbred mouse mature oocytes and is compatible with normal development.

**368. Effect of castration period to testes size, hormone and growth factor in elk.**

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The objective of this study was to evaluate size, testes size and hormone factor after surgically castration by biochemistry analysis. For such purpose, the study was conducted on 12 surgically castrated deer bulls(Elk) aged approximately 6 years old. For each hormone, all samples were assayed at the same time. Testes size and weight was increased from April which was growth period of velvet antler to October, there was significant(p<0.05) difference for decreasing size of testes and losing weight in April. Moreover, castrated in March before casting sperm concentration was 27.9 x 10^6 mL and motility was 54.6 % in October, concentration of sperm was 127.8 x 10^6 mL and motility was 71.9 %. There was no significant difference in elk’s concentration of plasma testosterone between pre- and post- castration group but it tended to little bit high in pre-castration group. After castration, the concentration of plasma testosterone in T1, T2, and T3 group were lower than 0.025 ng/mL which were very low level however, T4 that not castrated, concentration of plasma testosterone was 10.53 ng/mL, which was much higher than post-castration group. Plasma IFG-I concentrations increased in velvet antler growth period (T1) a similar pattern as concentration of IFG-I with antler growth curve. In conclusion, the results of the present study demonstrate a biochemistry characteristics of velvet antler growth related with castration.

**369. Analysis of sperm metabolic flux using 13C-based fluxomics in felids.**

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In order to better understand sperm physiology and improve assisted reproductive technologies in endangered felids, basic information regarding how their sperm metabolize various energy substrates is needed. This study represents the first analysis of metabolic flux in feline spermatozoa using a 13C-based fluxomics approach. The specific metabolic utilization of 13C-labeled substrates can be
detected and quantified using gas-chromatometry mass spectrometry (GCMS), thus allowing identification and quantification of downstream products. Cheetahs (Acinonyx jubatus) (n=4, ages 3 - 11 years) were electroejaculated according to previously established protocols, and sperm were incubated (3.0 x 10^6 sperm/mL) in media containing 1 mM of either U^{13}C-Glucose, U^{13}C-Fructose, or U^{13}C-Pyruvate. At 2.0 and 4.5 hours of incubation, percent motility (%M) and forward progressive status (FPS, scale 0-5) were measured to calculate the sperm motility index (SMI = [%M + (FPS*20)] / 2), and sperm were stored at -80°C until lyophilization. Freeze-dried sperm were chemically extracted with a 1:1.2 (v/v) mixture of MeOH:H₂O:CHCl₃ and the upper fraction was collected after quick-spin centrifugation for 30 seconds. Dried extracts were processed to form the tert-Butylmethylislyl derivatives of the various metabolites within each sample. Preliminary evaluation of metabolites following GCMS analysis revealed the uptake and utilization of labeled glucose and fructose in cheetah sperm, as indicated by the presence of heavy ions in sperm-metabolized lactate and pyruvate. Quantifying the ratios of these heavy ions to those that persist naturally in sperm reveals the relative importance of glycolysis to cellular function. In the cheetah, 53.26% of sperm-produced lactate came from labeled glucose in the absence of any other energy substrates, and 31.60% came from labeled fructose under the same conditions. Additionally 68.18% of sperm-produced pyruvate was derived from labeled glucose, and 54.53% from labeled fructose, when no other energy substrates were available. Preliminary results in domestic cat (Felis catus) epididymal sperm (n = 7; all ≥ 1 year of age) indicate similar rates of glucose and fructose utilization for lactate production (58.25% and 20.58%, respectively), and pyruvate production (63.65% and 37.57%, respectively). Despite the high rates of substrate utilization detected, neither glucose nor fructose had an effect on cheetah SMI (P > 0.05), but fructose significantly improved domestic cat epididymal sperm by 32.5 SMI units (P = 0.0184). While the addition of hexose substrates did not contribute to the calculated motility index in cheetah sperm, these data confirm that the glycolytic pathway is active. Additionally, this study provides chemical evidence that the intake and cycling of glucose and fructose does occur in the sperm of both cheetahs and domestic cats. This improved understanding of the metabolic cycling of nutrients in sperm will allow for the development of species-specific media and techniques for assisted reproduction.

370.  Ovary-mimetic 3D printed follicle niches (3DP-FNs) support survival, hormone production and oocyte maturation.

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Iatrogenic cancer treatments, such as radiation and chemotherapies, can impair fertility and endocrine function. The field of oncofertility creates options for preserving and restoring fertility in patients at risk of iatrogenic ovarian failure. Autotransplantation of cryopreserved ovarian cortical tissue has resulted in human live births, with short-term restoration of endocrine function. However, this technique carries the risk of reintroducing cancer cells since the tissue is removed prior to treatment. To minimize cancer transplant risk we bioengineered an artificial ovary consisting of follicles seeded within a 3D printed hydrogel scaffold modeled from the extracellular matrix (ECM) composition and architecture of decellularized bovine and human ovaries. The scaffolds were printed with gelatin in an intricate microporous pattern, engineered to accommodate multi-layered secondary ovarian follicles. Scaffolds having ‘tortuous’ architectures and porosities were investigated, including those printed with 30, 60 and 90 degree advancing angles. Ovarian follicles were supported in the artificial scaffold setting, as measured by survival, and were functional, based on measurement of secreted estradiol over 8 days and their ability to respond to luteinizing hormone (LH). The follicles settled within 3D printed follicle niche (3DP-FN) created by the overlaid struts and somatic cells along the follicle periphery made contacts with the struts through cell adhesion molecules, such as vitronectin. An essential feature of an ideal 3DP-FN was the number of engineered contacts; scaffolds that allowed for 2 or more contacts of the follicle within the niche out performed configurations that supported only 1 contact (81±1.7% versus 31±15.6% survival, n=113 follicles). Follicles within a tortuous 3DP-FN also released a meiotically competent egg in response to LH. Characterizing the ideal features of a transplantable engineered ovarian follicle niche is the first step in creating a safer artificial ovary that will provide both endocrine support and fertility to young women who have survived cancer.

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371.  Albumin concentration and uterine hemodynamics correlation after artificial insemination in Nellore cows.

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Deposition of semen in the uterus causes a local inflammatory reaction. Inflammation can be characterized by many processes that occurs in the animal body, among them is the hemodynamics and the production of acute phase proteins changes. In inflammatory processes is expected vascularization increase and acute phase proteins show a different pattern than when there is an absence of inflammation. There are two types of acute phase proteins, positive and negative. The positive ones are characterized by presenting concentration increase during inflammatory processes, while negative ones behave in a contrary manner. Considering that albumin is a negative acute phase protein, the aim of this study was to correlate the serum blood albumin concentration of cows and the data of the uterine hemodynamics, assessed by Doppler ultrasonography (uterine horns vascularization score (VS) and uterine artery resistance index (RI)). Were used 18 calved Nellore cows that were divided into two groups: inseminated (AIG, n = 9) and non inseminated (CG, n = 9). Uterine hemodynamics were evaluated using Doppler ultrasound in color mode, for the VS evaluation, and spectral mode, for RI evaluation, in five moments: 30 hours before artificial insemination (AI), 4, 24, 48 and 168 hours after AI. In the same moments, blood samples from the external jugular vein were collected. The serum samples were evaluated by automatic biochemical analyzer, with the aid
of specific kits, for the determination of albumin concentration. Data were analyzed using PROC CORR of SAS (SAS, 9.2, 2010). Statistical significance was considered when p < 0.05. No correlation was observed between albumin concentration and SV (R = 0.03, p = 0.077). A statistical trend with low negative correlation between albumin concentration and RI was observed (R = -0.20, p = 0.05). In these circumstances and using this amount of animals (n = 18), there is no correlation between serum albumin concentration and uterine hemodynamics data, assessed by Doppler ultrasonography. Research supported by FAPESP (process 2011/22833-9).

372. **Ovine oocytes from vitrified isolated secondary follicles grown in vitro are able to meiosis resumption after in vitro maturation.**

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Ovarian tissue cryopreservation has been an alternative for preservation and after recovery of reproductive ability at women subjected to cancer chemotherapy. However, reports shown indications of carcinogenic cells reintroduction after transplantation. In this context, the in vitro culture of secondary follicles after cryopreservation presents a viable tool to avoid cancer reintroduction and obtain fertilize oocytes. The goal of this study was to evaluate the development of secondary follicles vitrified included or free on ovarian tissue through their meiotic resumption. Sheep ovarian cortices (n=30) were cut into fragments of 1-2 mm and split into three different groups: a) Unfrozen Control Group (Unfrozen Control): secondary follicles were isolated without any previous vitrification; b) Follicle-Vitrification group (Follicle-Vit): secondary follicles were vitrified in isolated form c) Tissue-Vitrification group (Tissue-Vit): secondary follicles were vitrified within fragments of ovarian tissue, in situ form and subsequently subjected to isolation. Follicle isolation was performed by microdissection to recover secondary follicles with an average diameter around 280 µm. Solid Surface Vitrification (SSV) was performed with a cryoprotectant mixture of 2.60 M acetamid; 1.31 M 1,2-propanediol and 0.0075 M Polyethylene glycol in Minimum Essential Medium plus 10% fetal bovine serum solution. From all groups, isolated secondary follicles were submitted to in vitro cultured for 18 days. At the end of the culture period, the cumulus-oocyte complexes (COCs) were harvested from follicles. As an In Vivo Grown Control, in vivo-grown COC’s were collected from antral ovarian follicles. The COC’s were matured and the chromatin configuration was evaluated (calcine-AM, ethidium homodimer-I and Hoescht 33342). Data were analyzed by ANOVA means were compared by Student-Newman-Keuls test, and by Chi-square, the differences were considered to be significant when P<0.05. The number of oocytes recovered per cultured follicle were similar between both vitrified and unfrozen control (58.9%). Nonetheless, Follicle-Vit (66.6%) shows higher results (P<0.05) when compared to Tissue-Vit (40%). Oocyte diameters recovery from antral follicles grown in vitro (In vivo Grown Control) were significantly higher (131.0 ± 23.0 µm) then oocytes grown in vitro in all groups. The number of dead follicles were higher in Tissue-Vit group (33.3%) when compared with In Vivo Grown (6.6%), Unfrozen Control (8.6%), and Follicle-Vit (7.6%) groups. Concerning chromatin configuration, percentages of meiosis resumption (vesicle germinal breakdown – VGBD) in Unfrozen Control (34.7%), Follicle-Vit (43.3%) and Tissue-Vit (25%) groups were similar, nevertheless significantly lower to In Vivo Grown (86.6%). We emphasize such as In Vivo Grown and Unfrozen Control, only Follicle-Vit group shows oocytes in metaphase I (MI). Notwithstanding, were not obtained oocytes in metaphase II in all groups submitted to vitrification. Furthermore, only 4.3% (n=1) of oocytes in MI were observed in Unfrozen Control group, this value were significantly lower to In Vivo Grown group (53.3%). In conclusion our results indicate that sheep preantral follicles can be successfully vitrified and maintain the preservation of meiosis resumption after in vitro culture. However, using these in vitro culture conditions, only the follicles cultured without early vitrification achieve metaphase II stage.

373. **Evaluation of the expression of sperm proteins in normozoospermic and asthenozoospermic sperm samples using monoclonal antibodies, flow cytometry and fluorescence microscopy.**

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Asthenozoospermia (low percentage of motile sperm in an ejaculate) is one of the main seminal pathologies underlying male infertility. Previous proteomic studies have demonstrated the significant differences in the protein profiles between normozoospermic and asthenozoospermic sperm samples. Since these studies were primarily focused on the identification of differentially expressed proteins by mass spectrometry, we aimed to evaluate the ability of specific antibodies to detect the differential expression of selected protein markers by fluorescence microscopy and flow cytometry techniques. Therefore we analyzed sperm samples from 30 men with normal spermograms (>40% motile spermatozoa, average viability 78%) and 30 men with asthenozoospermia (<40% motile spermatozoa, average viability 74%) by the panel of our diagnostic anti-human sperm (Hs) antibodies. These antibodies were prepared in our laboratory and some of them are used in clinical practice as a tool for the differential diagnosis of various sperm pathologies. These pathologies do not include only decreased expression of proteins important for sperm energy metabolism, but also changes in protein composition of acrosome matrix related to the inability to undergo proper acrosome reaction, or similar changes related to surface proteins and to the inability to bind and fertilize an egg. Both fluorescence microscopy and flow cytometry analysis revealed quantitative differences in the protein abundances between normozoospermic and asthenozoospermic sperm samples, namely, in GAPDHs (glyceraldehyde phosphate dehydrogenase), evaluated with Hs-8 MoAb, VCP (valosin-containing protein), evaluated with Hs-14 MoAb, and ATP synthase (cAMP-dependent protein kinase II, PRKAR2A), evaluated with MoAb Hs-36. On the other hand no statistically significant differences were found in the expression of the sperm surface protein clusterin, evaluated with Hs-3 MoAb, and semenogelin, evaluated with Hs-9 MoAb. From the methodological point of view, we observed very high correlation between the data obtained by fluorescent microscopy and flow cytometry techniques (for example using Hs-8 antibody, r = 0.938, p ≤ 0.001) and therefore both methods are useful for evaluation of protein differences associated with asthenozoospermia. From the clinical point of view, we observed the strong association of the low sperm motility in the sample not only with the expression of proteins playing an important role in sperm energy metabolism (expected), but also with the expression of all tested intra-acrosomal proteins. These findings further demonstrate asthenozoospermia as a complex semen disorder frequently associated with other semen pathologies which are not diagnosed by basic semen analysis and the possibility to use monoclonal antibodies as a tool for diagnosis of protein associated sperm pathologies in the semen with the low sperm motility.

2015 Abstracts - Page 149
374. Mitochondrial dysfunction is associated with defective human sperm.
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Approximately 15% of sexually active couples are infertile. Male factor contribution is responsible of about 50% of the infertility cases. In fertile men usually produce a high amount of defective sperm cells that are unable to fertilize. Moreover, semen samples from infertile men are usually characterized by low count of sperm cells, which typically present low motility and abnormal morphology. Defective sperm function is a defined cause of human infertility, but the causes that produce malfunctioning sperm are unknown in 30 to 50% of cases. Mitochondrial dysfunction has been involved in the pathogenicity of several diseases, but its role in the production of defective human sperm remains scarce explored. Although plenty of functions are now known to be involved in the organelle, the principal role of mitochondria is to generate adenosine triphosphate (ATP) through the oxidative phosphorylation metabolic pathway, needed for a variety of cellular processes that assure the quality and the fertilizing ability of sperm cells. The correct production of mitochondrial ATP is challenged during mitochondrial dysfunction. High-resolution respirometry is able to detect variation of mitochondrial ATP in permeabilized and intact cells and tissues. In this study, we analyzed mitochondrial function by high-resolution respirometry in intact human sperm cells from 110 semen samples from men attending a fertility clinic. The samples analyzed presented a broad range of semen parameters. Since mitochondria is a well-known source of reactive oxygen species (ROS) that may lead to oxidative stress and dysfunction, the production of ROS in sperm was also analyzed. Sperm vitality, motility and morphology correlated with mitochondrial dysfunction. Sperm motility correlated with ROS production. Defective sperm cells evidenced oxidative stress as revealed by protein 3-nitrotyrosine, and the formation of protein-hydroxynonenal adducts. Moreover, pre-incubation of sperm with semen—that possess antioxidant properties—prevented the production of these species. These data suggest that dysfunctional mitochondria generate ROS and ROS in turn participate in the production of defective sperm cells. In spite of the diversity of mechanisms that may lead to mitochondrial dysfunction, the correlations found in this study suggest sperm mitochondrial alterations could contribute to male infertility. Furthermore, these data provide the rationale to test the potential of substances that improve mitochondrial function to treat male infertility.

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375. Non-invasive detection of metabolic heterogeneity in cow embryos as a predictor of developmental competence.
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The success of IVF depends on selecting the “best” embryos for transfer. While invasive and expensive technologies such as pre-implantation genetic diagnosis and time-lapse equipment have increased pregnancies rates in human clinics, selection based on morphology remains the most commonly utilised technique worldwide. Metabolic heterogeneity between individual blastomeres has been proposed within pre-compaction embryos; the degree of which is associated with quality but this is not measurable with current non-invasive assays. Therefore, we investigated if (non-invasive) fluorescence microscopy could determine embryo quality when cultured under optimum vs. stressed (7% vs. 20% O2) conditions. Cattle cumulus oocyte complexes were matured and fertilised as per standard methodologies. Between D1-8, presumptive zygotes were cultured in 7% or 20% O2. On-time blastocyst development (D8) was significantly reduced in embryos cultured in high O2 levels (7% O2 = 43.7±1.6% vs. 20% O2 = 32±2.9% blastocysts/cleaved, P<0.05). D5 embryos were stained with fluorophores sensitive to reduced glutathione (GSH; MCB), reactive oxygen species (ROS; PF1) and active mitochondria (Mitotracker Red). Poor quality/ arrested embryos had more active mitochondria and higher levels of ROS (P<0.05) compared to 7% O2 morula. Furthermore, compromised development was associated with decreased viability in blastomere metabolic phenotypes, with erratic localisation of fluorescence emissions between blastomeres within embryos (textural analyses of staining patterns), indicating variations in blastomere metabolism. Conversely, more consistent patterns of fluorescence were associated with on-time embryo development (morula staged embryos). This was particularly evident with higher uniformity of Mitotracker Red expression in 7% O2; morula compared to 20% O2 cleaved/arrested embryos (P<0.05). We used non-invasive hyperspectral fluorescence microscopy to analysis embryo autofluorescence (AF). The spectral images of D5 embryos were taken in 18 spectral channels and were analysed by unmixing contributions of individual metabolic co-factors. Variation in the molecular content between cells in each individual embryos were significantly higher in arrested, poor quality embryos compared to morula cultured in 7% O2 (P<0.05), further demonstrating that metabolic heterogeneity was associated with compromised embryo health. The AF (FAD and NAD(P)H) profiles of morula on D5 were determined as a non-invasive predictor of D8 development. Individual culture conditions and brief exposure to imaging at <1 microwatt did not affect developmental competence (main effect: -AF = 59.2±9.2 %, +AF = 76.9±7.4% blastocyst/morula; P<0.05). Morula that progressed to blastocyst stages on D8 had significantly higher levels of NAD(P)H and FAD AF compared to arrested morula (P<0.05). Furthermore, more advanced stages of blastocyst development (expanded and hatched) displayed less variability in the intensity of AF at the morula stage, compared to unexpanded blastocysts and arrested morula groups. In summary, metabolic heterogeneity exists within pre-implantation embryos, with increased heterogeneity associated with compromised development. Furthermore, non-invasive AF measurement of blastomere metabolism and its quantitative analysis offers a new tool for determining embryo health.
376. Embryonic secretome of bovine embryos produced in vitro with different developmental kinetics. 
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The ability to select embryos with greater viability is a crucial step to the success of assisted reproduction techniques. Currently, morphological assessments are used to select embryos with the highest implantation potential and, although it is a non-invasive and relatively successful method, it still has a number of limitations. In recent years, the development of new technologies has enabled quantitative and qualitative analysis to determine embryo viability and improve its selection. In this context, the evaluation of culture media secretome allows the identification of molecules that can be correlated with the potential of embryonic development and with embryonic viability biomarkers. Mass spectrometry (MS) technologies have been used, in particular, as a powerful tool for embryo characterization. Thus, this study aimed to perform a non-invasive secretome characterization of embryos with different cleavage kinetics at different embryonic stages in order to obtain specific patterns based on embryonic phenotype. For this purpose, bovine embryos were produced in vitro by standard protocols. The zygotes were transferred to individual culture media drops and divided into two groups: Fast (4 cells - 40 hour post insemination [hpi]) and Slow (2 or 3 cells - 40hpi). The culture media were collected at 40, 96 and 168hpi, transferred to cryotubes and kept frozen under -80°C until the time of analysis. The embryonic secretome analysis was performed by Matrix Assisted Laser Desorption Ionization (MALDI-TOF-MS). Metabolites were extracted through Bligh-Dyer technique and spectra were acquired in positive ionization mode on a mass range of 100-900 m/z. Univariate and multivariate analysis (Fold-Change and Partial Least Squares Discriminants Analysis, respectively) were performed using MetaboAnalyst 3.0 online software. Results show that embryos with different developmental kinetics also present distinct secretomic profiles during development, indicating a differential consumption and/or production of aminoacids metabolism. Thus, considering the results obtained, we propose that culture media analysis by MALDI-MS-TOF can be used for qualitative characterization of embryos secretome during in vitro culture.

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377. Complete disruption of RAG2 gene during porcine embryogenesis utilizing CRISPR/Cas9 system. 
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Severe combined immunodeficiency (SCID) large animals, such as RAG2 mutants, can serve as a useful animal model in stem-cell transplantation research field. Previously, RAG2 null pigs have been reported to show the capability of adopting human stem cells. One major shortcoming of these models is that they are sensitive to pathogen thus extremely difficult to maintain a line of homozygous mutants. Breeding heterozygous male and female can be an option, however, only a quarter of progeny will show the desired phenotype. Moreover, considering the longer gestation period of large animals, establishing founders and generating progeny by breeding is not cost effective. Targeted modification of the gene during embryogenesis could be an ideal solution, but no technology had been available until now. Recent development of the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR associated 9 (Cas9) system suggests that the technology is an effective tool to generate genetically engineered animals. CRISPR/Cas9 system causes a double-stranded break on a specific locus on genome then either insertion or deletion of the target gene can be introduced during DNA repair process. In this study, we examined the effectiveness of CRISPR/Cas9 system in inducing mutations on the RAG2 gene during porcine embryogenesis. Pig ovaries were collected at a local slaughter house and medium-size follicles were aspirated by 18-gage needle attached to a 10mL sterile syringe. Cumulus oocyte complex were then matured in vitro in a TCM-199 based maturation media containing 0.5IU/ml FSH, 0.5IU/ml LH, 0.82mM cysteine, 3.02mM glucose, 0.91mM sodium pyruvate, and 10ng/ml EGF. After 40hours of maturation, cumulus cells were removed by exposing the oocytes into a media containing 0.1% hyaluronidase. Oocytes extruded the first polar body were used for IVF. Gametes were co-incubated for five hours then presumable zygotes were transferred to Porcine Zygote Media 3 (PZM-3). Two sgRNAs, designed to target exon 1 of pig RAG2 gene, and mRNA coding for Cas9 were delivered into the presumable zygotes via microinjection. Embryos were then washed in PZM-3 and cultured for 7 days in an incubator at 38.5 °C, 5% CO2 and 5% O2. A total of 266 embryos, generated from 5 independent replicates, were used for this experiment. Cleavage stage embryos were 164 (61.6%) and the number of blastocysts on day 7 was 28 (17%). After 7 days, blastocysts were selected randomly and DNA was collected in a lysis buffer. We analyzed 20 blastocysts out of 28 for this experiment. The DNAs were used to amplify a target region, flanking projected cutting site by CRISPR/Cas9 system on the RAG2 genome, by PCR. Subsequent PCR amplicons were sequenced to identify targeted modification of RAG2. We have found that modification on RAG2 genome on all blastocysts examined demonstrating 100% targeting events; 9 with homozygous mutations, 9 with biallelic mutations, and two embryos with mosaic genotype. No wild type RAG2 sequence was found, indicating that any development from these embryos would result in the lack of functional RAG2 in founders. Our results on pig embryos suggest that potentially all founders produced through the CRISPR/Cas9 technology can be a null model. We demonstrate here that CRISPR/Cas9 system is an efficient system in generating genetically modified animals in livestock.

378. Distribution of Follicles in Canine Ovaries. 
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The cryopreservation of ovarian tissues is a technology with significant potential for the preservation of the genetic resource materials of working dogs, including guide dogs for the blind. Although possible vitrification and follicular survival subsequent xenotransplantation of canine ovarian tissues was reported, degree of density and distribution of follicles in ovarian tissues seems to affect interpretation of results after transplantation of the ovarian tissues. It has been reported that the follicles are not homogeneously distributed within the ovarian cortex in humans. However, to our knowledge, no studies have so far assessed the distribution pattern of follicles in dog ovary. In this study, we evaluated the distribution of follicles within canine ovarian cortex to estimate follicular homogeneity that should be analyzed to limit the impact of follicular heterogeneity on experimental results. Ovaries were collected from 14 immature bitches. For each bitch, five samples of the ovarian fragments (1mm³) were embedded in OCT compound. Sections (5-µm-thick) were cut perpendicularly to...
the ovarian surface on a cryostat. After drying for 2 min, the sections were stained with 0.05% of methylene blue. To evaluate density and distribution of follicles, the mean number of follicles per mm² was calculated under a microscope (BZ-9000, KEYENCE). Subsequently, variance, median and distortion for density of follicles were calculated using Microsoft Excel. The mean number follicles ranged from 3.7 to 15.6/ mm² in 14 ovaries examined. The variance and distortion ranged from 2.05 to 144.30 and -2.09 and 2.01, respectively. When variance was less than 10, or more than 10 less than 16 with absolute value less than 1 of distortion, the distribution of follicles was defined as even. As a result, distribution of follicles within ovarian cortex in 10 of 14 bitches was judged uneven. These results indicated that follicles were not homogeneously distributed within the ovarian cortex in majority of bitches. To maximize the reproducibility of experimental results, it is essential to assess the degree of density and distribution of follicles in ovarian tissues before transplantation experiments.

379. Generation of transgenic cattle using transposon system.
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380. Expression and Function of Toll-Like Receptors in Human Embryos.
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381. Invasion of trophoblasts derived from human embryonic stem cells through Collagen type I.
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preeclampsia. Studying TB invasion in human placentation in vivo is difficult. However, human embryonic stem cells (hESCs) can be efficiently differentiated into TB in vitro by treatment with BMP4 along with Activin/Nodal signaling inhibitor A83-01 and the FGF2 signaling inhibitor PD173074 (BAP treatment), providing a potential means for studying the mechanisms whereby early TB invade. We have previously demonstrated invasion through Matrigel by BAP-treated hESCs, but in vitro invasion of TB into the maternal decidua and spiral arteries involves a larger range of extracellular matrix materials than Matrigel- a mixture of laminin, collagen IV, and a number of bound growth factors. Collagen type I is the toughest fibrillar structural component of the extracellular matrix that TB cells encounter, degrade and modify in order to invade into spiral arteries. The goal of the present study is to test whether TB created by treating hESCs with BAP are capable of invading through type I collagen. Initial experiments demonstrated that hESC plated directly on collagen type I substratum spontaneously differentiate to a non-TB lineage. As an alternative, we cultured cells first on a Matrigel substratum and treated them with BAP for 1 day before transfer to Boyden chambers equipped with membranes (8 µm pores) coated with collagen type I from human placenta. In this case, plating efficiency was low. The BAP-treated cells were then passaged onto a thin layer of Matrigel coating the collagen layer to facilitate attachment. This strategy was successful, possibly because Matrigel is more analogous to what is confronted initially by invading TB, which must change their attachment and proteolytic capabilities as they encounter different matrix materials surrounding maternal cell layers. After 6 days cultured under these conditions in the presence of BAP, cells on the top of the membrane were scraped off and the invaded cells on the bottom of the membranes counted and assessed for TB markers by immunohistochemistry. BAP-treated hESCs were able to invade through a layer of collagen prepared at concentrations of 60, 235 and 470 µg/ml. At the highest concentration of collagen (470 µg/ml), preliminary results show approximately four-times more BAP-treated cells invaded than the undifferentiated control cells maintained in the same medium without BAP and with FGF2. Further, invaded cells from BAP-treated hESCs cultures stained positively for TB markers cytokeratin 7 (KRT7), whereas the small number of invaded cells in control cultures did not. Future aims include investigating BAP-treated hESC interactions with arterial endothelial cells and comparison of invasion between BAP-treated, induced pluripotent stem cells derived from umbilical cord tissue of preeclamptic pregnancies and normal pregnancies. Development of these in vitro models of TB invasion show great promise in the pursuit of the underpinnings of placental disorders such as preeclampsia. Supported by NIH grant HD067759

382. Replacing Serum In Culture Media For Bovine Embryos. Are We Producing Better Embryos?
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When mammalian embryos are produced in vitro, an increased incidence of overgrowth syndromes, such as the Beckwith-Wiedemann syndrome in human and the “Large Offspring Syndrome” in ruminants has been reported. More specifically, the “Large offspring syndrome” has been associated with the addition of serum to the culture medium of ruminant embryos. For this reason, bovine serum albumin (BSA) has been used to replace serum for cattle embryo production. Culture medium supplemented with BSA yields slower blastocyst development, with better freezability but still lower pregnancy rates than in vitro derived embryos, which remain the gold standard for embryo development. In this study we used RNA-sequencing to evaluate if supplementation with BSA is generating more in vivo-like embryos as compared to serum. To this end, we compared the global gene expression pattern of in vivo derived bovine embryos to that of embryos produced in two different culture conditions, which were either supplemented with serum or with BSA. Twenty-four blastocysts were used in the study, 8 obtained in vivo from superovulated cows, 8 produced in vitro in serum-free conditions (TCM-199 + 20 ng/ml EGF for maturation and Synthetic Oviduct Fluid (SOF) + 0.4 % BSA + 5 µg/ml Insulin + 5 µg/ml Transferrin + 5 ng/ml Selenium for culture) and 8 blastocysts produced in serum conditions (TCM-199 + 20 % Fetal Bovine Serum (FBS) for maturation and SOF + 5 % FBS for culture). The sex of the blastocysts was determined by PCR before RNA-seq, and at least 3 blastocysts of each sex per group were selected for the study. Blastocysts produced in the presence of serum had ten times more genes differentially expressed with respect to in vivo embryos (1106), than the blastocysts produced in serum-free conditions (110). Most genes which were differentially expressed between in vivo and both in vitro conditions were up-regulated under in vitro conditions, supporting the theory of the quiet embryo being the best. The genes differentially expressed in the embryos produced in the presence of serum were mainly involved in RNA processing and mitosis while the genes differentially expressed between embryos produced in serum- free conditions and in vivo embryos were involved in lipid metabolism. Surprisingly, male embryos of both conditions were more affected by in vitro conditions than their female counterparts, showing a gene expression pattern which was more deviant from in vivo derived embryos. This phenomenon was especially evident in serum conditions, where male embryos had eight times more genes differentially expressed compared to in vivo derived embryos than the female embryos. In conclusion, in vitro culture conditions for bovine embryos are not optimal yet, even after serum exclusion. The replacement of serum by BSA had a positive effect in the gene expression pattern of the embryos, although their lipid metabolism was still altered. Interestingly, embryos of different sex respond differently to the environment, the male embryos being more sensitive to suboptimal conditions than the females.

383. Procaine Induces Parthenogenetic Cytokinesis In Horse Oocytes Via A Ph Dependent Mechanism.
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Previous reports have suggested that co-incubating equine gametes in the presence of 5 mM procaine facilitates in vitro fertilization, with cleavage rates exceeding 60% of incubated oocytes. It has been suggested that procaine promotes sperm penetration by inducing hyperactivated motility to accompany other sperm capacitation events, triggered by other medium conditions like elevated medium pH and evidenced by tail-associated protein tyrosine phosphorylation. In this study, we demonstrate that while procaine does
trigger hyperactivation in tail-associated protein tyrosine phosphorylated sperm, it also activates equine oocytes. First, by both pre-labeling sperm with MitoTracker Green FM (MTG) and by lacmoid staining post-fixation, we showed that equine sperm do not penetrate equine oocytes, either in the absence or presence of 0.1, 2.5 or 5 mM procaine. Instead, procaine induced a sperm-independent activation of oocytes followed by cleavage. In our hands, 2.5 mM procaine induced 56 ± 4% of oocytes to cleave after 2.5 d in culture. However, cleaved oocytes did not develop further than the 8-16 cell stage and the daughter cells either lacked nuclei or contained condensed DNA fragments rather than normal nuclei. By contrast, oocytes fertilized by ICSI were able to develop to the blastocyst stage (15 ± 6% of injected oocytes) and all embryonic cells contained normal nuclei. Next, we assessed the role of calcium in oocyte activation during 6 h incubation with 2.5 mM procaine using the ratiometric fura-2 AM probe, and LCA-FITC staining to visualize the cortical reaction after 6 h incubation. In marked contrast to ICSI and ionomycin-activated oocytes, oocytes exposed to procaine showed no increase in cytoplasmic calcium, and neither was the cortical reaction initiated. Moreover, the ratiometric pH probe BCECF-AM indicated a procaine-dependent ooplasmic alkalization. This was followed by a procaine-dependent cortical F-actin depolymerization after 18 h incubation, as demonstrated by reduced actin phalloidin-FITC intensity. We conclude that procaine induces parthenogenetic cytokinesis in equine oocytes and, despite its ability to induce hyperactive sperm motility, does not support in vitro fertilization. (Supported by IWT-Flanders, grant number 101521).

384. The use of cholesterol-loaded cyclodextrin in low freezability sperm improves cryopreservation in rams, Leonardo Batissacco1, André F. Andrade2, Rubens P. Arruda1, Maira B. Alves1, Mariana A. Torres1, Kleber M. Lemes1, Tician G. Leite1, Roberto R. Prado Filho1, Tamie d. Guibu1, Bruna M. Oliveira1, Eneiva C. Celeghin1.

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Frozen ram semen has been the subject of many researches, especially due to the loss of post-thaw sperm viability caused by osmolarity and conformation changes the sperm undergoes, resulting in reduced fertility. Moreover, there is a major problem in defining a freezing technique, especially due to freezability differences between animals and even between ejaculates. The addition of cholesterol through cyclodextrin increases the cholesterol: phospholipids proportion on sperm membranes, leading to a state similar to those sperm unharmed from thermal shock caused by freezing. Based on this information, ram semen was treated with cholesterol-loaded cyclodextrin prior to cryopreservation and observed its viability post thawing in different sperm freezability groups. Five ejaculates from six rams were divided in the control (only extender) and treatment (extender + cholesterol-loaded cyclodextrin) groups. These samples were cryopreserved. The same samples were divided by freezability based on the total post-thaw motility and the percentage of reduction in total motility when compared fresh to post-thaw semen (only extender), being then divided into the high, intermediate and low freezability groups. The samples were analyzed for motility (CASA), plasma (PI-H342) and acrosomal (FITC-PSA) membranes integrity, and mitochondrial potential (JC-1). Comparisons were performed by analysis of variance (ANOVA) in a 2 X 3 factorial arrangement, with a main effects of treatment (control and treatment) and freezability (low, intermediate and high) and their interaction. Data were analyzed using PROC MIXED procedure of SAS software. Means were separated by the Tukey test (p <0.05) or non-parametric statistics (Kruskal-Wallis), when necessary. Data showed that the treatment group had higher values of sperm preservation when compared with the control group in the low and intermediate freezability ejaculates, observed in total (intermediate = 45.77 ± 2.82 vs. 73.47 ± 3.18; low = 14.47 ± 2.84 vs. 47.01 ± 6.54) and progressive (intermediate = 25.98 ± 2.54 vs. 40.58 ± 4.01; low = 5.53 ± 1.11 vs. 23.15 ± 5.40) motility, rapid cells (intermediate = 28.33 ± 2.99 vs. 47.25 ± 6.13; low = 5.06 ± 1.06 vs. 23.07 ± 6.40), plasma (intermediate = 13.07 ± 2.33 vs. 27.15 ± 3.64; low = 6.23 ± 2.78 vs. 14.92 ± 4.26) and acrosomal (intermediate = 40.69 ± 4.45 vs. 65.81 ± 4.75; low = 29.23 ± 6.89 vs. 50.47 ± 4.16) membranes and mitochondrial potential (intermediate = 34.05 ± 4.83 vs. 57.30 ± 5.69; low = 21.87 ± 6.71 vs. 30.73 ± 8.58). No significant differences were observed in the high freezability group. Through these results is possible to conclude that treatment with cholesterol-loaded cyclodextrin contributes to better preservation of sperm parameters in ram semen, moreover, has different results depending on the freezing ability of each ejaculate.


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The financial incentive to produce high quality animals has led to major improvements in assisted reproduction techniques in horses. A fairly new technique in Brazil for use in equine is intracytoplasmic sperm injection (ICSI). In 2012, In Vitro Clonagem produced two foals by ICSI, the first in Brazil. In this initial year, our maturation, blastocyst and pregnancy rates were 55%, 42%, and 25.4% respectively. We cultured embryos in DMEM/F12 medium with 10% serum after ICSI until the day of transfer. We also transferred embryos using the same medium. Our objective was to build on this result to improve oocyte maturation, blastocyst and pregnancy rates by altering the embryo culture medium and embryo transfer medium. For the 2014 season, 16 mares with an average age of 19 years were utilized. Immature oocytes, from follicles ranging from 15 to 30 mm in diameter, were collected by follicular aspiration. Oocytes were matured for 24 hrs in equine maturation media at 38.5°C in 5% CO2. ICSI was performed using fresh/frozen semen and injected using a beveled pipet. The embryos were cultured in C4 media for 4 days and transferred into DMEM/F12 medium with 5% knockout serum replacement and 5% serum until transfer on day 7. A total of 94 oocytes from 165 follicles (57%) were collected, giving an average recovery of 5.8 oocytes/mare. Maturation, cleavage and blastocyst rates were 45.7% (43/94), 41% (18/43) and 34.9% (15/43), respectively. All 15 blastocysts were transferred into synchronized recipients resulting in 3 pregnancies (20%). We did not see an improvement in embryo production or pregnancy rates compared to our 2012 results. Although work in equine present many obstacles, the high economic value of each animal justifies the costs necessary for foals through the use of assisted reproduction techniques such as ICSI and further research and development aimed at embryo culture and transfer medium should be done to increase performance.

386. DEVELOPMENT OF HUMAN INSULIN SECRETING PIGS FOR XENO-ISLET CELL TRANSPLANTATION.
Diabetes mellitus is a metabolic disease caused by impaired insulin secretion from the pancreatic beta cells and increased insulin resistance in peripheral tissues. Islet transplantation is considered an effective therapy of diabetes mellitus type 1. Recently, attempts of xenograft cell transplantation using pig islet cells are in the spotlight of transplantation research due to anatomical and physiological similarity between pig and human. Since porcine insulin secreted from xenograft islet is derived from the pig, an extended therapy period in the human body might cause side effects such as antibody formation, allergic response and immune rejection. So, secretion of human insulin from transplanted xenograft islet cells might solve these immunological problems derived from long period transplantation. First, an expression vector harboring the full sequence of human insulin was constructed. The vector was transfected into porcine fibroblasts by electroporation. After transfection, cells were harvested through antibiotic selection for two weeks. Transgenic porcine fibroblasts containing the gene for human insulin controlled by the human insulin promoter, as well as an antibiotic resistance cassette, the neomycin resistance gene, were established. These fibroblasts provided a source of cells for somatic cell nuclear transfer to generate a pig model that produces human insulin in response to stimuli similar to the human islet. Somatic cell nuclear transfer was performed, followed by embryo transfer. A single surrogate established a pregnancy and is due to farrow next month. If transgenic piglets are produced, their offspring could be useful sources of islet cells for xenotransplantation. Our next goal will be to knock out swine insulin so that these pigs produce only human insulin.

387. Cryopreservation of canine spermatozoa with skim milk based extender by brief equilibration.

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Cryopreservation of canine spermatozoa provides potential exchange of genetic materials, and may lead to improvement in the breeding management programs used to produce working dogs. In particular, application of transcervical artificial insemination using cryopreserved canine semen may contribute to adequate supply of guide dogs for the blind. We previously reported that skim milk is available as cryoprotectant for cryopreservation of canine spermatozoa instead of egg yolk that disturbs the transportation of frozen semen whenavian influenza is prevalent. However, long term equilibration after dilution with skim milk based extender is required before freezing of spermatozoa. In this study, we examined the optical equilibration term, and showed the delivery of pups derived from spermatozoa frozen by a brief equilibration. Collected ejaculates were diluted with the first extender composed of skim milk and raffinose (SR extender) to give a sperm concentration of 2 × 10^7 sperms/ml at 4C or room temperature (RT), and then they were cooled to 4C (first equilibration). After the first equilibration for 0 to 180 min, the equivalent volume of the second extender, which was SR extender supplemented with 14% (v/v) of glycerol at 4C, was added to the semen aliquots. The semen samples were left at 4C for 15 min (second equilibration), loaded into a 0.25-ml straw, and then were immersed into liquid nitrogen. The post-thaw semen samples were examined the sperm motility parameters by computer-assisted sperm analysis. The proportion of total motile spermatozoa of the samples without equilibration after extending with SR extender at RT (26.7 ± 12.9%) was lower than those of equilibrated samples (30min, 32.5 ± 3.7%; 60 min, 32.2 ± 10.5%; 120 min, 43.7 ± 10.5%; 180 min, 28.5 ± 17.2%). However, when ejaculates were diluted with cooled extender (4C) without the first equilibration, proportion of total motile spermatozoa improved to 38.3 ± 11.8%. To examine the fertilizing capacity, frozen spermatozoa after extending at RT or 4C without the first equilibration were transcervically inseminated into 4 or 5 recipient bitches, respectively. The cooled extender group yielded 7 pups from 4 recipient bitches, and the delivery rate was same with standard manner that was equilibrated in SR extender for 180 min. However, any recipients did not become pregnant when semen was extended with the SR at RT without the first equilibration. These results showed that when skim milk based extender at 4C is used, the first equilibration is not essential for the cryopreservation of canine spermatozoa. This improvement may contribute to both efficient exchange of genetic materials and production of guide dogs for the blind.

388. Characteristic analysis of a donor cell affecting an efficiency of bovine cloning by somatic cell nuclear transfer.

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For successful animal cloning by somatic cell nuclear transfer (SCNT), not only the quality of oocytes or handling techniques but the quality of donor cell is also an important factor. Although there have been various studies on the effects of donor cell quality cloning success rate, the selection criteria of donor cell is still deficient. Hence, we aimed to investigate the distinct characteristics of donor cells with different developmental competency. Two fibroblast cell lines (Line 1 and Line 2) derived from ear skin of different individual adult cow were analyzed. In the evaluation of the development following SCNT, a formation rate of blastocyst derived from Line 1 was significantly higher compared to Line 2 (34.7% vs 20.9%). By contrast with the full-term pregnancy, following SCNT embryo transfer, rate were rather the opposite (0.0% vs 15.2%). This interesting finding demonstrated that high in vitro developmental competency cannot always ensure high production efficiency of animal cloning. To investigate genetic factors affecting successful cloning, the differential expression genes (DEGs) were identified by the comparison of mRNA of two cell lines using next generation sequencing (NGS), and DNA methylation pattern was characterized by bisulphite sequencing. By combining the two information, several genes were identified that showed significant difference in their expression profile. Additionally, the oxygen consumption of the respective cell lines were measured to determine mitochondrial function by XF cell mito stress test. We confirmed that the results of oxygen consumption rate were consistent with efficiency of full-term development. The present results suggest that the success of animal cloning is critically dictated by the quality of donor cell, and some differential expression genes and oxygen consumption have an applicability as a novel marker of donor cell which has high cloning efficiency. This research was supported by a grant of Research Program (No. 307-02) Gyunngi-do Project, Republic of Korea. This work was also supported by the grant of Bio-industry Technology Development Program of iPET (Korea Institute of Planning.
389. **Intracytoplasmic injection of deionized bovine serum albumin immediately after somatic cell nuclear transfer enhances developmental potential of cloned mouse embryos.**

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In mouse somatic cell nuclear transfer (SCNT), the Honolulu method, polyvinylpyrrolidone (PVP) is usually added to a nuclear donor injection medium to increase its viscosity, thereby facilitating handling for intracytoplasmic injection. However, it was previously reported that PVP introduced into bovine embryos by the intracytoplasmic sperm injection technique is not digested by lysosomal enzymes and suppresses their developmental capacity. Thus, concerns over toxic effects of PVP on embryos prompted us to use bovine serum albumin (BSA) as an alternative to PVP in SCNT. As a result, we found that nuclear donor injection medium with 6% deionized bovine serum albumin (d-BSA) instead of 6% PVP is applicable for the production of mouse SCNT embryos using the Honolulu method, and unexpectedly, 6% d-BSA injected together with a donor cumulus cell nuclei into an enucleated oocyte significantly increased the rates (blastocyst formation rates, BFRs) of blastocysts developing from cloned 2-cell embryos that had been produced from the reconstructed oocytes after oocyte activation (activation), including the inhibition of second polar body formation, with strontium ion and cytochalasin B treatments, compared with those by 6% PVP (43.1%, 28/65 vs. 23.4%, 19/81, respectively). In the present study, we aimed to more precisely define the effects of d-BSA injection into reconstructed oocytes on the in vitro and in vivo development of B6D2F1 cloned mouse embryos. We firstly performed experiments in which, immediately after the production of reconstructed oocytes by fusion of an enucleated oocyte at the MII stage and a donor cumulus cell using hemagglutinating virus of Japan envelop (HVJ-E), around 10 pl of various concentrations (0 to 12%) of d-BSA containing Hepes-CZB medium was injected into the cytoplasm of the reconstructed oocytes before or after activation, followed by the in vitro culture of cloned embryos for 96 h in KSOM medium. It was found that 6% d-BSA injected into the oocyte cytoplasm before activation significantly increased the BFR (65.1%) compared with those in 0, 1, and 12% d-BSA injection (34.8, 29.4, and 48.7%, respectively) and the numbers of inner cell mass (ICM) cells in the resultant blastocysts from the 0 or 12% d-BSA injection group (10.6 and 12.1, respectively) were significantly higher than those from the 0 or 1% d-BSA injection group (6.7 and 8.1, respectively), and that the BFRs were not promoted by the d-BSA injection 6 h after activation, in the pronuclear (PN) stage. Furthermore, we found that the enhancing effects of 6% d-BSA injection were exerted to a similar extent by 6% deionized human serum albumin (d-HSA) injection but not by 6% deionized bovine gamma globulin (d-gamma G) injection (BFRs; 60.0, 52.1, and 32.8%, respectively). Surprisingly, immunofluorescence staining revealed that 6% d-BSA injection before activation had an ability to increase the acetylation levels of histone H3K9 and H4K12 in cloned PN and 2-cell embryos, compared with 6% d-BSA injection at the PN stage and in the non-injection control. Finally, to examine the in vivo developmental competence of cloned embryos, embryo transfer of 2-cell embryos was conducted. Live offspring were obtained from the 6% d-BSA injection before activation group (n=110, 2.7%), but not from the 0% d-BSA injection group (n=130). Based on the results, d-BSA injection into reconstructed oocytes before oocyte activation may improve the nuclear reprogramming of somatic cells.

390. **Knock-in Fibroblasts and Transgenic Blastocysts for Expression of Human FGF2 in the Bovine β-Casein Gene Locus using CRISPR/Cas9 Nuclease-Mediated Homologous Recombination.**

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Many transgenic domestic animals have been developed to produce therapeutic proteins in the mammary gland, and this approach is one of the most important methods for agricultural and biomedical applications. However, expression and secretion of a protein varies because transgenes are integrated at random sites in the genome. In addition, the distal enhancers are very important for transcriptional gene regulation and tissue-specific gene expression. Development of a vector system regulated accurately in the genome is needed to improve production of therapeutic proteins. The objective of this study was to develop a knock-in system for expression of human fibroblast growth factor 2 (FGF2) in the bovine β-casein gene locus. The F2A sequence was fused to the human FGF2 gene and inserted into exon 3 of the β-casein gene. We detected expression of human FGF2 mRNA in the HC11 mouse mammary epithelial cells by RT-PCR and human FGF2 protein in the culture media using western blot analysis. We transfected the knock-in vector into bovine ear fibroblasts and produced knock-in fibroblasts using the clustered regularly interspaced short palindromic repeats (CRISPR/Cas9) system. Moreover, the CRISPR/Cas9 system was more efficient than conventional methods. In addition, we produced knock-in blastocysts by somatic nuclear cell transfer using the knock-in fibroblasts. Our knock-in fibroblasts may help to create cloned embryos for development of transgenic dairy cattle expressing human FGF2 protein in the mammary gland via the expression system of the bovine β-casein gene.

391. **Global gene expression in slow-freezing and vitrified bovine embryos produced in vitro.**

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Embryo transfer technology in cattle exploits the genetic potential of superior dams and sires. In the present scenario of food markets’ focus on few high-producing breeds as well as emerging animal diseases, conservation of genetic diversity among livestock species is important. Cryopreservation is a common and useful tool in both embryo transfer and conservation programs. However, it is a harmful procedure and has known adverse effects on bovine embryonic development. To understand the mechanism of cryo-damage at molecular level, we compared the global gene expression in in vitro produced bovine embryos following cryopreservation using vitrification (VIT) and slow-freezing (SF) methods versus the unfrozen embryos (control group, CON). Bovine morulae (D6; D0=IVF)
were randomly assigned to VIT (n=61), SF (n=64) or CON (n=64) groups. CON and post-warm VIT and SF morulae were further cultured to blastocyst stage (D8). RNA was extracted from a pool of 3-7 viable blastocysts per group in each replicate. Four biological replicates were used to compare VIT and SF embryos with CON embryos (reference group) using dye-swap method on EmbryoGene platform BESTv1-4X44K microarray slides. The microarray data were analyzed using FlexArray (1.6.3) and Ingenuity Pathway Analysis softwares. Differential gene expression from microarray data was further confirmed with quantitative real time PCR. Data revealed a total of 64 genes expressed differentially between VIT and CON embryos (p<0.05). Vitrification changed the expression of genes related to lipid metabolism, cell adhesion, cell movement and molecular transport pathways. In VIT embryos, PTGS2, CYP11A1 and FADS2 genes related to eicosanoid signaling and fatty acid biosynthesis were downregulated. Other downregulated genes in VIT group included IFI30, CLDN23, Muc1 (involved in early embryonic growth and adhesion), SLCO1A1 and SLC44A4 (involved in inter-cellular molecular transport). These genes are directly or indirectly involved in embryo implantation. Likewise, a total of 162 genes expressed differentially between SF and CON embryos (p<0.05). SPP1 and VIM genes related to cell morphology, signaling and maintenance were downregulated but apoptosis-related genes (TRAF1, P4HB, KRAS and HSPB8) were upregulated. In conclusion, cryopreservation using vitrification and slow freezing methods affected different groups of genes and pathways during morula to blastocyst transition. Results from this study will help to modify and improve the existing cryopreservation media and methods for animal and human assisted reproductive technologies. Funded by research grants from NSERC Canada and Agriculture and Ag-Food Canada.

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Sal-like 1 is essential for kidney development and kidney abnormalities caused by this gene mutation. Defects in Sal-like 1 were caused Townes-Brocks syndrome in humans. Mice deficient in Sal-like 1 show kidney agenesis or severe dysgenesis. In this study, we were produced knock-out cell for Sal-like 1 gene locus using TALENs in the porcine. The Sal-like 1 targeting vector consisted 5.5kb of 5’ arm region and 1.8kb of 3’arm region, and neomycin resistance gene (neo) as a positive selection marker gene. Knock- out vector and TALEN were introduced in the porcine fibroblasts by electroporation. Antibiotics selection was performed 11 days using 300 ug/ml G418. After selection, PCR analyzed for G418-resistance colonies and 3’arm, 5’arm PCR analyzed for genomic DNA of G418-resistance cells. In addition, mono- allelic was isolated and identified by PCR-RFLP analysis. TALEN-assisted homologous recombination induced mono-allelic knock out of an endogenous gene.

393. Possibility of production of cloned lycaon by inter-genus somatic cell nuclear transfer.
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Reproductive cloning, or the production of offspring by interspecies nuclear transfer, is often recognized as having potential for conserving endangered species of wildlife. The African wild dog (Lycaon pitus) is one of endangered canid. We have attempted to clone the lycaon by intergenus somatic cell nuclear transfer (iSCNT) using domestic dog oocytes as recipient cytoplasts. Primary cells were established from femoral region skin biopsy of 3-years-old male and 1-year-old female lycaon. Oocyte donor dogs in natural estrus were subjected to surgical oviduct flushing for in vivo matured oocyte collection following ovulation detection by evaluation of serum progesterone level and ultrasonographic examination. In vivo matured oocyte were collected from bitches and were enucleated at the metaphase II stage, electrofused with lycaon somatic cells, and simultaneously activated a second time to induce genome activation. Total, 578 male and 625 female lycaon-domestic dog iSCNT embryos were reconstructed. Subsequently, these embryos were respectively transferred to 40 and 44 surrogates. Transabdominal real-time ultrasonography was used to detect the pregnancy at 30 days. Two surrogates were identified as pregnant. However, fetal retardation with no cardiac activity was observed by repeated ultrasound examination for 6 weeks. An exploratory laparotomy was performed at 46 day and 39 day gestation period, respectively and revealed mid-term pregnancy loss. One pregnancy was resulted in fetal mummification, and the other pregnancy was observed in fetal absorption and the growth retarded fetus with a massive hemorrhage (crown rump length: 2cm). Microsatellite analysis confirmed that the cloned lycaon abortus were genetically identical to the lycaon cell, whereas mitochondria analysis revealed that mtDNA of the lycaon abortus is transmitted by oocyte donor dog. In this study, we produced cloned lycaon embryos by iSCNT and confirmed mid-term pregnancies. Unfortunately, production of cloned lycaon by iSCNT was not fully success. However, this result has presented new possibilities of production of endangered canid by iSCNT for conservation.
This work was supported by a grant from the Next-Generation Bio Green 21 Program, Rural Development Administration, Republic of Korea.

394. Seasonal influence on reproductive parameters for production of clone dog.
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Canine cloning is less developed compared to other species due to unique characteristics. Canids are monoestrus, polyovulatory and polytocous with a non-seasonality due to long domestication in the dog. The aim of this study was to investigate the influence of season on recovery of matured oocytes by oviduct flushing, the oocytes maturation period after ovulation, and the pregnancy rate after cloned embryos transfer via surgical transfer. The year is classified into four seasons viz., spring (March-May), summer (June-August), autumn (September-November) and winter (December-February). These analyses are conducted for oocyte donor dogs and surrogates transferred cloned embryos for a year. In first survey, 999 oocyte donor dogs were evaluated the number of oocyte in vivo retrieved by oviduct flushing. The number of recovered in vivo oocyte was significantly higher in spring than other seasons (spring n=184, 12.6±3.6, summer n=364, 11.2±3.6, autumn n=255, 10.7±3.6, winter n=196, 10.8±3.8). Oocytes maturation time after ovulation were significantly elongated in spring and winter than summer and autumn (spring n=93, 110.8±8.8h, summer n=212, 106.3±8.6h, autumn n=159,
395. Supplementation with trehalose during in vitro maturation increases preimplantation embryonic development in porcine. Yubyeol Jeon¹, Yeon-Ik Jeong¹, Yeon Woo Jeong¹, Yeun-Wook Kim¹, Sang-Hwan Hyun², Il-Seok Yang¹, Woo Suk Hwang¹.

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Autophagy is a critical process for the maintenance of cellular homeostasis and mammalian early embryogenesis. Autophagy can be regulated by various chemical inducers. However, there are few reports about effect of autophagy inducer in vitro maturation (IVM) of porcine oocyte. The present study investigated the effects of supplementary trehalose, a novel mTOR-independent autophagy enhancer, on oocyte maturation and embryonic development after parthenogenetic activation (PA). Immature oocytes were treated with various concentration (0, 25, 50 and 100mM) of trehalose in M-199 (Invitrogen, Carlsbad, CA) supplemented with 10 ng/mL epidermal growth factor (EGF; Sigma-Aldrich Corp.), 1 ug/mL insulin (Sigma-Aldrich Corp.), 4 IU/mL of pregnant mare serum gonadotropin (PMSG; Intervet, Boxmeer, Holland), 4 IU/mL of human chorionic gonadotropin (hCG; Intervet) and 10% (v/v) porcine follicular fluid (pFF) for 10hr, and transferred to another IVM medium without trehalose. Osmolality of each groups (0, 25, 50 and 100M trehalose) was in the 290-295, 310-315, 330-335 and 375-380 osmol range, respectively. After 44hr of IVM, trehalose treatment during IVM did not improve nuclear maturation rates of oocytes in any group (90.7, 92.1, 92.7 and 90.1%, respectively). The developmental competence of oocytes matured with different trehalose concentrations was evaluated after PA. There were no significantly different in cleavage rates. However, blastocyst (BL) formation were different. Oocytes treated with 25mM of trehalose during IVM had a significantly higher (P < 0.05) BL formation rate (64.2%) after PA compared with the control (52.0%). BL quality was also improved in 25mM of trehalose treated group. Early BL rate significantly reduced in 25mM of trehalose treated group as compared to control (19.6% vs. 29.9%, P < 0.05). By contrast, expanded BL rate significantly increased in 25mM of trehalose treated group that of control (27.7% vs. 11.0%, P < 0.05). Total cell numbers of BL were significantly higher (P < 0.05) in 25mM of trehalose treated group compared to those in the control group (52.2 vs. 36.8). However, BL rate and quality of oocytes treated with 50 and 100mM of trehalose were similar with control group. In conclusion, these results indicate that 25mM of trehalose during IVM improved the developmental potential of porcine embryos. Trehalose will be useful for large scale production of BL with good quality in porcine in vitro production.

This work was supported by a grant from the Next-Generation Bio Green 21 Program, Rural Development Administration, Republic of Korea.

396. Efficient production of pigs overexpressing 11β-HSD1 by re-cloning. Eun-o Park¹, Yeon-Ik Jeong², Yubyeol Jeon¹, Kyeong Hee Ko¹, Yeon Woo Jeong¹, Yeun-Wook Kim¹, Sang-Hwan Hyun³, Il-Seok Yang¹, Woo Suk Hwang¹.

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The incidence of metabolic syndrome is increasing globally, as prevalence of obesity continues to rise. However, the basic mechanisms of metabolic syndrome are not completely known yet. Therefore, animal disease models are required for the study of metabolic syndrome. The overexpression of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) in mice leads to metabolic syndrome. So, we tried to produce the pigs with overexpression of 11β-HSD1 gene by somatic cell nuclear transfer (SCNT). However, the low transgenic (TG) efficiency has been an obstacle to the production of TG cloned pigs. And, a SCNT method in which somatic cells derived from TG pig are used as the nuclear donor (re-cloning method) is an effective technique for TG pig production. In this study, we attempted to increase TG efficiency by re-cloning method. Pregnancy efficiency, production efficiency and TG efficiency were compared with sources of donor cells (transfected TG fetal fibroblast vs. TG fibroblast derived from newborn TG cloned pig). A total of 1382 and 881 TG SCNT embryos were produced from fetal fibroblast vs. cloned fibroblast, and then transferred to 13 and 10 recipients. The pregnancy rate was not significantly different (30.8% vs. 20.0%). Seventeen live piglets and five stillborn piglets were born from four recipients in the fetal fibroblast used group and eight live piglets, two stillborn piglets and three mummies were born from two recipients in the cloned fibroblast used group. There were no significant difference in the production efficiency (3.7% vs. 5.0%). All of the thirteen re-cloned piglets showed reporter and target gene integration. But, of twenty two fetal fibroblast-cloned piglets, reporter gene integration was confirmed in nine, only three clone piglets showed reporter gene integration. TG efficiency was significantly increased in re-cloning group (13.6% vs. 100.0%). In this study, TG efficiency of 11β-HSD1 overexpressed pigs was improved by re-cloning method. These results indicate that re-cloning is an efficient method for production of TG cloned pigs. This work was supported by a grant from the Next-Generation Bio Green 21 Program, Rural Development Administration, Republic of Korea.

397. Leukogram profile of SCNT cloned calves is elevated compared with calves produced by artificial insemination. Estela R. Araujo¹, Aline T. Queiroz¹, Barbara Monteiro¹, Eduardo H. Birgel Junior¹, Luciano A. Silva¹.

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Bovine cloning by SCNT is a promising technique for research and livestock production, but still has presented low efficiency. About 1% of reconstructed oocytes result in healthy neonates. After birth, mortality rates run between 12.5 and 42%. The hematological evaluation is a tool to aid the diagnosis and therapy of SCNT cloned calves. The objective of this study was to evaluate the leukogram parameters in cloned calves produced by SCNT. Ten SCNT cloned and eight AI-derived Girolando breed calves delivered by cesarean section were used. Blood from the newborns was collected by jugular venipuncture at 1, 12, 24, 36, 48, 60, 72, 96 hours of age. The leukocytes and platelets were counted by an automatic blood analyzer (PocH-100i Vet, Roche) and the relative population of the white
blood cells determined manually by optical microscopy (E-100, Nikon). Data were examined for normality with the Shapiro-Wilk test and the variances homogeneity by Welch-Bartlett test. Comparisons between cloned calves and control calves at times 1 to 96 hours were analyzed for main effects of group and age and the interaction of group by age. The mixed procedure of SAS (version 9.2, SAS Institute) was used. When the main effects were significant (P<0.05), differences between selected ages and/or between groups on a given age were further examined by Student’s unpaired t-tests. All the main effects were significant (P<0.05) for the total number of leukocytes. Cloned calves presented higher amount of leukocytes (2 folds, P<0.0001) compared with control calves during all period of this study. For the analyses of the leukocyte subpopulation classes, the main effect of age and interaction (P<0.05) were significant for segmented neutrophils. Similarly to the total leukocytes, the segmented neutrophils presented 2 folds higher (P<0.0001) in cloned calves compared with the control calves. All the main effects were significant (P<0.05) for band neutrophils, however, only at 12h of age the number of band neutrophils were higher in the cloned calves compared with the control group (P<0.05). For lymphocytes the main effect of age and interaction were significant (P<0.05). The lymphocytes population was elevated in the clone group at 1h age returning to similar values of the control group at 12h of age (P<0.05). For monocytes only the main effect of group was detected (P<0.05). At 12h of age the cloned group presented more monocytes than the control group (P<0.05). Eosinophils maintained constant in the cloned and control group in all time points (P>0.05). For platelets, the main effect of age and interaction were detected (P<0.05). In the first 12h of life, control calves presented higher platelets counts. Similar counts were found from 24h to 60h for both groups, and higher platelets counts were observed in the cloned calves after 72h. These results are in contradiction with a previous literature work. The leukogram evaluation in SCNT cloned calves newborns is an important tool to determine their immunological status collaborating with the clinician decision of the best medical approach to treat and keep the calves alive.

398. A model system for syncytiotrophoblast generation from human pluripotent stem cells.
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Syncytiotrophoblast (STB) is a multi-nucleated, terminally differentiated syncytium that covers the surface of the villous placenta and forms the major interface with maternal blood. It releases placental hormones and plays a primary role in exchange of gases, nutrients and waste products. Alterations in STB development and turnover have been implicated in placental diseases, including preeclampsia (PE). In vitro cell models are badly needed to study STB development and physiology. Here we describe the in vitro generation of STB and its mononuclear precursors from human embryonic stem cells (hESC) that have been treated with BMP4, the ALK4/5/7 inhibitor (A83-01), and the FGF2 signaling inhibitor (PD173074) (BAP), a protocol that directs hESC to unidirectional commitment to the trophoblast lineage and provides both STB and extravillous trophoblast. Production of hCG began around d 5 of BAP treatment and peaked around d 8 before a subsequent decline. Multiple areas of what appears to be early syncytium became visible in the colonies around d 5 as placental hormone production began to rise. Syncytial areas at d 7-8 ranged in diameter from ~40 mm to >100 mm. The larger areas stained positively for KRT7, CGA, CGB, GATA3 and other STB markers. Such colonies were completely dispersed using a Gentle Cell Dissociation Reagent. The syncytial areas were then isolated intact by sieving successively through 70 μm and 40 μm mesh cell strainers. The captured cells were recovered by inverting the strainer and rinsing with culture medium. The fraction that passes through both sieves represents cells of smallest diameter, presumably cytotrophoblast. To characterize the three size- fractioned populations, the fractions were 1) immunostained after depositing on glass slides by Cytoospin; 2) examined by transmission electron microscopy; 3) assessed for DNA content; 4) profiled for RNA content by RNAseq. The larger >70 μm areas stained positively for STB markers while ultrastructural analysis clearly revealed multi-nuclear cells with an extensive cytoplasm containing many prominent secretion granules. The larger STB areas also had a DNA content consistent with the average presence of 17.13 ± 3.06 (n=3) diploid nuclei/cellular unit. Compared to the < 40 μm cell fraction, these larger cells over-expressed a full repertoire of genes characteristic of STB, e.g. CGA, CGB, PGF, ERVW1, GCMI. The 40-70 μm fraction also contained multi-nucleated cells, but nuclei number/cell was lower than in the larger fraction. Markers for STB and extravillous trophoblast were also less prominent. The smallest cell fraction had a DNA content consistent with mononuclear diploid cells, contained few secretory granules, and were only weakly positive for STB markers. A more complete characterization of these three cell fraction continues, but the data are consistent with the > 70 μm cells being mature STB, while the intermediate fraction may represent a precursor population. In addition, we are comparing the transcriptomic profiles of our cell populations with STB populations isolated after in vitro culture of primary human cytotrophoblasts derived from term placenta. We conclude that hESC directed along the trophoblast lineage by BAP treatment offers a consistent with the > 70 μm cells being mature STB, while the intermediate fraction may represent a precursor population. In addition, we are comparing the transcriptomic profiles of our cell populations with STB populations isolated after in vitro culture of primary human cytotrophoblasts derived from term placenta. We conclude that hESC directed along the trophoblast lineage by BAP treatment offers a

399. The requirement of glycerol increase for sperm banking of Ankole semen.
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Semen from Uganda native Ankole cattle was cryopreserved with glycerol and equex STM paste (STM). Ankole semen was collected by electrostatic stimulation in Nshaara Ruhengere ranches. Ankole semen was cooled to 20–25 °C after 10 min of adaptation at room temperature of 26–28 °C. The Ankole semen was diluted with 6%, 8%, or 10% of glycerol and 0.3% or 0.4% STM containing Citrate-Tris-Egg yolk extender. Diluted semen was packaged into 0.5 ml straws and frozen for 10 min by exposure to liquid nitrogen vapor 5 cm above the surface of liquid nitrogen in a styroform box followed by plunging into liquid nitrogen at ~196 °C. The rate of survival of spermatozoa after thawing at 37 °C for 1 min was increased significantly with higher concentrations of glycerol and STM (p<0.05). The survival rates of Ankole spermatozoa with 6%, 8% and 10% glycerol were significantly increased with 21±8.9%, 42±10.4% and 60±7.6%. To confirm protective effects of STM on sperm membrane, 0.3% and 0.4% were added to 6% and 10% glycerol containing Citrate-Tris-Egg yolk diluents respectively. The survival rate of Ankole sperm frozen with 6% glycerol and 0.3% STM containing diluent was...
19±10.7% and with 10% glycerol and 0.4% STM was 75±7.6%. In general case for cattle semen, glycerol concentration for successful cryopreservation was 5.6–6%. However, the commercial diluted form for cattle semen could not be used successfully for the semen of Ankole. With these results, the utilization of higher glycerol concentration and STM on the freezing media could offer an effective cryopreservation method of Africa-originated big horned cattle semen.

400. Heparin potentiates the oviduct fluid effect in controlling polyspermym during porcine in vitro fertilization, but reduces the efficiency of monospermic zygotes production.

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Although the prevalence of polyspermy under natural conditions is moderate, in IVF systems polyspermy remains as major obstacle to successful production of viable embryos in several species, mainly in porcine. In vivo, the oviduct provides appropriate microenvironment conditions for monospermic fertilization and early embryo development. In addition, proteoglycans such as heparin are present in the ovuduct and have been shown to modulate the activity of oviduct-secreted proteins on the regulation of sperm parameters. Thus, the present study was designed to examine the effect of the association of heparin (10 ng/mL) with oviductal fluid (OF) in the incidence of polyspermy during porcine IVF. Cumulus-oocyte complexes (COC) obtained from abattoir oocytes by aspiration of the follicles of 3–5 mm in diameter were matured in vitro for 44 h in TCM199 medium supplemented with 10% FBS, cysteamine (570 mM), EGF (10 ng/mL), and FSH (400 ng/mL). For IVF in medium TBM (tris-buffered medium), COCs were denuded and randomly allocated in one of the following conditions: i) 10% OF + Heparin (n = 150) – IVF medium supplemented with 10% OF coming from sows oviducts classified as late follicular phase (based on the appearance of the ovaries) plus heparin; ii) 10% OF (n = 136) – IVF medium supplemented with 10% OF; iii) Heparin (n = 146) – IVF medium plus heparin, and iv) Control (n = 145) – IVF medium. Co-culture with spermatozoa (4.5×10⁴ cell/mL) was performed for 20 h. Putative zygotes were fixed (alcohol-chloroform-acetic acid, 80: 10 v/v), stained (1% eosin), and examined under phase contrast microscopy. Penetration and monospermy rates, number of bound spermatozoa per ZP, number of pronuclei per oocyte and IVF efficiency (percentage of monospermic oocytes from total inseminated) were assessed. The results of five replicates were tested for normality using the Kolmogorov-Smirnov test, which indicated the normality of the data. Therefore, the parameters were compared using one-way ANOVA followed by Tukey’s test. Data are expressed as the mean ± SD. The combination of 10% OF with heparin (10% OF + Heparin) led to a decrease (P<0.05) in the percentage of penetration in comparison with 10% OF, Heparin and Control groups (35 ± 5%; 69 ± 11%; 97 ± 3% and 94 ± 10%, respectively). However, the percentage of monospermy was similar between 10% OF + Heparin (10% OF) (94 ± 7% and 75 ± 10%), while both were significantly higher than the Heparin and Control groups (15 ± 2% and 19 ± 2%). This resulted in a significant difference (P<0.05) in the rate of monospermic zygotes from total oocytes between the two groups (33 ± 6% and 52 ± 8%, for 10% OF + Heparin and OF, respectively). Despite this, both were higher than those not exposed to OF (14 ± 2% and 18 ± 4% for Heparin and Control). In addition, in groups exposed to OF (10% OF + Heparin and 10% OF) the number of promenocytes/spERMatozoon per oocyte decreased in comparison to not-exposed groups (Heparin and Control) (1.8 ± 0.1 and 1.1 ± 0.4 vs. 7.3 ± 1.2 and 8.6 ± 1.3), as well as the number of spermatozoa bound to the ZP (14 ± 2 and 21 ± 7 vs. 96 ± 6 and 89 ± 9). These results revealed that incubation of oocytes with OF during IVF contributed to the reduction of polyspermy in porcine species, but the association of OF with heparin reduces the efficiency of monospermic zygotes production in IVF.

401. Modulating Sperm Membrane Lipids in Domestic Turkeys to Improve Semen Cryopreservation for Valuable Genetic Stocks.

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There is a pressing need for preserving germplasm from research poultry lines, which are valuable genetic stocks for the research community and poultry industry. Semen cryopreservation can be a feasible strategy for maintaining threatened poultry lines, but the quality of frozen/thawed poultry semen with current methods is not reliable enough for germ-line retrieval, especially from turkeys. It has been suggested that poultry sperm cryosurvival is affected by the lipid composition of plasma membrane, which can be modified by inclusion of polyunsaturated fatty acids (PUFAs) in the diet. We tested the hypothesis that diets enriched with n-3 or n-6 PUFAs can modify the lipid composition of turkey sperm membranes and improve sperm cryosurvival. Using a standard formulation as the base diet, the following control and treatments were evaluated: no additives (Group 1, control); additional (120 mg/kg) vitamin E (Group 2); 5% Arasco oil (n-6 PUFAs) + vitamin E (Group 3); 5% Dhasco oil (n-3 PUFAs) + vitamin E (Group 4); 5% soybean oil (n-6 PUFAs) + vitamin E (Group 5); and 2.5% soybean/2.5% Arasco oil + vitamin E (Group 6). Four unique research turkey lines from Ohio State University (OSU) were evaluated. RBC1 and RBC2 are random-bred control lines; the E and F lines are sub-lines of RBC1 and RBC2, respectively, that have been selected for production traits. Sixty males from each line (n = 10/group) were fed the control or modified diets for 6 wks. Semen was frozen (8% dimethylacetamide; 0.5-mL straw; 2-min vapor freeze) after 3 and 6 wks of diet consumption and subsequently thawed (30 sec; 5°C) to assess membrane integrity, lipid peroxidation (LPO) and fertility. Sperm membrane integrity was determined using the SYBR/PI dual stain and assessed by flow cytometry. In RBC2 line, the percentage of sperm with intact plasma membranes was greater (P<0.05) for Groups 2-6 (range: 33.9–47.0%) than the control (31.0%). In RBC1 line, three diet treatments (Groups 4-6) improved (P<0.05) sperm membrane integrity (range: 32.5–36.8%) compared to the control (28.3%). For the E and F lines, not all diets improved sperm membrane integrity compared to control. Sperm that were subjected to LPO were detected by C11-BODIPY581/591/PI stain and flow cytometry. In RBC2 line, all diet treatments reduced (P<0.05) the percentage of peroxidized sperm (range: 10.1–33.0%) compared to the control (37.2%); a similar trend was seen in E line (control: 66.0%; Groups 2-6: 32.7–46.1%). For the RBC1 and F lines, only Groups 5 and 6 reduced (P<0.05) LPO in sperm compared to the control. For fertility trials, commercial hens (n=5/diet group; 30/OSU line) were inseminated with 300x10⁶ sperm for two consecutive days. Eggs were collected for 9 wks, candled on Day 7 of incubation and allowed to hatch. Line-specific differences in fertility rates were observed for the first 4 wks of egg.
production. For RBC2 line, Groups 5 and 6 supported higher fertility (P<0.05) than the control, Group 2 and Group 4. None of the diets improved (P>0.05) the fertility of frozen/thawed E line semen. For RBC1 line, more viable embryos (P<0.05) were observed for Group 5 than the control, Group 2 or Group 3; whereas no differences (P>0.05) occurred among diet treatments for the F line. Fertility persisted after wk 5, however, all embryos died within 48 hr of incubation. Taken together, the results suggest that PUFA- enriched diets may provide an alternative strategy to meet the immediate needs of poultry cryoconservation. Research supported by USDA Grant 2013-67015-2108.

402. The perfect follicle for the perfect oocyte.
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The understanding of oocyte quality has been in sight since the development of assisted reproduction technologies. It is known that the follicle is associated to oocyte’s competences. The recent ability to investigate how genes are expressed by transcriptomics leads to new perspectives in exploring the link between follicle environment and oocyte. The major goal of this project is to define the best follicular scenario to improve oocyte quality for a better success rate in the assisted reproductive technique. The methodology for this project consists in a collection of granulosa sample from a first group of 100 animals going through ovarian stimulation for commercial embryo production by ovum pick-up, IVF and in vitro culture of embryos. Samples will be pooled for each animal and snap-frozen and kept for RNA extractions, microarray and qPCR. Based on our microarray experiment and on previous analysis from the datasets EmbryoGENE, we found a list of genes differently expressed between animals with good responses (≥75% blastocyst) compared to animals with weak responses (< 35% blastocyst) to ovarian stimulation (15 upregulated and 63 downregulated). Based on the differential expression of key genes in conditions leading to good or poor development 21 biomarkers have been chosen to assess the timeless of the control of the oocyte, which will be a functional tool that can be used in different locations to monitor the efficiency of veterinarians in generating the best eggs possible. The main outcome of this project will increase the capacity of the industry to customize ovarian treatments to improve outcome.

403. Mitochondrial dynamics in slow and fast growing preimplantation bovine embryos.
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The use of metabolomics in the assessment of bovine embryo viability is a potential tool to non-invasively predict early embryo mortality. The early embryo primarily depends on the mitochondria for energy production. As the embryonic genome is activated around the 8-cell stage the embryo begins to rely more heavily on glycolysis. We have shown that the metabolite levels of pyruvate and lactate differ in the culture media of slow and fast growing embryos at various stages of development. It is therefore our goal to observe mitochondrial function during the preimplantation stages of development to elucidate potential molecular mechanisms behind impaired embryonic development. Firstly, in vitro fertilization (IVF) of bovine oocytes was carried out and subsequent zygotes were randomly selected for staining with live probes TMRM (mitochondrial membrane potential) and CM-H2DCFDA (reactive oxygen species; ROS). Time-lapse images were obtained using a live confocal microscope equipped with an on-stage incubator. Embryos that arrested at the zygote stage displayed higher TMRM and CM-H2DCFDA fluorescence than embryos that continued to cleave. The second experiment observed embryos that were separated into slow and fast growing groups at time stages (2-cell, 4-cell, 8-cell), stained with TMRM and CM-H2DCFDA. The time-lapse data indicated that embryos arrested at the 2-, 4- and 8-cell stages displayed higher TMRM and CM-H2DCFDA fluorescence than embryos that continued to develop. The results from both experiments indicate that early arresting embryos may be associated with overcompensation in the OXPHOS pathway. Energy production via the electron transport chain in the OXPHOS pathway generates reactive oxygen species as a side product. The high levels of ROS found in slow growing embryos may be a result of the higher than normal mitochondrial activity. Furthermore, fast growing embryos displayed a uniform distribution of mitochondria, whereas slow developing and arrested embryos displayed distribution about the periphery. This pattern was associated with embryos with lower developmental potential. The third experiment used qPCR to determine the GLYCOX genes involved in developmental delay and arrest of embryos. The expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an essential protein involved in the GLYCOX pathway, was 2-fold higher in slow growing blastocysts than in fast growing blastocysts. This suggests that embryos with slow or arrested development have to up-regulate the GLYCOX pathway in response to impaired OXPHOS pathways. In summary, our results indicate that delayed embryo development is associated with high mitochondrial activity in the early embryo and high expression of GAPDH in the blastocyst. Further studies will use GLYCOX gene hexokinase (HK) and OXPHOS genes ATP synthase (ATP5b) and cytochrome C oxidase (COX5a) to delineate glycolytic and oxidative activity in early embryos. This research supported by the Natural Sciences and Engineering Research Council of Canada and Dairy Farmers of Ontario.

404. Quantitative Proteomics Analysis of Nitrosoproteomes in Normotensive and Preeclamptic Placentas using SILAC and LC MS/MS.
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Introduction: Addition of a nitric oxide (NO) moiety to cysteines termed S-nitrosylation (SNO) is emerging as a key route by which NO directly regulates protein function. NO plays a key role in normal placental biology and abnormal NO homeostasis has been recognized to a causative factor in placental pathology including preeclampsia. However, how NO directly regulates placental function is unclear. Hypothesis: Protein SNO is differentially regulated in preeclampsia vs. normotensive human placentas. Methods: Human placentas of normotensive and preeclamptic pregnancies (n=7/group) were collected and their nitrosoproteomes were compared by a quantitative
405. Polyethylene Glycol Hydrogel Optimization to Support Vascularization and Folliculogenesis In Vitro. 
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Cancer therapies have saved countless lives and their efficiency continues to improve every year. These therapies however, come at a substantial cost to the patients. Cancer treatments have been shown to cause premature ovarian failure in prepubescent girls, causing lifelong sterility. A proposed solution for this problem is to remove a small portion of the patients’ ovarian cortex prior to treatment, followed by cryopreservation. When the patient is disease free the stored ovarian tissue can be auto-transplanted to restore endocrine function and fertility. Although this approach resulted in 24 live births in non-oncologic patients, there is a substantial risk of reintroducing cancer cells, especially in hematological cancers. To overcome this risk, we propose to engineer an artificial ovary that is free of cancer cells. This approach requires isolating ovarian follicles from the tissue and providing a supportive artificial matrix that, upon transplantation, will allow remodeling of the graft and vascularization. Polyethylene glycol (PEG) has been previously characterized as a bio-material that is able to support folliculogenesis and cell growth in-vitro so it was identified as the ideal candidate for matrix design. We hypothesized that vascularized hydrogels would increase follicle survival after implantation. To test our hypothesis we tested the effect of the presence of blood vessel precursor cells and the hydrogel stiffness on follicle development. PEG hydrogels were prepared at concentrations ranging from 3%-5% PEG, cross-linked with a protease-sensitive peptides and modified with 0.5 mM of the integrin binding peptide RGD. The design of the PEG hydrogel allowed for cell migration and follicle growth in vitro confirmed by microscopic imaging. Two crosslinking peptides were designed to be either Matrix Metalloproteinase (MMP) sensitive (GCLG-PACCLG-PACG) or plasmin sensitive (GCYK-NSGCYK-NSCG) and analyzed to identify the ideal hydrogel design. Human umbilical vein endothelial cells (HUVECs) and normal human lung fibroblasts (NHLFs) were co-encapsulated in 10 µL gels along with a secondary follicle isolated from 14 day-old mice and cultured for 12 days. PEG hydrogels at 3% were too soft to support follicle architecture but encouraged cell migration; however, 5% hydrogels were ideal to support follicle growth and cell migration. Hydrogels cross-linked with MMP-sensitive cross-linker facilitated cell spreading through the material but restricted follicle growth while the plasmin sensitive cross-linker allowed for proper follicle growth but reduced cell migration through the material. To optimize both follicle growth and cell migration, a mixture of both crosslinking peptides was used and found to be ideal for co-culture. Furthermore, secondary follicles co-encapsulated with HUVECs and NHLFs increased cell migration through the matrix and allowed for tubule formation during the 12 days of the hydrogel culture. In conclusion, through the fine-tuning of the chemical and physical properties of the PEG hydrogel we identified the matrix that can support follicle and capillary development. The described system will be further characterized as a candidate matrix for transplantation, will allow remodeling of the graft and vascularization. Polyethylene Glycol (PEG) has been previously characterized as a bio-material that is able to support folliculogenesis and cell growth in-vitro so it was identified as the ideal candidate for matrix design. We hypothesized that vascularized hydrogels would increase follicle survival after implantation. To test our hypothesis we tested the effect of the presence of blood vessel precursor cells and the hydrogel stiffness on follicle development. 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Hydrogels cross-linked with MMP-sensitive cross-linker facilitated cell spreading through the material but restricted follicle growth while the plasmin sensitive cross-linker allowed for proper follicle growth but reduced cell migration through the material. To optimize both follicle growth and cell migration, a mixture of both crosslinking peptides was used and found to be ideal for co-culture. Furthermore, secondary follicles co-encapsulated with HUVECs and NHLFs increased cell migration through the matrix and allowed for tubule formation during the 12 days of the hydrogel culture. In conclusion, through the fine-tuning of the chemical and physical properties of the PEG hydrogel we identified the matrix that can support follicle and capillary development. The described system will be further characterized as a candidate matrix for in-vivo implantation and artificial ovarian graft remodeling.

406. Fibroblast cell cycle synchronization to obtain bovine handmade cloned embryos. 
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In the cloning process it is compulsory to have synchrony and spatial/temporal compatibility between the oocyte cytoplasm and the fibroblast or donor cell nucleus. Thus, the nuclear reprogramming and the early embryonic developmental are able to initiate and culminate with properly. Ideal situation is that donor cells are in cell cycle G0/G1 phase; to achieve this, many methodologies have been used changing components or performing culture modifications. The objective of this study was to evaluate three fibroblast primary culture synchronization methods (serum restriction-SR, high cell confluence-HC, combination-SR+HC) for 24, 48 and 72h. These cells were used as nuclei donors to evaluate the in vitro potential embryo progress and quality produced by hand made cloning (HMC). All the results from embryos produced by HMC were compared to results from embryos obtained by in vitro fertilization (IVF) and by parthenogenesis. No difference was found related to the 3 methods used to synchronize fibroblasts in G0/G1 phase (p>0.05). Clones were produced with nuclei from fibroblast from 0h, SR, HC and SR+HC for 24h culture resulting in high cleavage rate in the 4 groups with no difference between them (p>0.05). Embryo production data were similar between 0h, SR and SR+HC for 24h culture groups (37.9%, 29.5% and 30.9%) and HC group showed significant difference compared to other 3 groups with only 19.3% of embryo production (p<0.05). Concerning embryo quality parameter, no difference between groups was found. Blastomeres number from IVF embryos were greater compared to blastomeres from other 3 groups of HMC, partenotes with and without zona pellucida (117% ; 80%; 75.9% and 67.1% respectively). Finally, global embryo production of HMC was very suitable and comparable to partenotes with or without zona pellucida and to IVF embryo (30.2%; 38.6%; 35% and 33.3% respectively); comparing HMC and partenotes with zona pellucida resulted in...
significant difference (p<0.05). This research was supported by the Estrategia de Sostenibilidad, University of Antioquia, to Biogenesis Group 2013-2014, and 2014-2015

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The use of adaptive reproductive technologies (ART) is common and up to 5% of children in developed countries are conceived with the use of ART. Retrospective studies have suggested that ART-conceived children are more likely to develop the loss-of-imprinting (LOI) overgrowth syndrome Beckwith-Wiedemann (BWS). BWS is characterized by macrosomia, macroglossia, abdominal wall defects, ear malformation, and predisposition of childhood tumors. In ruminants, ART is used to decrease the time required to produce genetically superior animals and for experimentation. Bovine embryos cultured in serum-supplemented media are prone to develop an overgrowth condition similar to BWS, which is known as large offspring syndrome (LOS). We have shown that LOS recapitulates the epigenetic (i.e. LOI at the KvDMR1) and phenotypic features of BWS making LOS an appropriate animal model to study the etiology of these ART-associated LOI overgrowth conditions. We recently determined allele-specific expression and transcript abundance of imprinted genes previously identified in human and/or mouse in bovine control and LOS fetuses using RNAseq. We found variable LOI and differential expression of imprinted genes in LOS when compared to control. For this study, we hypothesized that LOS fetuses have global misregulation in gene expression when compared to controls. To test this, RNAseq data from brain, kidney, liver, and skeletal muscle of eight (4 control and 4 LOS) day ~105 Bos taurus indicus X Bos taurus taurus F1 female fetuses were analyzed. The range in bodyweight was 392-416g for controls and 514-714g for LOS fetuses. Read counts for each gene were normalized to the RNAseq library size to determine the gene expression level. Differential gene expression between each LOS individual and the average of four control fetuses was determined using EdgeR package. The number of differentially expressed genes (DEGs) varied between tissues for different LOS fetuses. For example, in the largest LOS fetus analyzed (weight = 714g) the number of DEGs was 296, 517, 1845, and 4199 for brain, kidney, liver, and muscle, while in the smallest LOS fetus (weight = 514g) the number of DEGs for those tissues were 1239, 174, 3706, and 2780. The number of DEGs is not associated with fetal weight, but the fetuses with LOI at the KvDMR1 had more DEGs in liver and muscle than LOS fetuses with correct imprinting at this locus. Notably, our results show that all LOS fetuses shared 69 DEGs in brain and 7, 9, 19 DEGs in kidney, liver, and muscle, respectively. Of these, IGFBP1, an anti-apoptosis factor, was upregulated >2-fold in liver of all LOS fetuses. RCAN1, an inhibitor for skeletal muscle hypertrophy, was downregulated in muscle of all LOS fetuses. DAVID bioinformatics resources (v6.7) were used to gain biological insights of the DEGs. For the upregulated genes in muscle of the largest LOS fetus, the top enriched KEGG pathways included focal adhesion, extracellular matrix receptor interaction, and pathways in cancer, suggesting the over-proliferation of muscle cells in this individual. Given that only a limited number of genes (i.e. ~150 reported imprinted genes) were studied for their allelic expression in previous studies, another goal is to determine the global allelic gene expression in both control and LOS fetuses. To determine the parental origin of the expressed alleles in RNAseq, the sire of the eight fetuses was genotyped using DNASeq. The allele-specific expression of the expressed genes is currently under investigation.

408. Cryopreservation of epididymal dog sperm by two different extenders.
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Post-mortem recovery and cryopreservation of viable sperm from the epididymis of genetically valuable animals or endangered species is an important technique for preserving male gametes and thus for maintaining germ-plasm banks. The extender type used to freeze sperm is important to avoid spermatozoal membrane damage and to preserve semen quality after cryopreservation. The objective of this study was to determine the effects of a commercial bovine extender (BullXcell®; IMV, L’Aigle, France) and TRIS-citric acid-fructose-egg yolk-7% glycerol extender on cryopreservation of canine epididymal sperm. The testicles of ten adult Egyptian domestic dogs were kept at 5 °C in saline solution, and transported to laboratory via 24 h. Diluted samples were cooled slowly to 5°C over 2 h and equilibrated at that temperature for 2 h. Aliquots of samples were loaded into 0.25 ml straws and frozen in liquid nitrogen vapor for 15 min and stored in liquid nitrogen. The semen parameters, Post-thaw progressive motility, viability index, membrane integrity (HOST) and acrosomal integrity (silver nitrate stain) were evaluated. Results indicated that all assessed semen parameters were statistically higher in BullXcell® than TRIS. Despite the high percentage of sperm defects in epididymal cells, regardless of the extender, we concluded that RCAN1, an inhibitor for skeletal muscle hypertrophy, was downregulated in muscle of all LOS fetuses. DAVID bioinformatics resources (v6.7) were used to gain biological insights of the DEGs. For the upregulated genes in muscle of the largest LOS fetus, the top enriched KEGG pathways included focal adhesion, extracellular matrix receptor interaction, and pathways in cancer, suggesting the over-proliferation of muscle cells in this individual. Given that only a limited number of genes (i.e. ~150 reported imprinted genes) were studied for their allelic expression in previous studies, another goal is to determine the global allelic gene expression in both control and LOS fetuses. To determine the parental origin of the expressed alleles in RNAseq, the sire of the eight fetuses was genotyped using DNASeq. The allele-specific expression of the expressed genes is currently under investigation.

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Over the last decades, substantial progress has been made to improve IVF systems. However, IVP embryos are still more sensitive to cryopreservation than their in vivo-derived counterparts. This issue remains a key factor limiting the widespread use of IVP technology in the cattle industry. In the present study, we analyzed the changes in gene expression induced by the Cryotop vitrification procedure in bovine blastocysts using Agilent EmbryoGENE microarray slides. Bovine in vitro-produced embryos at the blastocyst stage (144 to 156 hours post insemination) were vitrified using the Cryotop system and compared with non-vitrified (control) embryos. After vitrification, the embryos were warmed and cultured for an additional 4 hours. Embryos that re-expanded or developed to the expanded blastocyst stage were used for microarray analysis and for qPCR quantification. The survival rates were higher (P<0.05) in the control embryos (100%) than in the vitrified embryos (87%). Global gene expression analysis revealed that only 43 genes exhibited significantly altered expression in the vitrified embryos compared to the control embryos, with a very limited fold change (P<0.05). A total of 10 genes (KRT8, CD38, HSPB1, PAG2, SLC1A4, HSPA5, TXNIP, FOSL1, MSH6 and GAPDH, included as housekeeping gene) were assessed by qPCR. Only FOSL1 presented differential expression (P<0.05) according to both the array and PCR methods, and this gene was over-
expressed in the vitrified embryos. This result can be explained by the observation that some embryos present a phenotype after freezing, but most do not, resulting in variations in the PCR results. Nevertheless, the only consequence of freezing seems to be the activation of the apoptosis pathway in some cells. Indeed, FOLs1 is part of the activating protein-1 (AP-1) transcription factor complex and is implicated in a variety of cellular processes, including proliferation, differentiation and apoptosis. Therefore, our results suggest that a limited increase in the rate of apoptosis was the only detectable response of the embryos to vitrification.

410. **Differentiation of trophoderm in the expanded human blastocyst.**

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Trophectoderm (TE) biopsy is being used more commonly for preimplantation genetic diagnosis and screening. Although level 1 evidence exists for its safety and efficacy, prior studies also indicate that TE from early blastocysts may have induced potential to dedifferentiate and contribute to the inner cell mass (ICM). However, the extent to which the TE has differentiated from pluripotent stem cells remains to be evaluated in fully expanded human blastocysts. This study seeks to investigate the differential expression of pluripotent and differentiated cell markers in the TE relative to the ICM in expanded blastocysts. Human preimplantation embryos which were fully expanded (Gardner grade B5 and B6) were utilized to determine differential expression. These embryos had been determined to be aneuploid during clinical care and had been donated to research. The differential expression of selected markers was determined by real-time quantitative polymerase chain reaction (qPCR). There were a total of 18 biopsies analyzed in triplicate from 6 blastocysts. Two biopsy strategies were utilized. For 3 blastocysts, a standard TE laser biopsy was obtained and placed in a PCR tube and this was compared to the remaining whole embryo. This is representative of clinical biopsy techniques were the TE cells were taken opposite of the ICM. In 3 blastocysts, 3 standard TE laser biopsy were obtained—opposite the ICM and the other two equal distances from the ICM and the first biopsy. Subsequently, the ICM was dissected free of TE cells and placed in a PCR tube for separate analysis. In the control marker group 18S, ACTB, EEF1A1 and GAPD were analyzed as housekeeping genes and were found to be present in all embryo biopsies from TE and ICM as well as control fibroblast cultures. NANOG, POU5F1, and SOX2 were analyzed as a marker of pluripotency and showed a 6-fold, 2-fold, and 5-fold decrease respectively when comparing TE biopsy to whole embryo, although not statistically significantly different. When comparing TE biopsy to pure ICM there was a 40-fold, 9-fold, and 22-fold decrease respectively, all comparisons p<0.05. Finally, CDX2 was analyzed as a marker of differentiation and showed a 1.7-fold increase in TE versus whole embryo and a 2.6-fold increase in TE versus pure ICM, although this was not statistically different. The data show that cells from TE biopsy have begun differentiation and no longer express markers of pluripotency at levels seen in the inner cell mass destined for embryonic development. This data is clinically important when it comes to the safety of obtaining cellular material obtained for preimplantation genetic diagnosis in expanded blastocysts.

411. **Effects of Heat Stress on In Vitro Maturation Rates of Oocytes from Heat Resistant Cows in Puerto Rico.**

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Thermal heat stress in tropical areas severely hinders year round farm animal reproduction. Depending on the season, cattle are under moderate to severe thermal stress. It is well established that heat stress has detrimental effects on cattle reproductive efficiency. The objective of this study was to evaluate the in vitro maturation rate of bovine oocytes collected from thermo vs. non-thermo tolerant cows. We collected ovaries from heat resistant cows (e.g. Senepol and Zebu crossbreds) and Holstein cows (temperate climate cattle). Ovaries from 87 Holstein cows and 120 heat resistant cows were obtained from abattoirs throughout the year: Dec. – Feb. (S1; ranging 21 - 31°C), Mar. – May (S2; ranging 20 - 31°C), Jun. – Aug. (S3; ranging 20 - 32°C) and Sep. – Nov. (S4; ranging 22 - 32°C). A total of 804 oocytes were collected from follicles ranging from 2-8 mm in diameter. Collected oocytes were described morphologically as complete cumulus oocyte complex (COC; i.e. at least one cumulus cell layer) and semi-denuded/denuded oocytes (SDO). Those with complete COCs were randomly placed into different media and matured in vitro for 22 hrs. at 37°C in an atmosphere of 5% CO2-95% air in a humidified environment. Although the adjusted total number of oocytes with a complete COC was not different between Holstein and heat resistant cows, Holstein cows presented a greater number of SDOs (P>0.05). During the period of moderate heat stress (S1 and S2), oocytes from Holstein cows presented higher maturation rates when compared to heat resistant cows (70.4% vs. 56.0% and 71.4% vs. 54.6%, respectively; P < 0.0001). However, during severe heat stress (S3 and S4), oocytes from Holstein cows exhibited a noticeable decline in maturation rates (50.1% and 59.9%, respectively) as opposed to heat resistant cows, which had significantly higher maturation rates (68.5% and 79.8%, respectively; P < 0.0001). These results could suggest that oocytes from heat resistant cows have a thermo tolerance mechanism that responds predominantly during periods of severe heat stress. Further research is necessary to elucidate putative mechanisms involved in the activation of bovine oocyte thermal tolerance.

Key words: bovine, oocyte, in-vitro maturation, thermo tolerance, heat stress, reproductive efficiency

412. **Generation of transgenic pig carrying both amyloid precursor protein precursor and presenilin1 transgenes.**

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Familial Alzheimer's disease (AD) is caused by genetic mutations of genes such as amyloid precursor protein (APP) and presenilin1 (PS1). Here we reported the production of transgenic pig carrying both the APP695sw (Swedish mutation) and mutant PS1 transgenes using the somatic cell nuclear transfer (SCNT) technology. Donor cells were collected from a transgenic cloned pig modified genetically with human synapsin1 promoter–APP695sw (SYN-APP) gene (Oh et al., 2014). Human synapsin1 promoter–PS1 (SYN–PS1) was introduced into SYN-APP cell, both APP and PS1 genes expression could be controlled through neuron-specific expression by
synchrony of ovulation (residuals: 0.24±0.07 vs. 0.68±0.13; P<0.01). The day on which letrozole treatment was initiated did not affect the proportion of heifers that ovulated or the interval to ovulation. Plasma estradiol concentrations were lower at the time of removal of the letrozole device in the Day 0 and 16 groups (12.8±0.58 mm and 11.7±0.59 mm, respectively), and intermediate in the Day 8 and 12 groups (14.5±0.83 mm and 14.2±0.48 mm, respectively; P<0.001). Compared to control data, the percentage of heifers that ovulated following letrozole treatment was greater (87.1% vs. 69.4%, respectively; P<0.05) as was the synchrony of ovulation (residuals: 0.24±0.07 vs. 0.68±0.13; P<0.01). The day on which letrozole treatment was initiated did not affect the proportion of heifers that ovulated or the interval to ovulation. Plasma estradiol concentrations were lower at the time of removal of the letrozole device in the Day 0 and 4 groups than in the respective controls (P<0.05). Estradiol concentrations in the Day 8 and 12 groups did not differ from already low concentrations in the respective controls. Corpus luteum diameter profiles and plasma progesterone concentrations were not different among Day-groups. In summary, an aromatase inhibitor-based protocol involving a letrozole-impregnated intravaginal device for 4 days, combined with PGF treatment at device removal and GnRH 24 h later, resulted in a greater ovulation rate and greater synchrony of ovulation than in heifers not given letrozole. Results suggest that treatment may be initiated effectively at random stages of the estrous cycle, and provide impetus for further studies to assess the efficacy of a letrozole-based synchronization protocol for fixed-time insemination. Research supported by the Natural Sciences and Engineering Research Council of Canada and the University of Saskatchewan.

414. Can soybean extract be a viable alternative to egg yolk for bovine semen cryopreservation? Search for a practical plant based semen extender.

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The rising issue of bio-security and cross boundary disease transmission has unveiled new concerns over disease transmission of some diseases with the use of egg yolk based semen extenders. This concern has resulted in initiatives to develop a plant based alternative to egg yolk for bovine semen cryopreservation. This study was designed to standardize a soybean extraction procedure and assess its effectiveness for preserving post-thaw sperm function and fertility. Soybean (Boyd variety) extracts were prepared using a modified Illinois method, and then different levels of soybean extract based extender (25%, 30% and 35%, v/v,) were compared to 20% tris-egg yolk extender. Sperm motility, kinematic parameters, membrane permeability and membrane lipid scrambling parameters were evaluated using two ejaculates from each of five Holstein bulls during storage at 4°C (0, 3, 6, 12, 24 and 48 hr) and post-thaw (0 hr and after 2 hr post-thaw incubation at 37°C). In vitro fertility was assessed by comparing 35% soybean and 20% tris-egg yolk (EYT) extenders. Cleavage of zygotes was observed at 48 hr post-fertilization using Hoechst 33342 nuclear staining. Total motility in 35% soybean and EYT extender did not differ significantly up to 24 hours at 4°C (69.6±2.9 and 79.5±4.8, respectively), whereas progressive motility remained comparable up to 12 hr of storage (50.98±2.3 and 59.0±3.7, respectively). Sperm velocity parameters, lateral head displacement, and beat fluidity parameters improved with increasing level of soybean extract. Hyperactivated motility was evident in soybean extenders following...
liquid storage at 4°C and in post-thaw sperm upon rewarming. Cleavage rate was significantly higher following in vitro fertilization (IVF) for sperm frozen in EYT than 35% soybean extender (72.6±0.7 vs. 50.5±1.2%). IVF results also indicate bull variability in compatibility to soybean extender. In conclusion, a standardized soybean extract was effective in protecting bull sperm during cryopreservation but post-thaw semen quality was generally somewhat less than with EYT following post-cooling or post-thaw incubation. Modifying the soy extender by adding membrane stabilizing compounds may further improve its sperm cryoprotective potential and make it a viable and cost-effective alternative to egg yolk as an extender for cryopreservation of bull semen.

415. **WITHDRAWN.**

416. **Mitochondrial Oxygen Consumption Indicates Functional State of Fresh and Cryopreserved Stallion Spermatozoa.**

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Cryopreservation significantly damages spermatozoa after thawing and leads to substantial decreases in total sperm motility and viability. Post-thaw sperm motility is typically less than fifty percent of initial motility and this proportion is lower and more variable in stallions than other species. Cryopreservation damage in sperm primarily results from reactive oxygen species production from the mitochondria and from the lipid peroxidation of membranes. This oxidative cryoinjury subsequently generates downstream organelle damage that contributes to decreased motility, membrane integrity and mitochondrial efficiency. We hypothesize that oxidative cryoinjury leads to mitochondrial damage resulting in decreased activity, measured as mitochondrial oxygen consumption. Since the mitochondria are responsible for essentially all of the oxygen utilization by the cell, measurement of oxygen consumption is a dependable indicator of mitochondrial activity and overall cell health. Sperm mitochondrial oxygen consumption was measured in fresh and frozen semen samples utilizing high-throughput oxygen biosensor plates (BD Biosciences) which fluoresce as oxygen is consumed from the media. Triplicates of 10 × 10⁶ sperm for both fresh and frozen semen from the same stallion (n=3) were aliquoted into biosensor plate wells and oxygen consumption, expressed as random fluorescent units (RFU), was determined over a period of two hours at 37°C. Fresh and thawed sperm were treated with Carbonyl cyanide-p-trifluoro-methoxyphenylhydrazone (FCCP, 1μM), a mitochondrial uncoupler, and Antimycin (1μM), an inhibitor of oxidative phosphorylation, to determine maximal oxygen consumption and non-mitochondrial respiration, respectively. Sodium sulfite (100mM), which removes oxygen from the media, was used as a positive control while dead sperm (snap frozen in liquid nitrogen) were used as a negative control. Concurrently, motility (CASA) and viability (Propidium iodide exclusion, flow cytometry) were monitored for the control, the FCCP treated and the Antimycin treated samples of both fresh and frozen semen. Differences were observed in the basal level of oxygen consumption between fresh and frozen samples at 1 hr (3,703 ± 333 RFU and 2,454 ± 289 RFU, mean ± SEM, respectively), but larger differences were revealed between fresh and frozen sperm with FCCP-stimulation. FCCP-stimulated fresh samples had 5 times more oxygen consumption than FCCP-stimulated frozen samples (19,527 ± 2,972 RFU and 3,727 ± 518 RFU, P<0.01) suggesting diminished mitochondrial oxygen consumption and decreased ability of FCCP stimulation of cryopreserved semen. This indicates that there may be irreversible damage to the machinery of oxidative phosphorylation occurring during sperm cryopreservation in addition to oxidative membrane damage. This direct measure of mitochondrial health may indicate a mechanism of cell cryoinjury and help elucidate the development of novel methods of cryopreservation that improve mitochondrial function in stallion semen after thawing. Future studies will assess the relationship among oxygen consumption and motility, membrane potential, ATP production and mitochondrial DNA status. The authors wish to thank the American Quarter Horse Foundation for their generous support of this work.

417. **Production of Elks (Cervus Canadensis) by Multiple Ovulation and Embryo Transfer.**

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Although multiple ovulation and embryo transfer (MOET) have been used to produce more offspring from genetic elites in industrial animal, its application is very limited in cervine species. In this study, we evaluated the feasibility of producing offspring from MOET in elk. Super-ovulation and synchronization were induced by progesterone realizing device, FSH, PMSG and PGF2α. In the super-ovulated donor hinds, embryos were collected by non-surgically flushing the uteri. From eleven donor hinds, a total of 71 embryos were recovered. Twenty one embryos were transferred freshly into recipients and 17 frozen- thawed embryos were transferred into recipients by conventional embryo transfer gun or modified embryo transfer gun with curved plastic catheter. Twenty three recipients were carrying pregnancy and delivered of seventeen fawns. In conclusion, this result demonstrated that after treating super-ovulation, transferrable embryos were collected, transferred by non-surgical approach, implanted and produced offspring. Success of MOET in elk can be a useful way to improve genetic background. This study was financially supported by Korea Industrial Complex Corp (SNU550-20140084).

418. **Coupling of Porcine Artificial Insemination with Intrauterine Mucosal Vaccination.**

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Successful mucosal vaccination is made difficult by a general tendency toward tolerance at mucosal sites which support a healthy microbiome. However, the upper genital tract is thought to be maintained in a nearly sterile state, and should therefore be naturally predisposed to an immunogenic response. In addition, this tendency should be augmented during insemination by the natural inflammatory response induced by the introduction of the insemination dose. The goal of this study was to provide proof of concept that an appropriate vaccine formulation delivered into the uterus during artificial insemination (AI) would produce an antigen specific immune response without compromising reproductive performance. The serpimicrobial effects of a wide range of commercially available vaccines against Porcine Parvovirus (PPV) as well as of adjuvants and other vaccine components were evaluated using flow cytometry. These in vitro experiments found the existing veterinary vaccine formulations to be spermicidal and identified the antimicrobial compounds and classical
adjuvants as the causative agents. In contrast minimal effects were observed for either inactivated viruses or a series of novel adjuvant compounds. Based on this information a vaccine formulation consisting of 1x10^7 TCID equivalent inactivated PPV and a next generation tri-adjuvant combo (polyphosphazene, poly I:C and a Host Defense Peptide) was mixed with commercially extended semen and delivered via post-cervical AI in sows during a natural standing estrus. Necropsy at 30 days of gestation found all vaccinated sows were pregnant with an average rate of fertilization and embryo survival of 72%. In addition all animals showed increased in PPV specific IgG, IgG1 and IgG2 titers at Day 15 and 30 following vaccination. Taken together, these results suggest intrauterine delivery is a viable approach to mucosal immunization and can be successfully combined with AI, the most widely used assisted reproductive technology in the swine industry. Research Funding provided by the Alberta Livestock and Meat Agency. J.A.P. is supported by postdoctoral fellowships from the National Science and Engineering Research Council of Canada and Saskatchewan Health Research Fund.

419. Use of Trolox and docosahexaenoic acid (DHA) as additives for cryopreservation of sperm from Alouatta caraya. Fernanda M. de Carvalho¹, Fernanda M. de Carvalho¹, Marceliio Nichi¹, João D. Losano¹, Camilla M. Mendes¹, Mayra E. Assumpção¹, José A. Muniz², Marcelo A. Guimarães¹, Rodrigo R. Valle³.

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About 50% of non-human primate species are considered endangered at some level. Therefore, there is increasing need for conservation efforts, among which is the use of assisted reproductive technologies (ARTs). In the present study, 12 semen samples from six adult male black-and-gold howler monkeys (Alouatta caraya) were cryopreserved using four different extenders (treatments): 1) Control – Test-yolk buffer (TYB) with 4% glycerol, 2) DHA – TYB + Docosahexaenoic acid (10 ng/ml), 3) Trolox – TYB+ vitamin E analogue (40 μM), and 4) DHAT – TYB+ DHA + Trolox. Semen samples were analyzed before (fresh) and after cryopreservation. Results (mean±SD) obtained for fresh sperm were: volume=199.08±111.36 µl; pH=8.51±0.24; concentration=837.31±685.53 sperm/ml; total motility=72.50±17.65%; progressive motility=62.50±17.65%; intact plasma membrane (IMP) =60.83±16.38; Intact acrosome (IA)=65.08±10.41; Mitochondrial activity (MA) class I=23.42±8.87% and class II=56.92±10.35%; Lipid peroxidation (LP)=83.27±80.16 Thiobarbituric acid reactive species (TBARS)/10^6 sperm; and DNA fragmentation index (DFI)=4.02±4.88%. When comparing fresh versus cryopreserved sperm using ANOVA, there were significant differences (p<0.05) only for total and progressive motility, and lipid peroxidation. The LSD test revealed significant differences between percentage of sperm with IPM and susceptibility to LP. There were significantly (p<0.05) more sperm with IPM in fresh sperm (60.83±16.38%) than in treatments DHA (50.25±9.27%) and DHAT (49.50±13.08%). Moreover, fresh sperm (83.27±80.16 TBARS/10^6 sperm) were significantly less susceptible to lipid peroxidation than cryopreserved sperm (from 260.53±38.79 TBARS/10^6 sperm – Trolox to 313.46±147.72 TBARS/10^6 sperm – DHAT), regardless of treatment. When comparing the effect of the different treatments on frozen-thawed sperm using ANOVA, there were no significant differences (p>0.05) for any of the parameters. However, the LSD test revealed a difference in the percentage of IA, in which control samples (70.67±12.18%) were significantly better than DHAT treated samples (57.92±14.99%). Therefore, DHA seemed to have a negative effect on plasma membrane integrity, while the combination of DHA and Trolox had a negative effect on acrosome integrity. Moreover, neither of the treatments were effective in protecting sperm from lipid peroxidation. In conclusion, addition of DHA and Trolox in these doses were not beneficial for cryopreservation of sperm from Alouatta caraya. Research supported by São Paulo Research Foundation (FAPESP process #2012/24709-6).


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421. Efficacy of progesterone - prostaglandin - PMSG combination for estrus synchronization in crossbred (Holstein Friesian X Zebu) dairy cattle in and around Mekelle, Tigray, Ethiopia.

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A total of 122 apparently healthy crossbred (Holstein Friesian X Zebu) dairy cattle (75 cows greater than 90 days postpartum and 47 heifers of 2 years old and above) with reproductive history of anestrus and repeat breeding were included in the study. Animals were grouped in to three according to their reproductive history, rectal palpation and On-Farm milk (lactating cows) and serum (dry cows and heifers) progesterone enzyme-linked immunosorbent assay (ELISA) into anestrus (smooth ovaries with low progesterone, n=62), repeat-breeders (normally cycling and observed estrus, but never conceived after multiple inseminations, n=11) and silent estrus (cycling but no visible estrus signs, n=46). Eazi-Breed Controlled Internal Drug Release, 1.38gm device was inserted into vagina and allowed to remain in-situ for 10 days. On 8th day of device insertion, a dose of 500µg of prostaglandin F2-alpha was administered intramuscular. On day of device removal (Day 10), 500 i. u. of pregnant mare serum gonadotropin was given intramuscular. Animals were inseminated/naturally mated by bull 48 and 72 hrs post device removal. Animals were examined for presence of pregnancy 20-24 days post insemination by milk (lactating cows) or serum (dry cows and heifers) On-Farm progesterone ELISA and pregnancy was confirmed by rectal palpation/ultrasonography 44-90 days post insemination. Overall estrus response and conception rat were, 97.5% and 73.8%, respectively with no significant differences in parity, pre-treatment reproductive status as well as farming system (backyard unorganized vs organized farms). Conception rate at induced/synchronized estrus was 82.9% and 72.1% in cows and heifers, respectively. Out of 90 pregnant animals 85 (94.4%) have calved, with no significant difference between parity, pre-treatment reproductive status of animals and farming system. Although the estrus response, conception rates and calving rate is quite high, a larger trial needs to be conducted to confirm the findings.

FERTILIZATION AND PREIMPLANTATION DEVELOPMENT: Program Numbers 422–475

422. Neither N- nor O-glycans in the N-terminus of ZP2 are essential for fertility in mice.

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At fertilization, mouse sperm bind to the extracellular zona pellucida that surrounds ovulated eggs and consists of three glycoproteins (ZP1, ZP2, ZP3). Which zona glycoprotein mediates gamete recognition has been a long-standing controversy and multiple glycans have been proposed as candidates zona ligands for a yet to be defined sperm receptor. Recently, the N-terminus of ZP211146 has been implicated as the sperm binding domain for mouse sperm. An earlier report using microscale mass spectrometry did not detect O-glycans on native mouse ZP2. However, one of the six N-glycans on ZP2 identified by mass spectrometry is attached to asparagine 83 that falls within the proposed sperm binding domain. To investigate the role of this N-glycan in fertilization, DNA recombineering was used to construct a mutant Zp2N83Q transgene and establish transgenic mouse lines. The Zp2N83Q transgenic mice were crossed into the Zp2null background to remove endogenous ZP2. These Zp2N83Q rescue lines form a normal zona matrix with ZP2 that lacks the targeted N-glycan. In vitro assays document the ability of sperm to bind and penetrate through the zona pellucida and female mice are fertile in vivo. These data indicate that neither O- nor N-linked glycans in the N-terminus of ZP2 are essential for fertility in mice.

423. Creating a 3D Culture System to Study Bovine Oviduct Physiology.

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The oviductal lumen provides the physicochemical microenvironment for gamete interaction and early embryo development, and is lined by a columnar epithelium containing ciliated and secretory cells. Because of its size and location, it is difficult to study intra-ovoidal events in vivo; instead, in vitro models that retain essential morphological and functional characteristics are being developed. Bovine oviduct epithelial cells (BOECs) in 2D culture rapidly lose differentiated cell properties (i.e. secretory activity or cilia) while cells in suspension have a limited lifespan. Our objective is to develop a 3D culture system in which BOECs maintain or re-establish in vivo-like differentiation status and function. Oviductal epithelial cells were harvested by scraping, washed and then cultured for 24h. The cells agglomerated into polarized floating vesicles with external ciliary activity. The vesicles were harvested and plated. After 1 week of culture, the resulting cell monolayers were scraped and washed, and the cells seeded onto; (i) hanging inserts (Poly carbonate with 0.4 µm pores) containing 150 µL of Matrigel (3D culture) and cultured at an air-liquid interface; after 21 days, the monolayer’s apical surface was washed with 100 µL of PBS to harvest secreted proteins, and the cells were fixed for immunocytochemistry. (ii) Alternatively, cells were seeded onto coverslips as monolayers (2D culture); after 20 days, the cells were washed and cultured in serum-free medium; 24 h later the medium was collected for protein analysis. For both systems, 20 µg aliquots of protein were separated on SDS-PAGE gels; protein bands were visualized by silver staining, and Non-stained gels were Western blotted to detect oviductal specific glycoprotein (OVGP1). Next, washed and diluted sperm were added to 2D or 3D cultures, or to floating vesicles. Sperm binding was monitored using bright field contrast microscopy, while sperm activation was monitored by fixation followed by immunostaining against PY-20 for protein tyrosine phosphorylation. Establishment of a polarized state in 3D cultures was demonstrated by immune detection of laminin and the presence of primary cilia. The epithelial phenotype was confirmed by detection of the cilia (acetylated α tubulin) and secretory activity (OVGP1), and was comparable to freshly collected BOECs. Cell polarization was consistently (n=3) lost in 2D culture. In the silver stained SDS-PAGE
gels, at least 16 extra protein bands were observed in the 3D compared to the 2D culture (46 vs 30 total bands, respectively; n=3). The differences in secreted proteins between the two cultures is under investigation. Preliminary results suggest that protein tyrosine phosphorylation in the sperm tail (15% in control sperm not exposed to oviductal cells) was not induced by the 2D oviduct epithelial cell culture (18.9%) but was induced to a similar degree by both the 3D culture (35.9% for isthmus, 27.8% for ampulla) and the floating vesicles (29.5%). In conclusion, 3D culture promoted the polarization and differentiation of BOECs in vitro such that they resembled freshly collected BOECs. Further studies will assess the possible role of the 16 additional proteins secreted in the 3D culture in sperm activation. We aim to develop a reliable oviduct-like environment to study gamete activation, fertilization and early embryo development.


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Mammalian oocytes and embryos are very sensitive to their environment. Therefore temperature, gas phase and humidity levels for culturing oocytes in vitro are usually very strictly regulated. To produce bovine embryos in vitro, oocytes are matured, fertilized and cultured in a water-jacketed CO2/O2 incubator. Gas phases in incubators are usually maintained at 5% CO2 in air for in-vitro maturation (IVM) and fertilization (IVF) of oocytes and at 5% CO2, 5% O2 and 90% N2 for in-vitro culture (IVC) of embryos, and temperature is maintained at 38.5 to 39 °C throughout in-vitro production (IVP) of embryos. We successfully used chemical packets that regulate CO2 and O2 (which are normally used for culturing bacteria) for gas regulation for producing bovine embryos in vitro (Saeki and Fuji1, 2015 IETS, Versailles, France). One packet (Anaero Pack(AP)-CO2, (Mitsubishi Gas Chemical (MGC), Tokyo, Japan) maintains a CO2 level of about 5% and consequent O2 level of about 15%, and the other (AP- MicroAero, AP-MA, MGC) maintains a CO2 level of 5-8% and an O1 level of 6-12%. Here we investigated whether a temperature-adjustable water bath (WB, Thermomate BF200, Yamato Scientific Co., Ltd, Tokyo, Japan) or a warming plate (WP, Te-Her Hot plate SP-45D, Hirasawa Works Inc., Tokyo, Japan) can be used to control temperature for in-vitro production (IVP) of cattle embryos. We also investigated two simpler methods for controlling the gas phases: no gas control for IVM/F and AP-CO2 for IVC. Bovine cumulus oocyte complexes (COCs) were collected from slaughterhouse ovaries, matured in 25mM HEPES-buffered TCM-199 (Gibco, Invitrogen Life Technologies, Tokyo, Japan) supplemented with 10% FCS (BioWest, Paris, France), 0.02 A/µm FSH (Antrin: Kyoritsu Pharmaceutical, Tokyo, Japan) and 1 µg/ml estradiol-17b (Sigma-Aldrich Japan, Tokyo, Japan) for 22 h (IVM), and fertilized in medium IVF100 (Research Institute for the Functional Peptides Co, Ltd (IFP), Yamagata, Japan) with Japanese Black bull frozen-thawed sperm (4x106 cells/ml) for 6 h (IVF). Sperm and cumulus cells were removed from the oocytes. The denuded oocytes were cultured in IVD101 (IFP, 20 to 30 embryos/50µL) for 8 days (IVC). Medium droplets covered with mineral oil (Nacalai Tesque Co., Ltd, Kyoto, Japan) were placed on the bottom of a 60-mm plastic culture dish (Falco 1007, Conring Life Sciences Japan, Tokyo, Japan). The dishes were placed in a 2.5L small plastic container (MGC), which also contained AP-CO2 for IVM and IVF, and AP-MA for IVC. About 20 ml of water was added into the container to maintain high humidity. In the first experiment, The containers were submerged in a 39 °C WB or placed on a 39 °C WP covered with a Styrofoam box. For a control group, IVM/F was done in a CO2 incubator (5% CO2, 39 °C ), and IVC was done in a CO2/O2 incubator (5% CO2, 5% O2 and 90% N2, 39 °C ). In the second experiment, COCs were matured and fertilized without gas phase control (100% air), then they were cultured with AP-CO2 or with AP-MA. Experiments were repeated 3 times. Data were analyzed by ANOVA followed by Fisher’s PLSD test. Maturation, normal fertilization, cleavage and blastocyst rates were examined at each endpoint. In the first experiment, maturation, cleavage and blastocyst rates with WB were 71%, 75% and 40%, respectively, and the same as those in the control group (P>0.05). However, the rates with WP were 28%, 37% and 2%, respectively, and significantly lower than those in a control group (82%, 69% and 33%, respectively, P<0.05). In the second experiment, blastocyst rates with AP-CO2 (30%) and with AP-MA (37%) were the same as that in the control group (34%, P>0.05). These results indicate that a temperature-adjustable water bath can be successfully used for IVP of bovine embryos in combination with use of chemical packets that regulate CO2 and O2. In addition, only AP-CO2 is needed during IVC for IVP of bovine embryos for gas phase control.

425. Screening for new receptor-ligand pairs involved in mammalian gamete recognition.

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Sexual reproduction is widely used by eukaryotes to ensure that a new, genetically distinct, organism is created at each generation. Male and female gametes have the sole task of bringing their unique genetic recombinations together in the new organism. In mammals, millions of spermatozoa compete to reach the few oocytes released at each ovulation, until finally one sperm binds and fuses with one egg leading to successful fertilization. Although we have a good cellular description of fertilization, surprisingly, the molecular basis of sperm-egg recognition and fusion remain poorly characterized. Recently, we identified Juno as the egg receptor that bound sperm Izumo1 and showed that this interaction is essential for mammalian fertilization. The identification of the Izumo1-binding partner revealed including PDIA4, ACE, BSG, CRISP1, SSLP1, SORT1, ADAM15, LMAN2, CANX, CDH, QSOX, and TEX101. These proteins are now being assessed for their ability to bind the oolemma and zona pellucida. For the sperm proteins that bind, we will attempt to determine the molecular identity of the egg receptor using an expression cloning approach with a mouse oocyte cDNA library that was successfully used.
to identify Juno as the Izumo1 receptor. It is very likely that proteins other than Izumo1 and Juno are involved in this fascinating step of reproduction, and we hope that our approach will help to further elucidate the mechanism of sperm-egg recognition.

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426. Supplementation in vitro maturation medium with L-carnitine reduces the lipid content in bovine oocytes without impairing the subsequent embryo development.

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This study aimed to evaluate the effects of supplementation with different concentrations of L-carnitine (L-car) during in vitro maturation (IVM) of bovine oocytes on their intracytoplasmic lipid content and acquisition of competence for the embryonic development. Cumulus-oocyte complexes (COCs) were matured during 22h at 38.5 °C and 5% CO2 in air; on IVM medium (TCM-199 with bicarbonate, hormones and one type of macromolecule: 10% FCS or 0.6% BSA) supplemented with 1, 5 or 10 mM of L-car, or without supplementation (Control).

For determination of relative amount of lipid oocytes were denuded from their cumulus cells and, immature (0h = analyzed immediately following aspiration; n=90) and matured oocytes (22h IVM; n=726) were stained with the lipophilic dye Sudan Black B. Stained oocytes were examined in light microscope and images were analyzed by Q-Capture Pro image software after gray scale conversion and oocytes delimitation to determine the area and the average intensity (pixels) by area. The immature group was chosen as the calibrator, and each treatment value was divided by the mean of the calibrator to generate the relative lipid content. In a second trial, a sample of COCs (n=1875) was IVM as above and then fertilized. The presumptive zygotes were cultured in SOF medium at 38.5 °C and 5% CO2 in air, for 7 days (Day 0 = 1VF), when the embryonic developmental rates were evaluated. The study was a 2x4 factorial design, including the effect of the culture media supplement (FCS and BSA), L-car concentration (0, 1, 5 and 10 mM) and interactions. The averages were compared by Tukey’s test and data are presented as least squares means ± standard error mean. The relative amount of lipid content was lower (P<0.05) in groups FCS + 1 mM L-car (0.96±0.01), FCS + 5 mM L-car (0.95±0.01), FCS + 10 mM L-car (0.96±0.01), BSA + 0 mM L-car (0.94±0.01), BSA + 10 mM L-car (0.96±0.01) compared to the FCS + 0 mM L-car (1.01±0.01), BSA + 5 mM L-car (1.01±0.01) and 0h (1.00±0.01) groups. The group BSA + 1 mM L-car (0.97±0.01) did not differ from the others (P>0.05). As there were no interactions (P>0.05) in the embryonic development analysis, only the main effects were presented: blastocyst rates were affected (P<0.05) by the macromolecule (FCS: 19.0±1.5 vs BSA: 33.0±1.5), but were not affected (P>0.05) by L-car concentration (27.3±2.1; 27.8±2.1; 27.7±2.1; 21.1±2.1, respectively for 0, 1, 5 and 10 mM). The acquisition of the developmental capacity was impaired in COCs matured in BSA-supplemented IVM medium, and the addition of L-car in this medium promoted a reduction in oocyte lipid content only when used in the highest concentration tested in this study (10 mM) but not in smaller concentrations. On the contrary, embryonic developmental rates were higher when COCs were matured in the presence of FCS, and L-car supplementation (1, 5 or 10 mM) reduced oocyte lipid content. In conclusion, supplementation with L-car in medium supplemented with FCS reduced the lipid content in oocytes without impairing the subsequent embryo development. Financial Support: FAPESP (#2012/10084-4 and #2013/07382-6).

427. The proteasome is involved in the regulation of protein phosphorylation on serine and threonine residues during the capacitation of human sperm.

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Sperm capacitation is a process that involves the early activation of protein kinases (PKs) and inactivation of protein phosphatases (PPs). The balance between the activity of PKs and PPs alters the pattern of protein phosphorylation on Ser and Thr residues during the beginning of this process; however, the participation of sperm proteasome in this process is not known. The goal of this work was to evaluate the role of the proteasome during the beginning of human sperm capacitation. Motile human sperm, free of seminal plasma, were obtained through a double percoll gradient and then incubated in a capacitating medium (Tyrode’s medium supplemented with 2.6% bovine serum albumin and 25 mM bicarbonate). Then, different aliquots were incubated at 37 °C and 5% CO2 for different periods (0, 1, 5, 15, 30 and 60 min). The percentage of capacitated sperm was evaluated using the chlortetracycline (CTC) assay. The chymotrypsin-like activity of the sperm proteasome was evaluated using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC. The pattern of protein phosphorylation on Ser and Thr residues was evaluated by means of western blots. The results indicate that the enzymatic activity of the proteasome increases during capacitation in agreement with the increase in the B pattern in the CTC assay. Sperm incubated in the presence of epoxomicin (proteasome inhibitor) exhibit a significant decrease in the B pattern according to this assay. In addition, incubation in the presence of epoxomicin changes the pattern of protein phosphorylation on Ser and Thr residues. These results suggest that the sperm proteasome has an important role during the beginning of capacitation and that its mechanism of action involves changes in protein phosphorylation on Ser and Thr residues. Acknowledgment to FONDECYT N°1120056

428. Physiological oxygen tension rescues poor developmental outcome of human in vitro matured oocytes through reducing the requirement for Sirt3 expression.

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Development deficiencies of in vitro matured (IVM) oocytes restrict their further application in the clinic. To date, limited studies have focused on the effects of oxidative stress and its controllers on immature human oocyte development. To identify a rapid clinically translatable method, we initially used mouse oocytes to establish potential mechanisms disrupting IVM oocyte development, tested the molecular mechanisms in human IVM oocytes, and finally established a feasible improvement method. Excessive production of reactive oxygen species (ROS) and impaired anti-oxidative stress protection ability were observed in IVM oocytes, possibly attributable to insufficient Sirt3 gene expression. Upon inhibition of Sirt3 gene expression via RNAi technology in in vivo matured (IVO) oocytes, similar development was observed to IVM oocytes. Furthermore, IVM development was improved upon upregulation of Sirt3 gene expression.
429. The correlation of zygotic genome activation and reactive oxygen species (ROS) on in vitro cultured embryos from different mouse strains.
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Zygotic genome activation (ZGA) is critical for normal embryonic development, and embryos development block may be related to the delay or failure of ZGA. 2-cell mouse embryos from KM strain cultured in M16 medium present 2-cell block, whereas B6D2F1 strain embryos mostly proceed to the 4-cell stage. Furthermore, medium components can also affect the block of embryos from KM strain mice, the reactive oxygen species (ROS) levels had an obvious change in different mouse strains. Under the medium of M16 and CZB media, The expression level of genes related to ZGA was significantly increased in embryos cultured in CZB media, but the expression patterns of genes related with ROS levels was not significant change for embryos cultured in different medium components. Consequently, this study demonstrates that ROS levels is not involved in the ZGA initiation and have no effect on normal embryo development.

430. The optimal concentration of 2', 3'-dideoxycytidine (ddC) for depletion of mtDNA in bovine oocytes.
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The method of current widely used somatic cell nuclear transfer (SCNT) causes heteroplasmic status because cloned embryos are a mixture with the nuclear DNA of donor cell and the mitochondrial DNAs of recipient oocyte. During normal fertilization, sperm’s mitochondria are destroyed and only maternal mitochondria are transmitted to the offspring. In this study, we used an inhibitor of 2', 3'-dideoxycytidine (ddC) for the depletion of mitochondrial DNAs (mtDNA) in recipient oocyte. To examine the optimal concentration of ddC, we evaluated various parameters related to oocyte maturation (the stage of the second meiotic metaphase (MII)) and embryo developmental competence (blastocyst formation). Bovine oocytes were matured in different concentrations of ddC (0, 1, 5, 10 or 20 μM) for 22-24h at 39°C in a humidified atmosphere of 5% CO2. In the first experiment, the bovine oocyte maturation was examined by the staining of Aceto-orcein. The rate of oocyte maturation in 20 μM ddC treated group (MII; 60.0±1.1) was significantly decreased when compared to different concentrations (0, 1, 5 and 10 μM; 79.2±1.2, 67.4±5.9, 68.1±6.4, 68.9±4.9, respectively) (P<0.01). In the second experiment, the developmental competence of parthenogenetically activated embryos following the treatment of various ddC concentrations for oocyte maturation was examined. The bovine oocytes (52.2%) treated with 20 μM ddC was reduced in the rate of cleavage as compared to the oocytes treated with different concentrations (0, 1, 5 and 10 μM; 65%, 71.1%, 73.0%, 60.9%, respectively). Additionally 20 μM ddC (4.2%) treated bovine oocytes were significantly lower in blastocyst formation rates when compared to other groups (0, 1, 5 and 10 μM; 16.3%, 15.1%, 11.7%, 9.9%, respectively) (P<0.05). Finally, the mitochondria of the bovine oocytes treated with 10 ddC for oocyte maturation were negatively labeled with Mito Tracker dyes. Based on the results of these experiments, we can determine the optimal concentration (10μM ddC) for the depletion of mitochondrial DNAs in bovine oocytes during oocyte maturation.

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Several embryonic and extraembryonic lineages emerge during early embryonic development. One such event is the specification of the primitive endoderm (PrE), which gives rise to the yolk sac, endothelium (blood vessel lining), and several organ systems. Human and mouse PrE cell lines have been used extensively to study early embryogenesis in these species. However, the efficiency of obtaining bovine endoderm cultures has been considerably low, thus impeding our ability to study this important feature of embryonic development in cattle and other ruminants. It remains unclear if PrE specification and differentiation in cattle is similar to the events observed in human and mouse models. The goals of the following research were to establish a protocol for obtaining bovine PrE outgrowth cultures, delineate the type(s) of endoderm lineages represented in these cultures, and characterize bovine PrE cell lines derived from these cultures. Bovine oocytes were collected from slaughterhouse-obtained ovaries, matured and fertilized in vitro, and then cultured until day 8 post-fertilization. Single embryos were then transferred to 3.8 cm² wells treated with MatrigelTM Basement Membrane Matrix and cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 5.5 mM glucose, 20% fetal bovine serum (FBS), and 10 ng/ml recombinant bovine fibroblast growth factor-2 (FGF2) at 38.5°C in 5% CO2, 5%O2, 90% NO2. Medium was replaced on day 12 post-fertilization, and embryos were assessed visually for the presence of endoderm outgrowths on day 15. Outgrowths were then maintained in DMEM supplemented with 5.5 mM glucose and 10% FBS at 38.5°C in 5% CO2 in air. When the cultures reached ~90% confluency, they either were processed for RNA extraction or passaged for further culture. This procedure yielded an endoderm formation rate of 80.3 ± 5.6% at day 15 (n=7 replicate studies). The outgrowths harvested before passage possessed markers for PrE (GATA4, GATA6), visceral endoderm (VE) (BMP2, VEGFA), and parietal endoderm (PE) (CXR4, THBD, HHEX). Transcript markers for trophoblast (IFNT, CDX2) and inner cell mass (NANOG) also were detected in cultures, albeit at low levels. Two endoderm lines were maintained in culture, and both lines were propagated for approximately 6 weeks (6 to 8 cell passages) before becoming quiescent. Both cell lines maintained endoderm-like patterns of genes related with ROS levels was not significant change for embryos cultured in different medium components. Consequently, this study demonstrates that ROS levels is not involved in the ZGA initiation and have no effect on normal embryo development.

429. The correlation of zygotic genome activation and reactive oxygen species (ROS) on in vitro cultured embryos from different mouse strains.
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Zygotic genome activation (ZGA) is critical for normal embryonic development, and embryos development block may be related to the delay or failure of ZGA. 2-cell mouse embryos from KM strain cultured in M16 medium present 2-cell block, whereas B6D2F1 strain embryos mostly proceed to the 4-cell stage. Furthermore, medium components can also affect the block of embryos from KM strain mice, the reactive oxygen species (ROS) levels had an obvious change in different mouse strains. Under the medium of M16 and CZB media, The expression level of genes related to ZGA was significantly increased in embryos cultured in CZB media, but the expression patterns of genes related with ROS levels was not significant change for embryos cultured in different medium components. Consequently, this study demonstrates that ROS levels is not involved in the ZGA initiation and have no effect on normal embryo development.
behavior, as both lines expressed GATA4 and GATA6 throughout culture. An increase in PE- and VE-specific transcripts was observed with continued time in culture, suggesting cells may be differentiating. Frozen stocks of each line were successfully thawed and possessed the same pattern of culture progression as the non-frozen cell lines. In summary, this work identified a scheme that improves PrE outgrowth efficiency in cattle. It also validated an endoderm expression profile in initial and extended cultures. Cultures contained VE and PE markers, suggesting spontaneous endoderm differentiation. This newly developed PrE culture protocol will have applications for understanding endoderm specification, differentiation, and function during embryonic and extraembryonic development in cattle.

432. Development of pre-implantation porcine embryos cultured within a three-dimensional alginate hydrogel system either conjugated with Arg-Gly-Asp (RGD) peptide or supplemented with secreted phosphoprotein 1 (SPP1).

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Many uterine specific factors have been shown to be increased within the uterine milieu as the porcine embryo initiates elongation. Secreted phosphoprotein 1 (SPP1) is increased during this time and contains an Arg-Gly-Asp (RGD) peptide sequence that has been shown to bind to cell surface integrins on the uterine endometrium and trophectoderm promoting cell adhesion and migration. We have previously developed a three-dimensional culture system using alginate hydrogels that provides mechanical support to maintain embryo architecture and facilitate morphological changes of pre-implantation embryos in vitro. The objective of the current study was to evaluate embryo development of pre-implantation porcine embryos cultured within alginate hydrogels either conjugated with RGD or supplemented with SPP1. White crossbred gilts (n = 32) from nine replicate collections were bred at d 0 and 1 of the estrous cycle. At d 9 of gestation, reproductive tracts were collected and flushed with RPMI-1640 media. Embryos were recovered and washed with RPMI-1640 media. Embryos (n = 256) were randomly assigned to be encapsulated using a standard double encapsulation technique (0.7% sodium alginate and 1.5% calcium chloride solution) or used as non-encapsulated controls. Evaluations of embryo development were made for: 1) the standard hydrogel system; 2) the standard hydrogel system conjugated with 2.3 mg/g RGD peptide; 3) the standard hydrogel system supplemented with 0.1 μg/ml SPP1; and 4) non-encapsulated controls. Embryos were cultured individually within 4-well NUNC plates for 96 h in CO2-pretreated RPMI-1640 with 10% FBS media, 5% CO2 in air and 100% humidity. Every 24 h, half of the media was replaced with fresh media and the embryos were imaged to assess cell survival by blastocyst fragmentation and evaluate morphological changes throughout the culture period. All data were analyzed with GLM procedure for ANOVA, reported as least-squares means, and means were separated using a series of orthogonal contrasts. At termination of culture, embryo survival was greater (P < 0.05) for embryos encapsulated in hydrogels conjugated with RGD (42.2 ± 5.9%) and those supplemented with SPP1 (42.2 ± 5.9%) compared to non-encapsulated controls (21.9 ± 5.9%). In addition, survival of embryos encapsulated in the standard hydrogels (35.9 ± 5.9%) tended (P = 0.10) to be greater compared to non-encapsulated controls. Throughout the culture period, all non-encapsulated control embryos remained spherical with no significant morphological change. In contrast, a significant (P < 0.01) proportion of embryos encapsulated in the standard hydrogels (10.9 ± 3.9%), in hydrogels conjugated with RGD (21.9 ± 3.9%), or supplemented with SPP1 (14.1 ± 3.9%) had morphological changes compared to non-encapsulated controls. These changes were characterized by minor to moderate flattening and tubal formation of the embryo through the gel. Furthermore, the proportion of embryos undergoing morphological changes was greater (P = 0.05) for those encapsulated in hydrogels conjugated with RGD compared to the standard hydrogels. These results demonstrate that embryos encapsulated in alginate hydrogels conjugated with RGD or supplemented with SPP1 develop similarly to those in our standard alginate hydrogel system. Furthermore, RGD conjugation within the hydrogel improved embryo development in terms of proportion of embryos undergoing morphological changes.

*USDA is an equal opportunity provider and employer.

433. Role For CC Cytokines In Differentiation Of The Inner Cell Mass Of The Bovine Embryo.

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Previous research indicates that the chemokine (C-C motif) ligand CCL24 is overexpressed in the inner cell mass (ICM) of the blastocyst as compared to the trophectoderm (TE) (BMC Dev Biol 12:33). Given the potential importance of cell migration for establishment of the hypoblast adjacent to the blastocoele on the outer edge of the ICM, we hypothesize that CCL24 or another embryo-derived chemokine plays a role in differentiation of the ICM. The first objective was to evaluate temporal expression of CCL24 throughout early development. Steady-stage amounts of mRNA were evaluated for pools of 10-40 embryos collected at the oocyte, 2 cell, 3-4 cell, 5-8 cell, 9-16 cell, morula (collected separately on Days 5 and 6), and blastocyst (collected separately on Days 6, 7, 8 and 9) stages using quantitative real-time PCR with YWHAZ, SDHA, and GAPDH as internal control genes. Expression of CCL24 was non-detectable until Day 6 of development, at the morula stage, peaked in the Day 7 blastocyst, and declined thereafter and became non-detectable by Day 9. Thus, CCL24 expression is maximal at a time coincident with formation of the blastocyst. The second objective was to determine whether inhibition of CCR3 (receptor for CCL24) alters the pattern of blastocyst formation. Embryos were treated with a CCR3 antagonist (SB328437) beginning at Day 6. Embryos (n=25-26 per group) were collected at Day 8, fixed with 4% paraformaldehyde and subjected to immunolabeling for GATA6 (hypoblast) and NANOG (epiblast). The number and location of cells positive for NANOG and GATA6 was determined using epifluorescence microscopy. SB328437 decreased the percent of GATA6+ cells that were in the outer part of the ICM (61±1.7%) as compared to controls (66±1.6%; P=0.057). The experiment was repeated with additional embryos (n=8-9 per group) using confocal microscopy. Again, treatment with SB328437 decreased the percent of GATA6+ cells that were on the periphery of the ICM (77±2.6% for treated vs 86±2.9% for control; P=0.04). Although the CCR antagonist altered localization of GATA6+ cells, it is likely that the antagonist affects an alternative receptor because CCR3 mRNA was non-detectable in embryos at all stages of development through the blastocyst stage. In summary, the bovine embryo expresses a C-C chemokine, CCL24, at a time coincident with blastocyst formation and the first differentiation of the ICM. Moreover, inhibition of a C-C receptor disrupted localization of hypoblast cells in the ICM.
hypothesize that CCL24 acts through a SB328437-sensitive mechanism to regulate position of hypoblast cells in the ICM. USDA AFRI Grant No. 2011-67015-30688.

434. CRISPR/Cas9-mediated Inactivation of Glucocorticoid Receptor (NR3C1): Effect on Ovine Embryo Development. Kelsey Brooks1, Gregory Burns1, Thomas E. Spencer1.
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In sheep, the blastocyst hatches from the zona pellucida on Day 8 and develops into an ovoid or tubular conceptus (embryo/fetus and associated extraembryonic membranes) by Day 12. The ovoid conceptus then elongates to 14 cm or more by Day 16 and synthesizes and secretes interferon tau (IFNT), the maternal recognition of pregnancy signal, as well as prostaglandins (PGs) and cortisol. The enzymes, hydroxysteroid (11-beta) dehydrogenase 1 (HSD11B1) and HSD11B2, interconvert inactive cortisol and active cortisol. In sheep, HSD11B1 is expressed and active in the conceptus trophectoderm as well as in the endometrial luminal epithelia; in contrast, HSD11B2 expression is most abundant in conceptus trophectoderm. Cortisol is a biologically active glucocorticoid and ligand for the glucocorticoid receptor (NR3C1) and mineralocorticoid receptor (NR3C2). NR3C2 is not detectable in either the endometrium or conceptus of the ovine uterus during early pregnancy. In tissues that do not express NR3C2, HSD11B2 protects cells from the growth-inhibiting and/or pro-apoptotic effects of cortisol, particularly during embryonic development. Cortisol readily passes across cell membranes and activates NR3C1, which is a transcriptional regulator that modulates gene expression and cellular physiology. Our previous study found that HSD11B1, but not HSD11B2, is essential for successful conceptus development in vivo (Endocrinology 2013; 154:931). Recent advances in the bacterial clustered regularly interspaced short palindromic repeat (CRISPR)/Cas9 system has enabled precise editing of mammalian genomes. In the present study, we used the CRISPR/Cas9 system to test the hypothesis that NR3C1 has an essential role in conceptus elongation and development in sheep. Superovulated ewes were bred at estrus (n=5), and one-cell zygotes were recovered 36 hours post-mating. Zygotes were injected with either: (1) wildtype Cas9 RNA alone (Control); or (2) wildtype Cas9 RNA along with 4 guide RNAs targeting the ovine NR3C1 gene (NR3C1-targeted). Injected zygotes were developed to the blastocyst stage in culture and then transferred to day 8 recipient ewes. The uterus was obtained 6 days post-transfer (Day 14). Elongating, filamentous type conceptuses (12-14 cm in length) were recovered from ewes gestating control embryos (n=7/7). Similarly, conceptuses recovered from ewes gestating NR3C1-targeted embryos were fully elongated and filamentous (n=6/7). DNA was isolated from recovered conceptuses, and the targeted region of NR3C1 was amplified and Sanger sequenced. All control conceptuses and one of the NR3C1 targeted conceptuses had no sequence alterations. However, the other 6 NR3C1 targeted conceptuses were edited, causing a frame shift mutation resulting in a premature stop (n=2/6) or deletion of the essential zinc finger binding domain (n=4/6). These results support the ideas that: (1) NR3C1 is dispensable for ovine embryo survival, blastocyst development, and peri-implantation conceptus elongation; and (2) the effects of HSD11B1-derived cortisol on conceptus elongation are indirectly mediated by the endometrium. This project was supported by AFRI competitive grant 2012-67015-30173 from the USDA National Institute of Food and Agriculture.

435. Synaptobrevin Puncta are Present in the Apical Ridge but do not Co-Localize with Syntaxin Puncta to form Trans-SNARE Complexes during Mouse Sperm Capacitation. Momal Sharif2, Elena P. Da Silva2, Syed Tahir Abbas Shah2, David J. Miller2.
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The principal role of SNARE proteins is to arbitrate vesicle fusion to a target membrane. Formation of tripartite protein complexes between SNARE proteins on apposing membranes is the minimal requirement for membrane fusion. A member of the SNARE family, syntaxin, is found on the sperm plasma membrane while synaptobrevin, is found on the outer acrosomal membrane. During the sperm acrosome reaction, the outer acrosomal membrane fuses at hundreds of points with the overlying plasma membrane, resulting in release of the acrosomal contents. We hypothesize that, during capacitation, syntaxin and synaptobrevin shift within their respective membranes to form trans-SNARE complexes that promote membrane fusion at hundreds of specific points during the acrosome reaction. Immunofluorescence was used to localize both syntaxin and synaptobrevin in mouse epididymal sperm before and after capacitation. Sperm were fixed, permeabilized, and incubated with antibodies to syntaxin, synaptobrevin and then fluorescent secondary antibodies. Super resolution Structured Illumination Microscopy (SR-SIM) was used to examine samples collected at 0-120 min of capacitation time, to obtain 3D images of SNARE localization. At 60 min of capacitation, syntaxin was localized in puncta that were mostly restricted to the apical ridge of the plasma membrane overlying the acrosome in over 90% of sperm. Syntaxin was localized in this restricted pattern in less than 20% of the sperm that were not capacitated or were incubated in medium lacking albumin. In contrast, synaptobrevin was already found in the puncta at the apical ridge of the sperm head in 83% of sperm prior to capacitation, where it remained during capacitation. When co-localization was assessed, the puncta containing syntaxin and synaptobrevin did not precisely co-localize at the beginning or end of capacitation. Our results demonstrate that, in contrast to syntaxin, the acrosomal SNARE synaptobrevin is already localized to the apical edge of capacitated sperm. But the puncta that contain synaptobrevin do not co-localize with those containing syntaxin, even after 60 min of capacitation time. Therefore, the formation of trans-SNARE complexes does not occur until after capacitation, during acrosomal exocytosis. Supported and funded by COMSATS Institute of technology, Pakistan.

436. Pre-implantation Ovine Conceptus Growth is Stimulated by Uterine Histotroph. Thomas E. Spencer1, Kelsey Brooks1.
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In sheep, the blastocyst hatches from the zona pellucida on Day 8 and develops into an ovoid or tubular conceptus (embryo/fetus and associated extraembryonic membranes) by Day 12. The ovoid conceptus begins to elongate on Day 12 and grows to 14 cm or more by Day 16. The elongating conceptus synthesizes and secretes interferon tau (IFNT) as well as prostaglandins (PGs). The uterine gland knockout ewe established that conceptus survival and elongation is dependent on secretions in the uterine luminal fluid (ULF), termed histotroph, from the endometrial epithelia. Indeed, ovine blastocysts can be developed in vitro, but will only elongate in utero. Available evidence supports the idea that conceptus IFNT and PGs act in a paracrine manner on the endometrium to modulate expression of progesterone-induced elongation-related genes expressed in the endometrial epithelia. In turn, the products of those genes modify ULF to...
stimulate trophoderm survival and proliferation for conceptus elongation. Study One tested the hypothesis that ULF contains essential factors that stimulate conceptus growth and elongation. First, ULF was obtained from Day 14 cyclic (C) and pregnant (PX) ewes (n=5 per status) by gently flushing the uterus with 5 ml sterile phosphate buffered saline (PBS). The conceptus was removed from the PX ULF, and the recovered ULF was clarified by centrifugation and stored at -80°C. Next, cycling ewes were mated at estrus (Day 0). A preloaded and equilibrated Alzet 2ML1 osmotic pump was secured to the mesosalpinx, and the attached catheter inserted into the uterine lumen. Osmotic pumps (n=5 ewes with 2 pumps per treatment) contained one of the following treatments: (1) C ULF; (2) PX ULF; or (3) recombinant ovine IFNT (roIFNT; 101 μg). On Day 12, the uterine lumen was gently flushed with 20 ml PBS to recover the conceptus, and the ULF and endometrium was collected for analyses. Concentrations from ewes receiving PX ULF were larger (P<0.05) than those from C ULF (9.9 vs 2.3±1.8 mm³) and roIFNT (9.9 vs 3.6±1.8 mm³) ewes and longer (P<0.05) than those from C ULF (3.5 vs 1.5±0.5 mm) and roIFNT (3.5 vs 1.9±0.5 mm) ewes. The amount of total PGs in the ULF was not different (P>0.10). The amount of IFNT amount in the ULF was not different (P>0.10) in roIFNT-infused ewes. Expression of classical IFNT -stimulated genes (CXXCL10, ISG51, RSAD2) was 3- and 4- fold higher (P<0.01) in the endometria of ewes receiving PX ULF or roIFNT compared to C ULF, but not different (P>0.10) in endometria of PX ULF compared to roIFNT ewes. Expression of several elongation-related genes (CST3, HSD11B1, IGFFBP1, PTGS2, SLCT2A1, SLC7A2) were not different (P>0.10) in the endometrium, but two genes (CST6, LGALS15) were more abundant (P<0.10) in ewes infused with PX ULF than C ULF or roIFNT. In Study Two, proteomic profiling of the ULF from Day 14 C and PX ewes (n=4 per status) was conducted using liquid chromatography-tandem mass spectrometry. Over 1,400 proteins were detected. Spectral counting analysis identified 261 proteins more abundant in or unique to PX ULF and 92 proteins less abundant or unique to C ULF (>2-fold, P<0.05). Collectively, these results support the ideas that: (1) factors other than IFNT and PGs present in the ULF of Day 14 pregnant ewes stimulate pre-implantation conceptus growth and development; and (2) IFNT is not directly embryotrophic. This project was supported by AFRI competitive grant 2012-67015-30173 from the USDA NIFA.

437. Seminal vesicle protein SVS2 acts as a protectant of sperm sterols and prevents ectopic sperm capacitation.
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Mammalian sperm must “capacitate” to fertilize an egg in the female reproductive tract. Plasma membrane of the ejaculated mammalian sperm contains high levels of cholesterol, and efflux of cholesterol is required for sperm capacitation in vitro. Thus, sperm capacitation in vivo seems to be regulated by some cholesterol liberator such as albumin or high-density lipoprotein in the fluid of the female reproductive tract, though concentration of albumin in any places of the female reproductive tract is enough to induce capacitation. On the other hand, since contamination of seminal plasma prevents sperm capacitation in vitro, seminal plasma is considered to contain a “decapacitation factor” that prevents sperm capacitation. We previously demonstrated that seminal vesicle secretion 2 (SVS2), which is a seminal plasma protein secreted from the mouse seminal vesicle, acts as a decapacitation factor in in vitro. On the other hand, the SVS2-KO male mice are infertile in vivo, and SVS2 protects spermatozoa from some spermicidal attack in the uterus. In this study, we tried to investigate the mechanisms by which SVS2 controls in vivo sperm capacitation. SVS2-deficient males that mated with wild-type partners resulted in decreased cholesterol levels on ejaculated sperm in the uterine cavity. SVS2 prevented cholesterol efflux from the sperm plasma membrane and incorporated liberated cholesterol in the sperm plasma membrane, thereby reversibly preventing the induction of sperm capacitation by bovine serum albumin and methyl-b-cyclodextrin in vitro. Thus, binding of SVS2 to the sperm prevents cholesterol efflux and retrieves free cholesterol from the medium. After mating, SVS2 enters the uterus and the uterotubal junction, arresting sperm capacitation in this area. SVS2 is disappeared from the sperm in the oviductal ischemia where sperm capacitation occurs. Therefore, sperm capacitation in in vivo condition seems not to be mediated by any cholesterol liberators such as albumin, but by the cholesterol protector, SVS2; the “decapacitation factor” SVS2 plays a key role in unlocking the ectopic sperm capacitation in vivo.

438. WITHDRAWN.

439. The Role of Galectin-3 on Sperm-Zona Pellucida Interaction.
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Human spermatozoa leaving the testis can fertilize an oocyte only after post-testicular maturation and capacitation. These processes involve modification and reorganization of molecules on the sperm plasma membrane, resulting in acquisition of the binding ability of the spermatozoa to the outer coat (i.e. zona pellucida) of the oocyte followed by zona pellucida-induced acrosome reaction. Defects in the sperm-zona pellucida interaction are major causes of low fertilization rates in clinical in-vitro fertilization. Galectin-3 is a secretory lectin that can cross-link glycoproteins together as a complex on the cell surface. It is known for its action on cell adhesion, cell surface receptor trafficking and clustering. In the male reproductive tract, galectin-3 has been detected in seminal plasma and ejaculated spermatozoa, but not in testicular germ cells, indicating that the ejaculated spermatozoa acquire galectin-3 post- testicularly. However, the role of galectin-3 in sperm functions is unknown. In this study, we hypothesize that galectin-3 plays a modulatory role in spermatozoa-zona pellucida binding. Our result showed that galectin-3 immunoactivity was localized to the apical region of capacitated spermatozoa, a region known to bind zona pellucida. Blocking the function of sperm galectin-3 by functional blocking antibody or galectin-3 competitive substrate (i.e. N-Acetyl-D-lactosamine) inhibited spermatozoa-zona pellucida binding as demonstrated by hemizona assay. On the other hand, no effect can be observed for the antibody/N-Acetyl-D-lactosamine on sperm motility, viability and acrosome reaction as demonstrated by computer assisted sperm analysis, trypan blue staining and FITC-Pisum sativum agglutinin staining respectively. Furthermore, galectin-3 was demonstrated to be present in seminal plasma in form of extracellular membranous vesicles, suggesting that galectin-3 may be transferred to sperm surface by extracellular vesicles during post-testicular maturation. The resided galectin-3 can then modulate spermatozoa-zona pellucida binding after capacitation. Our results provide evidences that protein from the male reproductive tract may be important for fertilization. Continued investigation of the area will provide considerable understanding of the regulation of
fertilization that will be useful for practical application in human contraception and reproductive medicine. Acknowledgements: This work was supported in part by grants from the Committee on Research and Conference Grant, Hong Kong Research Grant Council (764512M and 17105214).

440. Ultrastructural comparison of nucleus-associated organelle in porcine embryonic stem cells derived by in vitro fertilization and somatic cell nuclear transfer.

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The ultrastructure of porcine embryonic stem cells (pESCs) and porcine fetal fibroblasts (PFFs) was analyzed by transmission electron microscopy (TEM). The aim of this study was to compare the features of protein synthesis and intracellular digestion-associated organelles in in vitro fertilization-derived pESCs (IVF-pESCs) and somatic cell nuclear transfer-derived pESCs (SCNT-pESCs). The important function of ribosomes, rough endoplasmic reticulum (rER) and the Golgi apparatus is protein synthesis. The rER was rarely observed in PFFs and SCNT-pESCs. Furthermore, the cytoplasm of these cells contained few free ribosomes and polysomes. By contrast, long stalks of ribosome-studded rER were observed in IVF-pESCs. The rER was often extensive and rich in free ribosomes/polysomes. On the other hand, the Golgi apparatus was rarely observed in the cytoplasm in all cell lines. Intracellular digestion-associated organelles include phagocytic vacuoles, autophagic vacuoles and lysosomes. Round phagocytic vacuoles containing membranous structures were frequently seen in PFFs and SCNT-pESCs, but not in IVF-pESCs. Autophagic vacuoles containing dense irregular bodies were also observed, although they were not seen in PFFs and SCNT-pESCs and were rare in IVF-pESCs. In addition, lysosomes were frequently seen in PFFs and SCNT-pESCs as round electron-dense cytoplasmic structures. By contrast, lysosomes were not prominent in IVF-pESCs. This conclusion studies that the ultrastructural characteristics of pESCs differ depending on their origin. These ultrastructural characteristics might be useful in biomedical research using pESCs. Furthermore, these results could be used to establish naïve pESC lines. This work was supported, in part, by a grant from the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ011288), Rural Development Administration, and the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2012R1A1A4A01004885, NRF-2013R1A2A2A04008751), Republic of Korea.

441. The role of Sialyl-Lewis(x)-binding protein and sialidase on spermatozoa-zona pellucida interaction.

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A spermatozoon cannot fertilize an oocyte immediately after ejaculation, but acquire the fertilization capacity within the female reproductive tract by a process termed capacitation. This process involves relocalization of molecules in the plasma membrane of the sperm head resulting in acquisition of the binding capacity of the spermatozoon to the outer coat (zona pellucida, ZP) of the oocyte. Capacitated spermatozoa initiate fertilization by binding to the carbohydrate sequences on the ZP, which induces acrosome reaction of the ZP-bound spermatozoa. Defects in this binding are the major causes of reduced fertilization rates in assisted reproduction. Despite the importance of spermatozoa-ZP interaction, its regulatory mechanisms are unclear partly due to lack of knowledge on the identity of the ZP receptor on spermatozoa. Accumulated evidences indicate that the ZP receptor is a multi-molecular structure involving coordinated actions of several proteins assembled during capacitation. Consistently, we find that molecules carrying the major carbohydrate chain of the ZP known as sialyl-Lewis(x) (SLeX) can suppress spermatozoa-ZP binding but do not affect acrosome reaction, showing that other molecules are involved in the induction of acrosome reaction during spermatozoa-ZP interaction. By using affinity chromatography followed by mass spectrometric analysis, chromosome 1 open reading frame 56 (C1orf56) was identified to be one of the SLeX-binding proteins in the membrane protein extracts of capacitated spermatozoa. Interestingly, immunohistochemical staining of various human tissues shows strong C1orf56 immunoreactivities only in cells of the seminiferous tubules. Treatment with antibody against C1orf56 inhibited spermatozoa-ZP binding and ZP-induced acrosome reaction dose-dependently. Anti-C1orf56 antibody did not affect sperm viability, motility and spontaneous acrosome reaction. The acrosomal region of spermatozoa possessed C1orf56 immunoreactivity and its intensity increased after capacitation in vitro, suggesting translocation of C1orf56 to the cell surface during capacitation. Our data also show that capacitated human spermatozoa possess cell surface sialidase activity. Sialidase-1 and -3 immunoreactivities can be detected on the acrosomal region of spermatozoa. Two observations support the involvement of sialidase in spermatozoa-ZP interaction. First, blocking the sialidase activity reduces the capacities of the spermatozoa in binding to ZP and undergoing ZP-induced acrosome reaction. Second, sialidase-treated ZP has increased sperm binding capacity and acrosome reaction-inducing activity. Our studies provide evidence that human sperm-ZP interaction involves coordinated actions of different proteins. Continued investigation of the area will provide considerable understanding of the regulation of fertilization that will be useful for practical application in human contraception and reproductive medicine.

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442. Effects of L-carnitine and Nicotinic acid on In Vitro Maturation in Porcine Cumulus-Oocyte Complexes.

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The objective of this study was to investigate effects of L-carnitine (L) and/or nicotinic acid (N) on nuclei maturation and mitochondria activity during in vitro maturation in porcine cumulus-oocyte complexes (COCs). The COCs in maturation medium (TCM-
443. Phage-Peptide Constructs for Immunocontraception of Wild Pigs: Effects on Events Associated with Fertilization In Vitro.


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Overpopulation of wild pigs is an expanding economic, health and welfare problem in many parts of the world. Wild pigs are increasing in number throughout the United States, with populations currently found in 47 states. The annual cost of agricultural damage due to wild pigs in the United States in recent years was estimated to be over $1.5 billion. This conservative estimate does not include costs associated with diseases transmitted from wild pigs to other livestock or humans. Current control programs that focus on wild pig eradication through trapping and hunting are expensive, inefficient, and often unacceptable to the public. A promising strategy for population control of wild pigs is contraception. Filamentous phage can be used as delivery vectors for immunogenic peptides with contraceptive properties. In previous studies multiple phage-peptide constructs, when administered to pigs, stimulated production of anti-sperm antibodies detectable in blood, as well as in oviductal fluids. In this study, in vitro fertilization (IVF) was used to evaluate the ability of sera obtained from immunized pigs and containing anti-sperm antibodies to inhibit sperm-oocyte interactions and events associated with embryogenesis. Evidence of the inhibition of such events would support the functionality of phage-peptide constructs as immuno-contraceptive reagents. Serum samples from pigs immunized with three different phage-based constructs displaying GQQGLNGDS (GQQ), GEGGYGSHD (GEG), and GPNSSDADS (GPN) peptides, selected for their specificity to porcine zona pellicida, were tested. Both sperm and oocytes were incubated separately with immune sera before performing IVF in the presence or absence of immune sera in 100 μl reaction volumes. Generally, at least three replicates of 60 individual IVF reactions were performed for each antisem. Non-immune porcine serum was used in negative control reactions. Number of spermatozoa bound per embryo, percent (%), embryos cleaved, and % blastocyst development were determined. Overall, immune sera inhibited (P < 0.05) sperm binding and reduced both % cleaved and % blastocyst development. Effects were most pronounced at a serum dilution 1:50 (v/v) and sperm concentration of 0.25 x 10^6 cells/ml, with sperm exposure to anti-serum for 15 minutes and oocyte exposure for 30-40 minutes prior to IVF. Under such conditions, fewer sperm bound to the zona pellucida in the presence of anti-sera and the combined group (1.5 ± 0.08 pixels/oocyte) was compared with the control group (3.0 ± 0.1 pixels/oocyte), and N (3.0 ± 0.1 pixels/oocyte) at 22 h (P < 0.05). Moreover, we determined antioxidant levels in maturated oocytes, but stimulate mitochondria vitality. The level of hydrogen peroxide in the combined L and N group was increased (P < 0.05), but not glutathione level. In addition, mitochondria vitality in nicotinic acid (1.3 ± 0.02 pixels/oocyte) and the combined group (1.5 ± 0.08 pixels/oocyte) was stimulated (P < 0.05). In conclusion, cumulus cell expansion and the nuclei maturation in porcine oocytes are increased by L-carnitine and nicotinic acid, such as antioxidants. Also, both antioxidants inhibit hydrogen peroxide level in maturated oocytes, but stimulate mitochondria vitality. Together these results show that cumulus cells in porcine regulates oocyte ability with mitochondria vitality via inhibition of reactive oxygen species. This research was supported by Bio-industry Technology Development Program (IPET 312060-05-1-CG000), Ministry for Food, Agriculture, Forestry and Fisheries, Republic of Korea.

444. Zygote injection of CRISPR/Cas9 RNA successfully modifies the target gene without delaying blastocyst development or changing the male:female sex ratio in pigs.

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The CRISPR/Cas9 genome editing tool has increased the efficiency of creating genetically modified pigs for use as biomedical or agricultural models. CRISPR guide RNAs transcribed from gBlocks (IDT, Coralville, Iowa) along with Cas9 RNA (Sigma, St. Louis, MO) were co-injected into in vitro derived pig zygotes in order to modify the transmembrane protease, serine 2 (TMPRSS2) gene. The objectives of this experiment were to determine if DNA modification by CRISPR/Cas9 injection resulted in a delay in development to the blastocyst stage or in a skewing of the male/female sex ratio. Six gBlocks (1,2,3,4,5,6) containing a T7 promoter sequence, the CRISPR Guide RNAs (18-20 bp) and 85 bp of tracer RNA were PCR amplified with Q5 polymerase (NEB, Ipswich, MA) and in vitro transcribed with the MEGashortscript™ T7 Transcription Kit (Life Technologies, Grand Island, NY). Three CRISPR guide RNA pairs (1+5, 2+4, 3+6, 20 ng/ul) and polyadenylated Cas9 (20 ng/ul) were co-injected into the cytoplasm of zygotes (3 replicates) and cultured in vitro in PZM3 + 1.69 mM arginine medium (MU1) to the blastocyst-stage. Blastocysts were counted and collected as they formed on days 5, 6 or 7. DNA was isolated from each embryo and PCR was performed to determine genotype and gender of each embryo. Embryos were classified as modified if they contained an INDEL as measured by both gel electrophoresis and DNA sequencing. Two embryo transfers were also performed surgically into recipient gilts on day 4 of estrus (56 or 60 day 5 embryos/pig). Significant differences were determined by using Proc Freq or Proc GLM in SAS 9.4. The rate of blastocyst development was not significantly different between 2015 Abstracts – Page 176
Vitrification is further assessed with oocytes from Atg7f/f/Zp3-CRE mice, with specific deletion of Atg7 gene in oocytes. MII oocytes from these mice showed an increased autophagic response, suppression of mTOR potentially influencing other cellular processes. The role for autophagy during the initial stages of development, including the cleavage and blastocyst stages, is critical for embryo survival. Rapamycin is a selective inhibitor of mTOR, and it is shown to induce autophagy in various systems. Autophagy, a subcellular catabolic process, contributes to clearance and recycling of macromolecules and organelles in response to cellular stresses including starvation, infection, and environmental change. We previously reported that vitrified-warmed mouse oocytes show an acute increase in autophagy during warming and suggested that it is a normal response to cold stress. In this work, we aim to investigate if the modulation of autophagic response influences survival, fertilization, and developmental rates of vitrified-warmed mouse oocytes. We used rapamycin to enhance autophagy in phase II (MII) oocytes prior to vitrification. Autophagic response in MII oocytes was effectively increased at 100 nM rapamycin, as assessed by LC3 immunofluorescence staining. The oocytes were then vitrified for 2 weeks, warmed, and subjected to in vitro fertilization (IVF). We found that the fertilization rate of vitrified-warmed oocytes after 100 nM rapamycin treatment was significantly lower than that of the control (fresh oocytes) and DMSO-treated groups (33.18 ± 4.506% vs. 63.02 ± 7.649% and 62.30 ± 8.220%; P<0.05). Developmental rate to the blastocyst stage did not differ significantly between rapamycin-treated and DMSO-treated vitrified-warmed oocytes (70.75 ± 8.242% vs. 75.53 ± 6.403%; P>0.05). The results indicate that rapamycin treatment prior to vitrification increased autophagic response in MII oocytes and that these oocytes show reduced fertilization rate. Although one effect of rapamycin is the increased autophagic response, suppression of mTOR potentially influences other cellular processes. The role for autophagy during vitrification is further assessed with oocytes from Atg7+/Zp3-CRE mice, with specific deletion of Atg7 gene in oocytes. MII oocytes from Atg7+/Zp3-CRE were vitrified-warmed and subjected to IVF. The fertilization rate between Atg7+/ (control) and Atg7+/Zp3-CRE vitrified-warmed oocytes was not significantly different (51.10 ± 13.11% vs. 52.93 ± 8.87%; P>0.05). Interestingly, Atg7+/Zp3-CRE vitrified-warmed embryos showed a higher developmental rate than the Atg7+/Zp3-CRE fresh oocytes (77.77 ± 5.53% vs. 49.67 ± 3.3%; P<0.05), suggesting that the process of vitrification per se improves preimplantation embryonic development of Atg7+/Zp3-CRE oocytes.

Overall, complete absence or enhancement of autophagy in oocytes does not negatively influence fertilization, warming, and fertilization and these results indicate a dispensable role for autophagy during vitrification and warming of MII oocytes. This study was supported by a grant from the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI12C0055, https://www.hiddream.kr/).


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The mammalian target of rapamycin (mTOR), one of the downstream effectors of phosphatidylinositol 3- kinase and protein kinase B, regulates cellular processes such as cell survival, growth, metabolism, transcription, protein translation, and autophagy. Rapamycin is a selective inhibitor of mTOR, and it is shown to induce autophagy in various systems. Autophagy, a subcellular catabolic process, contributes to clearance and recycling of macromolecules and organelles in response to cellular stresses including starvation, infection, and environmental change. We previously reported that vitrified-warmed mouse oocytes show an acute increase in autophagy during warming and suggested that it is a normal response to cold stress. In this work, we aim to investigate if the modulation of autophagic response influences survival, fertilization, and developmental rates of vitrified-warmed mouse oocytes. We used rapamycin to enhance autophagy in phase II (MII) oocytes prior to vitrification. Autophagic response in MII oocytes was effectively increased at 100 nM rapamycin, as assessed by LC3 immunofluorescence staining. The oocytes were then vitrified for 2 weeks, warmed, and subjected to in vitro fertilization (IVF). We found that the fertilization rate of vitrified-warmed oocytes after 100 nM rapamycin treatment was significantly lower than that of the control (fresh oocytes) and DMSO-treated groups (33.18 ± 4.506% vs. 63.02 ± 7.649% and 62.30 ± 8.220%; P<0.05). Developmental rate to the blastocyst stage did not differ significantly between rapamycin-treated and DMSO-treated vitrified-warmed oocytes (70.75 ± 8.242% vs. 75.53 ± 6.403%; P>0.05). The results indicate that rapamycin treatment prior to vitrification increased autophagic response in MII oocytes and that these oocytes show reduced fertilization rate. Although one effect of rapamycin is the increased autophagic response, suppression of mTOR potentially influences other cellular processes. The role for autophagy during vitrification is further assessed with oocytes from Atg7+/Zp3-CRE mice, with specific deletion of Atg7 gene in oocytes. MII oocytes from Atg7+/Zp3-CRE were vitrified-warmed and subjected to IVF. The fertilization rate between Atg7+/ (control) and Atg7+/Zp3-CRE vitrified-warmed oocytes was not significantly different (51.10 ± 13.11% vs. 52.93 ± 8.87%; P>0.05). Interestingly, Atg7+/Zp3-CRE vitrified-warmed embryos showed a higher developmental rate than the Atg7+/Zp3-CRE fresh oocytes (77.77 ± 5.53% vs. 49.67 ± 3.3%; P<0.05), suggesting that the process of vitrification per se improves preimplantation embryonic development of Atg7+/Zp3-CRE oocytes. Overall, complete absence or enhancement of autophagy in oocytes does not negatively influence fertilization, warming, and fertilization and these results indicate a dispensable role for autophagy during vitrification and warming of MII oocytes. This study was supported by a grant from the Korea Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI12C0055, https://www.hiddream.kr/).


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Non-invasive embryo monitoring by time-lapse microscopy (Primovision time lapse embryo monitoring system, Vitrolife, Göteborg, Sweden) allows continuous embryo observation without removing the embryo from optimal culturing conditions. It is widely used in human IVF clinics to gain information on the cleavage pattern, morphological changes and embryo development dynamics. Embryos are cultured in multi-well dishes with 9-16 wells, which are covered by a single drop of culture medium. This system allows individual embryo observation while maintaining the benefits of group culture. The aim of our study was to monitor embryo development of domestic cat embryos produced after IVM/IVF. Cumulus- oocytes complexes were obtained from domestic cat ovaries and matured as described before (Hrihal et al, Theriogenology 2014). Within four experiments using cryopreserved epididymal sperm from three tomcats, 53 in-vitro matured oocytes were fertilized and cultured in 9-well embryo dishes (Vitrolife) covered with 50 µL medium. The computer software recorded images of each embryo every 30 minutes for 8 days (192 hours), allowing a detailed analysis throughout the whole culture period. From the 53 matured oocytes, 38 cleaved (72%), 15 stopped at morula stage and 9 reached blastocyst stage (17%). Embryos reaching only morula stage (Mo-group), cleaved from 1-cell to 2-cell stage at 21.3 ± 1.5 h post inseminationem, from 2 to 4-cell stage at 29.1 ± 1.7 h, from 4 to 8-cell stage at 44.0 ± 2.3 h, from 8 to 16 cell stage at 62.7 ± 4.3 h, and stopped their development at morula stage after 83.1 ± 2.3 h. The blastocyst group (Bla-group) showed first cleavage at 19.9 ± 1.1 h post insemination, followed by 4-cell at 28.4 ± 1.5 h, by 8-cell at 40.4 ± 3.1 h, by 16 cell at 54.6 ± 2.8 and morula at 73.1 ± 2.8 h. The time difference between Mo-group and Bla-group was steadily increasing. Bla-group embryos reached morula stage 10 hours earlier compared to Mo-group embryos (p < 0.05, t=2.128). After 170.4 ± 6.9 h they reached blastocyst stage and expanded after 170.4 ± 6.9 h. The maximum diameter of expanded blastocysts was 189 µm (150 ± 13 µm). Six of nine blastocysts showed a repeated collapse with a following re-expansion of the blastocoel, which occurred at 160.6 ± 4.9 h for the first time. Mean time between two collapse cycles was 6.6 ± 1.2 h. In summary, we were able to demonstrate that cat embryos that are able to develop to the blastocyst stage reach morula stage significantly faster compared to embryos arresting at morula stage. Furthermore, expanded blastocysts show repeatedly collapse and re-expansion, a pattern known already from...
embryos of other species. Time-lapse monitoring allows us to judge the quality of embryos noninvasively and to identify the ones with the biggest developmental potential.

447. **SLO3 K⁺ channels control Ca²⁺ entry through CATSPER channels in mouse sperm.**
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Mouse sperm possess two pH-activated sperm specific ion channels: the SLO3 K⁺ channel and the CATSPER Ca²⁺ channel. The genetic deletion of either of these channels confers male infertility characterized by a failure in sperm hyperactivation. During capacitation SLO3 hyperpolarizes the sperm while CATSPER allows the Ca²⁺ entry necessary for sperm hyperactivation. These channels might be functionally connected but it hasn't been demonstrated that SLO3 dependent hyperpolarization is required for Ca²⁺ entry through CATSPER channels nor has a functional mechanism linking the two channels been shown. Here we show that Ca²⁺ entry through CATSPER channels is deficient in the slo3⁻/⁻ KO sperm. Our experiments using the Ca²⁺ fluorescent indicator Fluo 4 show that 60% of wild type capacitated sperm respond with Ca²⁺ increases through CATSPER channels to KCl depolarization or to stimulation by ZP, while mutant slo3⁻/⁻ sperm fail to show Ca²⁺ increases. We found that Ca²⁺ responses can be rescued in the slo3⁻/⁻ sperm by alkaline depolarization or by hyperpolarizing the plasma membrane with the potassium ionophore valinomycin. According to these results we propose that the activation of CATSPER channels by SLO3 channels is indirect and may involve a voltage- dependent change in pH possibly through a Na/H exchange mechanism. In agreement with these results, we show that the decrease in Na⁺ inward driving force abolished Ca²⁺ responses in wild type sperm. In addition, pH measurements in mouse sperm using the pH-sensitive dye BCECF shows that membrane hyperpolarization produced by valinomycin, induces intracellular alkalization. Taking into account these results we propose a mechanism in which sperm encountering the more alkaline environment of the female genital tract will experience an increase in pH that would cause the initial activation of SLO3 channels leading to sperm hyperpolarization. This hyperpolarization would in turn activate a Na/H exchanger causing a further increase in pH sufficient to activate CATSPER channels and causing the Ca²⁺ increase needed for sperm hyperactivation.

448. **Characterization of the WW-domain containing ubiquitin ligase NEDL2 in porcine oocytes and preimplantation embryos.**
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The NEDD4-like ubiquitin ligase 2 (NEDL2) is a HECT type ubiquitin ligase that contains two WW domains/protein-protein interaction modules and a lipid/Ca 2⁺-binding (C2) domain. Beyond its predicted ubiquitin-ligase activity, cellular functions of NEDL2 remain largely unknown. Because the abundance of NEDL2 in the cytoplasm of porcine metaphase II (MII) oocytes, its WW-domain could be engaged during fertilization by the WW-binding motifs of the sperm-released post-acrosomal sheath WW-domain binding protein (PAWP). A candidate component of the sperm-borne oocyte-activating factor, PAWP elicits calcium oscillations, activates oocytes and promotes pronuclear development following fertilization or microinjection of PAWP protein or human/porcine Pawp CRNA. Thus, to our knowledge, our group is the first team suggesting that NEDL2 alone or upon stimulation by sperm PAWP could play a role in fertilization and development. In order to understand its role in fertilization, oocyte activation and development, our initial objectives, in this ongoing study, were to seek the presence of NEDL2 protein in germinal vesicle- (GV), MII- stage oocytes and preimplantation embryos up to blastocyst and then to investigate its potential interaction with PAWP, the sperm-borne oocyte activating factor. Besides diffuse cytoplasmic localization at all stages, immunofluorescence studies showed that the NEDL2 protein accumulated in distinct foci in the germinal vesicles of immature oocytes, in maternal and paternal pronuclei of zygotes, and in the blastomere nuclei of 2-, 4-, 8-cell, morula- and blastocyst-stage embryos. To study its role in pronuclear development, anti-NEDL2 antibody was injected into MII oocytes before IVF while normal serum served as the control. There was no difference in the percentage of embryos cleaved at 48 h post IVF (NEDL2 antibody: 69.0±27.0% vs control: 53.2±10.7%), or percent blastocysts on day 7 (4.5±4.5% vs 6.1±1.3%), although the said antibody does not recognize the critical functional domains of NEDL2. In the third experiment, human or porcine Pawp cRNA was injected into MII oocytes to activate them. Oocytes were examined for activation and NEDL2 localization at 24 or 48 h post injection (human and porcine cRNA, respectively), or cultured to day 7 to obtain parthenogenetic blastocysts (porcine cRNA only). Some recent studies propose that embryo viability is associated with a “quieter” metabolism rather than an “active” one. Therefore this proposition, an elevated metabolism leads to cellular stress and to an increased oxidative state that could impair embryos
quality. Previous studies from our group support the hypothesis that blastocysts with slow developmental kinetics are more similar to in vivo embryos. It has also been proposed that sperm could play an important role for promoting functional quietness in early embryos. Therefore, this study aimed to investigate the role of the spermatozoa on developmental kinetics of cleavage in bovine embryos produced in vitro. Furthermore, we performed a preliminary study to characterize spermatozoa lipid profile on the search for possible differential features among semen samples. For this purpose, bovine embryos were produced in vitro by standard protocols using semen from three different bulls (B1, B2 and B3). The embryos originated from each semen sample were evaluated and divided into two groups: Fast (4 cells at 40 hours post insemination) and Slow (2 or 3 cells at 40hpi). Cleavage and blastocysts rates were compared among the 3 samples. Lipid fingerprinting was also performed in all semen samples through Matrix Assisted Laser Desorption Ionization Mass Spectrometry. Spectra were acquired in positive ionization mode and analyzed through the online software MetaboAnalyst 3.0. Preliminary results show that B3 originated significantly higher amounts of Slow Blastocests (18.5±2.53) and also presented the lowest rates of Fast Blastocysts (20.85±1.86) when compared to B1 (Slow=3.85±1.72 and Fast=36.65±1.49) and B2 (Slow=10.84±1.12 and Fast=30.15±2.3). The lipid fingerprinting obtained by mass spectrometry confirms a differential lipid profile on B3 and indicates an imbalance on phosphatidylcholene (PC) profile when compared to the other bulls. In general, PC is one of the main components of membranes lipid bilayer and it is related to maintenance of cell integrity, membrane dynamics and regulation of molecular machineries. This lipid subclass has already been related to the efficiency of motility in human sperm. Our focus now goes on increasing the number of samples to confirm the pattern of lipids found on the semen sample which was able to originate the highest numbers of blastocysts with a quiet metabolism. Then, we expect to find possible biomarkers of embryo viability as well as to hypothesize the mechanisms involved with the dynamics of cleavage in early embryos. Financial support by Fapesp 2012/10351-2 and ABC Federal University.

450. The roles of epigenetic factors in the development of mouse preimplantation embryos. Shimnosuke Suzuki¹, Yusuke Nozawa¹, Takehito Kaneko¹, Satoshi Tsukamoto¹, Hiroshi Imai², Naojiro Minami².¹Kyoto University, Kyoto, Kyoto, Japan; ²Graduate School of Agriculture, Kyoto University, Kyoto, Kyoto, Japan; ³Graduate School of Medicine, Kyoto University, Kyoto, Kyoto, Japan; ⁴National Institute of Radiological Sciences, Chiba, Chiba, Japan
Epiagenetic regulation is considered to be one of the most important mechanisms by which changes in gene expression occur without changes in the underlying DNA sequence. Methylation of histones that plays a dual role is a special process in epigenetic regulations. Some activate gene expression and others inhibit it. Especially, it has been reported that the level of trimethylated H3K4 (H3K4me3) is gradually increased throughout oocyte growth and is maintained at a high level until zygotic gene activation (ZGA) in maternal chromosomes, while it is drastically increased in paternal chromosomes after fertilization. However, the molecular mechanisms of H3K4me3 during oocyte maturation and ZGA are unknown. In the present study, we investigated the roles of epigenetic factors (Smyd3; an H3K4 methyltransferase and Chd1; a chromatin remodeling factor) in the development of mouse preimplantation embryos and showed that loss of SMYD3 or CHD1 function led to embryonic lethality after implantation. Smyd3-knockdown embryos global H3K4me3 levels appeared unchanged. However, in Smyd3-knockdown embryos global H3K4me3 levels appeared unchanged. It is possible that SMYD3 is involved in the methylation of H3K4 of only the lineage-specific genes during preimplantation development. Chd1 -knockdown also reduced the expression of Oct4 and Cdx2 from MGA. Additionally, Chd1-knockdown reduced the expressions of Hmgn1 and Klf5 at ZGA. With respect to Hmgn1, the suppression of Hmgn1 expression continued until the blastocyst stage in Chd1-knockdown embryos. On the other hand, Klf5 expression was suppressed at the 4- and 8-cell stages and the expression gradually recovered after the morula stage in Chd1-knockdown embryos. Accordingly, we investigated the effects of Hmgn1-rescue in Chd1-knockdown embryos on Oct4 and Cdx2 expression and postimplantation development. We observed that Oct4 and Cdx2 expression, normal ICM-derived colony formation, and the numbers of live offspring were all restored in Chd1-knockdown-Hmgn1-rescue embryos, suggesting that CHD1 plays important roles as a trigger for both Oct4 and Cdx2 expression through the regulation of Hmgn1 expression at ZGA. Phenotype of Smyd3-knockdown and Chd1-knockdown is very similar. Therefore, we hypothesize that SMYD3 methylates H3K4 within the promoter regions of the lineage-specific genes, and then CHD1 recognizes the H3K4me3, resulting in transcriptional activation of these genes. In conclusion, our results indicate the possibility that an elucidation of the epigenetic factors involved in H3K4me3 may shed light on the molecular mechanisms of ZGA in the mouse.

451. Prostaglandin I2 Analogue as a useful supplement for early development of porcine embryos. Seung-Bin Yoon¹, Pil-Soo Jeong¹, Hae-Jun Yang¹, Seong-Eun Mun¹, Seon-A Choi¹, Young-Ho Park¹, Bo-Woong Sim¹, Ji-Su Kim¹, Sun-Uk Kim¹, Kyu-Tae Chang¹.¹Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongju-si, Chungcheongbuk-do, Korea, Republic of
Despite the several evidences concerning the presence of prostaglandin I2 (PGI2) in mammalian oviducts, its role in early embryonic development was largely unknown. Thus, the current study was carried out to examine the effect of PGI2 on in vitro developmental competence of porcine early embryos and underlying mechanism(s) by supplementing iloprost, a PGI2 analogue, in vitro culture (IVC) medium. To determine the optimal treatment conditions, the parameters for developmental competence, such as blastocyst formation rate, ICM/TE proportion and cellular survival, were investigated using in vitro-fertilized (IVF) embryos cultivated in the presence of increasing iloprost concentrations (0, 0.01, 0.1, and 1 µM). Unlike the results from the treatment of 0.01 and 0.1 µM iloprost, rates of blastocyst formation and expanded blastocyst were significantly increased by addition of 1 µM compared to control. Especially, trophectoderm cell numbers were greatly increased, and cell survival was considerably improved in the blastocysts of 1 µM iloprost treatment group. Interestingly, Western blotting analysis showed that phosphorylation of Akt was markedly increased by treatment with 1 µM iloprost, suggesting the activation of phosphatidylinositol-3, 5-bisphosphate 3-kinase (PI3K) signaling pathway. In addition, blastocyst formation rate, cell numbers and cellular survival were greatly reduced by Wortmannin, a potent PI3K inhibitor, which were significantly ameliorated by 1 µM iloprost treatment. Consistent with results from the IVF embryos, 1 µM iloprost improve the developmental competence in both parthenogenetically-activated and somatic-cell-nuclear-transferred embryos. Taken together, these results demonstrated that iloprost efficiently enhances the early embryonic development via Akt activation in pigs. Therefore, the current
study strongly suggests that iloprost can be defined as a useful IVC supplement for massive production of porcine early embryos with high developmental competence

452. Fetal bovine serum promotes early development of porcine embryos by reduction of oxidative stress.
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Application of fetal bovine serum (FBS) has been frequently used to improve in vitro culture (IVC) conditions of mammalian cells, a few or no have been studied concerning the effect of fetal bovine serum (FBS) on early development of mammalian embryos. Thus, the current study was conducted to investigate the effect of FBS on the developmental competence of porcine embryos and to establish the optimal treatment conditions using parthenogenetic activation (PA), in vitro fertilization (IVF), somatic cell nuclear transfer (SCNT) embryos. Unlike the treatment during full period (1-6 days) or early-phase (1-2 days) of IVC, the beneficial effect of FBS supplementation into medium was found only in late-phase (4-6 days) of IVC in PA embryos. The rates of blastocyst formation and hatching were significantly increased by addition of FBS during the late-phase of IVC compared to control, which was further evidenced by the improvements in cellular survival, total cell number and trophoderm (TE) proportion. Moreover, reactive oxygen species (ROS) levels were markedly reduced in FBS treatment group compared to control, which appeared to be closely associated with increase of TE cells in blastocysts. Indeed, addition of hydrogen peroxide greatly reduced the developmental competence parameters including TE cell proportion, whereas the defects were significantly restored by supplementation of FBS. More interestingly, the beneficial effects of FBS in late-phase of IVC were consistently found in the embryos produced by IVF or SCNT embryos. Taken together, these results suggest that FBS can be efficiently used as a useful IVC supplement for massive production of porcine embryos with high developmental competence.

453. Beneficial role of 6-diazo-5-oxo-l-norleucine in in vitro oocyte maturation and subsequent early embryogenesis in pigs
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Activators of protein kinase A (PKA) signaling pathway have been frequently used to transiently stall meiosis at early phase of in vitro maturation (IVM) for balancing between cytoplasmic and nuclear maturation of mammalian embryos. Despite the tight association of 6-diazo-5-oxo-l-norleucine (DON), an inhibitor of hyaluronan synthesis, with PKA cascade, few evidences has been provided concerning the role of DON as an IVM supplement with meiosis-stalling activity during IVM of porcine oocytes. Thus, the current study was conducted to examine the effect of DON on nuclear/cytoplasmic maturation of porcine oocytes and subsequent early embryonic development. From the observation of nucleus patterns, metaphase II (MII) oocyte production rate at 44 h of IVM was not significantly different among groups, although retardation in meiosis progress was found in 22 h of IVM. Unlike 5 µM and 10 µM DON treatment groups, however, 1 µM DON greatly improves the developmental competence parameters, including monospermic fertilization, blastocyst formation rate, TE proportion, and cell survival compared to control. Consistent with this, the beneficial effect of 1 µM DON on early embryogenesis was reproducible in pathenogenetically-activated or somatic cell nuclear transferred embryos. In particular, meiosis-stalling activity was maximal at 6 h after 1 µM DON treatment, which resulted in reduction of cell cycle-related pathway, such as decreases in phospho-extracellular signal-related kinase 1/2 (pERK1/2) and CCNB1, CDK1, HAS1, HAS2 transcript levels. Together with the effects of DON-assisted IVM and fertilization and early embryogenesis, similar level of pERK and clear rearrangement of cortical granules in perimembrane of MII oocytes further provided the evidences for balanced meiosis progression between nuclear and cytoplasmic maturation during IVM in 1 µM DON treatment group compared to control. From the results, I concluded that 1 µM DON can be efficiently used to massively yield MII oocytes with high nuclear/cytoplasmic maturation degree and developmental competence.

454. Effect of Lactation on Conceptus Gene Expression at the Initiation of Implantation in Cattle.
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The aim of this study was to characterize differences in the transcriptome of the bovine conceptus recovered at the initiation of implantation (Day 19) from a cohort of age-matched postpartum (pp) primiparous Holstein Friesian dairy cows that were either milked post-calving (i.e., lactating (LACT)) or were dried off immediately at calving (i.e., never milked, non-lactating (DRY)) as well as a group of contemporaneous nulliparous heifers. At approximately 65-75 days pp late morula/early blastocyst stage embryos recovered from superovulated Holstein Friesian heifers were transferred into synchronized pp recipients (DRY; n=12: LACT; n=13) on Day 7 of a synchronized estrous cycle. As a control, a subset of nulliparous heifers (HEIF, n=8) were synchronized as per the recipients and inseminated to a standing heat. All animals were blood sampled daily from Day 0 until day of slaughter (Day 19) for serum progesterone (P4) analysis. At slaughter, the ipsilateral uterine horn was flushed with 10 ml of phosphate -buffered saline to recover the conceptus, which were snap frozen in liquid nitrogen. High quality RNA was extracted from trophectoderm tissue and was used to construct sequencing libraries using the Encore Complete RNA-Seq library system of NuGEN. All libraries were sequenced on the HiSeq 1500 as 100 b single reads and mapped by Tophat2 to the reference genome (bos Taurus). An appropriately developed conceptus was recovered from 66% of heifers (4/6), 66% of dry cows (7/11) and 45% of lactating cows (5/11). Circulating concentrations of P4 increased as the estrous cycle progressed (P<0.01) in all three groups but were not different between groups (P>0.05). No significant differences were observed in conceptus gene expression between LACT and DRY cows. Two hundred and twenty four genes were differentially expressed in conceptuses recovered from LACT cows compared to those recovered from HEIF. The genes AQP7, IRF2, SLC2A4, SHOX2, IQCD, AIF1L, PGM2L1, TRIM47, CLDN3, and HEXIM2 were up-regulated while PER2, HHEP, EFNA3, TINAG, NGEF, DEG52, MAS1P1, PLXNA3, MLXIPL, and SLC38A11 were down-regulated in the LACT group to the greatest extent. Forty differentially abundant transcripts were detected between conceptuses recovered from HEIF and DRY cows. Of these, RELN and AIF1L were decreased to the greatest extent, while abundances of SFRP1, SERPINE1, GRK5, SLC10A1, and PLEKHF1 were higher in the DRY compared to HEIF group. Comparison
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It is well known that in vitro culture can affect gene expression, embryo quality and epigenetic processes in bovine embryo. However, it is not clear if the changes caused by in vitro culture on embryos are maintained after they are transferred to recipient uteruses. The aim of this study was to evaluate the effect of in vitro culture until day (D) 7 of development on bovine embryo transcriptome at D14 of development. Embryos were produced from oocytes obtained by follicular aspiration of slaughterhouse ovaries. On D7 after in vitro fertilization only grade I blastocysts were selected and were transferred non-surgically to the uteruses of previously synchronized recipients (VT group). As a control group, in vivo embryo were produced (VV group), and on D7 were recovered by uterine flushing and after selection for the same stage and quality of the VT they were also transferred to recipient uteruses. Embryos from both groups were collected on Day 14. From all embryos a biopsy of the trophoblast was removed for sex determination. After sex identification 3 pool of 4 embryo from the same gender were formed for each group. Total RNA was extracted using RNAasy plus micro kit (Qiagen) and used for RNA sequencing (Illumina, HiScanSQ). After sequencing the data were analyzed by DESeq2 software and the differential expression between VV and VT group was performed using the generalized linear model (P<0.05). The P value was adjusted by FDR Benjamini-Hochberg method to avoid false positive. A total of 29,005 genes were found to be expressed, from which 900 were differentially expressed in both DRY and LACT cows compared to HEIF. These data indicate that the transcriptional profile of embryos derived from the same source (superovulated nulliparous heifers), is altered by the maternal environment to which the developing conceptus is exposed during conceptus elongation; however, the consequences of these alterations on the successful establishment of pregnancy remain to be elucidated. The research leading to these results has received funding from the European Union Seventh Framework Programme FP7/2007-2013 under grant agreement n° 312097 (‘FECUND’).

457. CDK9 Inhibitor, Flavopiridol, Disrupts Global Transcription, Oocyte Maturation and Embryo Development in the Mouse.
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Positive transcription elongation factor b (P-TEFb) is an RNA polymerase II kinase that phosphorylates Ser2 of the carboxy-terminal domain and promotes the elongation phase of transcription. In this study, we present evidence indicating that P-TEFb Kinase activity is essential for completion of mouse oocyte maturation and embryo development. Treatment with CDK9 inhibitor, flavopiridol...
resulted in metaphase I arrest in maturing oocytes. Inhibition of CD9 kinase activity did not interfere with in vitro fertilization and pronuclear formation. When zygotes or 2-cell embryos were treated with flavopiridol in their G2 phase of the cell cycle, development to the blastocyst stage was impaired. Exposure of embryos after genome activation resulted in failure to form normal blastocysts and aberrant phosphorylation of RNA polymerase II CTD. In all stages analyzed, treatment with flavopiridol abrogated global transcriptional activity. Using immunocytochemical analyses, we find that the P-TEFb components, CD9 and Cyclin T1, are localized to nuclear speckles, as well as in nucleoli, in mouse and pig germline vesicle oocytes. In mouse oocytes, Cyclin T1, CD9 and its T-loop phosphorylated form are colocalized with the SC35 splicing factor. Moreover, using fluorescence in situ hybridization, we show that in absence of CD9 activity, nuclearolar integration, as well as production of 28S rRNA, is impaired. Collectively, our data suggest that P-TEFb kinase activity is crucial oocyte maturation, embryo development and regulation of RNA transcription in mouse.

458. Correlation between glutamate, pyruvate, lactate and glutamate for fast or slow bovine embryos. Marcella Milazzotto1, Jessica Ispadá2, Carlos A. Soares2, Glaucia P. Alves3, Kelly Annes3.

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In vitro production (IVP) of embryos is a biotechnic who allow increase the production of embryos from animals. This technic had been improved through the years on cattle, however, each step of this procedure decrease the success rates obtained, so the ratio gestations/oocytes had value below the optimal. IVP embryos have different kinetics of development and this may be a parameter to predict embryo quality. Recent data had shown that bovine embryos with slow kinetic of development may be more similar with in vivo produced embryos and have high quality. The aim of this study was to elucidate the relationship between development kinetic and the consumption correlation of embryonic metabolites. Slaughterhouse derived cumulus-oocyte complexes (COCs) were subjected to in vitro maturation (IVM) in maturation medium for 22-24 hours followed by in vitro fertilization (IVF) for 18 hours. After this, the presumptive zygotes were individually cultured for 22 hours (40 hours post-insemination - hpi) in medium 1 [KSOM (Millipore) supplemented with 10 % of FCS, 0.25 µL/mL of gentamicin and 4.5 µL/mL of nonessential amino acids] and classified as fast (4 or more cells) or slow embryos (2-3 cells). After this, the medium 1 was replaced by medium 2 [Synthetic fluid oviduct (SOF) containing 5 % of FCS, 10 µL/mL of nonessential amino acids and 20 µL/mL essential amino acids]. All in vitro steps were performed on incubator at 38.5°C and 5% of CO2 and high humidity. The amount of glucose, pyruvate, lactate and glutamate in the culture medium were analyzed from fast and slow groups after 40hpi (FCL and SCL groups), 96hpi (FMO and SMO groups) and 168hpi (FBL groups and SBL) by fluorimetric analysis. All the evaluations were made by commercial kits, for glucose was used Amplex Red Glucose/Glucose Oxidase Assay, for pyruvate was used EnzyChrom Pyruvate Assay, for lactate used EnzyChrom Lactate Assay and for glutamate was used Amplex Red Glutamic Acid/Glutamate Oxidase Assay. The results obtained by each metabolite concentration were submitted to correlation analysis using Spearman test on Prism® 5.01 with significance level of 5%. There was only a tendency of negative correlation in the consumption of glucose X glutamate at the cleavage stage for slow (p=0.62) and fast (p=0.62) embryos. At morula stage, there was a negative correlation in the consumption of glucose X pyruvate just for slow embryos (p=0.78). At the blastocyst stage, there was a tendency of positive correlation in the consumption of glucose X lactate for slow (p=0.62) and pyruvate X lactate (p=0.56) for fast embryos and a negative correlation in the consumption of glucose X pyruvate (p=0.92) and pyruvate X lactate (p=0.78) for slow and positive correlation in the consumption of glucose X pyruvate (p=0.81) and glucose X lactate (p=0.78) for fast embryos. In conclusion, there was difference in the correlation of metabolites consumption after the morula stage, and this difference is more pronounced at the blastocyst stage. The patterns obtained by our group suggest that slow and fast embryos follow different pathway after the cleavage stage and this difference could be a parameter to determine embryo quality.


459. Micronucleus Formation Causes DNA Damage And Perpetual Unilateral Chromosome Inheritance In Mouse Embryos. Cayetana Vázquez1, Shardul Trivedi1, Kazuo Yamagata1, Greg Fitz-Harris1.

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Aneuploidy in the preimplantation embryo is associated with infertility, but the causes of aneuploidy are poorly explained. In somatic cells, lagging chromosomes in anaphase result in the formation of micronuclei (MN), small cellular bodies enclosing chromosomal DNA separate to the main nucleus, and it is well known that micronuclei are common in human preimplantation embryos. However, the impact of micronuclei remains elusive. Here we show that MN commonly occur in in vitro cultured mouse embryos and ultimately result in DNA damage and further chromosome segregation errors. MN quantification at different developmental stages revealed MN to occur most frequently at the morula 32-cell stage, where over 70% of embryos will have at least one MN. Nuclear properties of MN at this stage were investigated by immunofluorescence and confocal imaging. Increased chromatin condensation (30% increase, P<3x10^(-6)) in the MN is accompanied by significantly increased levels of DNA damage, as indicated by phosphorylated histone variant H2AX (2-fold increase, P<2x10^(-5)). Further, we show that the abundance of the of the nuclear lamina component LaminB1 is reduced in MN (~50% decrease, P<4x10^(-5)), and the nuclear import substrate LSD1 fails to localize to micronuclei, indicating that nuclear structure and function is impaired in MN, providing a possible explanation for the DNA damage. By using a combination of live imaging and fixation techniques we show that although DNA within micronuclei does condense into mitotic-like chromatin during cell division, it fails to attach to spindles microtubules, and is therefore unilaterally inherited into one daughter cell rather than being segregated correctly. Strikingly, this series of events can occur repeatedly over several generations of cell divisions. This perpetual unilateral DNA inheritance may present a novel mechanism whereby chromosome segregation errors cause DNA damage within micronuclei and disables chromosomes from segregating, which drives aneuploidy and increases the likelihood of further DNA damage in the next embryonic division.

460. Effects of Human Recombination Granulocyte–Colony Stimulating Factor (hrG-CSF) Treated during in vitro Culture on Viability of Porcine Embryonic Development. Lian Cai1, Eun O. Park1, Yong X. Jin1, Sang H. Hyun2, Il S. Yang1, Woo S. Hwang1.

12015 Abstracts – Page 182
In human, G-CSF is produced in the decidual tissue and it could increase trophoblasts cells proliferation. In addition, it could be a predictor of embryo implantation for IVF outcome and also have important role in maintain pregnancy. In the current experiment, the cumulus oocyte complexes (COCs) were aspirated from superficial follicles from 3 to 6 mm. Then, we performed PA and IVF using MII matured oocytes and subsequently PA and IVF embryos were cultured in FBS free-culture medium supplemented with various concentrations of hrG-CSF (0, 10, 50 and 100ng/ml, respectively). The embryos were evaluated under a stereomicroscope for cleavage on Day 2 (48 h) and for blastocysts formation on Day 7 (168 h) after PA and IVF. A total of 585 oocytes were used for the PA. The cleavage rates and blastocysts formation rates have no significant difference. However, cell numbers of blastocysts were significantly \( p<0.05 \) higher in 10ng/ml (48.08%) and 50ng/ml (49.22%) compared with the control group (44.03). A total of 478 oocytes were used for the IVF. The cleavage rate and the blastocysts formation rate were significantly \( p<0.05 \) higher in 10ng/ml group (70.53% and 22.79%) compared with the control group (58.82% and 13.72%). However, the groups of 50ng/ml (44.20%) and 100ng/ml (43.50%) showed a significantly \( p<0.05 \) lower cleavage rates compared with the control group (58.82%). Furthermore, there were no significant difference in cell number of blastocysts. These results indicated that 10ng/ml hrG-CSF supplemented during IVC improved the porcine IVF embryos development competence. Nevertheless, the high concentrations of hrG-CSF supplemented during IVC have negative effects on the viability of porcine IVF embryos. Next, the embryos were cultured with 10ng/ml hrG-CSF for Day 0 to 3 (early stage), Day 4 to 7 (late stage), and Day 0 to 7 (whole stage) after performing PA and IVF. During the experiment, the IVF blastocysts formation rates significantly increased in early, late and whole stage groups (17.65%, 18.32% and 21.98%) compared with the control (14.81%). In conclusion, the above data indicated that 10ng/ml hrG-CSF may have a beneficial physiological role during porcine in vitro embryo development.


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In vitro maturation (IVM) in the mouse results in poor oocyte competence and reduced quality of subsequent blastocysts. Our objective was to examine specific components of maturation medium to determine alterations that might better support oocyte competence. In total, 12 treatments were replicated twice in 4 separate experiments (52-74 oocytes/treatment). We examined the effects of 1) citrate (0.1 and 0.5 mM), hyaluronan (0.125 and 0.25 mg/ml), and glycine (0 and 2 mM); 2) PO4 (0 and 0.5 mM), taurine (1.0 and 5.0 mM), and MEM vitamins (0.25x and 1x); 3) taurine (0.1 and 1.0 mM), glycine (1.0 and 2.0mM), and insulin (0 and 0.1 mM); and 4) glucose (0.5 and 1.5 mM), alanyl-glutamine (ala-gln, 0.5 and 1.0 mM), and insulin-transferrin-selenium (ITS, 0 and 0.5 ug/mL I, 0.275 ug/mL T, 0.25 ng/mL S). Following IVM, cumulus expansion was observed and IVF performed. Resulting embryos were cultured in a defined, sequential (48 h + 64 h) media system and evaluated for blastocysts development and cell allocation. Cumulus expansion was improved in the presence of reduced concentrations of vitrmins (0.25x) and increased glucose (1.5 mM) concentrations. None of the components evaluated affected cleavage . Blastocyst development (/oocyte at 96 h) was improved in reduced concentrations of citrate (0.1 mM) and hyaluronan (0.125 mg/ml). Development of hatching blastocysts was improved by reducing (0.25x) the concentrations of vitamins (/oocyte at 96 h) and eliminating PO4 from the medium (/cleaved embryo at 112 h). The use of 0.25x vitamins \( p<0.05 \) and 1.5 mM glucose \( p<0.08 \) tended to increase the number of trophectoderm cells and % inner cell mass cells, respectively, compared to the control. Based upon these initial results, we reformulated our maturation medium to contain 0.1 mM citrate, 0.125 mg/ml hyaluronan, 2.0 mM glycine, 0 mM PO4, 0.25x MEM vitamins, 1.5 mM glucose, 0.5 mM a-gln and ITS, in addition to our standard components (0.5 mM pyruvate, 4.0 mM lactate, 1x NEAA, 0.25x EAA, 10 ng/mL EGF, 1.5 mg/mL fetuin and 2.5 mg/mL recombinant human albumin). Compared to the original medium, our reformulated maturation medium significantly increased embryo cleavage \( (82.7\pm3.2\% \) and 93.8±2.0%, respectively) and blastocyst development at 96 hr \( (66.9±4.0\% \) and 80.8±3.3% /oocyte). More glucose was taken up by COCs in the reformulated medium \( (1790.7±184.2 pmol/COC) \) compared to control \( (780.4±301.7 pmol/COC) \). COCs in both media used less than a third of the glucose provided, indicating the reduced uptake of glucose by COC in the control medium was not due to substrate depletion. In summary, our improved maturation medium results in better cumulus expansion, potentially due to increased glucose uptake. In addition, embryo cleavage and blastocyst development were increased following IVM/IVF. As these differences were noted when development was evaluated per total oocyte and not per cleaved embryo, we suggest that the reformulated maturation medium increases the ability of mouse COC to mature, fertilize properly, and/or undergo the first cleavage division.

462. Involvement of ooplasmic autophagy receptors SQSTM1 and GABARAP in the regulation mitochondrial inheritance after fertilization. Won-Hee Song1, Young-Joo Yi2, Miriam Sutovsky1, Peter Sutovsky1.

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Maternal inheritance of mitochondria and mitochondrial DNA (mDNA) prevents heteroplasmy and assures normal embryo development in humans and most animals. Post-fertilization sperm mitophagy is mediated by the ubiquitin-dependent proteasome system (UPS) via the ubiquitin-binding autophagy receptors SQSTM1 and GABARAP, and the proteasome-interacting protein dislocase VCP. In particular, we found that SQSTM1 directly binds to ooplasm-incorporated sperm mitochondrial sheath and the GABRAP containing autophagosomes accumulate in its immediate vicinity. Components of canonical mitophagy/autophagy pathway (LC3, BCL2, BNIP3L, PRK8) were not detected in/near sperm mitochondria, suggesting that the zygotic sperm mitophagy differs from somatic cells. Three SQSTM1- binding sperm mitochondrial proteins, AC02, ATP5B and HADHA, were identified. The GABRAP- positive autophagosomes accumulated around the intact/mitophagy-delayed mitochondrial sheath in the zygotes treated by a specific proteasomal inhibitor. Thus, we examined the post-fertilization effect of the microinjection of antibodies specific to SQSTM1 and GABARAP into mature metaphase II (MII) oocytes. We designed two strategies for pre-injection of oocytes with antibodies followed by in vitro fertilization (IVF) and grading of sperm mitophagy: 1) Separate groups of MII oocytes were pre-injected with mouse anti-SQSTM1 or rabbit anti-VCP antibodies before...
Multiple generations in a paternally-mediated manner. Populations are persistently contaminated. The body burden of Inuit is considerably higher than in southern, non-Aboriginal groups. OC exposure is associated with decreased sperm quality and we have reported that prenatal exposure to an environmentally relevant OC mixture impacts fertility parameters and the sperm proteome across multiple generations using the rat model. We compared sperm proteins from paternally non-exposed and exposed rat lineages over three generations (F1, F2, F3). Isobaric tags for relative and absolute quantitation (iTRAQ) labelling, and 2D-LC-MS/MS were conducted to identify differentially expressed proteins. One to two such proteins were identified in F1, F2 and F3 OC exposed lineages. Cytochrome C, Superoxide Dismutase 1 (SOD1) and Glutathione Peroxidase were among the upregulated proteins in F3 OC lineages. These results were confirmed by immunoblotting. F1 males exposed during early development presented decreased sperm motility \((P \leq 0.0001)\), lower daily sperm production per testis \((P=0.006)\), and decreased epididymal sperm concentration \((P=0.0001)\). F2 OC sons were subfertile \((\text{P} \leq 0.0001)\), and F3 OC grandsons had fewer pups \((P=0.0001)\). Seven, 19 and 37 differentially-expressed proteins were identified in F1, F2 and F3 OC exposed lineages. Cytochrome C, Superoxide Dismutase 1 (SOD1) and Glutathione Peroxidase were among the upregulated proteins in F3 OC lineages.
Peroxidase 4 (GPX4) were 3 of the reduced proteins in F1 OC males. In their F2 OC sons, Citrate Synthase, Solute Carrier Family 2 member 3 (SLC2A3) and Calcin were decreased. In F3 OC grandsons, Citrate Synthase, IZUMO and Zona Pellucida Binding Protein (ZPBP) were reduced. Immunoblotting confirmed that SOD1, GPX4, Citrate Synthase and ZPBP were significantly reduced in F1, F2 and F3 OC males. This is the first study to compare sperm protein levels due to paternal OC exposure across multiple generations using iTRAQ technology and immunoblotting confirmation. In sum, OC exposure induced a decrease in key proteins implicated in sperm motility and cell death (SOD1 and GPX4- F1), a reduction in proteins involved in gamete fusion and sperm head cytoskeleton (Citrate Synthase, SLC2A3 and Calcin- F2), and a decrease in proteins playing a role in fertilization (IZUMO and ZPBP- F3).

465. **Heparan Sulfate Is Present In Bovine Oocytes: A Possible Protamine Acceptor During Sperm Decondensation After Fertilization In Vivo.**

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Treatment of sperm with heparin and reduced glutathione (Hep-GSH) induces nuclear sperm decondensation in vitro. GSH is an endogenous disulfide bond reducer which plays a critical role in sperm decondensation and male pronuclear formation in mammals, but its action needs to be followed by the remotion of protamines from DNA. In amphibians and Drosophila melanogaster, nucleoplasmin has been shown to exert this role. In human, heparan sulfate (HS) has been demonstrated to function as protamine acceptor during sperm decondensation in vitro, suggesting that decondensation mechanisms are not completely conserved across the evolutionary scale. Heparin induces the release of histone 1 and protamines (critical step for decondensation), and it is widely used in bovine to induce sperm capacitation before or during in vitro fertilization. Moreover, Hep-GSH treatment has been successfully applied for ICSI by our group and other laboratories. Given that heparin is not present in bovine oocytes, and there is a high similarity between heparin and HS molecules, we hypothesize that HS could act as a protamine acceptor during bovine sperm decondensation in vivo, as has already been postulated by others both in human and mice. To this aim, we studied the decondensation kinetics of semen samples from 6 different bulls after treatment with Hep-GSH, and tested the presence of HS in bovine oocytes. Methods: Frozen sperm from six bulls were thawed individually, and incubated in BO medium containing 80 µM Hep and 15 mM GSH for 1, 3, 7 and 20 h under standard IVM conditions. For each bull, a control group incubated in BO medium was also included. After incubation, treated sperms were fixated with glutaraldehyde and observed under bright light at 400X magnification. The percentage of decondensed spermatozoa was determined, considering as decondensed those sperm with head size > 7 µm and altered membranes. A minimum of 200 cells from each sample were evaluated in duplicate. To investigate the presence of HS in the bovine oocyte, cumulus oocyte complexes were collected from slaughtered cow ovaries and matured in vitro. After 22 h, cumulus cells and zona pellucidae were removed from oocytes at metaphase II (MII) stage. After fixation, indirect immunofluorescence was performed. Briefly, MII oocytes were permeabilized with 0.2% Triton X-100 for 15 min at room temperature. Following incubation in blocking solution for 30 min, oocytes were immunolabeled with anti-HS monoclonal antibody overnight at 4°C. A FITC–goat polyclonal anti-mouse IgM was used as second antibody for 90 min at 37°C. Negative controls did not contain first antibody. Nuclei were counterstained with propidium iodide. Oocytes were mounted on glass slides and observed under confocal microscopy. Data was statistically analyzed by Fisher’s exact test (p<0.05). Results: After 3 h of Hep-GSH treatment, sperm from all bulls assayed showed higher decondensation levels than control groups, not showing differences between them (34% ± 6 vs. 3% ± 3, respectively). However, decondensation kinetics varied between bulls. Some of them reached the maximum decondensation level at 7 h, and others at 20 h, ranging from 37 to 83%. Immunofluorescence showed a strong cytoplasmic fluorescent label in all oocytes incubated with the anti-HS antibody (n=62 in four different experiments), which was not observed in negative controls (n=12). Conclusions: Hep-GSH treatment induces decondensation kinetics that varies between bulls, reaching maximum decondensation levels between 7 and 20 h. Our results evidence for the first time that HS is present in bovine oocytes and allows us to propose it as a putative protamine acceptor during nuclear sperm decondensation after fertilization in cattle. Research supported by PICT-2010-2716 grant.

466. **Investigating the molecular mechanisms involved in embryotrophic actions of follistatin on bovine early embryonic development.**

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Maternal (oocyte-derived) follistatin is obligatory for bovine early embryonic development. Exogenous follistatin treatment during d 1-3 (pre-compaction period) of in vitro embryo culture promotes early cleavage, embryonic development to blastocyst stage and embryo quality via enhancing blastocyst cell allocation to trophectoderm (TE). Follistatin is a binding protein for TGFβ-superfamily ligands with actions mediated via modulation of SMAD dependent and SMAD independent signaling pathways. However, mechanism of action of follistatin linked to above embryotrophic effects is unclear. Our objectives were to determine the effects of follistatin treatment on abundance of total (t) and phosphorylated (p) forms of SMAD2/3 (linked to activin, TGFβ and nodal signaling) in bovine embryos (Experiment 1), differences in SMAD2/3 phosphorylation in early versus late cleaving embryos (Experiment 2) and effects of follistatin treatment during d 1-3 (pre-compaction stage), d 4-7 (post-compaction stage) or both stages (d 1-7) on protein abundance for CDX2 and BMP-4, factors known to regulate cell lineage determination to TE (Experiment 3). In Experiment 1, in vitro produced zygotes were cultured in KSOM containing 0.3% BSA with and without follistatin (10ng/ml; maximal stimulatory dose). At 1, 2, 5 and 10 hours (h) later, untreated control and follistatin treated embryos were harvested and subjected to Western blot analysis (n = 5 pools of 20 embryos/pool) with antibodies against pSMAD2/3, tSMAD2/3 and ACTIN. Results demonstrated that follistatin treatment significantly reduced (P<0.01) tSMAD2/3 and pSMAD2/3 abundance at 10 h post treatment, but pSMAD2/3 and tSMAD2/3 were not different between follistatin treated versus control embryos at 2 and 5 h. Abundance of tSMAD2/3, but not pSMAD2/3 was also decreased (P<0.05) at 1 h in follistatin treated embryos. We have previously demonstrated increased follistatin mRNA in early cleaving (with higher rates of development to blastocyst stage) than late cleaving 2-cell embryos, but the relationship between time of first cleavage and SMAD2/3 phosphorylation is not known. In Experiment 2, early and late cleaving 2-cell embryos were collected at 30 and 36 h post insemination respectively (n = 20 embryos at time point; n = 5 replicates) and subjected to Western blot analysis for pSMAD2/3 and tSMAD2/3.
Results demonstrated lower abundance of pSMAD2/3, but not tSMAD2/3 in early cleaving embryos compared to their late cleaving counterparts. In Experiment 3, abundance of BMP4 and CDX2 proteins was determined via Western blot analysis of bovine blastocysts derived from untreated control embryos and embryos treated with 10 ng/ml follistatin during d 1-3 pre-compaction stage, d 4-7 (post-compaction stage) and entire duration (d 1-7) of embryo culture in vitro. Results demonstrated higher (P<0.05) amounts of BMP4 protein in blastocysts from d 1-3 and d 1-7 follistatin treatment groups compared to the untreated control and d 4-7 treatment group. However, abundance of CDX2, a TE cell marker, was higher (P<0.05) in blastocysts from all the follistatin treatment groups compared to the untreated controls. Collectively results provide evidence supporting effects of follistatin treatment on abundance of total and phosphorylated forms of SMAD2/3 during bovine early embryonic development and divergent effects of follistatin treatment pre versus post-compaction potentially linked to its embryotrophic effects. Supported by NIH grant HD072972 (GWS, JK).

467. Effects of inhibiting DNA repair pathways during early embryo development.
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DNA double-strand breaks (DSBs) are known to affect early embryo development in multiple species. In our previous studies we have shown that DSBs affect embryo cleavage kinetics, somatic cell reprogramming, gene expression and blastocyst formation in porcine embryos. Two main DNA damage repair pathways, the homologous recombination (HR) and the non-homologous end-joining (NHEJ), are activated for DSBs repair. Although genes and proteins participating in the two repair pathways have been shown to be regulated in response to DSBs in early developing embryos, it has not been determined if both pathways are required for DSBs repair in cleavage stage embryos before blastocyst stage. In this study we used specific inhibitors of the HR and the NHEJ repair pathways to investigate the importance of each pathway on embryo development, presence of DSBs and cell death. In the first experiment, porcine embryos were in vitro cultured for 1, 2, 4 or 7 days in the presence of inhibitors of both HR (ATM inhibitor, 10µM KU-55933 and ATR inhibitor, 10µM VE-821) and NHEJ (DNA-PK inhibitor, 1µM NU-7441) DNA repair pathways. The cleavage rate at 24 and 48 h of culture was not affected by the inhibition of DNA-damage repair. However, embryo development to blastocyst stage was significantly reduced when embryos were cultured in the presence of the inhibitors for 4 or 7 days. In the second study, we used control and UV-treated embryos (exposed to UV light for 10 seconds to induce DSBs) to assess the effects of each repair pathway. For this experiment, control and UV-treated embryos were cultured in the presence of HR (KU-55933 and VE-821), NHEJ (NU-7441) or HR+NHEJ inhibitors for 7 days. Blastocyst rates were significantly reduced in UV-treated embryos treated with HR+NHEJ inhibitors and in UV-treated embryos treated with HR inhibitors. There was no significant reduction in blastocyst rates neither in control or UV-treated groups exposed to the NHEJ inhibitor nor in control embryos exposed to HR inhibitors. We then assessed the effect of the inhibitors on the number of cell apoptosis in day 5 embryos that were UV-treated and cultured in the presence of HR+NHEJ inhibitors. The presence of cleaved-caspase 3 (CC3) detected by immunofluorescence, indicated an increased rate of cell apoptosis in day 5 embryos that were UV-treated and cultured in the presence of HR+NHEJ inhibitors. At the blastocyst stage, the proportion of CC3 positive cells was higher in both control and UV-treated embryos that were cultured with HR and HR+NHEJ inhibitors. In summary, our findings indicate that: a) both HR and NHEJ DNA repair pathways are important for early embryo development; b) the inhibition of DNA repair pathways results in DSBs accumulation during embryo development; c) the inhibition of DNA repair pathways increases cell apoptosis in developing embryos; and d) the HR pathway seems to be more important than the NHEJ pathway for DSBs repair in developing embryos, especially in embryos with higher number of DSBs.

468. Analysis and characterisation of bovine IVP and TE embryos biomarkers by matrix-assisted desorption ionisation mass spectrometry imaging.
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The lipid composition of pre implantation embryos is important for mammalian development and affects the success of embryo freezing in animal production systems facilitating the storage of valuable animal genetic resources. Bovine IVP (In Vitro Production) embryos have more cytoplasmic lipid droplets than ET (Embryo Transfer) embryos and as a result have reduced post cryopreservation survival. This study was conducted to compare the lipid profile in pre implantation IVP and ET embryos (Bos taurus indicus - Nellore). It was used a mass spectrometry based analytical approach, and the images and mass spectra were acquired in a MALDI-LTQ-XL instrument with imaging feature (Thermo Scientific, California, USA). It was equipped with a UV laser (Nd:YAG, 355 nm) at “minimum” focus.

Analysis and characterisation of bovine IVP and TE embryos biomarkers by matrix-assisted desorption ionisation mass spectrometry imaging.

In blastocysts from d 1-3 and d 1-7 follistatin treatment groups compared to the untreated control and d 4-7 treatment group. However, abundance of CDX2, a TE cell marker, was higher (P<0.05) in blastocysts from all the follistatin treatment groups compared to the untreated controls. Collectively results provide evidence supporting effects of follistatin treatment on abundance of total and phosphorylated forms of SMAD2/3 during bovine early embryonic development and divergent effects of follistatin treatment pre versus post-compaction potentially linked to its embryotrophic effects. Supported by NIH grant HD072972 (GWS, JK).

469. Plasminogen Improves Mouse IVF By Interactions With Inner Acrosomal Membrane-Bound MMP2 And SAMP14.

2015 Abstracts – Page 186
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Spermatozoa must penetrate the outer investments of the oocyte, cumulus oophorus and the zona pellucida in order for fertilization to take place. This process may require regulation or activation of enzymes on the sperm’s inner acrosomal membrane (IAM) by factors in oviductal fluid. Plasminogen is present in oviductal fluid and is an activator of matrix metalloproteinase 2 (MMP2) in somatic tissues. The objectives of this study were 1) to examine possible interaction between plasminogen and IAM-bound MMP2, 2) demonstrate plasminogen’s presence in the extracellular environment at the site of fertilization, and 3) provide evidence that it plays a role in fertilization. Zymographs of sonicated sperm extracts incubated without or with plasminogen showed acceleration of inhibition of MMP2 activity in concentrations as low as 1 µg/mL. Immunohistochemical analysis of plasminogen expression in superovulated mouse female reproductive tracts revealed that it is present in the cytoplasm of ovarian and oviductal oocytes, the oviductal epithelium, and extracellularly in the cumulus. We modified the standard IVF approach to more closely mimic natural fertilization by reducing the sperm concentration during insemination by ~100x. When inseminating in a low-sperm environment, addition of plasminogen in IVF medium significantly improved fertilization, while MMP2 antibody significantly inhibited fertilization. The MMP2 antibody inhibition was coincident with a failure by spermatozoa to disperse the cumulus oophorus. IVF improvement by plasminogen was blocked by the presence of SAMP14 antibody (kindly donated by Jagathpala Shetty and John Herr), which is an antibody raised against an IAM-bound plasminogen activator receptor. We provide evidence of a non-cysteine switch related mechanism by which plasminogen improves the ability of cumulus-free oocytes to be fertilized by sperm and demonstrate its effect in sperm penetration of oocyte investments. (This research was supported by grants to RO from NSERC RGPIN/192093)

470. Regulation of Expression of Interferon-tau by Ovine Trophoderm Cells via a Toll-Like Receptor Signaling Pathway. Jing Xu¹, Irene Ruiz-Gonzalez², Kathrin Dunlap³, Beiyan Zhou¹, Fuller w. Bazer¹.
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Interferon-tau (IFNT), the maternal pregnancy recognition signal in ruminants, is synthesized and secreted by oviducal mononuclear trophoderm (oTr) cells between Days 10 and Day 21 of pregnancy. We reported that: 1) IFNT acts in an autocrine manner to regulate oTr cell proliferation, gene expression, and resistance to viral infection; 2) toll-like receptors (TLR) 7/8 likely mediate the cell signaling pathway for induction of expression of IFNT as for other type I IFNs in response to single stranded RNA such as the endogenous Jaagsiekte Sheep Retrovirus (enJSRV); and 3) oTr cells express cell surface markers common to macrophages (CD11B, CD14, CD68) and plasmacytoid dendritic cells (TLR7 and CD205), pDC and oTr cells secrete type 1 IFNs. Therefore, we hypothesized that enJSRV expressed by macrophage epithelia in response to progesterone and present in exosomes transactivate TLR7/TLR8 cell signaling via MyD88/IRF7 to induce expression of IFNT. We reported that oTr cells increase production of IFNT in response to exosomes that contain the enJSRV envelope protein. In the present study, we inserted the 5’ upstream 1kb fragment of the IFNT gene which contains 14 GAAA sites (significant homology DNA binding domain of all IRFs) into the pGL3-promoter vector and used this plasmid to transfect oTr cells. oTr cells treated 12 h later with either Loxoribine, a TLR7 agonist or exosomes at 0.5mM and 0.1ug/ul, respectively and analyzed 24 h thereafter using the Bright-Glo Luciferase Assay did not exhibit increases in IFNT secretion. We next determined whether IRF7 is associated with response elements in the 5’ promoter region of the IFNT gene using chromatin immunoprecipitation assays. The pull-down DNA PCR results identified two of six IRF1/IRF2 response elements within 10 kb of the transcriptional start site in the 5’ promoter region of the IFNT gene which were associated with significant stimulation of IFNT gene expression. The two active IRF2 response elements identified are at -3699bp and -3707 bp upstream of the start site for the IFNT gene. IRF6 may be downstream of IRF7 in the type 1 IFN signaling pathway; however, results from an assay using a plasmid to over-express IRF6 in oTr cells did not reveal an effect of IRF6 on expression of IFNT mRNA. We also used RT-PCR to analyze oTr cells and Day 15 and Day 16 ovine conceptuses to confirm that they only express IFNAR1. It is known that interferon-β induced cell signaling is mediated via IFNAR1. Our current results support our hypothesis that enJSRV acts on ovine Tr cells via TLR7/8 to induce secretion of IFNT in a manner similar to that for innate immune responses of macrophages and plasmacytoid dendritic cells to viral pathogens. Defining the role of IRF7 and its response elements in the 5’ promoter region of the IFNT gene requires further investigation.

471. Physiological concentrations of glycine affect embryonic development and metabolic activity of bovine embryos. Jason R. Herrick¹, Sarah M. Lyons², Adam L. Heuberger², William B. Schoolcraft³, Rebecca L. Krisher¹.
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Although the beneficial effects of glycine (Gly) on embryonic development are widely acknowledged, the concentration of Gly used in most culture media (0.1 mM) is much lower than that reported in bovine uterine fluid (>1.2 mM). The objective of this study was to evaluate the effects of physiological concentrations of Gly (0.1, 1.1, 2.1, and 4.1 mM) on embryonic development (blastocyst formation and hatching), cell allocation to the trophoderm (CDX2-positive) and the inner cell mass (ICM, SOX2- positive), and metabolic activity. Cumulus-oocyte complexes (COC) were recovered from slaughterhouse ovaries and matured for 24 h in a defined maturation medium (5.0 mM glucose, 0.6 mM cysteine, 0.5 mM cysteamine, 0.2 IU/ml bovine FSH, 50 mg/ml EGF, 0.25 mg/ml hyaluronan, and 2.5 mg/ml recombinant human Albumin). Frozen-thawed spermatozoa were processed by density gradient centrifugation and coincubated (2 x 10⁶/ml) with COC (10 COC/50 µl; 7.5 µg/ml heparin, 2 mM caffeine, and 8.0 mg/ml fatty-acid free (FAF) BSA) for 20 to 22 h. After removing cumulus cells, zygotes were cultured (10 embryos/20 µl) in a medium for cleavage stage bovine embryos (0.5 mM glucose, 0.3 mM pyruvate, 6.0 mM lactate, 0.25 mM citrate, 1.0 mM alanyl-glutamine, 0.25x MEM nonessential and essential amino acids, 5 µM EDTA, and 8.0 mg/ml FAF BSA). After 72 h, embryos with >4-cells were randomly allocated (5 embryos/20 µl) to a culture medium for compaction and blastocyst formation (3.0 mM fructose, 0.1 mM pyruvate, 6.0 mM lactate, 0.5 mM citrate, 1.0 mM alanyl-glutamine, 1x MEM nonessential amino acids, 0.5x MEM essential amino acids, 0.075 mM myo-inositol, and 8.0 mg/ml FAF BSA) containing 0.1, 1.1, 2.1, or 4.1 mM Gly. Embryonic development was evaluated at 192 h post- insemination (96 h in the second medium containing Gly treatments), and hatching blastocysts were fixed for analysis of cell allocation. For metabolomic evaluations, embryos were cultured in groups (5 embryos/20 µl) for 48 h in the second medium and then transferred to individual drops (12 µl) of the same medium for the final
472. ANALYSIS OF THE WHOLE TRANSCRIPTOME OF HUMAN PREIMPLANTATION EMBRYOS USING THIRD GENERATION SEQUENCING TECHNOLOGY.

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Human preimplantation embryos mark the first stages of development. Understanding how this program gets established is paramount to more broadly understand early events of differentiation, pluripotent stem cells biology and ultimately early gestational failures. Though much is known about mouse embryo development we know little about the specifics of human preimplantation development. We have recently developed a new and sophisticated “hybrid” RNA-seq method based on the combined analysis of short reads and long reads generated, respectively, by second- and third-generation sequencing technology. By combining the strengths of the two sequencing platforms it is possible to identify the full length of long transcripts and at the same time gain deep information on exon-intron boundaries, splicing variants and isoforms abundance. We applied this method to obtain a comprehensive characterization of the transcriptome of human embryonic stem cells and identified hundreds of novel pluripotency-specific genes for which we have detailed information both at the genomic and at the transcriptomic level.

The aim of this project is to adopt a similar approach on single embryos and single blastomeres, with the goal to build a transcriptome map of human preimplantation development to accurately determine the transcriptional program from the zygote (soon after fertilization) to the blastocyst stage. This work will give us invaluable information about early development in humans and will be instrumental to the development of early diagnostic tools to assess the quality of embryos obtained by Assisted Reproductive Technology.

473. Combinatorial Functions of GATA2 and GATA3 are Essential For Early Trophoblast Development and to Balance the Stem vs. Differentiation and Angiogenic Equilibrium in Trophoblast Lineage.

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GATA transcription factors are important regulators of mammalian development. In this study we established a mouse model, in which both Gata2 and Gata3 genes could be conditionally deleted. We found that GATA2/GATA3 double knockout embryos do not mature to the blastocyst stage due to defective trophoderm development. Global analyses of gene expression (RNA-seq) and Chromatin targets (ChIP-seq) in GATA2/GATA3-null mouse trophoblast stem cells revealed that loss of GATA factors instigates expression of genes that are associated with trophoblast differentiation and regulates angiogenesis. Gene Expression analyses in GATA2/GATA3-null preimplantation embryos and primary trophoblast progenitors of developing mouse placentas also revealed GATA factor-dependent maintenance of stem-state and angiogenic balance in developing trophoblast lineage. Our study indicates that GATA2/GATA3-dependent transcriptional program is essential for temporal fine-tuning of trophoblast lineage development and to balance angiogenic equilibrium at the maternal-fetal interface.

474. Function of Tight junction member Coxsackie Virus And Adenovirus Receptor(CXADR) in Porcine Early Embryonic Development.

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Coxsackievirus and adenovirus receptor(CXADR) is a member of tight junction protein at a cell surface. Here, we determined the temporal and spatial expression patterns of CXADR in porcine parthenotes during preimplantation development. The porcine CXADR were expressed at all stages of preimplantation development, with highest expression at the blastocyst stage. After compaction in morula stage, CXADR protein organized in the cell to cell membrane. In the blastocyst stage, CXADR located in the tight junction by localization with another tight junction protein, TJP1 and OCLN, and also localization with cell adheses junction protein, β-catenin and E-cadherin. Interestingly, CXADR protein co-localization of OCT4 detected in the blastocyst. In the loss of function study using by dsRNA injection into zygote, the majority of CXADR depleted embryos were arrested prior to compacted morula stage. The developmental failure is attributed to abnormal expression of genes involved in cell adhesion, tight junction biogenesis, and cell proliferation. Interestingly, 40kDa dextran get into knock-down of CXADR blastocysts, suggesting depletion of CXADR cause breakdown of tight junction in the porcine embryos. In summary, our results suggested that CXADR is necessary for cell to cell membrane formation by compaction to expansion and further development, and expression of CXADR in porcine blastocyst stage may present pluripotent cells during preimplantation embryo development.

475. EFFECT OF FIBROBLAST GROWTH FACTOR-18 (FGF18) ON EMBRYO DEVELOPMENT IN-VITRO.

2015 Abstracts - Page 188
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It has previously been shown that the theca-derived fibroblast growth factor-10 (FGF10) can enhance embryo development in cattle. In this study, we tested the hypothesis that theca-derived FGF18 impacts cumulus expansion, oocyte maturation and early embryo development. The experiments used ovaries from a slaughterhouse and cumulus-oocyte-complexes (COC) were aspirated from follicles and underwent in-vitro maturation (IVM) with FSH in drops of 50 COC for 24 hours in the presence or not of FGF18. The oocytes were fixed and stained with Hoechst 33342 to evaluate the rate of oocytes that reached metaphase II. In subsequent experiments, after IVM, oocytes were fertilized and transferred to IVF medium for 8 days. The addition of FGF18 (100, 500, and 1000 ng/mL) during IVM did not affect cumulus expansion or progression through meiosis at any dose (P<0.05, n=4). We then assessed the effect of FGF18 on embryo development by adding FGF18 at different stages of in vitro production. Three groups were established: control (absence of FGF18 at any stage), FGF-IVM (COCs treated only during IVM with 100 ng/mL FGF18) and FGF-IVF (zygotes treated with FGF18 during IVF). Blastocyst rates were assessed at day 8. Exposure to FGF18 during IVM or during IVF reduced the number of oocytes developing to blastocysts but only FGF18 treatment at IVF was significant (Absence of FGF18, 43%; FGF-IVM, 33% and FGF-IVF, 24%; P < 0.05). To begin to explore the mechanism of FGF18 action, COC or zygotes were treated or not with FGF18 during IVF or during IVM and abundance of mRNA encoding development genes (GADD54B, 53BP1, RAD52, IFN-T2 and COX2) was determined by qPCR in the embryos derived on day 8 of embryo development. Addition of FGF18 during IVM significantly inhibited levels of COX2 mRNA in embryos, but did not affect the abundance of the other mRNA measured (P<0.05). In conclusion, although FGF18 did not influence the processes of cumulus expansion and oocyte maturation, it significantly reduced the rate of embryo production, through as yet unidentified mechanisms. Supported by FAPESP, Capes, Brazil and NSERC Canada.

Poster Topic Area 4: Small noncoding RNAs, Oogenesis, Male germ cell development & Meiosis

SMALL NONCODING RNAs: Program Numbers 476-485

476. miR-122, a microRNA that Mediates Ligand-Mediated Luteinizing Hormone Receptor (LHR) Downregulation, Acts by Regulating SREBPs in the Ovary.
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We have identified and characterized a microRNA, miR-122, in the ovary that regulates the expression of LHR mRNA by controlling the tissue levels of LHR mRNA binding protein (LRBP). This mRNA binding protein, identified as mevalonate kinase, is an oxysterol responsive gene product controlled by Sterol Regulatory Element Binding Proteins (SREBPs). In the present study, we have examined the mechanism by which miR-122 regulates the expression of LRBP which, in turn, controls the levels of LHR mRNA in the ovary. Since initial experiments showed that LH/hCG activates SREBPs in the ovary, we examined whether SREBP (SREBP-1a and SREBP-2 isoforms), serves as a downstream target of miR-122. Superovulated rats were injected with a dose of hCG to induce LHR mRNA downregulation and ovaries were collected after different time intervals. To inhibit miR-122, rats were injected with a specific antagonist directly into the bursa of the ovary. 24h before hCG treatment. cAMP/PKA and ERK pathways were inhibited by pretreating rats with H-89 and U0126, 1h before hCG treatment. Western Blot analysis of the ovary lysates showed that hCG stimulated increases in the activation of both SREBP -1a and SREBP-2. Pretreatment with H-89, U0126 or miR-122 antagonist abrogated these hCG-mediated increases. SREBP activation was then inhibited by pretreating the rats with a pharmacological agent, fatostatin, and changes in LRBP were examined. Fatostatin pretreatment abrogated hCG-induced upregulation of LRBP mRNA and protein. Fatostatin also significantly inhibited ligand-induced LRBP- LHR mRNA complex formation examined by RNA electrophoretic mobility shift assay (REMSA) and LHR mRNA downregulation. Chromatin immunoprecipitation (CHIP) assay showed that binding of SREBP-1a to the promoter region of LRBP increased during LH/hCG - induced downregulation and this increase was suppressed by miR-122 antagonist. These results conclusively show that SREBP plays an intermediary role in LH/hCG- induced LHR mRNA downregulation. The activated SREBP binds to the promoter regions of LRBP gene and increases its expression which, in turn, leads to an increase in its association with LHR mRNA, leading to the translational suppression and degradation of LHR mRNA. We conclude that miR-122 regulates LHR mRNA downregulation through SREBP-mediated activation of LHR mRNA binding protein. (Supported by NIH grant, R01-HD-06656).

477. MicroRNA abundance is temporally regulated by transcription and the level of Drosha expression throughout in vitro maturation in bovine oocytes.
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Oocyte maturation comprises a series of events that precede ovulation in vivo during which the egg becomes competent for fertilization and assembles the repertoire of mRNAs and proteins necessary for embryogenesis. In vitro oocyte maturation followed by in vitro fertilization (IVF) and embryo transfer is an assisted reproductive technology that has been implemented for embryo production in cattle in order to overcome declining fertility rates in this agricultural species and meet increasing global demands for beef and dairy products. In vitro oocyte maturation is also used to model many aspects of preovulatory maturation events that occur in vivo. In both systems it is strongly suspected that the original quality of the oocyte is a major determinant in the probability of successful embryo development. Two central events of oocyte maturation are the condensation of chromatin and a widespread decrease in transcriptional
activity. This suggests that many gene expression changes in the maturing oocyte are regulated post-transcriptionally. MicroRNAs (miRNAs) are non-coding RNAs that regulate gene expression. They are transcribed as precursors (pri-miRNAs) that are subsequently cleaved by the RNase III enzymes Drosha and Dicer into active forms that bind to specific sites on target mRNAs. Several miRNAs are abundant in oocytes, and we hypothesize that pri-miRNA transcription and processing represent key events required for successful maturation and subsequent embryo development.

Deep-sequencing analysis of small RNAs present in immature and in vitro matured oocytes of Bos taurus revealed a number of miRNAs that increase markedly in abundance over the course of maturation, including bta-miR-21. Mir-21 targets apoptosis-related miRNAs to promote cellular survival, which represents an important aspect of an oocyte’s final maturation and subsequent embryo development. We therefore quantified primary and mature forms of miR-21 during in vitro maturation by quantitative RT-PCR. Surprisingly, a marked increase in pri-miR-21 abundance suggestive of transcriptional activation was observed during the first 8-16 hours of maturation. Characterization of the oocyte pri-miR-21 transcript using 5' and 3' RACE revealed two isoforms, which were then cloned and sequenced. Using bioinformatic approaches, potential regulatory elements including FOS, STAT and TBP binding sites in the putative pri-miR-21 promoter were identified. While pri-miR-21 levels rose in the early portion of the maturation period, a significant increase in mature miR-21 was not observed until 16-23 hours of maturation (one-way ANOVA, pools of 20 oocytes in 3 independent experiments). To address the discrepancy between pri-miR-21 transcription and its processing to the mature form, we investigated the expression of the pri-miRNA processing enzyme Drosha. Western blot analysis revealed that Drosha protein is only expressed late in the maturation period, coinciding with increased levels of mature miR-21. In vitro culture of oocytes for 16 hours in 89μM cycloheximide effectively inhibited the biosynthesis of Drosha, depleting the protein in the ooplasm. This coincided with a significant decrease in the mature (cleaved) form of miR-21 and other miRNAs, suggesting that the presence of Drosha is required for miRNA accumulation in mature oocytes. This study is the first to describe a functionally-active miRNA-processing pathway in bovine oocytes that appears to be regulated during maturation. Our findings suggest that regulation of both transcription and processing of specific miRNAs may contribute to oocyte maturation, and that this pathway may provide useful insight into the factors determining oocyte competency and fertility. This work is supported by the Ontario Ministry of Agriculture, Food and Rural Affairs (OMAFRA) and the Natural Sciences and Engineering Research Council of Canada (NSERC).

478. WITHDRAWN.

479. Human microRNA Patterns Associated with Ovarian Reserve. Bahar Behrouzi1, Arshia Azizeddin1, Shlomit Kenigsberg1, Cliford Librach2.
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Intra-follicular communication is integral to the development of the oocyte within its isolated micro-environment in the human body. In recent years, an increasing body of research has acknowledged that exosomes present inside the follicular fluid, surrounding the oocyte, can be implicated in determining the progress of its development. Exosomes are typically 30nm-200nm membrane vesicles of endocytic origin that are secreted by all cell types into various biological fluids and known to partake predominantly in cell-cell communication, carrying a wide variety of coding/non-coding RNAs and modified proteins. Hence, profiling the contents of exosomes is promising for identifying genetic and protein biomarkers for various fertility phenotypes. Unfortunately, the specific isolation of these vesicles and their contents from within this milieu has proven to be the subject of heavy debate, and so in this study, we set out to isolate exosomes from the follicular fluid and sera of patients with varying ovarian reserve (normal ovarian reserve, n=3; high ovarian reserve, n=3; low ovarian reserve, n=3), who presented at the CReATe Fertility Centre in Toronto for fertility treatment. Ovarian reserve refers to the capacity of the ovary to provide competent oocytes for successful fertilization and pregnancy. Specifically, we aimed to profile and compare the total microRNA content of (1) follicular fluid-derived exosomes, with and without extra-vesicular RNA degradation treatment, (2) serum-derived exosomes, with and without extra-vesicular RNA degradation treatment, and (3) granulosa luteinized cells (GLCs). We compared these 5 microRNA profiles across the 3 patient groups, in order to identify distinct markers within each medium, with expressions differing based on ovarian reserve. This study was approved by the University of Toronto Research Ethics Board. Follicular fluid and GLCs from consenting patients (n=9), were collected by individual follicular puncture, during routine sonographically guided transvaginal follicular aspiration for oocyte retrieval. Serum was collected from the same patients on the day of oocyte retrieval as part of the routine patient admission procedure. Exosomes were enriched using ExoQuick from equal aliquots of follicular fluid and serum that were either treated or not treated with proteinase and RNases. Small RNA was isolated from these enrichments and from the GLCs using a commercial kit, with an optimized exosome lysis procedure, when applicable. These small RNAs were profiled and analysed using the commercial Human Cell Development & Differentiation miRNA PCR array that focuses on a select group of 84 microRNAs that are differentially expressed during development and cellular differentiation. We were able to identify microRNAs from this panel that are exclusively found in GLCs, or exosomes derived from the follicular fluid and sera of these patients. These microRNAs can serve as significant biomarkers in the development of minimally invasive clinical diagnostic tests for various fertility phenotypes, and can aid in enhancing our understanding of how exosomes function within the ovarian follicle, potentially enhancing oocyte development and embryo quality.

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Three cellular types are isolated by a basement membrane compose ovarian follicles: granulosa, cumulus and an oocyte. The consequence of the follicular growth is production of hormones and a viable oocyte capable of generate a pregnancy. Intercellular communication is crucial to induce cell proliferation and differentiation and part of crosstalk occurs in cavity filled by follicular fluid called
Antrum. Recently, extracellular vesicles such as exosomes and microvesicles were identified within the follicular fluid and suggested as cell communication mediators. Extracellular vesicles can transfer bioactive molecules such as lipids, proteins, mRNA and miRNAs. Our hypothesis is that cell-secreted vesicles originated from granulosa cells contain different miRNAs compared to vesicles originated from cumulus-oocyte-complex. In order to test this hypothesis and to determine the origin of exosomal miRNA bovine ovaries were collected from slaughterhouses and follicles between 3-6 mm were individually isolated. Follicle contents were separated under a stereomicroscope to allow the collection of granulosa cells and cumulus-oocyte-complex (COC). Granulosa cells and COCs were placed in culture; separately, for 48 hr without FSH and 48 h with FSH, supplementation cell-culture media was collected and extracellular vesicles (EV) were isolated. Following isolation we performed real-time PCR analysis of 365 miRNAs, in order to identify miRNAs signatures for cells and respective exosomes. Bioinformatics analysis was performed in order to identify possibly regulated pathways based on miRNAs presence in cells and respective exosomes isolated from culture media. Initially, we identified thirteen miRNAs present only in COCs and eight only in granulosa cells. Granulosa cells and COCs shared 305 common miRNAs. Similarly, we identified 30 miRNAs present only in vesicles isolated from COCs culture media and thirteen present only in vesicles isolated from granulosa cells culture media. A total of 24 miRNAs were commonly identified in EVs isolated from COCs and granulosa culture media. Bioinformatics analysis demonstrated that PI3K-AKT is a common pathway targeted by miRNAs enriched in the cells as well as EVs. Beside these results we investigated members of PI3K-AKT pathway present in EVs isolated from follicular fluid and in follicular cells as pIP3 lipid, AKT protein and PTEN mRNA that can have influence on oocyte competence acquisition during follicular development. Therefore, these results suggest that EVs present in follicular fluid are part of an intricate intercellular communication and most likely are involved in regulation of apoptosis and cell proliferation by regulation of PI3K-AKT signaling pathway. Supported by FAPESP GIFT-2012/50533-2; CEPID-CTC-2013/08135-2; BPD-2013/10473-3, 2014/21034-3.

481. Role of REST Target Genes in the Pathogenesis of Uterine Leiomyomas. Mina Farahbakhsh1, Faezeh Koohestan1, Vargheese Chennathukuzhi1.
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Uterine leiomyomas (ULs), also known as uterine fibroids, are smooth muscle cell tumors of the myometrium and are the most frequent reason for a hysterectomy. Patients with ULs suffer from symptoms of abnormal uterine bleeding, incontinence, recurrent pregnancy loss and infertility. Although ULs are the most common tumors of the female reproductive tract with an estimated annual cost of $34 billion to the US economy, there are currently no long-term therapies that will leave fertility intact. Hence, there is an urgent need to understand the pathogenesis of ULs in order to develop effective long-term drug therapies. ULs are characterized by increased cell proliferation and aberrant extracellular matrix deposition. Atypical activation of growth factor receptor signaling and downstream PI3K/AKT-mTOR pathway has been suggested to promote UL development. However, the molecular mechanisms that initiate the activation of this pathway in ULs are currently unknown. Our laboratory has recently shown that the loss of RE1 suppressing transcription factor (REST) promotes the activation of PI3K/AKT pathway in ULs. REST, a known tumor suppressor, is involved in long term silencing of many genes in the periphery. Analysis of gene expression datasets from GEO database (GSE13319) indicated that many of the most abnormally expressed genes in ULs are known targets of REST. Using matched human leiomyoma and myometrial tissue samples (n=12), we analyzed the expression level of REST target genes, SCG2, GRIA2, NEFH, SALL1, GRIN2A, STMN2, DCX and CBLN1 and found that these genes were overexpressed in ULs. Additionally, silencing of REST in primary myometrial cells (n=4) using REST-specific siRNAs resulted in significant increase in the expression of REST target genes, suggesting that the loss of REST leads to the activation of these genes in ULs. In addition to the gene targets above, REST is known to regulate the expression of miRNAs including miR-29b, which plays an important role in the pathogenesis of ULs. Interestingly, miRNA29 family has been shown to directly target the 3’UTR and regulate the expression of ADAM12, a-disintegrin and metalloprotease 12. In leiomyoma samples (n=12), we found an increase in ADAM12 transcript levels along with a decrease in miRNA29 expression compared to that in matched myometrial samples. Furthermore, silencing REST in primary myometrial cells (n=4) led to a loss in miRNA29 and a gain in ADAM12 transcript levels. In conclusion, our data suggest that the loss of REST in myometrium leads to altered gene expression resulting in the pathogenesis of ULs. This research was supported by R01HD076450-01A1.

482. WNT/PCP Pathways in the Pathogenesis of Uterine Fibroids. Faezeh Koohestani1, Michelle McWilliams5, Wendy Jefferson2, Kavya Shivashankar3, Mina Farahbakhsh1, Carmen Williams2, Vargheese Chennathukuzhi1.
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As the most common pelvic neoplasm in women of reproductive age, uterine leiomyomas (ULs) or fibroids affect up to 70% of women. In a quarter of this group, severe morbidity such as pelvic pain and pressure, menorrhagia, anemia, and complications in fertility and pregnancy requires medical attention. Available treatments for ULs are limited to surgical procedures or hormonal therapy, which are associated with high costs, significant side effects and loss of fertility. Therefore, there is an urgent need to understand UL pathogenesis to provide better treatment options for women. To address this need, we focused on the main cellular characteristics of ULs, altered cell proliferation and tissue architecture, and investigated the involvement of WNT/PCP pathways in UL pathogenesis. These complicated networks of signaling molecules play important roles in tissue and tumor development through modulating cell proliferation and tissue architecture. Using healthy myometrial and leiomyoma tissues from patients (n=7), we discovered the expression of PRICKLE1, a non-canonical WNT (ncWNT) component, was significantly (P<0.05) less in ULs at both RNA and protein levels. Surprisingly, the expression of DISHEVELLED1 (DVL1), which is negatively regulated by PRICKLE1, was also found to be reduced (P<0.05) in ULs. There was, however, no change in the expression of the other two isoforms of DVL1; DVL2 and 3 (P>0.05). Furthermore, gene expression analysis showed that compared to healthy myometrial tissues ncWNT target genes were dysregulated in ULs. To prove the involvement of ncWNT pathway in the pathogenesis of ULs, primary myometrial cells silenced for PRICKLE1 showed increased expression of collagens, which is a hallmark of altered tissue architecture in ULs. Conversely, overexpression of this gene in primary leiomyoma cells had an opposite effect. We then examined the status of canonical WNT (cWNT) or β-catenin-dependent pathway in fibroids. In both myometrial and fibroid
tissues, we observed the non-nuclear localization of β-catenin and the inactivated form of LRP5/6 as the main receptor involved in cWNT. In addition, in silico and in vivo on the suppressed expression of cWNT main target genes further indicated the off state of this pathway in ULs. Since environmental estrogens such as genistein or diethylstilbestrol (DES) are known risk factors for UL formation, we investigated the modulatory role of these agents on the status of WNT/PCP pathways. Neonatal mice (n=4) were treated with 50 mg/kg genistein or 1 mg/kg DES and uteri were collected at days 5 and 22. Immunohistochemical analysis of mice uteri showed an increase in the expression of collagens, reduced expression of PRICKLE1 and DVL1 along with cytoplasmic localization of β-catenin in genistein or DES-treated mice as compared to control mice. Taken together, our data suggest that ncWNT pathway is involved in the UL pathogenesis and may be modulated by environmental estrogens. These findings can link a major risk factor for ULs to a crucial signaling pathway that controls tissue architecture and tumorigenesis for future therapeutic purposes. (Supported by NIH-RO1HD076450-A1 and NIH-KINBRE P20GM103418)

483. Critical Involvement of miR-21 Star in the Cisplatin Resistant Ovarian Cancer Cells.
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MicroRNAs (miRNAs) are endogenously expressed small non-coding RNAs evolutionarily conserved that act as post-transcriptional regulators of gene expression. MiRNA are exported from nucleus to cytoplasm as immature pre-miRNAs where they are processed to miRNA duplex of 22 nucleotides in length (denoted miR-5p and miR-3p). One of the generated miRNA strands (guide strand) is incorporated into the RNA-induced silencing complex (RISC) for interaction with its mRNA targets. The other chain (passenger strand), which is denoted by an asterisk (miR*), is degraded. Although the regulation of miRNAs by the guide strand has been amply studied, recent evidence indicates that both strands of the pre-miRNA duplex are viable and become functional miRNAs that target different miRNA populations. The oncogenic miR-21 (guide strand or miR-21-5p), one of the best-studied miRNAs, is upregulated in almost all human cancers. Ovarian cancer is the deadliest gynecological cancer. Its poor prognosis has been related to the development of chemoresistance to platinum-base therapies. Reports indicate that miRNA-21 is increased in ovarian cancer and its target genes might be involved in the cisplatin resistance of ovarian cancer cells. This study aims to determine the role of the miR-21 passenger strand (miR-21*) in ovarian cancer cells. Cell growth and clonogenic experiments showed that targeting miR-21* reduced cell growth and decreased then invasion ability of cisplatin resistant ovarian cancer. In addition miRNA target prediction software’s in combination with real-time PCR and Western blot experiments, and luciferase reported assays identified to ZNF608, RBPMS, and RCBTB1 as potential targets of miR-21*. Together, these results suggest that miR-21-3p is also a functional miRNA in ovarian cancer and possibly in other tumor types.

484. Effects of ovarian superstimulation on luteinizing hormone receptor (LHR) mRNA-binding protein (LRBP) mRNA and mir-222 expression in granulosa cells from Nelore cows.
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Luteinizing hormone (LH) plays a key role in the control of physiological processes in the ovary acting via, LHR. The LHR expression in bovine granulosa cells is crucial for the transition of FSH/LH- dependency in antral follicles. Previous data showed that ovarian superstimulation with FSH (P-36 protocol) increases LHR mRNA abundance in bovine granulosa cells. Several factors regulates negatively the LHR expression, including microRNAs (miRNAs), e.g. mir222 and LHR mRNA-binding protein (LRBP). LRBP is responsible for the down-regulation of LHR mRNA and was expressed inversely compared to LHR mRNA in bovine granulosa cells during follicle deviation. Thus, the aim of present work was to verify the effects of superstimulation with FSH or FSH combined with eCG (P-36/eCG protocol) on the LRBP mRNA abundance and mir-222 expression in bovine granulosa cells from Nelore cows (Bos indicus). Thus, Nelore cows were submitted to two superstimulatory protocols: P-36 protocol (n=10) or P-36/eCG protocol (replacement of the FSH by eCG administration on the last day of treatment; n=10). Non-supersumulated cows were only submitted to estrous synchronization without ovary superstimulation (n=10). The animals were slaughtered 12 hours before the expected endogenous LH surge. The granulosa cells were harvested from follicles and submitted to total RNA and miRNA extraction. The mRNA abundance of LRBP and mir-222 expression were measured by real time RT- PCR using bovine-specific primers and probes. The data were normalized by the expression of endogenous gene, peptidyl isopropyl isomerase (PPIA). Effects of the superstimulatory treatments were tested by ANOVA and the mean values compared with orthogonal contrast, P<0.05 indicated significant difference. Data are presented by mean ± SEM. The LRBP mRNA abundance did not differ (P=0.05) between granulosa cells from non-supersomulated cows (10.56±2.43) and cows submitted to P-36 (16.49±3.05) or P-36/eCG (13.10±1.60) protocols. However, granulosa cells from Nelore cows submitted to P-36 protocol showed lower levels (P<0.05) of mir-222 expression (0.08±0.03) when compared with non-supersomulated cows (0.34±0.13), but not differ from granulosa cells cows submitted to P-36/eCG treatment (0.14±0.06; P>0.05). In conclusion, the ovarian superstimulation did not regulate the mRNA expression of LRBP, but P-36 protocol down regulates mir-222 expression in bovine granulosa cells. Moreover, the inverse correlation between LHR mRNA and mir-222 expression indicates that LHR mRNA abundance during granulosa cells differentiation is regulated by miRNAs, but not by LRBP. Supported by FAPESP

485. Detection of miRNAs in spent media as potential markers of bovine embryonic health.
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MicroRNAs consist of small, non-coding RNAs that regulate protein expression inside cells by binding to messenger RNA, therefore regulating important intracellular mechanisms. These molecules can be secreted from within the cell, packed within microscopic vesicles called the exosomes. Several miRNAs have been identified in body fluids and have also being detected in the spent media of in vitro cultured bovine embryos. In addition, miRNAs have also been detected within in vitro cultured embryos showing different patterns of miRNA expression between blastocysts and degenerated embryos, however, no study has as yet correlated the secreted miRNA expression to embryo health and viability. Therefore, we hypothesized that embryos developing at different rates and of different viability will have a different secreted miRNA expression pattern. To test this hypothesis, bovine embryos were cultivated in groups of 20 embryos until day 8 of development. The blastocyst yield for each group was recorded and the spent media collected for miRNA analysis. Drops with a blastocyst yield percentage of >20% were called as high yield group and drops which had a blastocyst yield of <20% were the low yield group. The media from the two groups were subjected to miRNA extraction using standard protocols. A media sample that never came in contact with embryos served as a control and all samples were spiked with cel-miR-39-3p (C. elegans) for qPCR normalization. The spent media was analyzed for miR-181a, snRNA U6, miR-370, and miR-196 expression. Expression of miR-181a and snRNA U6 transcripts was the highest in the spent media of low yield blastocysts as compared to the high yield and control groups. In contrast, miR-370 and miR-196 were not consistently detectible in either the low or the high yielding blastocyst groups. In conclusion, both miR-181a and snRNA U6 may prove to be potential markers of embryonic health in bovine IVF embryo media. Further studies are required to validate these findings and to substantiate the use of miRNA assessment in the media as a new and non-invasive potential method for classification of bovine embryos cultivated in-vitro.

486. **Oocyte-specific Pat1a in the frog *Rana rugosa*: molecular cloning and characterization.**

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The Pat1a protein, also called P100 was originally purified from *Xenopus* oocytes. In *Xenopus*, Pat1a is maternally expressed in immature oocytes, but disappears during oocyte maturation. Pat1a is a RNA-binding component of ribosome-free messenger ribonucleoproteins (mRNPs) in *Xenopus* oocytes. When this protein is over-expressed by microinjection of Pat1a miRNA, the kinetics of oocyte maturation are significantly retarded. However, it is still unknown when Pat1a first appears in the differentiating ovary of amphibians. To address this issue, we isolated the full-length *Pat1a* cDNA from the frog *Rana rugosa* and examined its expression in the differentiating ovary of this species. Among eight different tissues examined, the *Pat1a* mRNA was detectable in only the ovary. When frozen sections from the ovaries of tadpoles at various stages of development were immunostained for Vasa and Pat1a, Vasa-immunopositive signals were observed in all of the germ cells, whereas the Pat1a antibody immunoreactive with signals were restricted to the growing oocytes, and absent from the tiny germ cells. Forty days after testosterone injection into tadpoles to induce female-to-male sex-reversal, Pat1a-immunoreactive oocytes had disappeared completely from the sex-reversed gonad, but Vasa-positive tiny germ cells persisted. By determining the pattern of immunostaining with antibodies against three markers (Pat1a, Vasa and BrdU), germ cells in the ovary of juvenile frogs may be classified into 4 categories. Since the tiny germ cells in the sex-reversed gonad will differentiate into testicular germ cells, they are probably germline stem cells (GSCs). Thus, it is expected that immunosignals elicited by the Pat1a, Vasa and BrdU antibodies will be valuable for identification and localization of the GSCs in the gonad of amphibians. Supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to MN (No. 19370026).

487. **The effect of Lyosphosphatic acid (LPA) during porcine *in vitro* maturation.**

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The lyosphosphatic acid (LPA) is a signaling molecule derived from phospholipid known to have biological activities such as stimulating cell proliferation, differentiation and migration. In this study, we examined the effect of LPA on *in vitro* maturation (IVM) of porcine oocytes analyzing by nuclear maturation, intracellular glutathione (GSH) and reactive oxygen species (ROS) levels, and subsequent embryonic development following parthenogenetic activation (PA). After 44 h of IVM, the 30 μM LPA treated group showed significant (P< 0.05) increase in nuclear maturation (92.97%) compared with the 0 (control), 10 and 60 μM LPA treated groups (86.61%, 87.48% and 88.69%, respectively). The 30 μM LPA treated group exhibited a significant (P< 0.05) increase in intracellular GSH levels and decrease in intracellular ROS levels compared with other LPA treated groups. Oocytes matured with 30 μM LPA during IVM had significantly (P< 0.05) higher cleavage rates after PA (88.50%) than other LPA treated groups (79.42%, 83.87% and 83.65%, respectively). The blastocyst formation rates increased up to 30 μM LPA treated group, and then the rates decreased at concentrations of 60 μM LPA treated group (49.64%, 53.28%, 37.00% and 51.09%, respectively). However, statistical difference was not observed (P> 0.05). Furthermore, control and all LPA treated groups had no significant total cell numbers (43.92, 46.84, 48.48 and 42.12, respectively) after PA. Our results suggested that treatment with 30 μM LPA during IVM improved the developmental potential of PA porcine embryos by increasing the intracellular GSH level, thereby decreasing the intracellular ROS level during oocyte maturation. Further studies are required to examine subsequent embryonic development following *in vitro* Fertilization (IVF) and investigate the functional mechanisms of LPA, implicated in supporting the developmental competence in oocytes during maturation. Acknowledgements: This work was supported, in part, by a grant from the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ011288), Rural Development Administration, and the National Research Foundation of Korea Grant funded by the Korean Government (NRF-
488. A critical role of differential expression of LINE-1 (L1) in fetal oocyte attrition in mice.
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The oocyte reserve of resting primordial follicles is a critical determinant of the female reproductive lifespan. The size and quality of the oocyte reserve are defined during the second half of gestation by massive elimination of fetal oocytes, broadly defined as fetal oocyte attrition (FOA). Despite the evolutionary conservation of this phenomenon, its molecular and cellular mechanisms, and its biological purpose remain poorly understood. We have recently obtained evidence implicating retrotransposon Long Interspersed Nuclear Element-1 (LINE-1 or L1), the only active class of autonomous mobile element in the human genome, in FOA in mice (Malki et al., Dev Cell, 2014). Retrotransposons pose significant danger to the germline particularly during periods of their increased expression such as during epigenetic reprogramming. We showed that all fetal oocytes express L1 ribonucleoproteins (or L1RNPs) that can be reliably identified using antibodies to L1ORF1 protein, an RNA chaperon encoded by L1. Unexpectedly, we observed differential L1 expression between wild-type fetal oocytes before and during FOA. Importantly, increased L1 expression levels positively correlated with elevated DNA damage, meiotic prophase I defects and oocyte lethality. Interestingly, we also found that oocytes surviving elevated levels of L1 activity exhibited decreased chiasmata leading to increased incidence of aneuploidy and embryonic lethality. Furthermore, experimental overexpression of L1 resulted in even higher levels of these meiotic defects. Inhibition of L1 retrotransposition with an anti-retroviral drug AZT has dramatically altered the dynamics of FOA and increased homologous recombination even in wild-type mice. While AZT treatment did not prevented FOA due to continuous DNA damaging activity of L1RNPs, it suggested existence of a reverse transcriptase-dependent trigger of FOA, most likely L1 RNA/DNA hybrid molecules. Our ongoing studies are aimed at the elucidation of the molecular mechanism of differential L1 expression between fetal oocytes. To begin to address this difficult problem, we developed a fluorescence-activated cell sorting approach to isolate fetal oocytes based on their levels of L1ORF1p expression and are using recovered DNA and RNA samples to gain insights. In addition, we are also examining a "non-cell-autonomous" role of the germ-cell cyst architecture in differential L1 expression by studying the effects of abrogation of intercellular bridges in Tex14 mutant mice (generated previously by the Matzuk group). We will present results of these ongoing studies and the updated model of L1-driven FOA.

489. The activation of Akt signaling pathway in in-vitro culture of mouse ovarian follicles promotes the formation of spindle microtubule in nuclear maturation.
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The mammalian ovaries contain numerous follicles that include immature oocytes. In Vitro Growth (IVG) and In Vitro Maturation (IVM) culture technologies for these immature oocytes in mammals are attempted to induce a resumption of meiotic competence and normal embryo development after fertilization. These technologies serve as assistance to reproduction for domestic animals, preservation of genetic resources, and applications for assisted reproductive medicine in humans. In 2013, Mochida et al., reported to enable in vitro growth culture with early secondary follicles of mice. This multi two-step technique can culture from early secondary follicles and produce offspring. However, there are problems such as the low embryonic development rate in fertilization and the production rate of maturation oocytes after IVG-IVM, but there is potential for improvement. In the present study, we focused on improving oocyte maturation. Oocyte maturation consists of maturation of the nucleus and cytoplasm as it occurs germinal vesicle breakdown, aggregation of chromosomes, and the formation of the spindle. These are essential phenomena in oocyte maturation; we thought that perhaps the Akt signaling pathway is largely related. It is not clearly known what kind of effect Akt activity has on IVG-IVM on mice. Therefore, we added a small molecule to the medium that may have either activated or inhibited these signaling pathways, and we observed normal maturation and spindle formation after IVM and examined whether there was an effect on developmental competence after fertilization. In this study, we used 7-day-old BDF1 female mice and isolated the early secondary follicles with diameters of 60–95μm from ovaries and cultured them in collagen gel for 9 days (IVG1). On day 9, the follicles were transferred to a second culture with collagen coat membrane for 8 days (IVG2). At day 17, isolated cumulus oocyte complexes were cultured in the IVM medium with/without SC79 as the Akt activator for 19h. As a result, the production rate of maturation oocytes, pronuclear formation and second polar body after fertilization with the treated Akt activator improved compared with the untreated in vitro culture oocyte. Especially, in vitro culture oocyte had abnormal spindle microtubule, but treated activation of Akt in vitro maturation exhibited normal morphology of spindle microtubule. However, the difference in the rate of development after fertilization was not observed in the 2 cell stage onward. These results indicate that activated Akt signaling has an effect on maturation, but not on the development after fertilization. Therefore, we suggest that the highly activated Akt in vitro maturation is important for the formation of the spindle and the release of the second polar body after fertilization.

490. Discovery of a Novel Oocyte-Specific KRAB-Containing Zinc Finger Protein Required for Early Embryogenesis in Cattle.
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Zinc finger (ZNF) transcription factors are known to interact with DNA through zinc finger motifs and play important roles in a variety of cellular functions, including cell growth, proliferation, development, apoptosis, and intracellular signal transduction. One-third of ZNF proteins contain a highly conserved N-terminal motif known as the KRAB domain, which acts as a potent, DNA-binding dependent transcriptional repression module. To date, ZNF proteins expressed specifically in mammalian oocytes have not been reported. RNA sequencing of a bovine oocyte library uncovered a highly abundant transcript that matches an uncharacterized gene in the NCBI database. Through cDNA cloning of the novel gene (ZNFO) a transcript containing a 2,145 bp open reading frame that codes for a protein of 714
YAP (Yes-associated protein) is a transcriptional co-activator that drives growth and proliferation in a wide range of cell types. Pharmacological agents that activate YAP by shutting down inhibitory pathways promote follicle growth, suggesting a potential new therapeutic strategy to overcome human infertility. However, in the absence of knowledge of the normal expression and regulation of YAP in the follicle, our understanding of the mechanistic basis of these strategies remains very limited. We found that YAP and YAP are present in growing and fully grown oocytes. At both stages of oocyte development, however, YAP was predominantly located in the cytoplasm and thus unable to exert its transcriptional activity. In other cell types, phosphorylation of YAP at Serine (S)-127 by the protein kinase, LATS, prevents nuclear localization. We found that oocytes express Lats1 and that YAP is phosphorylated on S127 in both growing and fully grown oocytes. To identify the mechanism that regulates LATS1 activity and hence YAP phosphorylation, we manipulated the activity of the upstream regulator, protein kinase A (PKA). When we cultured fully grown oocytes in the presence of the PKA activator, dibutyryl cyclic AMP, YAP remained phosphorylated. In the absence of dbAMP, however, YAP became dephosphorylated at S127, indicating that phosphorylation is regulated by PKA. Dephosphorylation occurred even when the cyclin-dependent kinase inhibitor, roscovitine, was added to fully grown oocytes to maintain an intact nucleus. Surprisingly, YAP did not accumulate in the nucleus under these conditions, even in the presence of the nuclear export inhibitor, LMB, indicating that dephosphorylation at S127 is not sufficient to trigger YAP nuclear localization in fully grown oocytes. In growing oocytes, PKA inhibitors also decreased phosphorylation of YAP at S127 as in fully grown oocytes. Unlike fully grown oocytes, however, LMB induced nuclear accumulation of YAP in growing oocytes and this accumulation was enhanced by inhibition of PKA. We conclude that PKA in growing and fully grown oocytes phosphorylates YAP at
S127. Dephosphorylated YAP can enter the nucleus of growing but not fully grown oocytes, but is rapidly exported. In addition we found that YAP is excluded from the nuclei in granulosa cells around growing oocytes as well as in cumulus cells around fully grown oocytes. Our results suggest that the mechanism by which activators of YAP induce follicle growth is unlikely to involve activation of YAP-dependent transcription in the oocyte and granulosa cells.

493. IMD/ADM2 Promotes the Cell Interaction in Cumulus-Oocyte Complexes (COCs) and Enhances Oocyte Maturation to Develop competence In Vitro.
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Accurate chromosome segregation is critically dependent on formation of the spindle apparatus and the establishment of stable chromosomes-microtubule interactions. Yet, few mechanistic studies have addressed the control of meiotic spindle assembly and stability in mammalian oocytes. Spindle formation in oocytes differs from mitosis and is regulated by unique microtubule organizing centers (MTOCs) that lack centrioles. Our previous in vitro studies identified an essential role for a key conserved MTOC-associated protein, pericentrin (Pcnt), in mammalian oocytes. To test pericentrin function in vivo, we developed an oocyte conditional knockdown mouse model using a transgenic RNAi approach. Two ZP3-Pent RNAi mouse lines were established in which efficient knockdown of Pent was confirmed exclusively in oocytes from transgenic females. Less than 1% residual Pent transcript levels were detected by qPCR. Similarly, oocyte-specific loss of pericentrin protein expression was confirmed by immunofluorescence. Oocytes from transgenic females are ovulated at metaphase-II (MII), but show a high incidence of aneuploidy, indicative of chromosome segregation errors during Meiosis I. Moreover, Pent-deficient oocytes at MII also exhibit disrupted spindle structures with significant chromosome misalignment and a high frequency of lagging chromosomes that point to defects in chromosome- microtubule attachment. Notably, loss of pericentrin reduces the level of MTOC-associated γ-tubulin and NEDD1, which are essential for microtubule formation. These meiotic defects impair fertility, as transgenic females produce significantly fewer (P<0.05) pups per litter and exhibit an increased rate (~25%) of perinatal mortality relative to controls. In sum, these data demonstrate that ablation of maternal pericentrin disrupts oocyte MTOC function, leading to increased meiotic errors and significant embryonic loss. This unique mouse model provides a valuable genetic approach to assess the molecular mechanisms underlying oocyte MTOC function.

494. Testing the Functional Role of Microtubule Organizing Centers (MTOCs) in Oocytes.
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Accurate chromosome segregation is critically dependent on formation of the spindle apparatus and the establishment of stable chromosomes-microtubule interactions. Yet, few mechanistic studies have addressed the control of meiotic spindle assembly and stability in mammalian oocytes. Spindle formation in oocytes differs from mitosis and is regulated by unique microtubule organizing centers (MTOCs) that lack centrioles. Our previous in vitro studies identified an essential role for a key conserved MTOC-associated protein, pericentrin (Pcnt), in mammalian oocytes. To test pericentrin function in vivo, we developed an oocyte conditional knockdown mouse model using a transgenic RNAi approach. Two ZP3-Pent RNAi mouse lines were established in which efficient knockdown of Pent was confirmed exclusively in oocytes from transgenic females. Less than 1% residual Pent transcript levels were detected by qPCR. Similarly, oocyte-specific loss of pericentrin protein expression was confirmed by immunofluorescence. Oocytes from transgenic females are ovulated at metaphase-II (MII), but show a high incidence of aneuploidy, indicative of chromosome segregation errors during Meiosis I. Moreover, Pent-deficient oocytes at MII also exhibit disrupted spindle structures with significant chromosome misalignment and a high frequency of lagging chromosomes that point to defects in chromosome- microtubule attachment. Notably, loss of pericentrin reduces the level of MTOC-associated γ-tubulin and NEDD1, which are essential for microtubule formation. These meiotic defects impair fertility, as transgenic females produce significantly fewer (P<0.05) pups per litter and exhibit an increased rate (~25%) of perinatal mortality relative to controls. In sum, these data demonstrate that ablation of maternal pericentrin disrupts oocyte MTOC function, leading to increased meiotic errors and significant embryonic loss. This unique mouse model provides a valuable genetic approach to assess the molecular mechanisms underlying oocyte MTOC function.

495. Centrosome/ chromosome-free spindles in mouse oocytes are MPF and kinesin5 dependent.
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Centrosome segregation errors in oocytes are a major cause of infertility. Why segregation errors are common in oocytes remains poorly understood. Accurate chromosome segregation depends upon organizing microtubules into a spindle. In mitosis, microtubules are organized by centrosomes. In oocytes, on the other hand, the spindle forms independently of centrosomes and chromosomes play a pivotal role in microtubule generation. Classic experiments revealed that in mouse oocytes microtubules can form a spindle after chromosome removal. However, there has been no examination of this centrosome/chromosome-independent microtubule generation pathway, which likely offers unique insights into microtubule behavior. Here we show that centrosome/chromosome –free
spindles form even after enucleation of germinal vesicle stage oocytes. Centrosome/chromosome-free spindle formation requires an M-phase cytoplasm, as it is prevented by phosphodiesterase inhibitor, and spindles are disassembled by inhibiting cyclin-dependent kinases. Specific small-molecule inhibitors of Kinesin-5 and Dynein cause disassembly of the spindles, indicating that both motors are necessary for spindle maintenance. Surprisingly, cold-shock treatment revealed that the spindles are comprised of two different microtubule populations with distinct turnover rates. For further details on the mechanism of centrosome/chromosome-free spindle assembly we performed live microtubule imaging during assembly using a variety of probes. Notably, using the microtubule plus-end marker EB1:EGFP, MTOCs were seen to interact via antiparallel microtubule growth events during spindle assembly. We conclude that centrosome/chromosome-free spindles are formed by MTOCs generation and random interaction, and that spindle-like structures represent a stable steady-state MT conformation. Understanding the cause of chromosome segregation error in oocytes will ultimately depend upon unraveling the idiosyncrasies of microtubule function and spindle assembly in this unique cellular environment.

496. FSH regulates maternal mRNA translation in the oocyte and promotes developmental competence. Federica Franciosi1, Marco Conti2.
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The development of the ovarian follicle is under the control of the gonadotropins FSH and LH. FSH promotes granulosa cell growth and prepares the follicle to respond to LH, whereas the LH surge is the major signal for oocyte meiotic resumption and ovulation. However, an increased secretion in FSH accompanies the LH surge prior to ovulation in most mammalian species. The exact function of this FSH surge is unclear even though it is thought to be important for oocyte maturation and developmental competence. In rodents, FSH and the growth factor EGF have been shown to increase the developmental competence of cumulus oocyte complexes matured in vitro. Improvement in fertilization and pregnancy rates has been demonstrated in IVF patients stimulated with FSH/hCG compared to hCG alone. The signaling pathways and mechanisms by which FSH improves oocyte quality are unknown. Here we have tested the hypothesis that FSH improves oocyte quality by regulating the translation of maternal mRNAs in mouse oocytes. Experiments were conducted in cumulus oocyte complexes (CEOs) collected from ovaries of 21-24 days old PMSG-primed mice, and cultured in groups of 30-50. All the experiments were repeated 3-6 times. Data were analyzed by t-test, OneWay ANOVA or TwoWay ANOVA. P values <0.05 were considered statistically significant. Dual luciferase translation reporters were co-injected in oocytes still enclosed in the cumulus cells. The translation of prototypic luciferase reporters (Tpx2 and interleukin7 – I7 3'UTRs) was significantly increased when CEOs were cultured in the presence of FSH. Consistent with the increased translation, the IL7 secretion was significantly higher in the culture supplement of CEOs cultured with FSH compared to control. The FSH-dependent increase in translation was preceded by a transient phosphorylation of AKT in the oocyte, measured by western blot. Knockdown of the expression of the EGF receptor (Egfr) in the granulosa/cumulus cells using a genetic approach (Egfr<sup>+/<sup>loxP</sup>:Cyp19<sup>cre</sup>) prevented the oocyte AKT activation by FSH. These results indicate that the FSH signal transduction requires EGFR for its action. To further investigate the role of PI3K/AKT in the regulation of oocyte translation by FSH and its effect on developmental competence we used a genetic model where Pten (phosphatase and tensin homologue), which negatively regulates PI3K, is specifically ablated in oocytes. AKT was constitutively activated in Pten<sup>loxP/loxP</sup>:Zp3<sup>cre</sup> oocytes, independently of the culture treatment. In absence of any hormone, the constitutive AKT activation was sufficient to induce an increase in the translation of the luciferase reporters compared to controls. The fertilization rate was significantly increased in Pten<sup>loxP/loxP</sup>:Zp3<sup>cre</sup> oocytes in the absence of hormone treatment. These data strongly suggest that FSH promotes oocyte developmental competence by regulating translation in the oocyte, a regulation that requires intact EGFR signaling in cumulus cells. These findings provide a molecular rationale for the use of FSH to improve egg quality during assisted reproductive technologies. Supported by: FP7-PEOPLE-2013-IOF GA 624874 MateRNA and NICHD- NIH NCTRI P50HD055764

497. Characterization of NADH/NAD+ Ratio During Early Stages of Ovarian Follicular Activation in Live Murine Neonatal Ovaries. Rachel P. Cinco1, Enrico Gratton1, Michelle Digman1, Ulrike Luderer1.
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Follicular bioenergetics is a critical determinant of oocyte quality and ovarian aging. Ovarian bioenergetics has largely focused on defining follicular metabolism by measuring metabolic byproducts in cultured media. However, culture conditions required for these data compromise the native biological cell state. Furthermore, there are few data characterizing early follicular metabolism before the primary stage, likely due to limitations in small follicle isolation. Nicotinamide adenine dinucleotide (NAD) and its reduced form NADH play a central role in bioenergetics as one of the main energy currencies of the cell. The NADH/NAD+ ratio has significant impact on energy production, proliferation, cell survival, and aging. NADH is naturally fluorescent and its free and bound states can be measured by lifetime imaging microscopy using 740nm 2-photon excitation. The development of the phasor approach to fluorescence lifetime imaging microscopy (Phasor FLIM) offers a straightforward approach to interpretation of lifetime differences at the pixel level. Phasor FLIM analysis of relative free/bound NADH provides a quantitative measure that approximates the intracellular NADH/NAD+ ratio. This method has been extensively used to characterize the spatial distribution of free/bound NADH in living tissues. Here, we apply Phasor FLIM at 740nm 2-photon excitation to examine the spatial distribution of free/bound NADH during early stages of follicular development in live mouse neonatal ovary. We hypothesized dynamic changes in NADH/NAD+ ratio to parallel the developmental maturation of primordial thru activated primary follicles. We obtained subcellular resolution phasor FLIM 2D and 3D data and assessed the NADH/NAD+ ratio in oocyte nucleus and oocyte cytoplasm within primordial to primary follicles. Furthermore, we assessed the NADH/NAD+ ratio in granulosa cell nuclei of transitional follicles containing mixtures of morphologically flat and cuboidal granulosa cells. Primordial to primary follicles showed no statistically significant differences in NADH/NAD+ in the oocyte cytoplasm, suggesting little change in metabolism during these stages of development. However, we observed primordial follicle nuclei had significantly higher NADH/NAD+ ratios compared to activated transitional and primary staged follicles. We furthermore characterized the...
developmental expression of NAD+ dependent deacetylase SIRTUIN1 (SIRT1) in neonatal ovary by immunohistochemistry and found SIRT1 expression in oocyte nuclei to parallel observed decreases in free/bound NADH. Cell phasor analysis of free/bound NADH in transitional follicle granulosa cells revealed three populations of granulosa cells characterized by statistically significant differences in NADH/NAD+ ratios. Taken together, our data characterize a dynamic change in NADH/NAD+ and SIRT1 expression during early follicular development that has not been previously characterized. Supported by NIH ES020454 to UL and NIH/NIGMS 8P41 GM103540-28 to EG and MD.

498. Improved bovine oocytes retrieval using 2-step retrieval process.
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Embryological issue is still remains to be solved in conservation of wild animals, including endangered species. This is largely due to limited availability of ovaries for the retrieval of oocytes. If we can increase the number of oocytes from the same amount of ovaries, we can contribute to have more opportunity to restore and conserve a large number of wild animals. In this study, we attempted 2-step retrieval process in order to increase the number of oocytes retrieved from bovine ovaries. In 2-step retrieval process, the oocytes were first collected by aspiration. After this, the ovarian tissue was sliced and remain of the oocytes were collected (remainder-slicing method).

Number of oocytes recovered per ovary was 7.2 ± 0.9 from aspiration and 6.5 ± 1.4 from remainder-slicing method. Total number of oocytes recovered using 2-step retrieval process (13.8 ± 1.7) was more than that which was retrieved using general slicing process (10.7 ± 2.7). We investigated the grade distribution, maturation rate and reactive oxygen species level of the oocytes retrieved by aspiration and remainder-slicing method. Oocytes retrieved by aspiration contain 23.2 ± 2.2 % A-grade oocytes and gave rise to high maturation rate (81.8 ± 8.2 %, using A & B-grade oocytes). Incorporation of remainder-slicing method has increased the total number of oocytes retrieved from an ovary. However, most of the oocytes (70.7 ± 1.5 %) collected from remainder-slicing method were inferior oocytes (C grade oocytes) with high number of lysed oocytes (33.3 ± 4.8%). We investigated whether addition of resveratrol, an antioxidant would improve the quality of the C-grade oocytes. When 2 μM resveratrol was included in the IVM medium of the C-grade oocytes retrieved using remainder-slicing method, the maturation rate was improved. The maturation rate with and without 2 μM resveratrol was 48.9 ± 2.0% and 32.5 ± 3.3%, respectively. The relative ROS level of the oocytes with 2 μM resveratrol (1.20 ± 0.03) was lower than those without 2 μM resveratrol (1.35±0.02). This work was supported by a grant of Research Program (No. 307-02) Gyeonggi-do Project, Republic of Korea.

499. Involvement of cystatin C and its cognate target protease cathepsin B in the disassembly of post-ovulatory cumulus-oocyte complexes.
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Cystatin C (CST3) is one of the most abundant cysteine protease inhibitors expressed in reproductive tissues. In this study, we investigated the effect of CST3 on the structural integrity of cumulus cells on post-ovulatory cumulus-oocyte complexes (COCs). Three-week-old ICR mice were used as the animal model. To obtain COCs, mice were supervoluplated following standard procedures, and the COCs were retrieved post ovulation by flushing the oviducts with M2 medium. Cellular expression of CST3 in the oviduct and COCs was examined by immunohistochemical staining. COCs collected post ovulation were treated with or without CST3 for 4, 8, or 12 h at 37 C in DMEM medium. To evaluate the effect of CST3 on the structural integrity of cumulus cells surrounding COCs, COCs size and the activity of cathepsin B (CTSB), a cognate cysteine protease, were measured at various incubation time intervals. The CST3 protein was detected in the oviductal epithelium and the cumulus cells surrounding oocytes. Its expression levels in cumulus cells of post-ovulatory COCs were at a relatively high level 12-16 h after hCG treatment, gradually decreased from 20 h after hCG treatment, and to a low level 24 h after hCG administration. Parallel to expression levels of CST3, the size of post-ovulatory COCs gradually diminished and left a half size at about 24 h after hCG treatment owing to cumulus disassociation. In vitro culturing of post-ovulatory COCs for 12 h resulted in similar extent of cumulus disassociation. However, this disassociation was significantly suppressed by exogenously supplemented CST3 in a dose-dependent manner. CTSB activity was at a relatively high level 20 h and 24 h after hCG treatment, and its levels were positively correlated to the extent of cumulus disassociation. CST3 and a specific CTSB inhibitor both suppressed CTSB activity and also prevented cumulus disassociation. In summer, lower expression of CST3 protein and higher activity of CTSB in cumulus cells of post-ovulatory COCs were correlated with cumulus disassembly of COCs. Exogenously added CST3 prevented cumulus disassembly from COCs. The suppressive effect of CST3 on cumulus disassociation may be mediated by blocking the CTSB activity.

500. SOHLH1 and SOHLH2 interaction is essential for their cellular localization and oocyte differentiation.
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SOHLH1 and SOHLH2 are germ cell specific helix-loop-helix transcriptional regulators, essential in oocyte differentiation. Postnatally, SOHLH1 and SOHLH2 are co-expressed in small follicles and show identical expression, however their prenatal expression differs. A SOHLH2 protein expression starts at E12.5, and until E15.5 is predominantly located in the cytoplasm. At E15.5, a small subset of oocytes begins expressing SOHLH1 protein in the cytoplasm as well as in the oocyte nucleus. SOHLH1 appearance at E15.5 correlates with SOHLH2 translocation into the nucleus since cells that are positive for SOHLH1 are also positive for SOHLH2 nuclear staining, while SOHLH1 negative cells have SOHLH2 confined to the cytoplasm. SOHLH1 and SOHLH2 co-expression continues after birth, with SOHLH2 exclusively present in the nucleus. Global knockout of Sohlh1 does not abolish SOHLH2 expression. However, SOHLH2 is exclusively expressed in the cytoplasm of Sohlh1 deficient oocytes. These results indicate that SOHLH1 is essential for SOHLH2 nuclear localization and function. Despite their different patterns of expression in the embryonic gonad, Sohlh1 and Sohlh2 knockouts co-regulate identical sets of genes. These results support the interpretation that SOHLH1 and SOHLH2 work as a heterodimer rather than as
homodimers and explain why Sohlh1 and Sohlh2 single knockouts have an identical phenotype with each other and Sohlh1/Sohlh2 double knockouts. We studied at what stage Sohlh1 can rescue its deficiency, and whether it can rescue Sohlh2 loss. We generated Sohlh1 (CCS1) and Sohlh2 (CCS2) transgenic mice to allow their conditional expression under the control of the CAG promoter. Using germ cell specific Ddx4-Cre and Gdf9-Cre to induce CCS1 transgene expression at E15.5 or PD3, respectively, Sohlh1−/− phenotype was rescued. These results suggest that Sohlh1 is not essential prior to PD3. However, CCS2 transgene induced with Ddx4-Cre did not rescue Sohlh2−/− phenotype. These results indicate that SOHLH2 is essential prior to E15.5. We searched for signaling molecules that modulate SOHLH1 and SOHLH2 expression and localization. Retinoic acid binding sites were found in Sohlh1 and Sohlh2 promoter regions. We used in vitro culture of E13.5 and E15.5 gonad and treated them with AGN193109, a retinoic acid antagonist, or DMSO, the vehicle used to dissolve AGN193109. In the E13.5 embryonic gonad, in vitro culture for three days with AGN193109 suppressed SOHLH1 expression when compared to the DMSO treated ovary. In E15.5 embryonic gonads treated for three days with AGN193109 treatment, 5% of oocytes showed SOHLH2 localized in the nucleus while 38% oocytes showed SOHLH2 expression localized in the cytoplasm. In comparison, gonads treated with DMSO (vehicle control) showed that 40% of oocytes had SOHLH2 localized in the nucleus but only 10% of oocytes had SOHLH2 localized in the cytoplasm. We interpret these results that retinoic acid induces SOHLH1 expression, which in turn regulates SOHLH2 intracellular localization.

501. Gene expression profiles and functionality of bovine cumulus cells derived from oocytes with different chromatin configuration.
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During follicle development, interactions between somatic and germ cells are critical for the acquisition of oocyte competence. Moreover, granulosa cells serve a critical role in the modulation of chromatin configuration changes that occurs before meiotic resumption. Importantly, chromatin configurations within the oocyte germinal vesicle (GV) are indicative of oocytes metabolic state as well as developmental potential. However, the mechanisms acting in the somatic compartment that regulate oocyte competence are far from being understood. This study was aimed to assess gene expression profile by microarray platform in cumulus cells (CC) isolated from ovarian follicles, which are linked to the oocyte developmental competence. To this aim we determined the expression profile of CC of oocytes at different stage of differentiation and selected according to their chromatin configuration as GV0, GV1, GV2 and GV3. CC belonging to each group were collected, RNA was extracted, amplified and hybridized on a bovine embryo-specific 44K Agilent slide (EmbryoGene). The GV1, GV2 and GV3 classes were each hybridized against the GV0, which represents a stage of early oocyte differentiation with poor development competence. Data were normalized with Loess and Fold changes (FC) of differentially expressed genes were determined with Limma procedure. Finally, the regulation pattern genes and function predicted were studied with Ingenuity Pathway Analysis software. Transcriptome analysis (FC >1.5; p<0.05) reveals 1441 genes differentially expressed in GV1 vs GV0 (684 down and 757 upregulated), 1474 in GV2 vs GV0 (699 down and 775 upregulated) and 2047 in GV3 vs GV0 (981 down and 1066 upregulated). Data analysis indicated a deregulation of several transcripts associated to extracellular matrix formation and stabilization such as: Hyaularonan synthase 2 (HAS2), Serpine 2 (SERPINE2) and Progesterone receptor (PGR). Moreover, along with the increase in chromatin compaction, we noticed an upregulation of the gene solute carrier family 39 member 8 (SLC39A8), which is a zinc transporter, an upregulation of inhibin alpha subunit (INHA), which is implicated in hormonal regulation as well as a down-regulation of G-protein signalling 2 (GNG2), which is implicated in the G protein coupled receptor pathway. Importantly, another group of differentially regulated genes was represented by apoptosis-related transcripts such as apoptosis-related cysteine peptidase (CASP3), Thrombospondin-1 (THBS1) and proliferating cell nuclear antigen (PCNA).

Microarray data were validated by quantitative RT-PCR. Relative expression levels of target genes such as THBS1, SERPINE2, regulator of RGS2, INHA and SLC39A8 were calculated with the delta-delta Ct method using B ACTIN, GAPDH and HPRT1 as reference genes for normalization. Statistical analysis of the data were conducted by ANOVA followed by Newman-Keuls post hoc test. To further confirm that CC belonging to oocytes with increasing degree of chromatin compaction are more prone to apoptotic events, CC belonging to each GV group were cultured for 3hrs in defined conditions and apoptotic cells were assessed by CaspaTag Pan-Caspase in situ Assay kit. Our results indicated that a low percentage of CC from GV0 oocytes are undergoing apoptosis, while this percentage significantly increases in oocytes with more condensed chromatin, reaching the highest level in CC of GV3 oocytes (one way ANOVA p<0.05). This study further confirms how the process of oocyte competence acquisition is profoundly influenced by the somatic compartment and provides multiple non-invasive biomarkers that can predict oocyte developmental potential. This has important implications in treating human infertility as well as developing breeding schemes in domestic mammals. Work supported by the NSERC Strategic Network EmbryoGene, Canada.

502. Lhx8 interacts with a novel germ cell-specific nuclear factor containing an Nbl1/Borealin N terminal domain in rainbow trout (Oncorhynchus mykiss).
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Lhx8 is an important transcription factor that is preferentially expressed in germ cells. Lhx8 null mice are infertile due to lack of oocytes and impairment of the transition from primordial follicles to primary follicles. Lhx8 deficiency also affects the expression of many important oocyte-specific genes, such as Gdf9, Pou5f1, and Nobox. To date, no attempts have been made to investigate the existence of any cellular factors that might interact with Lhx8 protein in oocytes and early embryos. The objectives of this study were to characterize the rainbow trout Lhx8 gene, and identify cellular proteins that interact with and mediate the functions of Lhx8 during oogenesis and early embryonic development. Through database mining, we identified two Lhx8 genes, Lhx8 and Lhx8-like, in rainbow trout. They are located on two different chromosomes, with Lhx8 being on chromosome 28 and Lhx8-like being on chromosome 8. The open reading frames for Lhx8 and Lhx8-like are 1035 bp and 1086 bp in length encoding 344 and 361 amino acids, respectively, both of which contain two LIM domains and one homeobox domain. The two proteins share 83% sequence identity, and rainbow trout Lhx8 shares 79%, 74%, and 74% sequence identity with the zebrafish, mouse and human counterparts, respectively. Tissue distribution analysis of Lhx8 and Lhx8-like
By reverse-transcription PCR revealed that both transcripts are predominantly expressed in the ovary with minor expression in testis and gill, but undetectable in any other somatic tissues. To further analyze the function of Lhx8 and Lhx8-like during development, we performed quantitative real-time PCR (RT-qPCR) analysis using samples collected from different stages of oogenesis and embryogenesis. The expression of both genes is high in ovaries at early vitellogenic stages, as well as in early stage embryos until 3 days post fertilization (dpf) followed by a gradual decline to a undetectable level in the late stage embryos. To identify cellular proteins that might interact with Lhx8 proteins, we performed a yeast two hybrid screening using Lhx8-like as a bait. Two identical cDNA clones from a rainbow trout egg cDNA library were identified. Sequence analysis of the isolated clones revealed that they code for a novel protein of 255 amino acids containing an Nbl1/Borealin N terminal domain. The protein (named Borealin-2) was predicted to possess a typical nuclear localization signal (NLS), indicating that it is a nuclear factor. The interaction between Borealin-2 and Lhx8-like was confirmed by retransformation of Borealin-2-2 AD fusion construct into Y187 yeast cells followed by mating with the Y2Gold cells expressing Lhx8-like-BD fusion protein. Further confirmation of interaction between the two proteins was performed by a β-galactosidase CPRG assay. In addition, the interaction between Borealin-2 protein and Lhx8 protein was tested positive. Tissue distribution analysis revealed that Borealin-2 is specifically expressed in ovary and testis. RT-qPCR analysis showed that expression of Borealin-2 mRNA is high in early stage embryos until 18 h post fertilization followed by a gradual decline to an undetectable level after 5 dpf. Collectively, our results indicate that both Lhx8 and Lhx8-like function through interaction with Borealin-2, which may play an important role during oogenesis and early embryogenesis in rainbow trout (Supported by USDA-ARS Cooperative Agreement No. 58-1930-0-059).

503. Functional assessment of Progesterone Receptor Membrane Component 1 (PGRMC1) action during bovine oocyte meiosis by means of PGRMC1 inhibitor and small-interfering RNA mediated gene silencing.
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Previous studies suggest that Progesterone Receptor Membrane Component 1 (PGRMC1) plays an essential role during bovine oocyte meiosis, since it 1) localizes to the centromeres at metaphase-I and II and 2) concentrates between the separating chromosomes at anaphase-I. Moreover, injection of an antibody to PGRMC1 significantly impairs completion of meiosis. The aim of the present study is to expand these findings by using a PGRMC1 inhibitor (AG205) and small-interfering RNA (RNAi) mediated gene silencing. In a first set of experiments, bovine Cumulus-Oocytes Complexes (COC) were collected from 2-6 mm antral follicles. COC or denuded oocytes (DO) were in vitro matured (IVM) for 24h in the presence of 0, 10, 20 or 40 μM of AG 205. After IVM, the oocyte capability to extrude the first polar body (PBI) and the effect on the meiotic progression were assessed. Data were analyzed by two-way ANOVA followed by Tukey’s Post hoc test. Treatment with AG205 affected oocyte meiosis of both COC and DO in a dose dependent manner by: 1) decreasing the % of oocytes that extruded the PBI (p<0.05); 2) affecting nuclear meiotic progression by increasing the % of MI stage arrested oocyte, decreasing the % of oocytes that reached MII stage and increasing the % of oocytes showing aberrant meiotic figures (p<0.05) and 3) increasing the % of oocytes with clumps of scattered DNA within the cytoplasm (p<0.05). Importantly these effects were more severe in DO, indicating that AG205 directly acts on oocytes and cumulus cells do not mediate its negative effect. In order to finally confirm PGRMC1 function during bovine oocyte meiosis, a second set of experiment were conducted, in which COC were microinjected to deliver PGRMC1 or CTRL-RNAi into the oocytes cytoplasm, kept in meiotic arrest for 18h with 10µM cilostamide and then in vitro-matured for 24h. After IVM, efficacy in depleting PGRMC1 expression was assessed by quantitative RT-PCR and western blotting. As above described, the oocyte capability to undergo meiotic maturation and to extrude the PBI were considered as biological end points. Data were analyzed by t-test. PGRMC1 expression following PGRMC1-RNAi treatment was significantly reduced by 30% (p<0.05). This was accompanied by a 22% reduction of the oocytes that extruded the PBI (p<0.05). PGRMC1-RNAi treatment induced a significant reduction of PGRMC1 mRNA and protein expression. Overall, PGRMC1 down-regulation mirrored AG205 effects, by 1) reducing the % of oocytes that extruded the PBI (p<0.05); 2) increasing the % of oocytes showing aberrant meiotic figures (p<0.05) and 3) increasing the % of oocytes with clumps of scattered DNA. Surprisingly, although we observed a decrease of the % of MII stage oocytes in PGRMC1 RNAi treated group, this effect was not significant. Therefore, we further assessed the morphology of the MII plates. Data indicated that PGRMC1 down regulation induced a decrease of the % of MII plates with aligned chromosomes together with an increase of the % of oocytes with misaligned chromosomes. The present findings are consistent with PGRMC1 localization at the centromeres and at the midbody and with a putative role in both chromosomes separation and cytokinesis. We hypothesize that lower PGRMC1 expression impairs the process of chromosome separation and/or PBI formation. As a consequence, DNA that should be extruded with the PBI forms aberrant meiotic figures or is degraded. Funding: FP7-PEOPLE-2011-CIG, contract n.:303640-Pro-Ovum.

504. Primordial Follicle Formation and Oocyte Survival in Aromatase Deficient Mice.
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The correct assembly of primordial follicles is essential for reproductive success in mammalian females. Oocytes develop in connected clusters of cells called germ cell cysts during embryonic development. In late fetal and early neonatal development, cysts break down into individual oocytes that are surrounded by somatic pregranulosa cells to form primordial follicles. As cysts separate, a large number of oocytes are lost by apoptosis, however the mechanisms by which cyst breakdown and oocyte death occur are not well understood. Exogenous exposure to estrogen or estrogen mimicking chemicals delays cyst breakdown and follicle formation. Our current model is that estrogen produced in fetal ovaries keeps cells in cysts and that late in fetal development synthesis of estrogen stops, triggering cyst breakdown. Supporting this, we have previously shown that mRNA and protein for aromatase, the enzyme required to produce estradiol, is detected in fetal ovaries. In addition, we found that blocking aromatase activity in ovary organ culture had no effect on primordial follicle formation but resulted in a reduction in the number of oocytes. In the current study, oocyte number and primordial follicle formation were examined in aromatase knockout mice. Ovaries from wild type and homozygous mutant mice were collected at postnatal day 4 and labeled with the oocyte marker, Vasa, using whole mount immunocytochemistry (n=6 ovaries per genotype). Unlike
our organ culture studies, there was no difference in oocyte number in the aromatase mutant mice. However, primordial follicle formation and follicle development were reduced. Our results support the idea that alterations in the fetal hormonal environment affect the assembly and activation of primordial follicles. This research was supported by NSF IOS-1146940 to MP.

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3Growth differentiation factor8 (GDF8) is a member of the transforming growth factor-β that has been identified as a strong physiological regulator. The purpose of this study is to investigate the effects of GDF8 on porcine oocytes during in vitro maturation and ensuing in vitro fertilized (IVF) embryonic development. We first analyzed GDF8 concentration in each follicular size as follows small (smaller than 3mm), medium (larger than 3mm and smaller than 6mm) and large (larger than 6mm), and then examined the effect of GDF8 treatment during in vitro maturation (IVM) on nuclear maturation, intracellular glutathione (GSH), reactive oxygen species (ROS) levels, sperm penetration (SP) analysis, and IVF embryonic development. Each follicular fluid was aspirated from each follicular size and evaluated GDF8 concentration by ELISA. Data were analyzed by ANOVA followed by Duncan using SPSS (Statistical Package for Social Science) mean ± SEM. ELISA result showed concentration of GDF8 in each graded follicular fluid following small (0.479 ng/ml), medium (0.668 ng/ml), and large (1.318 ng/ml). Each concentration (0, 1, 10, and 100 ng/ml) of GDF8 was added in maturation medium during process of IVM. After 44 h of IVM, no significant difference was observed on nuclear maturation from the different concentrations (0, 1, 10, and 100 ng/ml) of GDF8 treatment groups (85.5%, 85.9%, 89.4%, and 87.6%, respectively) compared with the control (P>0.05). The 10- and 100 ng/ml GDF8 treated group showed a significant (P<0.05) decrease in intracellular ROS levels compared with other groups. The IVF embryonic developmental competence was affected with GDF8 treatment during IVM. The 10 ng/ml treatment group showed a significantly (p<0.05) higher blastocyst formation rates and total cell number compared with control (21.5% and 131.3 vs 15.0% and 92.6, respectively). Also in sperm penetration assessment, the 10- and 100ng/ml treatment groups showed higher mono sperm ratio and fertilization efficiency (32.7% and 27.1, 32.0% and 26.5 vs 22.6% and 19.7, respectively) than control in significant p < 0.05. In conclusion, the treatment of 10 ng/ml GDF8 during IVM improved the IVF porcine embryo developmental competence by decreasing the intracellular ROS levels. Acknowledgment: This work was supported, in part, by a grant from the Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ011288), Rural Development Administration, and the National Research Foundation of Korea Grant funded by the Korean Government (NRF-2013R1A2A04008751), Republic of Korea.

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We used serial section electron microscopy to examine mouse ovarian antral follicles. The granulosa cells around the follicle periphery and the cumulus cells surrounding the oocyte are in constant communication with each other and with the oocyte. We found long cytoplasmic projections from granulosa and cumulus cells that are likely to have an important role in cell-cell interactions. Similar filipodia-like projections called cytonemes have been particularly studied in Drosophila and there is evidence that suggests that they are involved in protein signaling between cells. In our preliminary observations, there are ~8-12 projections per granulosa / cumulus cell. They have a diameter of ~150 nm, are about one cell diameter in length (5-10 μm), and appear to have no preferred directional orientation within the follicle. They sometimes touch or make invaginations into neighboring cells without any apparent specialization at the end. The serial section electron microscopy was done using a new method developed by Lichtman and collaborators. Sections are cut on a conventional ultramicrotome, collected on tape using an "ATUM" instrument, and examined by field emission scanning electron microscopy. The sections are collected more reliably than with conventional methods. The zona pellucida is a thick acellular region surrounding the oocyte. Long filamcntous projections, called trans-zonal projections, originate from cumulus cells and cross the zona pellucida. At the oocyte, the ends of these processes form a specialized structure which are the sites of gap junctions between cumulus cells and the oocyte. We suggest that the projections we found have cell to cell functions between cumulus / granulosa cells, but if they are in close proximity to the oocyte, they can also traverse the zona pellucida and become induced to form a trans-zonal process when they contact the oocyte.

507. The role of RNA-binding protein Rbms2 in oocyte and embryonic patterning. Odelya Hartung1, Sophie Rothaemel1, Lianna Schwartz-Orbach1, Florence L. Marlowl1.
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Before fertilization, the egg is poised to provide the developing zygote with the materials required to guide its development into a complex multicellular organism. During oogenesis, numerous RNAs and proteins are produced and stored in ribonucleoprotein complexes (RNPs) that are chiefly responsible for translational control of early embryonic development while the zygotic genome is transcriptionally silent. In invertebrates and some vertebrate species like fish and frogs, the asymmetric localization of gene products within RNPs of the oocyte cytoplasm determines future embryonic axes and tissue specification. Many of these patterning molecules are localized in an asymmetric structure transiently present in stage I oocytes, the Balbiani body. The Balbiani body has been observed in oocytes of every species, including humans, and in fish and frogs has been implicated in localization of components required for axis formation. Despite the widespread conservation of the Balbiani body throughout the animal kingdom, little is known about its function or the factors that regulate its assembly. A screen for zebrafish genes required for proper egg formation identified the only vertebrate gene essential to Balbiani body formation, bucky ball (buc). Oocytes mutant for buc lack Balbiani bodies and their eggs and embryos lack the animal-vegetal axis. In humans and other mammals, mutations disrupting maternal-effect genes such as buc would likely lead to severe early developmental abnormalities resulting in failed implantation or miscarriage. Thus, the contribution of such genes to oogenesis and early development may escape detection in mammalian models. We use the zebrafish, a vertebrate in which external fertilization and embryonic development make every egg or zygote accessible to examination for developmental defects. The buc RNA, like other RNAs that regulate patterning of the
germline and embryonic axes, is localized to the Balbiani body along with Buc protein and the conserved RNA-binding protein Rbpms2 (RNA binding protein with multiple splicing 2). We have recently demonstrated that Buc protein directly interacts with Rbpms2 protein, and that Rbpms2 interacts with buc RNA. These interactions indicate a possible mechanism whereby Buc protein, whose asymmetric localization at zygote precedes Balbiani body formation can then recruit its own RNA to the Balbiani body by interacting with RNA-binding proteins such as Rbpms2, promoting a feedback amplification loop of brc RNA translation and localized translation. We have used CRISPR-Cas9 mutagenesis to disrupt zebrafish rbpms2, and have recovered germline mutant alleles to investigate rbpms2 functions in oocyte patterning and germline development. In addition, using a transgenic approach, we have identified the RNA interaction domain of Rbpms2 as essential to its localization to the Balbiani body, and are gaining further insights into the mechanisms of Rbpms2 localization in oocytes. These studies are expected to elucidate whether zebrafish rbpms2 is necessary and/or sufficient for Balbiani body formation, oocyte patterning and embryogenesis.

508. Interactions of YWHA (14-3-3) protein isoforms with CDC25B phosphatase in regulating mouse oocyte maturation.
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Immature mammalian oocytes are held in prophase I arrest by an inhibitory phosphorylation of the cyclin-dependent kinase 1 (CDK1) that, with the regulatory cyclin B1 (CCNB1), makes up the maturation promoting factor (MPF). Dephosphorylation of CDK1, and thus, resumption of meiosis resulting in germinal vesicle breakdown (GVBD), is performed by cell cycle division 25B (CDC25B). Evidence suggests that YWHA (14-3-3) proteins sequester phosphorylated CDC25B in the cytoplasm of immature oocytes, consequently preventing it from activating the MPF. There are seven mammalian isoforms of YWHA encoded by separate genes. To understand how release from meiotic arrest may be regulated by YWHA proteins, it is necessary to examine the expression of all YWHA protein isoforms and the interactions of each isoform with CDC25B. Using isoform-specific antibodies, we previously found that all seven mammalian isoforms of YWHA are expressed in immature oocytes and mature eggs. Here, we present results to confirm this observation and examine which of the seven isoforms may be associated with meiotic arrest. PCR results confirmed by sequence analysis show the expression of all seven YWHA isoform mRNAs in immature mouse oocytes and mature eggs. Each YWHA isoform was found to interact with CDC25B by co-immunoprecipitation experiments. To determine if the YWHA proteins are important in maintaining meiotic arrest, oocytes were microinjected with R18, a non-isoform-specific, YWHA-blocking peptide. Microinjection of R18 caused a significant increase in GVBD compared to the control oocytes. To determine which isoform(s) may be responsible for maintaining prophase I arrest, 0.1mM isoform-specific translation-blocking morpholino oligonucleotides were microinjected into the oocytes. The injected oocytes were held in prophase I arrest for 24 hours, and then incubated with media containing a threshold concentration of dbcAMP, which would normally maintain prophase I arrest. To determine if the YWHA proteins are important in maintaining meiotic arrest, oocytes were microinjected with R18, a non-isoform-specific, YWHA-blocking peptide. Microinjection of R18 caused a significant increase in GVBD compared to the control oocytes. To determine which isoform(s) may be responsible for maintaining prophase I arrest, 0.1mM isoform-specific translation-blocking morpholino oligonucleotides were microinjected into the oocytes. The injected oocytes were held in prophase I arrest for 24 hours, and then incubated with media containing a threshold concentration of dbcAMP, which would normally maintain meiotic arrest. A 70% increase in GVBD in the oocytes injected with the YWHAH (14-3-3η) morpholino was seen, despite the presence of dbcAMP, compared to control eggs including those injected with morpholinos against the other isoforms. The reduction of interactions with CDC25B or perhaps other target proteins by YWHAH resulted in the release of the oocyte from meiotic arrest. Although all YWHA isoforms were found to interact with CDC25B, these results suggest that YWHAH may be key in maintaining prophase I arrest.

509. Investigation of a role for germ cell cyst architecture in establishing differential L1 expression in fetal mouse oocytes.
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The differential expression of L1 retrotransposons among fetal oocytes determines the make-up and quality of the ovarian reserve in mice. Oocytes with increased L1 levels acquire greater amounts of DNA damage leading to their subsequent elimination in a process known as fetal oocyte attrition (FOA) (Malki et al., Dev Cell, 2014). Characterizing the molecular and cellular mechanisms driving differential L1 expression is critical for understanding oocyte specification and FOA. Interestingly, we observe that FOA takes place during a similar developmental time window as oocyte cyst breakdown, suggesting that cyst breakdown may be a product of oocyte death by FOA. Using the intercellular bridge marker, TEX14, to define connected oocytes within cysts, we have observed that expression of the L1 protein, L1ORF1p, is variable among connected cells in E15.5 fetal mice prior to FOA. Since intercellular bridges are thought to transport material to the specified mature oocyte within a cyst, an interesting idea arises that L1 transcripts or protein may be actively transported away from the mature oocyte during cyst development, contributing to the preferential elimination of recipient oocytes with higher levels of L1. To test this hypothesis, we are currently using a Tex14 mutant mouse model (generated by the Matzuk Lab) to examine differential L1 levels among oocytes when germ cell cyst structure is lost. By comparing levels of L1 among WT and Tex14 mutant and WT oocytes, we can determine whether the differential L1 expression observed in WT cysts is acquired cell autonomously or is a cell non-autonomous phenomenon influenced by germ cell cyst architecture.

510. The Role of GPR3 in the Acquisition of Oocyte Meiotic Competence.
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Mammalian oocytes acquire meiotic competence towards the end of growth, and when fully grown, mature spontaneously when removed from the follicle. In contrast, growing oocytes are unable to mature after removal from the follicle. The acquisition of meiotic competence from growing to fully grown oocytes has been shown to be modulated by cAMP, as dibutyryl cAMP (dcAMP) can promote meiotic competence in growing oocytes in a dose-dependent manner. The constitutively active G protein- coupled receptor 3 (GPR3) stimulates adenyl cyclase 3 (AC3) to produce cAMP within the oocyte. Previously, GPR3 has only been implicated in maintaining meiotic arrest in fully grown, follicle-enclosed oocytes; however, its role in growing oocytes is not known. Here we report that Gpr3 RNA is present in oocytes within very early stage follicles and throughout growth. In contrast to Gpr3−/− mice, both fully grown and growing oocytes from Gpr3−/− mice showed an impaired ability to resume meiosis when released from the follicle. Two hours after release from the follicle, only 57% of the fully grown oocytes from Gpr3−/− mice had resumed meiosis, compared to 95% of the Gpr3+/+ oocytes. This suggests that GPR3 promotes the acquisition of meiotic competence. The mechanism by which this occurs is unknown. The expression of
the cell cycle protein CDK1 increases during oocyte growth and has been linked to the acquisition of meiotic competence. However, CDK1 levels have only been measured using oocytes from mice of different ages. Here, we found that CDK1 expression increases from growing to fully grown oocytes within the same ovary. However, GPR3 does not regulate the amount of CDK1, as its expression is similar in fully grown oocytes from both Gpr3−/− and Gpr3+/+ mice. Also, incubating growing oocytes in the presence or absence of dcAMP does not stimulate expression of CDK1. These findings show that Gpr3 RNA is present during very early stages of oocyte development and that GPR3 plays a role in acquiring meiotic competence, although, not by regulating the expression of CDK1. This research was supported by the University of Connecticut Health Center Research Advisory Council.

511. DAZL and CPEB1 cooperate in the translation of maternal mRNAs during oocyte maturation.

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Oocyte maturation and early embryo development are dependent on translation control of maternal mRNAs. Transcripts synthesized during oocyte growth are stored in repressed complexes and recruited for translation at specific developmental stages. A genome-wide analysis of mRNAs regulated during oocyte maturation identified binding motifs for the cytoplasmic polyadenylation binding protein 1 (CPEB1) and Deleted in azoospermia-like (DAZL). CPEB1 is an RNA binding protein (RBP) component of repressive complexes blocking maternal mRNA translation in immature frog oocytes. Upon meiotic re-entry, CPEB1 becomes phosphorylated releasing repressing proteins and promoting polyadenylation and concomitant translation initiation. DAZL is a major regulator of gametogenesis, and deletion of this RBP results in loss of gametes in both sexes. Several functions have been proposed for DAZL but to date the only mechanistically validated role for this is as a translation activator. Using a mouse line expressing an epitope tagged ribosomal protein (Ribotag) under control of an oocyte specific cre recombinase, we were able to recover transcripts associated with ribosomes during meiosis. We use Ribotag immunoprecipitation together with mutagenesis and bioinformatics analysis to define the synergy between CPEB1 and DAZL, and their role as translation activators. We show that both proteins are required for oocyte maturation as the ability to transit to MII is impaired or completely prevented after depletion of these RBPs. We next used the mRNA coding for Tex19.1, a modulator of transponas, as a prototype of regulated transcripts since its association with polysomes increases more than 10 fold as oocyte progress through the cell cycle. Ribosome loading on the Tex19.1 was disrupted after depletion of either CPEB1 or DAZL; however the depletion of DAZL had a more profound effect on ribosome loading. Conversely, ribosome loading on CenB1 is affected only by CPEB1 depletion, demonstrating that the function of these RBPs is transcript specific. The role of the Tex19.1 3'UTR in translation was then investigated by injecting oocytes with reporter constructs where the luciferase coding region is fused to the 3' UTR of CPEB1 and DAZL binding sites in the 3'UTR of Tex19.1 abolished binding of the RBP and greatly decreased the rate of translation initiation. We showed that multiple DAZL binding sites are required to activate translation. Synergism between DAZL and CPEB1 requires the presence of a non-consensus CPE and, when this is a mutated to a consensus sequence, DAZL effects on translation are greatly decreased. Bioinformatic analysis of polysome associated transcripts shows that transcripts that become associated with the polysomes are enriched in both non-consensus and multiple DAZL sites. These data provide evidence that CPEB1 and DAZL function synergistically to promote a well-coordinated program of translational regulation necessary for the progression through the meiotic cell cycle. (This project is supported by NIH RO1-GM097165 and 1U54HD055764-07 grants.)

512. In vitro Differentiation of Porcine Induced Pluripotent Stem Cells into Primordial Germ Cells through Embryoid Body Formation, BMP 4 and Retinoic acid.

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It has been reported that induced pluripotent stem (iPS) cells are able to differentiate into primordial germ cells (PGCs) using a combined in vitro induction system such as embryoid body (EB) formation and bone morphogenetic protein 4 (BMP-4)-retinoic acid (RA) (Peng Li et al., 2013). Therefore, we investigated the capacity of porcine induced pluripotent stem (iPS) cells to differentiate into PGCs through a combination of EB formation, BMP 4 and RA in vitro. iPS cells were prepared from the latrival transduction of six reprogramming factors (hOct4, hNanog, h Sox2, h c-Myc, hKLF4 and hLin28) and mouse embryonic feeder cells (MEFs) pre-treated with mitomycin C (10 µl/ml) in DMEM/F12 contained 20% FBS, 1% MEM-nonessential amino acids, 1% penicillin/streptomycin, 2 mM glutamine, 0.1 mM β-mercaptoethanol and 1000 U/ml LIF. For in vitro differentiation to PGCs, iPSCs at passage 10 were harvested by trypsinization when using hanging drops at a density of 1.0 × 10³ cells/EB and hung to 20-30 µl of drops in differentiation medium for 2 days. iPSCs cells were aggregated to form EBs for 4 days and the aggregated EBs were plated on gelatin coated six-well plate (10 cm²) in differentiation medium supplemented with 50 ng/ml BMP 4 and 10 µM RA for 14 days. The BMP 4 and RA treated EBs were analyzed by the expression pattern of specific germ cell marker (DAZL and VASA) and meiotic marker (SCYP3) using reverse transcription polymerase chain reaction (RT-PCR). As a result, BMP 4 and RA treated EBs were expressed specific germ cell marker (DAZL) and meiotic marker (SCYP3) but not expressed VASA. This result demonstrated that iPSC cells are able to differentiate toward PGCs in response to BMP-4 and
513. Dissecting the structural basis of MEIG1 interaction with PACRG and the regulation of spermiogenesis.
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The product of the meiosis-expressed gene 1 (MEIG1) is a key regulator of spermiogenesis. MEIG1 is expressed in the cells bodies of spermatocytes. It is recruited to the manchette, a unique structure only present in the elongating spermatids which is believed to be essential for sperm flagella assembly, by PACRG, another regulator of spermiogenesis. Mouse MEIG1, an 88 amino acid polypeptide, has no conserved functional domains. To define the structural basis of MEIG1’s action in the regulation of spermiogenesis, its structure was resolved by NMR. These studies revealed that MEIG1 adopts a unique fold that provides a large contact surface for binding to other proteins. The shape of MEIG resembles a dumbbell, such that associated proteins can bind either of two opposing concave surfaces or the two convex ends of the dumbbell. One of the convex surfaces displays a hydrophobic patch comprised of tyrosine and phenylalanine residues that are often involved in protein-protein interactions. Based on the NMR structure, 12 solvent exposed, hydrophobic or charged residues were identified as potential mediators of protein-protein interactions. These 12 amino acids were mutated, and binding strength between PACRG with wild-type and these mutated MEIG1 proteins were tested by a direct yeast two hybrid experiment. It was found that binding strength was reduced by W50A, K57E, and F66A mutations, and nearly eliminated by a Y68A mutation. These four amino acids are on the convex surface of the globular region that displays the hydrophobic patch. Furthermore, we have found that PACRG is not stable when overexpressed in bacteria in isolation, but can be stabilized by co-expression of wild-type MEIG1. The four mutations we identified not only reduce binding strength but also reduce the ability of MEIG1 to stabilize PACRG in bacteria. In addition, the W50A/Y68A double mutation reduces binding strength and the ability to stabilize PACRG to a greater extent than either single mutation alone. Together, these studies establish the structure of MEIG1 and the amino acids that mediate interactions with PACRG, and open the possibility of exploring the structural mechanism for MEIG1 interaction with other proteins.

514. The stability of TEX101/Ly6k complexes is maintained by both factors during biosynthesis in testicular germ cells.
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Mammalian spermatozoa are formed in the seminiferous tubules through a complex morphological/physiological alteration that includes meiosis and transformation. These processes do not occur in any other somatic cell type, therefore, it is generally believed that unique molecular mechanisms regulate these processes. In fact, many molecules are specifically expressed during the germ-cell formation. However, the precise molecular mechanisms underlying remain unclear as to the germ-cell formation including functional maturations. We previously identified TEX101, a unique germ-cell-specific glycosyl-phosphatidylinositol (GPI)-anchored glycoprotein, belongs to the Ly-6/urokinase type plasminogen activator receptor-like protein (LU)-superfamily. Recent studies have demonstrated that TEX101 is essential for the production of fertile spermatozoa. In addition, this molecule is associated with Ly6k, a GPI-anchored protein that also belongs to the LU-superfamily. Although TEX101+ mice can produce morphologically intact spermatozoon, they are unable to migrate into the oviduct, resulting in an infertile phenotype. Since Ly6k specifically interacts with TEX101, Ly6k may also have an important role in the fertile spermatozoa production. Indeed, recent study showed that the Ly6k deficient mice represent similar male infertile phenotype in vivo due to disability of sperm migration into the oviduct. To characterize the role of Ly6k in the process of spermatogenesis more precisely, the present study is focused on the fate of Ly6k as a testicular protein in the presence or absence of TEX101. Data obtained from the present study showed that: 1) Although Ly6k mRNA was detected, the protein was present at very low levels in the mature testes of TEX101−/− mice by immunoblot and immunofluorescent analyses; 2) A qRT-PCR study indicated that the Ly6k mRNA level was within normal range in TEX101−/− mice, 3) Ly6k mRNA was translated into a polypeptide in the testes of TEX101−/− as well as TEX101+/+ mice, analyzed by polysome fractionation assay; and 4) Both TEX101 and Ly6k are mutual co-factors that contribute to the molecular stability of TEX101/Ly6k complex, as confirmed by gene knockdown experiments in a TEX101/Ly6k stable transfectant cell line. These results indicate that both TEX101 and Ly6k contribute to the post-translational stability of the TEX101/Ly6k protein complex at the cell membrane. The mechanism may be important in maintaining the production of fertile spermatozoa during spermatogenesis. (Supported in part by Grants-in Aid for General Scientific Research, Nos. 21592111, 23390389, 24592609, 25670702, 25462575, and "High-Tech Research Center" Project for Private Universities: matching fund subsidy from the Minister of Education, Culture, Sports, Science, and Technology, Japan)

515. Regulation of TSPO expression during germ cell differentiation.
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2Translocator protein 18kDa (TSPO) is a high affinity cholesterol- and drug-binding protein highly expressed in steroidogenic cells, such as Leydig cells, where it plays a role in cholesterol mitochondrial transport (among other functions). We have previously shown that TSPO is expressed in postnatal day (PND) 3 rat gonocytes, precursor cells to spermatogonial stem cells. Gonocytes undergo regulated phases of proliferation and migration, followed by retinoic acid (RA)-induced differentiation. Understanding these processes is important
since their disruption may lead to the formation of carcinoma in situ, precursor of testicular germ cell tumors (TGCTs). Previously, we showed that TSPO is expressed in gonocytes and in some adult germ cells, and it was not involved in gonocyte proliferation. In the present study, we found that TSPO expression is downregulated between PND3 gonocytes and PND8 spermatogonia, and in gonocytes undergoing RA-induced differentiation. Similarly, in F9 embryonal carcinoma cells, a TGCT cell line with embryonic stem cell properties, there was a significant decrease in TSPO expression during RA-induced differentiation. Furthermore, in normal human testes, one could locate TSPO not only in Leydig cells, but also in discreet phases of germ cell development such as the forming acrosome of round spermatids. By contrast, seminomas, the most common type of TGCT, presented strong expression of TSPO mRNA and protein. Thus, TSPO appears to be tightly regulated during germ cell differentiation, and its high levels in seminomas suggest that its dysregulation might have deleterious results in germ cells. These data suggest that TSPO has an important role in germ cell development.

516. Characterization of Two-type Colonies Derived from Epididymis of Tet on/off Piglet.
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Transgenic inducible system is required to minimize the miscarriage and mortality due to early expression of foreign genes in the production of transgenic animals. We already reported the production of transgenic pigs with tet on/off-eGFP systems in previous studies. In this study, we confirmed the expression level of the transgene in the germ cells and tried to establish spermatogonial stem cells obtained from epididymis of tet on/off-eGFP transgenic pig. The cells were obtained from the epididymis and then were cultured in a medium supplemented with FBS 10% in DMEM. 17 days after incubation, we could observe that the expression of GFP appeared wider range and round-type cells without the addition of antibiotics in the culture medium. These cells began to form dome-shape colonies. Flat-type of colonies formed in 23 days after incubation and the expression of GFP appeared their boundaries. 30 days after incubation, Flat-shape colonies were subcultured on Mitomycin C (MMC)-treated mouse embryonic fibroblast (MEF) and formed distinct borders. 24 days after subculture, flat-shape colonies were divided into several parts and their boundaries became blurred. Checking the result of GFP expression, cells in the boundary of flat-shape colony showed distinct expression even without treatment of doxycycline. The alkaline phosphatase staining was conducted to confirm the pluripotency of these cells, followed by a strong positive reaction appeared in the dorm-shape colonies. Interestingly, flat-shape colony also showed positive reaction but it was less than that of round colony, and there was no expression in the surrounding cells. Next, reverse transcript PCR was performed for characterization. As a result, pluripotent markers of Oct4 and Nanog were expressed in the dome-shaped colonies. However Nanog was not expressed in the flat-shaped colonies. Flat-shape colony was subcultured on MEF feeder that was expressed GATA4 (sertoli cell marker) and c-KIT (differentiating spermatogonial cells marker) gradually. PGP9.5 and integrin β1 (spermatogonial stem cells markers) were expressed in all four types of colonies. In conclusion, flat-type cells were differentiated from the round-type cells, which is thought to be a germ cell differentiation in the case of subcultured in MEF gradually. This is because it lacks the major factors in the culture medium probably necessary to keep the pluripotency. More than 100 days remaining cell culture have remained only as a result of cells and cell types that form a similar flat colony, GFP was expressed as a whole. Further studies, immunocytochemistry is needed for characterization of these cells from epididymis.

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Fluoride is abundant known as an environmental pollutant. The major sources of exposure to fluoride are drinking water, dental products, food, and pesticides. Fluorosis, a disease caused by deposition of fluoride in the body, affects skeletal tissue and teeth, but also soft tissues, such as liver, kidney, brain, pancreas and testes. Chronic fluoride exposure in drinking water has been associated with decrease in fertility. Diabetes mellitus is a well-recognized cause of male sexual dysfunction and impairments of male fertility. This alteration includes endocrine disorders, neuropathy, and deleterious effects on male reproductive function. The aim of the study was to investigate the influence of fluoride in streptozotocin-induced diabetic mice on the spermatozoa quality, mitochondrial transmembrane potential and caspase-3/7 activity. Male CD1® mice were divided into four groups of six animals each: control group, diabetic group, diabetic group exposed with fluoride and fluoride group. Animals were treated with a single injection of streptozotocin (150 mg/kg; ip). Blood glucose was estimated after one week and the animals with glucose levels ≥ 250 mg/dl were included in this study. Animals were kept with diabetes and exposed to fluoride (NaF at a dose of 100 ppm in their drinking water), during two months before the experiments. We evaluated several spermatozoa parameters divided in the four groups: i) standard quality analysis according to World Health Organization, ii) mitochondrial transmembrane potential, iii) caspase-3/7 activity and iv) urinary fluoride levels. Spermatozoa from diabetic mice fluoride-treated exhibited a significant decrease in motility (p<0.05), concentration (p<0.05), viability (p<0.01), a significant decrease in mitochondrial transmembrane potential (p<0.05), and a significant increase on caspase-3/7 activity (p<0.01), relative to spermatozoa from diabetic animals. Finally, diabetic mice fluoride-treated retained significantly greater amounts of fluoride (p<0.001), relative to fluoride group. These observations suggest that fluoride exposure in subchronic diabetes produces an important increase in alterations caused by diabetes on sperm quality, caspase-3/7 activity and loss of mitochondrial transmembrane potential.

518. Discovery of high-sensitive biomarkers for male fertility prognosis and diagnosis.
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Although, conventional semen analyses are commonly used to evaluate the male factor of fertility/infertility, their clinical value remains in argue. Therefore, new study for more accurate assessment of male fertility based on sperm function and mechanism of fertilization is on the rise. Currently, proteomic study is widely used and acceptable technique in order to investigate the protein containing in spermatozoa. To increase the accuracy of predictive biomarkers for male fertility, a comprehensive proteomic approach was applied in boar spermatozoa after capacitation. The results demonstrated that cytochrome b-c1 complex subunit 2 (UQRC2) was abundantly expressed in the high -litter size spermatozoa (>3-fold). On the other hand, equatorin, beta-tubulin, cytochrome b-c1 complex subunit 1 (UQRC1), sperolins, ras-related protein Rab-2A (RAB2A), speradhesin AQN-3, and seminal plasma sperm motility inhibitor were abundantly expressed in the low-litter size spermatozoa (>3-fold). Moreover, RAB2A and UQRC1 were negatively correlated with litter size, while UQRC2 was positively correlated with litter size. Finally, it has been proved that the litter size is significantly increased in AI trial using these novel predicted biomarkers. To the best of our knowledge, this is the first work that identify biomarkers for prognosis and diagnosis of male fertility with high sensitivity in animals and humans. This work was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ10160101), Rural Development Administration, Republic of Korea.

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The toxicology effects of the synthetic xenoestrogen bisphenol-A (BPA) have been studied extensively, and most of these reports are part of the open scientific literature. Considering mice spermatozoa as a potential in vitro model, we investigated the effects of BPA exposure (0.0001, 0.01, 1, and 100 µM for 6 h) on spermatozoa and the related mechanisms of action. The same doses were employed to identify the proteomic biomarkers of exposure by using two-dimensional gel electrophoresis and mass spectrometry analysis. Our results demonstrated that high concentrations of BPA negatively affect sperm motility, viability, intracellular ATP, and mitochondrial functions by activating the mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and protein kinase-A pathways. Moreover, a high concentration of BPA (100 µM) induced differential expression (>2-fold) of 24 proteins (16 down- and 9 up-regulated) that are putatively involved in the pathogenesis of several diseases, mainly cancer, carcinoma, neoplasm, and infertility, in spermatozoa. This study is the first to demonstrate the mechanisms of BPA by using potentially effective dose levels and to identify the biomarkers of BPA exposure in spermatozoa. Additionally, the protein biomarkers identified in the current study may provide excellent therapeutic targets to overcome BPA toxicity. This research was supported by a 2014 grant (14162MFDS661) from the Ministry of Food and Drug Safety of the Republic of Korea.

520. Immunohistochemical characterization of purinoceptor expression during spermatogenesis. Nadine Mundt1, David Fleck1, Patricia Almeida Machado2, Sophie Veitinger2, Corinna Engelhardt1, Marc Spehr1.
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Spermatogenesis is one of the most intricate processes of sequential cell proliferation and differentiation in postnatal life. Initially, spermatogonial stem cells divide mitotically into different types of spermatogonia, which finally give rise to haploid spermatozoa arising from successive mitotic, meiotic and postmeiotic divisions within the seminiferous epithelium. Thus, a fundamental regulatory aspect of spermatogenesis is cell-to-cell communication both between developing germ cells as well as germ and Sertoli cells. As previously shown, a candidate mediator of such communication is ATP. In both previous and on-going studies, electrophysiological characterization revealed the functional expression of P2X2 receptors in Sertoli cells as well as P2X4 and P2X7 receptors in spermatogonia. Here, we use molecular and immunohistochemical methods to obtain a more detailed profile of purinoceptor expression in murine seminiferous tubules at different stages of development. RT-PCR, western blotting and immunohistochemistry confirmed the presence of P2X2, P2X4, and P2X7 as well as BKca channels in murine testes. Focusing on the cell type-specific distribution of purinergic receptors, we performed co-localization studies with cell-specific markers. Together, our studies provide new insight into the complexity of purinergic cell-cell communication during spermatogenesis.

521. CD46 and β1 integrin relocation in the sperm head during capacitation and acrosome reaction.
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CD46 protein plays an important role during fertilization and its role is associated with acrosome stability. CD46 is probably involved in signaling pathways triggering the acrosome reaction (AR). It also associates, through membrane integrins, with specific MAP kinases involved in AR. The interaction of these two proteins in sperm can be predicted, however it still has not been shown. Our aim was to monitor the possible dynamics of relocation and movement of CD46 and β1 integrin during sperm maturation and its preparation for the fertilization. Our results show changes in the localization of these proteins associated with the AR and their mutual co-localization was observed. The original β1 integrin location in the freshly released epididymal sperm is in the acrosome and it relocates during the AR further through the sperm head compartments into the equatorial segment and over the whole sperm head. Its density over the equatorial segment is decreasing with the extended time of the capacitation. Also its presence in the perforatorium of the mouse sperm head is very prominent. The pattern for protein CD46 is extremely similar if not identical in both aspects such as compartment localization and time progress during capacitation and AR in vitro. Similarly to CD46, β1 integrin was observed progressing across the apical acrosome and the equatorial segment. However, its relocation carries on further to the postacrosomal region of the sperm head. The molecular interaction of CD46 and β1 integrin is being investigated using the Proximity Ligation Assay. The data were statistically analysed. The immunofluorescent staining pattern of CD46 and β1 integrin relocation in the sperm populations was monitored in 200 sperm in eight individual experiments. Statistical analyses show that the majority (80–95%) of freshly released and capacitated epididymal sperm...
We speculate that this relocation is of importance for the successful sperm-egg interaction, adhesion and subsequent gamete fusion. Information that proteins CD46 and β1 integrin were localized in the acrosome membrane in the majority of freshly released epididymal sperm (~95%). The similar results were detected in capacitated sperm (~83%). During the AR β1 integrin was detected in the whole sperm head in the majority (~89%) of sperm population. This process of proteins relocation was not detected during sperm capacitation. It was detected only in minority sperm population, which probably represents those after the spontaneous AR. These sperm showed acrosome vesicle disruption. In summary, our results deliver new information that proteins CD46 and β1 integrin undergo dynamic relocation towards the sites of sperm-egg fusion during the AR in vitro. We speculate that this relocation is of importance for the successful sperm-egg interaction, adhesion and subsequent gamete fusion. Research was supported by the Grant Agency of the Czech Republic GACR No. 14-05547S.

522. **PLZF overexpression is a novel molecular signature implicated in age-related spermatogonial dysfunction.**

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Spermatogonia maintain life-long sperm production but their activities decline with age. To study the underlying mechanism, we focused on promyelocytic leukemia zinc finger (PLZF) protein, which is specifically expressed in spermatogonia. PLZF deletion leads to spermatogonial depletion and results in a testicular “aging-like” phenotype with increased numbers of degenerative tubules. Therefore, we evaluated whether there is an age-associated loss of PLZF expression by comparing young (3 month old) and aged (24 month old) mouse testes. To our surprise, Plzf mRNA was significantly elevated in aged testes. Immunohistochemistry staining for PLZF revealed that, while there were differential levels of PLZF expression in young testes, there were more spermatogonia showing high levels of PLZF expression in aged testes and many of these cells were located in the degenerated tubules where spermatogenesis was already ceased. Moreover, we used flow cytometry (FACS)-based intracellular staining to quantify the frequency of PLZF-expressing (PLZF+) cells. We found that aged testes showed a 2.1-fold increase in the frequency of PLZF+ cells when compared with that in young testes, together with a higher intensity of PLZF expression. Next, to compare the cellular expression pattern of PLZF between young and aged testes, we isolated the undifferentiated (α6-integrin-high/c-Kit-negative) and differentiating (α6-integrin-low/c-Kit-positive) spermatogonia by FACS and conducted immunocytochemistry staining for PLZF. Consistently, we found an elevated level of PLZF expression in the undifferentiated spermatogonia from aged mice compared with those isolated from young mice. Additionally, in contrast to the differentiating spermatogonia from young mice that only showed weak or no PLZF staining, some differentiating spermatogonia from aged testes were clearly positive for PLZF. To examine the consequence of PLZF overexpression in aged testes, we noted that PLZF maintains spermatogonial self-renewal by suppressing mTORC1 activity via transcriptionally activating Redd1. To determine whether increase in PLZF could potentially affect this pathway, we found that Redd1 mRNA was significant elevated in aged testes. Next, we measured the phosphorylation of ribosomal protein S6 (p-RPS6), a downstream target of mTORC1. Dual-immunofluorescence staining for p-RPS6 and PLZF revealed that, while in young testes PLZF expression cells were surrounded by p-RPS6 cells (probably progeny of PLZF+ cells that start to express p-RPS6), there was a lack of p-RPS6 expression associated with strong PLZF expression in aged mice, particularly in degenerative tubules. Furthermore, we used FACS-based intracellular staining to measure p-RPS6 expression, in which aged testes showed a 50.1% decrease in p-RPS6-positive cells. Lastly, aged testes contained a comparable frequency of undifferentiated spermatogonia but a significantly decreased frequency of differentiating spermatogonia when compared with that of young testes (3.80% vs. 5.46%, P < 0.05), suggesting that an increase in PLZF expression disrupts spermatogonial differentiation. In fact, ectopic PLZF expression arrests retinoic acid-induced meiotic initiation reflected by Stra8 expression in F9 cells in vitro. Taken together, these data indicate that PLZF overexpression is a novel molecular signature underlying age-related loss of spermatogonial function. (Supported by NIH R00-AG039512 and MGH Vincent Department of OB/GYN)

523. **Isolation, identification, distinct global microRNA profiling and novel targets of human spermatogonia, pachytene spermatocytes and round spermatids.**

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Spermatogenesis comprises three main stages, namely mitosis of spermatogonia, meiosis of spermatocytes, and spermiogenesis of spermatids that transmit genetic information to next generations. Although much progress has been achieved on mechanisms underlying spermatogenesis in rodents, very little is known about epigenetic and genetic regulation of the mitosis, meiosis, and spermiogenesis in humans. Human spermatogenic cells have not yet been isolated, and notably, their global miRNA profiles remain unknown. Since cell types and biochemical phenotype of human male germ cells are distinct from rodents, it is of unusual significance to separate human spermatogenic cells and uncover miRNAs controlling different stages of human spermatogenesis. Here we have effectively isolated human spermatogonia, pachytene spermatocytes and round spermatids using STA-PUT velocity sedimentation. RT-PCR, immunocytochemistry and meiosis spread assays revealed that the purities of isolated human spermatogonia, pachytene spermatocytes, and round spermatids were 90%, and the viability of these isolated cells was over 98%. MiRNA microarrays showed distinct global miRNA profiles among human spermatogonia, pachytene spermatocytes, and round spermatids. Thirty-two miRNAs were significantly up-regulated whereas 78 miRNAs were down-regulated between human spermatogonia and pachytene spermatocytes, suggesting that these miRNAs are involved in the mitosis and meiosis, respectively. In total, 144 miRNAs were significantly up-regulated while 29 miRNAs were down-regulated between pachytene spermatocytes and round spermatids, reflecting potential roles of these miRNAs in mediating spermiogenesis. A number of novel binding targets of miRNAs were further identified using various software and verified by real-time PCR. Significantly, our ability to isolate human spermatogonia, pachytene spermatocytes and round spermatids and unveil their global distinct miRNA signatures and binding targets could provide novel small RNA regulatory mechanisms mediating three stages of human spermatogenesis and offers new targets for treatment of male infertility and for male contraception.

524. **Histone demethylases Fbx111 regulates balance between self-renew and differentiation of spermatogonia in mice.**
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Methylation and demethylation of histone residue are important modifications of epigenetics. Histone modifications fluctuate drastically during germ cell development or spermatogenesis. A number of gene knockout mice lacking gene(s) which catalyze these modifications show an infertile phenotype, suggesting that proper epigenetic modifications of histone residue are essential for germ cell development or spermatogenesis. Fbxl11 is a gene catalyzing demethylation of H3K4 or H3K36. Although these histone modifications has been reported to be important for normal germ cell development or sustainable sperm production, functional roles of Fbxl11 in male germ cell development or spermatogenesis are poorly understood until present. To determine the role of Fbxl11 in spermatogenesis, we have developed germ cell specific Fbxl11 knockout mouse (Fbxl11cKO) model by mating Nanos3-Cre mice in which Cre recombinase is expressed only in primordial germ cells from around embryonic day 7 (E7) with Fbxl11 floxed mice in order to escape embryonic lethal phenotype of conventional Fbxl11KO mice. Germ cell specific Fbxl11 mouse are totally infertile, and showed drastic abnormality in spermatogenesis, e.g., few sperm could be recovered from epididymis, or the average weight of testis is significantly reduced in the Fbxl11cKOs at 8 weeks old (33.9 ± 4.9mg in the Fbxl11cKO vs 76.5 ± 7.1mg in the control). Immunohistochemistry using anti-SCP3 antibody, a first meiotic spermatocyte marker, anti-cleaved Caspase3, an apoptotic marker, and anti-PLZF antibody, an undifferentiated spermatogonia marker, revealed that about 80 % of seminiferous tubules have abnormal spermatocyte layers (34 ± 3.2% tubules are without spermatocytes and 45.7 ± 5.8% tubules are with a few spermatocytes) and apoptosis occurred more frequently in the Fbxl11cKO testis than the control. Interestingly, of almost all the abnormal seminiferous tubules contained PLZF-positive undifferentiated spermatogonia in spite of the presence or absence of meiotic cells in the tubules, and the number of PLZF positive cells per tubule is rather greater in 3 weeks old Fbxl11cKO testis than the same-age control (average 13.8 ± 0.6 cells per tubule in the Fbxl11cKO vs 9.2 ± 1.4 in the control). Furthermore, flow-cytometrical analysis indicated that the number of EpCAM positive spermatogonia in Fbxl11cKO is comparable with the control at postnatal day 5 (P5), whereas development of c-KIT positive differentiating spermatogonia are strongly inhibited in the Fbxl11cKO at P12, suggesting that Fbxl11 might regulate phase-decision from self-renew to early differentiation in spermatogonia. In addition to the in vivo analysis, we have developed Fbxl11 KO germline cell (GSC) in vitro and compared transcripetime using Affymetrix GeneChip Mouse Transcriptome Array. By cutoff criteria of P<0.05 and fold differences greater than or less than 2 or 0.5, respectively, 475 genes are downregulated whereas 170 genes are upregulated in the Fbxl11 KO GSC compared with the control. Gene ontology (GO) analysis revealed that GO terms related with germ cell development or spermatogenesis such as ‘Male Gamete Generation’, ‘Spermatogenesis’, or ‘Meiosis’ are significantly accumulated in gene group downregulated in the Fbxl11 KO GSC. Further and detail analysis to discover gene(s) which directly regulate balance between spermatogonial self-renew and differentiation under control of Fbxl11 are undergoing.

525. Spermatogonial stem cell potential of CXCR4-positive cells from prepubertal bovine testis.

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Spermatogonial stem cells (SSC) are responsible for the continuous spermatogenesis throughout the male adult life. These cells have the potential to colonize and restore spermatogenesis when transplanted into testes depleted of germ cells. Due to this property, SSC could be used for breeding programs and also to generate transgenic sperm and consequently, transgenic animals. Particularly in bovine species, SSC are not as characterized as in the mouse or human. In the mouse, CXCR4 positive (CXCR4+) testicular cells have high spermatogonial stem cell potential. We hypothesized that CXCR4 is a marker of undifferentiated spermatogonia in bovine. We surgically removed the testicles of 8 prepubertal bull calves at 6 months of age. Samples were fixed in 4% paraformaldehyde for 24h and embedded in paraffin prior to mounting slides for immunohistochemistry. Expression of CXCR4 was detected by immunohistochemistry in few cells of the seminiferous tubules. Testicular cells were isolated after sequential enzymatic digestion with collagenase and trypsin and then cryopreserved. Cells were thawed and submitted to magnetic-activated cell sorting (MACS) using CXCR4 antibody. Flow cytometry analysis after MACS revealed that 68.51% of cells were positive for CXCR4, a 6.42-fold increase when compared to non-sorted cells (P<0.001, PROC GLM, Tukey post-hoc comparison, SAS 9.3). These results indicate that CXCR4 could be used as a marker to enrich bovine cells with spermatogonial stem cell potential. In the future, these cells could be used in breeding programs and for generation of transgenic livestock.

526. Chromatoid body assembly and nucleolar cycle: linking these two important events for spermatogenesis.

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Chromatoid body (CB) is a very intriguing cytoplasmic structure of male germ cells playing important roles in the spermatozoan formation, such as mRNA and protein regulation/storage for later germ cell. Recent proteomic analysis produced a list of more than 100 CB-associated proteins and confirmed that the most abundant proteins in the CB molecular composition are MVH, MIWI, DDX25, TDRD6, and TDRD7. Additionally, the CB proteome also includes many proteins with reported nuclear localization/function, whose close observation revealed that most of these proteins are shuttling and they can be translocated to the cytoplasm. Recently, the presence of the nucleolar protein fibrillarin was reported in the molecular composition of the CB. The relationship between the nucleolus and the CB has long been proposed. The translocation of fibrillarin from the nucleolus to the CB during the nuclear reorganization, which takes place during spermatogenesis, may play an important role in gamete and/or embryo development. It has been shown that nucleolar morphology during spermatogenesis, e.g., few sperm could be recovered from epididymis, or the average weight of testis is significantly reduced in the Fbxl11cKOs at 8 weeks old (33.9 ± 4.9mg in the Fbxl11cKO vs 76.5 ± 7.1mg in the control). Immunohistochemistry using anti-SCP3 antibody, a first meiotic spermatocyte marker, anti-cleaved Caspase3, an apoptotic marker, and anti-PLZF antibody, an undifferentiated spermatogonia marker, revealed that about 80 % of seminiferous tubules have abnormal spermatocyte layers (34 ± 3.2% tubules are without spermatocytes and 45.7 ± 5.8% tubules are with a few spermatocytes) and apoptosis occurred more frequently in the Fbxl11cKO testis than the control. Interestingly, of almost all the abnormal seminiferous tubules contained PLZF-positive undifferentiated spermatogonia in spite of the presence or absence of meiotic cells in the tubules, and the number of PLZF positive cells per tubule is rather greater in 3 weeks old Fbxl11cKO testis than the same-age control (average 13.8 ± 0.6 cells per tubule in the Fbxl11cKO vs 9.2 ± 1.4 in the control). Furthermore, flow-cytometrical analysis indicated that the number of EpCAM positive spermatogonia in Fbxl11cKO is comparable with the control at postnatal day 5 (P5), whereas development of c-KIT positive differentiating spermatogonia are strongly inhibited in the Fbxl11cKO at P12, suggesting that Fbxl11 might regulate phase-decision from self-renew to early differentiation in spermatogonia. In addition to the in vivo analysis, we have developed Fbxl11 KO germline cell (GSC) in vitro and compared transcripetime using Affymetrix GeneChip Mouse Transcriptome Array. By cutoff criteria of P<0.05 and fold differences greater than or less than 2 or 0.5, respectively, 475 genes are downregulated whereas 170 genes are upregulated in the Fbxl11 KO GSC compared with the control. Gene ontology (GO) analysis revealed that GO terms related with germ cell development or spermatogenesis such as ‘Male Gamete Generation’, ‘Spermatogenesis’, or ‘Meiosis’ are significantly accumulated in gene group downregulated in the Fbxl11 KO GSC. Further and detail analysis to discover gene(s) which directly regulate balance between spermatogonial self-renew and differentiation under control of Fbxl11 are undergoing.

Gamete Generation', Spermatogenesis', or 'Meiosis' are significantly accumulated in gene group downregulated in the Fbxl11 KO GSC. Further and detail analysis to discover gene(s) which directly regulate balance between spermatogonial self-renew and differentiation under control of Fbxl11 are undergoing.
reestablishment is an important factor in resetting the lifespan during gametogenesis because of its elimination of age-induced cell damage. Thus, the CB is presumed to act as a receptor of some nucleolar proteins that shuttle during this nucleolar morphology reestablishment process. The aim of the present study was: to follow the expression of the nucleolar protein fibrillarin in the seminiferous tubules at different stages of the spermatogenic cycle (I-III; IV-VI; VII-IX; X-XII); and to detect the presence of fibrillarin in the molecular structure of post meiotic CBs, discussing the possible roles that this protein may play for the CB physiology maintenance evidenced by which CB molecular markers fibrillarin is associated with (MIWI and/or HSP70). The analyses were performed by Western blot and immunofluorescence. Results showed a higher expression of fibrillarin at stage IV-VI and co-localization between fibrillarin and MIWI, a typical CB component which plays a role in the mRNA regulation and small-RNA mediated gene control (Pearson’s coefficient r = 0.559); it was also evident the co-localization between fibrillarin and HSP70, a chaperone important in the proteasome degradation in the CB (Pearson’s coefficient r = 0.613). Up to date, our data show higher fibrillarin expression in seminiferous tubules at stages IV-VI, which is composed of high number of cells containing active CBs; fibrillarin co-localizes with CB markers that play distinct functions for CB physiology maintenance, yet it is not possible to confirm its function in the CB at the present time. New experiments need to be performed to achieve this goal. This research is supported by São Paulo State Research Foundation (FAPESP). Grant numbers: 2012/22009-7; 2013/14102-0

527. The ID4+ Spermatogonial Stem Cell Population is Established During a Defined Period in Early Neonatal Life.
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Male fertility is dependent on the daily production of millions of genetically unique gametes, a process that relies on the activities of a regenerative subpopulation of undifferentiated spermatogonia, designated spermatogonial stem cells (SSCs). A foundational SSC pool develops from spermatogonial precursors during early neonatal life and impairment of this process results in severe fertility defects including complete elimination of the germline leading to Sertoli cell only syndrome. At present, the mechanisms underpinning formation of the SSC pool in mammalian testes is undefined. A major impediment in defining the kinetics of SSC pool formation has been lack of markers to identify the cells within the heterogeneous spermatogonial population in postnatal life. Recently, we demonstrated that the protein ID4 is a marker of SSCs and generated an Id4-Gfp transgenic mouse line to study the population in more detail. Here, we conducted whole mount immunostaining analysis using Id4-Gfp mice to establish the kinetics of the SSC pool formation from spermatogonia within the developmental timeframe of embryonic day 18 (E18) and postnatal day (PD) 6. Outcomes revealed that prior to birth, at E18, less than 1% of the spermatogonial population is ID4+ but increases in abundance by PD 0 and 3 comprising 18% and 28% of the undifferentiated spermatogonial population, respectively. By PD 6 when transition of all spermatogonia has occurred and the undifferentiated and differentiating spermatogonial populations are fully established, the ID4+ population is again a minor subset, making up 11% of the total population, whereas the ID4- progenitor population is the major subset. Collectively, these findings suggest that the ID4+/SSC pool begins to form within the spermatogonial population prior to birth and expands in number between PD 0-3, followed by transition to a rare subset of the undifferentiated population by PD 6 similar to the situation in adulthood. The rarity of ID4+ cells within the spermatogonial population at ED 18 suggests that a subset is preprogrammed in fetal life to serve as a seed population for generation of the SSC pool in neonatal development.

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528. Identification of microRNAs associated with sexual maturity in rainbow trout brain and testis through small RNA deep sequencing.
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Several recent studies in mammals have revealed the importance of microRNAs (miRNAs) in the regulation of spermatogenesis; in contrast, there is still scarce information about the role of miRNAs during spermatogenesis and sexual maturation in teleosts. In order to further the understanding of the miRNA structure in the reproductive axis of male teleosts, the present study was designed to characterize the miRNA expression in brain and testicular tissues from sexually immature juvenile and mature adult rainbow trout males and to identify their potential target genes.

Four small RNA libraries were constructed and sequenced from testis and brain dissected from pre-pubertal juvenile and post-spawned adults of rainbow trout (Oncorhynchus mykiss) using Illumina small RNA deep sequencing. The small RNA sequences identified were blasted against the Pisces miRBase database v.19, as well as with reported rainbow trout sequences. The differential expression analysis of miRNAs between mature and immature samples was performed using CLC Genomics Workbench software. The fold-change and P-value were calculated from the normalized expression data. When |log2Ratio| ≥ 1 and P-value ≤ 0.05, it was considered as differential expression. In addition, the 3’ UTR and coding regions of several genes involved in puberty, sex-determination, and hormone synthesis/regulation in the Salmonidae family were extracted from the NCBI database, and two software tools (PITA and RNA22v2.0) were used to predict target genes of the differentially expressed miRNAs. Finally we performed RT-qPCR analyses on several differentially expressed miRNAs to validate the deep sequencing results.

We obtained 56,632,987; 39,870,661; 82,454,370; and 53,143,465 high quality filtered reads for immature testis (IT), mature testis (MT), immature brain (IB), and mature brain (MB), respectively. Considering all libraries, 433 known mature piscine miRNAs were identified, with 124 and 116 significantly differentially expressed miRNAs found between sexually immature/mature testes and immature/mature brain tissues, respectively. Among the differentially expressed miRNAs in the testis libraries, let-7a was found to target the glucocorticoid receptor, cytochrome P450 19A (cyp19a), SRY-related high mobility group box 11 (sox11), and sox24 genes. Also, mir-210 was found to target the Androgen receptor beta (ar-beta), Kisspeptin receptor (kissr), and sox4 genes. As for the miRNAs differentially expressed in the brain libraries, mir-107b was found to target the activin receptor I (acvr1), adrenodoxin (adx), low density lipoprotein receptor (ldlr), and sox6 genes. In addition, mir-122 was found to target the activin receptor II b (acvr2b), androgen receptor alpha (ar-alpha), doublesex- and mab-3-related transcription factor 1 (dmb1), and steroidogenic acute regulatory protein (star) genes. Furthermore, mir-nov210 was...
Amy T. Desaulniers1, Rebecca A. Cederberg1, Ginger A. Mills1, Clay A. Lents2, Brett R. White1.

A putative role for GnRH-II and its receptor in spermatogenic function of boars.

For novel methods of puberty control in cultured fish. meiosis in rainbow trout. Ongoing experiments are being performed to confirm the details of this mechanism, and might provide the basis for novel methods of puberty control in cultured fish.

529. WITHDRAWN.

530. A putative role for GnRH-II and its receptor in spermatogenic function of boars.
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Unlike the classical gonadotropin-releasing hormone (GnRH-I), the second mammalian isoform of GnRH (GnRH-II) is ubiquitously expressed with the most abundant transcript levels found in tissues outside of the hypothalamus. Moreover, GnRH-II is only an inefficient stimulator of gonadotropin release. Interestingly, the pig represents the only livestock species to maintain the full-length coding sequence needed to produce a functional receptor specific for GnRH-II (GnRHR-II). Together, GnRH-II and its receptor appear to modulate the interaction between feed intake and reproductive behavior in female marmosets and musk shrews. Despite its ubiquitous expression, other functions of the GnRHR-II remain elusive. We recently demonstrated that GnRH-II levels were higher in the porcine testis than in the anterior pituitary gland or hypothalamus, corresponding to greater GnRHR-II abundance in the testis versus the anterior pituitary gland. Previous data in our laboratory also suggest that testicular GnRH-II is present on the plasma membrane of Leydig cells and has a role in localized steroidogenesis. Notably, we also detected GnRHR-II staining within the seminiferous tubules, suggesting that GnRHR-II may be involved in spermatogenesis and/or spermatogenic function. In the present study, our objective was to further localize GnRH-II and GnRHR-II in the reproductive tract of the boar. First, immunoblotting (n = 5 boars) revealed that GnRHR-II levels are 12-fold greater in the testis (P < 0.0001) compared to the epididymis, prostate, seminal vesicles or bulbourethral glands, suggesting an important role in testicular function. Next, we performed immunofluorescence on paraformaldehyde-fixed, paraffin-embedded boar testicular tissue (n = 6) using an antibody directed against GnRH-II and GnRHR-II. We identified GnRH-II immunostaining primarily within the tubular compartment, localizing to round germ cells. Moreover, we also observed intense GnRHR-II staining on elongating spermatids. In order to further explore the identification of the GnRHR-II on spermatids in fixed tissue, we collected Percoll-purified, ejaculated spermatozoa (n = 7) for use in immunoblotting and immunocytochemistry procedures. Indeed, a distinct band for GnRHR-II was detected by Western blot. Interestingly, the molecular weight of GnRHR-II differed in the testis (60 kDa) compared to purified sperm samples (54 kDa), indicating differential post-translational modification. We also performed immunocytochemistry on the purified spermatozoa samples and detected intense GnRHR-II signal on the connecting piece, suggesting a role in fertilization or acrosomal exocytosis. After discovering the GnRHR-II on mature spermatozoa, we next evaluated seminal plasma for the presence of GnR-II via ELISA. Indeed, GnR-II was detected in seminal plasma (n = 7; 122-332 pg/ml), which likely originates from the testis as GnR-II levels were highest in testicular tissue homogenates (26.9 ± 2.0 ng/g tissue; P < 0.0001) compared to the epididymis (14.5 ± 2.5 ng/g tissue), prostate (3.4 ± 2.0 ng/g tissue), seminal vesicles (3.0 ± 2.0 ng/g tissue), or bulbourethral glands (1.4 ± 2.2 ng/g tissue). Together, these data suggest that GnRH-II and its receptor may have a role in spermatogenesis and or spermatogenic function in boars. Partially supported by NIFA Hatch (NEB-26-199; BRW) and AFRI (2011-67015; CAL) funds. USDA is an equal opportunity provider and employer.

531. Relationships between disease and fertility: 4-hydroxynonenal interferes with AMP-kinase activation in stallion spermatozoa.
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Obesity, metabolic syndrome and other metabolic and inflammatory systemic conditions are increasingly shown to negatively impact on male fertility. These pathological states are often associated with elevated levels of 4-hydroxynonenal (4HNE), a reactive aldehyde that, in turn, is associated with poor sperm function through a number of mechanisms. AMP-activated-kinase (AMPK) is a major regulator of metabolism, recently identified in spermatozoa. This kinase is activated in response to cellular energy depletion and when phosphorylated, stimulates cellular processes that favour ATP production over ATP consumption. We have previously observed that AMPK can affect mitochondrial function and energy production in stallion spermatozoa, while direct adduction of 4HNE to AMPK in other cell types has been demonstrated elsewhere. This study investigated whether AMPK could provide another route via which 4HNE can negatively affect sperm function in the stallion.

Sperm samples were collected from three stallion stallions, transported in Kenney’s extender and processed on a Percoll gradient. Sperm were incubated in BWW media at 22°C or 37°C at a concentration of 2 x 10^5 sperm/mL, with or without 4-hydroxynonenal at various concentrations. Anti- 4HNE and anti-phospho-AMPK antibodies were used for immunocytochemistry and flow cytometry analysis, to determine the endogenous levels of 4HNE addition and AMPK phosphorylation in spermatozoa. AMPK activation in sperm subjected to incubation with exogenous 4HNE was also assessed. Sperm vitality and motility were analysed by Live-Dead stain/flow cytometry and CASA, respectively. Each experiment was repeated at least three times for each stallion.

Immunocytochemistry for phospho-AMPK localised the activated form of AMPK to the sperm midpiece, consistent with a mitochondrial distribution. Analysis of endogenous 4HNE and phospho-AMPK in sperm samples incubated in BWW identified an inverse relationship between levels of 4HNE and activated AMPK (R^2=0.68, P < 0.001) after 24 hours, and a positive relationship between AMPK activation and total motility (R^2=0.64, P < 0.001). Treatment of spermatozoa with exogenous 4HNE caused a decrease in the levels of AMPK phosphorylation (59.6 % in control vs 47.06 % in 10 μM 4HNE, P < 0.05) at 24 hours, while sperm vitality was not affected. These results suggest that interference with AMPK phosphorylation is one of the mechanisms via which 4HNE may negatively affect sperm function.
metabolism. Considering the systemic elevation of 4HNE seen with obesity, metabolic syndrome, diabetes, and inflammatory conditions, the emerging role of AMPK dysfunction in these pathological states, and the apparent involvement of both molecules in sperm function, our findings represent an important new connection between the systemic metabolic state and male fertility.

532. Expression of Estrogen Receptor Beta in the Male Reproductive Tract and Sperm in Mice.
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Estrogens are sex steroid hormones that play an important role in several aspects of male reproduction including sperm production and transfer through the male reproductive tract, as well as in later maturation steps, which spermatozoa undergo in the female genital tract. Generally, estrogen action is mediated via binding and activation of estrogen receptors (ERs). Activated ERs form dimers, bind to DNA and act as transcription factors. Estrogen binding can also lead to rapid activation of several signalling cascades. Currently, two types of classical ERs are known, namely ER alpha (ERα) and ER beta (ERβ). In addition to classical ERs, several splice variants that may differ in their ligand- or DNA-binding properties were detected in different tissues and cell lines. These variants may also form dimers and modulate the function of classical full-length ERs. Estrogen action seems to be a very complex and therefore it is necessary to know the exact expression pattern of all existing variants of ERs. Since ERβ is a predominant variant in testes, we focused our study on expression of ERβ and its potential splice variants in murine testes, sperm and epididymis as a starting point to understand mechanism of estrogen signalling in male reproduction. According to our results from mRNA analysis, there are at least two variants of ERβ in mice testes, epididymis and sperm. These variants differ in one exon in ligand binding domain and this variability leads to different affinity to estrogens. It seems that both variants are similar in abundance within the same organ or sperm. Furthermore, cauda epididymis and sperm from cauda epididymis contain fewer ERβ compared to caput epididymis and sperm from this region. To analyse these variants also at a protein level, we prepared specific monoclonal antibodies recognizing particular variant of ERβ. Both antibodies detected band(s) in protein extracts from testes and epididymis. The present study demonstrated that at least two variants of ERβ are expressed in male reproductive tract. Further studies are needed to determine whether these variants are present within a single cell and can thus interact, or whether one type of cells preferentially express one ERβ variant while another cell type express the second variant.

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533. Differentiation of zebrafish spermatogonial stem cells to functional sperm in culture.
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Spermatogenesis is a complex process in which spermatogonial stem cells (SSCs) self-renew, develop to differentiated spermatogonia, and then give rise to meiotic spermatocytes, haploid spermatids and fertile sperm ultimately. Cell culture methods representing this process will have a lot of advantage in analyzing molecular function and imaging molecules. However, a whole process of spermatogenesis has been represented only in organ culture systems in eel and mice. Here, we describe a grafting method for maintenance of a hypertrophied testis and culture methods representing proliferation of SSCs and differentiation to functional sperm by using the hypertrophied testis. The hypertrophied testis is a tumorous testis that happened to be found in zebrafish, and contains a large number of the early stage of spermatogonia. To use the hypertrophied testis for SSC culture constantly, we first developed the grafting method of a hypertrophied testis in zebrafish. When a fragment of the hypertrophied testis was transplanted under abdominal skin of the rag1 mutant, remarkable growth of the fragment was observed after 3 months of transplantation. Furthermore, the grafts kept on growing during the sequential transplantation. Then, we next performed SSC culture by using a hypertrophied testis of sox17 promoter::EGFP transgenic zebrafish that express EGFP in the early stage of spermatogonia. Changes of basal culture medium from L-15 to DMEM and supplementation of heparin resulted in a fivefold greater number of EGFP-positive spermatogonia than that in our previous conditions. Transplantation assay using testicular aggregates showed stem cell properties in the spermatogonia after 1 month of culture, suggesting that the EGFP-positive spermatogonia are SSCs. When SSCs after 1 month of culture were plated on feeder layers of the zebrafish Sertoli cell line, ZLA6-12, meiotic spermatocytes were observed after 10 days of the plating. Expression of a meiotic marker, Sycp3, was also detected in the spermatocytes. After 20 days of the plating, fertilized embryos were obtained by artificial insemination with differentiated sperm in culture. The embryo expressed EGFP as same as the sox17::EGFP line, and grew up normally, suggesting that normal haploid sperm were produced from SSCs under the culture condition. These results indicate that a whole spermatogenic process including proliferation of SSCs and differentiation to functional sperm is represented in our cell culture conditions in zebrafish. This research was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sport, Science and Technology, Japan.

534. The Effect of Tetrabromobisphenol A on Prostate Content and DNA Integrity in Mouse Sperm.
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Tetrabromobisphenol A (TBBPA) is commonly used as flame retardant and currently is among the most widely used; its consumption is around 210,000 tons/year and is still growing. TBBPA has negative effect on different mammalian cell lines in vitro and it is able to affect the thyroid hormone system as well it might be able to influence the androgen/estrogen hormone system in vivo. Thus TBBPA is considered as endocrine disruptor and its possible negative effect on human and wildlife health needs to be investigated. In our previous study we have observed that TBBPA is able to induce apoptosis of testicular cells and changes in the expression of genes playing
important roles during spermatogenesis. Moreover, we observed that certain effects can be transmitted to the second generation. In this study we examined in more detail the effect of low concentration of TBBPA (35 μg/kg b.w.) on reproductive parameters of male mice in vivo. C57Bl/6J mouse pups were exposed to TBBPA (experimental group) during the gestation, lactation, pre-pubertal and pubertal periods up to the age of 70 days and compared to unexposed control group. We mainly focused on the analysis of protamines, the most abundant sperm nuclear proteins, and on the sperm DNA integrity. We have shown that TBBPA is able to induce changes in sperm protamination, number of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positive sperm and serum testosterone level. We detected significantly decreased protamine 1/protamine 2 ratio (0.362 vs. 0.494; p < 0.001), increased total protamine/DNA ratio (0.517 vs. 0.324; p < 0.001) and increased number of TUNEL positive spermatozoa (39.5% vs. 21.2%; p < 0.05). Protamines have recently been implicated in the epigenetic marking of sperm chromatin in human and mouse spermatozoa. Thus, changes in sperm protamination induced by TBBPA, may explain the previously observed second generation effect. This research was supported by grants from the Spanish Ministry of Economy and Competitiveness (FEDER BFI 2009-07118 and PI13/00699), by the Grant Agency of the Czech Republic (No.GAP503/12/1834) and by BIOCEV project from the ERDF (CZ.1.05/1.1.00/02.0109).

535. Anti-GAPDH antibodies can inhibit in vitro sperm-oocyte binding.
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There are ten enzymes of the glycolytic pathway which are highly conserved and present in nearly all organisms. In male germ cells undergoing spermatogenesis, at least some somatic glycolytic enzymes are replaced with sperm-specific isoforms. The first enzyme of the second glycolytic phase, glyceraldehyde 3-phosphate dehydrogenase-spermatogenic (GAPDH) is encoded with different gene than somatic GAPDH and was shown to be essential for energy production and sperm motility. We hypothesized that GAPDHs, like its somatic counterpart, might be involved in other cellular processes in addition to glycolysis. In this study, we first characterized the sperm protein recognized by a monoclonal antibody Hs-8 that was prepared by immunization of BALB/c mice with human ejaculated sperms. In the immunofluorescence test, Hs-8 antibody recognized the protein localized in the acrosomal part of the sperm head and in the principal piece of the sperm flagellum in the human, boar and mouse spermatozoa. Hs-8 labeled the 45 kDa protein in the extract of human sperm and with sequence analysis it was identified as GAPDH. Commercial mouse anti-GAPDH MoAb was used to confirm our findings. Next, functional analysis of the GAPDHs from the sperm acrosome was performed using the boar sperm/zona pellucida binding assay. We tested the effect of both Hs-8 and anti-GAPDH antibodies, while anti-P4 (anti-progesterone) and ACR.2 (anti-acrosin) were used as negative and positive controls, respectively. There was a four- to five-fold decrease in the number of bound sperm cells to the oocyte, when ACR.2, Hs-8, or anti-GAPDH antibodies were present in the incubation medium. Anti-P4 had no effect on the sperm/oocyte binding. The outcome of the in vitro sperm/oocyte binding assay suggests involvement of the GAPDHs protein in the secondary sperm/zona pellucida binding. To sum up, GAPDHs is, at first place, the sperm-specific glycolytic enzyme involved in energy production during spermatogenesis and sperm motility. In addition, it seems to have a certain function in the sperm head as well. We confirmed GAPDHs localization in the apical part of the sperm head in addition to the principal piece of the flagellum. In an indirect binding assay, we showed that anti- GAPDHs antibodies interfere with the secondary sperm/oocyte binding.

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536. Regulation of GDNF-responsive genes by PLZF and SALL4 in mouse spermatogonial stem cells.
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Spermatogonial stem cells (SSCs) maintain spermatogenesis throughout adulthood through balanced self-renewing and differentiating fate decisions, yet little is known about how these fate decisions are controlled. The transcription factors Sal-like 4 (SALL4) and zinc finger and BTB domain containing 16 (ZBTB16, aka: PLZF) are known to be required for normal SSC self-renewal and differentiation. Previous ChIP-Seq studies in undifferentiated spermatogonia identified 4,201 PLZF- bound genes (3,075 binding sites) and 2,591 SALL4-bound genes (3,490 binding sites) of which 1,372 (1,116 binding sites) were bound by both factors. Subsequent gene ontology (GO) analysis of these binding repertoires identified an over-representation of GDNF-responsive genes among those bound by SALL4 and/or PLZF. That is, of the 269 genes shown to exhibit significant mRNA level changes in cultured THY1+ spermatogonia following GDNF manipulations, 92 were bound by SALL4 and/or PLZF (34%) including genes known to be involved in SSC self-renewal and differentiation (e.g., Bcl6, Er5, Fos, Foxa1, and Lhx1). To validate our ChIP-seq data and determine the relevance of these binding sites to transcription of putative target genes, we performed siRNA-mediated knockdown of PLZF or SALL4 in cultured THY1+ spermatogonia and measured mRNA levels of target genes. Results of these studies indicated that SALL4 and PLZF are required to maintain the levels of these target genes, suggesting that transcription factor binding to these genes at the positions identified by ChIP-seq activates their transcription. This raise the intriguing possibility that these two transcription factors, SALL4 and PLZF, which are required for normal spermatogenesis, are key mediators of GDNF regulation of SSC function.

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537. Functional Characterization of Ion Channels in juvenile Germ Cells in vitro and in situ.
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Spermatogenesis is a fundamental biological process that ensures male fertility. However, few physiological details are known about testicular cell communication during spermatogenesis. Since we and others have previously shown that Sertoli cells are able to communicate via ATP, we hypothesize a general role for purinergic signaling in the seminiferous cycle. Using wildtype C57BL/6 mouse pups, we first developed a coculture of Sertoli cells and spermatogonia. Next, we investigated ATP-dependent signaling in spermatogonia by whole-cell patch-clamp recordings. Pharmacological profiling and gene expression knockdown allowed identification of the underlying ion channels. Here, we report that cultured spermatogonia respond to extracellular ATP (1–100 µM). ATP-induced currents show fast activation and moderate desensitization. The current–voltage relationship reveals strong inward rectification. Current potentiation by ivermectin and inhibition by an acidic extracellular pH (6.3) and extracellular copper (100 µM) indicate a functional role of P2X4 receptors. Accordingly, knockdown of P2X4R expression by RNA interference significantly reduced currents activated by ATP concentrations ≤ 300 µM. Interestingly, an increased ATP concentration (>300 µM) activated an additional current with different kinetics. A similar current could be activated by 300 µM 3′-O-[(4-Benzyloxy)benzoyl] ATP (BzATP) and was blocked by the P2X7-Antagonist A-438079. Similarly, knockdown of P2X7R expression decreased this current. Combined with molecular evidence, our results indicate functional expression of at least two P2X receptor subunits (P2X7R and P2X4R) in spermatogonia of prepubescent mice. Downstream P2X receptor activation, a calcium–dependent potassium current functionally antagonizing the depolarizing effect of P2XR activation. To confirm these results in situ, we established an acute tissue slices preparation from prepubescent mouse seminiferous tubules. Electrophysiological recordings from both Sertoli and germ cells revealed ATP–induced currents that strongly resembled our in vitro results. Together, we established a toolkit to measure sort termed responses from identified testicular cell types in a physiological setting. Our data thus represent a first important step towards a deeper understanding of cellular purinergic communication during spermatogenesis.

538. The role of TAF4b in transcripional regulation during mouse spermatogonial stem cell development and renewal. Eric A. Gustafson1, Lindsay Lovasco1, Kathryn Grive1, Kimberly Seymour1, Dirk de Rooij2, Richard Freiman3.

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Long-term mammalian spermatogenesis requires proper development of spermatogonial stem cells (SSCs) that replenish the testis with germ cell progenitors during adult life. TAF4b is a gonad-enriched component of the general transcription factor complex TFIID, and several lines of evidence suggest that TAF4b is essential for SSC establishment and maintenance. Mice lacking TAF4b have fewer gonocytes during late embryogenesis, a deficient neonatal gonocyte expansion and several SSC-related abnormalities during the initial waves of spermatogenesis. These data indicate that TAF4b is required for SSC development before their establishment and may direct a crucial gene regulatory network in embryonic gonocytes. In order to identify genes in this network, we performed a comparative transcriptome analysis on E18.5 whole testes from Taf4b +/- and Taf4b -/- individuals. This analysis identified 879 upregulated and 520 downregulated genes in Taf4b -/- E18.5 testes, several of which are reproduction-associated genes. To distinguish between direct and indirect TAF4b target genes, we performed chromatin immunoprecipitation (ChIP) to analyze TAF4b proximal promoter occupancy. To gain further mechanistic insight into how TAF4b coordinates SSC-specific gene expression, we identified a TAF4b-interacting protein cofactor that can function as a transcriptional activator, characterized its expression during development and examine its potential role in SSC transcriptional regulation.

539. RHOX13 is Required for Normal Spermatogenesis and Fertility in Male Mice. Jonathan T. Busada1, Christopher B. Geyer2.

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Spermatogenesis is a stem cell-based system. Spermatogonial stem cells (SSCs) balance self-renewal with the consistent production of progenitor spermatogonia that proliferate and differentiate in response to retinoic acid (RA) to enter meiosis and eventually become spermatozoa. This results in the daily production of millions of spermatozoa throughout the male reproductive lifespan. Currently, little is known about the essential program of spermatogonial differentiation, and the pathways and proteins involved are poorly defined. We previously described a novel germ cell-specific X-linked reproductease homoeyocyte (Rhox13) that is upregulated at the level of translation in response to RA in differentiating spermatogonia and preleptotene spermatocytes prior to their entry into meiosis. To elucidate the role of RHOX13 in male germ cell differentiation, we created a conventional (whole-body) knockout (KO) mouse model. Rhox13 KO mice were born in expected Mendelian ratios, and adults had normal testis weights with a full complement of spermatogenic cell types. However, KO mice had ≈50% reduced daily sperm production and epididymal sperm counts. Careful histological analysis of adult KO testes revealed a number of defects. These included increased apoptosis and disruption of the precise stage-specific association of germ cells within the seminiferous tubules, indicating that the synchrony of spermatogenesis was disrupted. A 2-month breeding study revealed that KO males produced normal- sized litters, but they took significantly longer to sire litters (29 days) as compared to wild type littermate controls (22 days). Taken together, our results reveal that RHOX13 is not essential for mouse fertility in a controlled laboratory setting, but it is required for normal fertility. A mutation in the Rhox13 gene would certainly be selected against in the wild based on its negative effect on fecundity. This project was supported in part by a grant from the NIH/NICHD (HD072552) to C.B.G.

540. WITHDRAWN.

541. Subcellular localization of the PRAME protein in bovine spermiogenesis. Wansheng Liu1, Yaqi Zhao2, Gang Ning3.

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The PRAME (preferentially expressed antigen in melanoma) proteins are cancer-testis antigens (CTA) with leucine-rich repeat (LRR) domains that fold into a horseshoe shape for protein-protein interactions. The PRAME gene family has been amplified in the
genome of eutherian mammals. In bovid lineage, PRAME has been transposed to the Y chromosome through an ‘autosome-to-Y’ transposition, an evolutionary mechanism that usually produces novel sex-linked gene to enhance male reproduction. The Y-linked PRAME gene, named PRAMEY, has expanded on the bovine Y with a median copy number of 13 - 26 among different cattle breeds. The copy number variations (CNVs) of PRAMEY were found to be associated with testicular size, semen quality and male fertility. Our previous western blot analysis with a custom-made anti-PRAMEY antibody on the bovine testes at different developmental stages (20d, 3m, 8m and 2y) indicated that PRAMEY was first expressed at the age of 8 months. Immunofluorescent staining further revealed that PRAMEY was expressed in the acrosome of spermatids and mature spermatozoa, as well as in the sperm flagellum. The objective of the present study was to investigate the subcellular localization of the PRAMEY protein by immunogold electron microscopy (IEM). Adult testis tissues and mature epididymal spermatozoa were collected from a local abattoir and used in the IEM experiments. The results indicated that the immunogold particles of PRAMEY are restricted to the ground substance/matrix of the pre-acrosomal granule (or the acrosome) without evidence of any membrane association in the Stage I round spermatids and the Stage II round spermatids when the spermatids just begin to elongate. It appears that the gold particles were nonrandomly distributed in the pre-acrosomal granule and were associated with the matrix structure. Compared to the very low (almost no) background labels across the cell organelle, gold particles were also found in regions around Golgi complex. Along the formation and expansion of the acrosomal vesicle (spreading over the nucleus) during the differentiation of the spermatids, enriched gold particles were distributed in the acrosomal matrix at the top of the head (near the apical ridge) and a tendency of association with the inner acrosomal membrane (IAM) on the bottom of the head was observed in the elongated spermatids and mature spermatozoa. The gold particle labels were also seen in the flagellum of sperm. Our preliminary IEM data suggests that the PRAMEY protein is localized in Golgi complex, the pre-acrosomal granule that is enveloped in the Golgi vesicle, and acrosomal matrix, signifying a functional role of PRAMEY in acrosome formation during spermiogenesis.

542. Development and assembly of the compartmentalized Ca$^{2+}$ signaling domains in sperm flagella.
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Sperm-specific Ca$^{2+}$ channel, CatSper, and associated Ca$^{2+}$ signaling molecules form a multi-linear domains in sperm flagellar membrane. How this compartmentalized domains are developed and why they form the quadrilateral structures are unknown. Here, we studied the developmental mechanism of this multi-linear structure. We found that this structure emerged early during flagella development when flagellum imparts the nucleus and starts as primary flagellum. Both the pore-forming and the auxiliary subunits of CatSper channel are recruited to the plasma membrane enveloping the axoneme before the peri-axonemal structures such as the fibrous sheath and the outer dense fiber assembly in the flagella. When CatSper1 is knocked out, CatSper delta was targeted to the principal piece of some developing spermatids but did not display the linear arrangement. These results demonstrate that CatSper delta is important to target the channel complex to the flagella but the presence of CatSper1 in the flagellar membrane is required for the formation of the quadrilateral structure in sperm flagella.

543. The dominant lethality of acrylamide correlates with CYP2E1 expression within the epididymis of male mice.
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Acrylamide is a toxicant which has been found to be present in many food sources; particularly fried foods cooked at temperatures above 120°C. Hence humans are chronically exposed to acrylamide. Previous studies in our lab have chronically administered acrylamide via the drinking water to male mice at doses of 0-2mg/kg bw/day, which includes doses relevant to human exposure, for periods of 1, 3, 6, 9 or 12 months (n=6 per group). We have established that such exposure leads to DNA damage in the germ cells as assessed using a modified version of the comet assay which detects the presence of specific adducts on the DNA. Analysis revealed a time and dose dependent increase in glycidamide adducts in the DNA of spermocytes of mice exposed to acrylamide. Oxidative adducts were also detected in germ cells of mice with chronic acrylamide exposure in a time and dose dependent manner measured by probing testis sections with an antibody raised against the modified base 8-hydroxydeoxyguanosine. Glycidamide is the metabolite of acrylamide and the main contributor to its associated toxicity. Acrylamide is converted to glycidamide solely via the enzyme CYP2E1. We demonstrated by immunofluorescence that CYP2E1 is expressed within spermatoctyes and at higher levels within the principal cells of the epididymis. Hence we propose that these are the locations within the male reproductive tract where acrylamide can be converted in situ to glycidamide. Using an in vitro model system, we have isolated spermatoctyes and treated them with acrylamide or glycidamide (1 or 0.5µM respectively, 18h). Using the modified comet assay we have determined that acrylamide and glycidamide treatment led to an increase in glycidamide adducts as well as oxidative adducts, agreeing with the findings of our chronic in vivo study. When spermatoctyes were cotreated with an inhibitor of CYP2E1; resveratrol (0.1µM), acrylamide induced DNA damage was reduced. In the current study, we treated male mice with acute doses of CYP2E1 (12.5 and 25mg/kg bw/day for 5 consecutive days) to establish the sensitivity of different stages of spermatogenesis to acrylamide’s reproductive toxicity. The same exposed male mice (n=3 per group) were mated with unexposed females at different times such that the fertilising sperm were exposed to acrylamide at the spermatogonial, spermocyte or spermatid stage of development, or spermatozoan stage during transition through the testis, epididymis or vas deferens. Female mice were euthanased approximately 13 days after each mating period and live embryos and embryo resorptions were counted. Our study used narrowed windows of fertilisation and we were able to determine that dominant lethality was most significant when acrylamide exposure occurred during the spermatozoan stage and highest during transit through the epididymis with 72.2% dominant lethality observed with the highest dose of acrylamide. These results show that CYP2E1 is highly expressed within the epididymis, and that sperm are most sensitive to acrylamide induced dominant lethality when exposed during storage in the epididymis. Hence expression of CYP2E1 matches dominant lethality. This emphasises the role of conversion to glycidamide in acrylamide induced dominant lethal mutations.

544. WITHDRAWN.
545. Isoform-Specific Roles for PP1γ2 and GSK3α for Normal Sperm Function and Male Fertility in mammals.

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The four protein phosphatase isoforms, PP1α, PP1β, PP1γ1, and PP1γ2, are interchangeable in most cells and tissues. The PP1γ2 isoform with its unique C-terminus, present only in mammals and predominant in testis, is the only PP1 isoform in sperm. Targeted disruption of the Ppp1cc gene, which results in the loss of both PP1γ1 and PP1γ2, results in male infertility. We have previously shown that transgenic expression of PP1γ2 in testes of Ppp1cc null mice restores normal sperm function and male fertility, provided a threshold level of the enzyme is present. Efforts to determine whether PP1γ1 can substitute for PP1γ2 in sperm were hampered by the inability to transgenically express PP1γ1 in developing germ cells. Removal of a 1 kb region of the 3'UTR in the PP1γ1 CDNA permitted high levels of transgenic expression of PP1γ1. We show that testsis expression of PP1γ1, driven by the Pgk2 promoter, in Ppp1cc null mice affects sperm motility resulting in subfertility. Surprisingly transgenic expression of PP1γ1 along with PP1γ2 in wild type mice also impairs sperm motility and fertility. Thus, the essential function of PP1γ1 for optimal male gamete function and fertility cannot be effectively substituted by the PP1γ2 isoform. Two isoforms of glycogen synthase kinase 3, GSK3α and GSK3β, have numerous roles as a signaling enzymes regulating a wide spectrum of cellular and tissue functions. The two GSK3 isoforms are interchangeable in most tissues except in the developing embryo. Both isoforms, present in testis and sperm, were proposed to play a role in regulating sperm PP1γ2. We recently discovered that targeted disruption of GSK3α results in compromised sperm function and male infertility. The GSK3β isoform, present in testis and sperm, does not substitute for the loss of GSK3α. Using a Cre-Lox approach we generated mice lacking GSK3β in testes. Mice lacking GSK3β in pre- and post-meiotic germ cells are normal and fertile. Sperm characteristics of mice lacking GSK3α are similar to those in mice expressing sub-optimum levels of sperm PP1γ2. The activity of PP1γ2 increases in sperm lacking GSK3α. Moreover, the down-stream effects of sperm cAMP dependent protein kinase (PKA) are compromised in sperm from GSK3α null mice. Thus GSK3α, which has a highly conserved N-terminus in mammals, has an essential isoform-specific role in sperm. Our data show that PP1γ2 and GSK3α, along with PKA, are parts of a sperm signaling pathway essential for male fertility.


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Complex N-glycans are differentially expressed in germ cells during spermatogenesis in the mouse. The synthesis of complex N-glycans is initiated by MGAT1 (also known as GlcNAcT-I or N-acetylgalcosaminyltransferase I), encoded by the Mgat1 gene. We previously generated a conditional deletion of Mgat1 and complex N-glycans in spermatogonia, and identified a germ-cell autonomous block in spermatogenesis (Batista et al., 2012). Other glycosylation-defective and basigin-null mice have a similar block in spermatogenesis but are mutant in both germ and somatic cells of the testis. Conditional deletion of Mgat1 in spermatogonia results in a complete loss of sperm and the formation of aberrant, giant, multinucleated syncytia that contain either round or elongated spermatids. Ldhc promoter (in spermatocytes), and the Prm1 promoter (in spermatids) have been generated.

Stage MGAT1 and complex N-glycans are required for spermatogenesis, mice expressing a

PS (RO1 CA 36434 and RO1 GM 105399).

There is an emerging interest in the study of exosomes as potential biomarkers for disease. These nanosized, cell-derived-vesicles are packed with protein and RNA and have been identified in every human bodily fluid. Seminal plasma is a complex fluid secreted from several tissues including testis, epididymis and accessory sex glands. As such, it also contains several populations of exosomes. Assessing the protein content of these vesicles can provide information on their biogenesis and possibly serve as biomarkers of disease or subfertility. The purpose of this study was to design a novel HTFC method to determine the presence of 373 proteins on the surface of human seminal fluid exosomes from normozoospermic patients. With REB approval, exosomes were isolated from 10 seminal fluid samples by differential centrifugation to remove sperm and cellular debris, followed by immunoprecipitation using anti-human CD9-coated magnetic beads enabling detection by flow cytometry. In total, 251 proteins were detected on the surface of exosomes; 12 of them were abundantly expressed on 51 to 96%. In support of previous studies, we detected CD13, CD63, CD81, CD256, CD82, CD10, CD177, CD326 that are known to play vital roles in cell to cell communication, protein metabolism and immune regulation. We also identified two highly abundant
proteins not previously reported on seminal fluid exosomes: CD175s (alpha-N-acetylgalactosaminide alpha-2,6-sialyltransferase) and CD66e (caricinoembryonic antigen-related cell adhesion molecule 5). These proteins are involved in cell to cell communication and immune response regulation, respectively. Our results validate the utilization of an HTFC method for the detection of surface proteins on exosomes and for the discovery of new non-invasive biomarkers of male infertility.

548. A Novel Non-surgical Method For Male Sterilization Using An Inhibitor Of the SHP2 Tyrosine Phosphatase.
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A single treatment, non-surgical method to sterilize men and male animals has been an unmet need for centuries. For men, a single treatment sterilization method would eliminate the need for vasectomy surgeries. For animals, a non-surgical sterilization technique would help control the population of feral cats and dogs, thus preventing the deaths of millions of animals in shelters each year. Furthermore, new sterilization ideas are needed to humanely control the populations of deer, groundhogs and other destructive wild animals. Toward these goals, we found that knockout of the PTEN11 gene encoding the SHP2 tyrosine phosphatase in mouse germ cells permits the first wave of spermatogenesis but then results in the complete absence of sperm due to blocking the proliferation of spermatogonial stem cells and the death of these stem cells that are required to produce sperm. Similarly, we found that injection of the SHP2 small chemical inhibitor NSC-87877 into the testes of adult mice results in the loss of nearly all germ cells within 15 days. Supporting Sertoli cells and testosterone producing Leydig cells were maintained after NSC-87877 treatment. Initial toxicity studies indicate that there were no deaths, health problems or damage to stem cell dependent processes in the spleen or liver due to injection of NSC-87877 into mouse testes. A pilot study was performed to translate our studies to pigs having larger, external testes that are better models for spermatogenesis in men. The testes of a 12 week-old (prepubertal) male pig were injected at three sites with either NSC-87877 (40 mM, 1 ml, right testis) or vehicle (left testis). The pig was castrated 11 weeks later (about 7 weeks after the first sperm are normally detected in the testis) and testis tissue sections were assessed for spermatogenesis. These studies revealed regions of the NSC-87877 treated testis in which initial round(s) of spermatogenesis appear to have occurred but subsequent spermatogenesis was blocked due to the inability to produce additional developing germ cells. Although the system for delivering NSC-87877 remains to be optimized, our results indicate that the SHP2 inhibitor permanently eliminates spermatogonial stem cells and sperm production in the pig testis while maintaining the supporting somatic cells. These results suggest that the stem cell-directed NSC-87877 could be a low cost, non-surgical solution for controlling animal populations and potentially replacing vasectomy surgeries while maintaining the internal structure of the testis and normal hormone profiles.

549. Analysis of human Aurora kinase C variants in oocyte meiosis.
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Chromosome segregation is an extensively choreographed process yet errors still occur frequently in females, leading to miscarriage or offspring with developmental disorders. The Aurora protein kinases are well-established regulators of mitotic and meiotic chromosome segregation. Aurora kinases A and B are expressed in most cell types, whereas Aurora kinase C (AURKC) is only highly expressed in gametes. Our studies in mouse oocytes indicate that AURKC is required to regulate chromosome segregation during meiosis I (MI); however, little is known about the functional significance of AURKC in human oocytes. Here we show that human AURKC functionally complements mouse AURKC when expressed in Aurkc−/− oocytes. In this study, we use this oocyte system to address the function of 2 types of AURKC variants: splice variants and sterility-associated variants. To determine which splice variants human oocytes express, we performed qRT-PCR using single oocytes and found that oocytes express three splice variants. To evaluate the functional differences between the splice variants, we created GFP-tagged constructs to express in oocytes from Aurkc−/− mice. By quantifying metaphase chromosome alignment, cell cycle progression, phosphorylation of INCENP, and microtubule attachments to kinetochores, we found that AURKC_v1 is the most capable of the 3 variants at supporting MI chromosome segregation although AURKC_v2 is the most stable splice variant. AURKC_v3 localized to chromosomes properly and supported cell cycle progression to metaphase II, but chromosome segregation was not as accurate compared to the other 2 variants as indicated by its inability to correct erroneous spindle attachments. Therefore, oocytes express 3 variants of AURKC that are not functionally equivalent in supporting meiosis. Three mutations in the coding region of Aurkc (L49W, C229Y, and Y248X) are correlated with male infertility. These men have 100% tetraploid sperm with multiple flagella. The majority of these men (85%) were homozygous for L49W, while the remainder was compound heterozygous for L49W and one other mutation. L49W is a single base pair deletion that creates a frameshift and premature stop codon after 19 missense residues. This premature stop codon occurs at the beginning of the kinase domain and is therefore hypothesized to be a loss-of-function mutation. C229Y is a residue conserved in all three human aurora kinases and therefore likely critical for kinase function. Y248X introduces a premature stop codon towards the end of the kinase domain and results in a loss of the C-terminus. We found that neither the L49W or Y248X mutants localized to the chromosomes when expressed in Aurkc−/− oocytes; however, C229Y localized to centromeres in most oocytes and phosphorylated INCENP indicating that C229Y retains partial kinase activity. Therefore we infer that while C229Y retains some catalytic activity, it is not sufficient to support meiosis when the other copy is non-functional. AURKC plays a critical role in maintaining ploidy in oocytes. Here we show 2 splice variants that support this role in separate ways: v1 through error correction and v2 through protein stability. We also show that when its function is altered by mutation in the kinase domain, that fidelity of
chromosome segregation is impaired. This study was supported by an ASRM Research grant and a Busch Biomedical Foundation grant to K.S.

550. Spatial regulation of kinetochore microtubule attachments by destabilization at spindle poles in meiosis I.
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To ensure accurate chromosome segregation in cell division, erroneous kinetochore-microtubule (MT) attachments are recognized and destabilized. Improper attachments typically lack tension between kinetochores and are positioned off-center on the spindle. Low tension is a widely accepted mechanism for recognizing errors, but whether chromosome position regulates MT attachments has been difficult to test. We exploited a meiotic system in which kinetochores attached to opposite spindle poles differ in their interactions with microtubules, and therefore position and tension can be uncoupled. In this system homologous chromosomes are positioned off-center on the spindle in oocytes in meiosis I, while under normal tension, as a result of crossing mouse strains with different centromere strengths, manifested by unequal kinetochore protein levels. We show that proximity to spindle poles destabilizes kinetochore-MTs, and that stable attachments are restored by inhibiting Aurora A kinase at spindle poles. During the correction of attachment errors, kinetochore MTs detach near spindle poles to allow formation of correct attachments. We propose that chromosome position on the spindle provides spatial cues for the fidelity of cell division.

551. Regulation of expression of oocyte quality control factor, ATRX, in mammalian and amphibian oocytes during meiotic maturation.
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In humans, mutation of the ATRX gene leads to improper methylation of repetitive DNA sequences. In this case, failure to establish the necessary epigenetic modifications essential for inactive X chromosome results in genomic and chromosome instability and leads to X-linked α-thalassaemia with mental retardation (ATRX) syndrome. A meta-analysis of published mammalian oocyte microarray data across several model species led to the identification of ATRX as a potential biomarker of oocyte quality. The aim of the present study was to determine the expression and regulation of ATRX at a protein level, in both Xenopus laevis oocytes and the bovine cumulus oocyte complex (COC). ATRX protein was found to be conserved during oogenesis and to be dramatically downregulated during oocyte maturation (meiotic cell cycle) in both model systems. Treating Xenopus oocytes with MG132 (proteasome inhibitor) during maturation led to stabilization of the protein, while co-immunoprecipitation studies showed ATRX to be ubiquitinated in germinal vesicle (GV) oocytes; thus indicating ATRX is degraded via the proteasome. Injection of Xenopus oocytes with a PKA inhibitor induced maturation and caused ATRX protein to be cleaved and degraded, showing ATRX to be downstream of PKA in the oocyte maturation signaling cascade. We have previously shown that inhibition of progesterone signalling during bovine in vitro oocyte maturation has a detrimental effect on subsequent in vitro embryonic development. Here we show that this is characterized by an increase in ATRX expression and a parallel increase in the expression of a known marker of apoptosis (active Caspase-3) during oocyte maturation, in both oocytes and cumulus cells. Our findings suggest that ATRX expression may be linked to the apoptotic pathway; playing a role in progesterone regulation of oocyte quality. Mechanical fractioning of GV Xenopus oocytes revealed ATRX to be nuclear localized. Immunohistochemistry studies performed on bovine oocytes at different stages of meiotic maturation showed ATRX to be localized to the chromosomal area of GV oocytes, but undetectable in MI oocytes. Inhibition of progesterone signaling during maturation resulted in the stabilization of ATRX in bovine oocytes, with ATRX remaining localized to the chromosomal area of mature MI oocytes. In conclusion, ATRX protein expression and localization appears to be progesterone regulated and associated with oocyte quality. However, further work is needed to determine how the regulation of ATRX corresponds to specific epigenetic regulations and subsequent developmental competence.

552. Aurora Kinase C Organizes Bipolar Spindle Assembly Through Regulating Eg5-kinesin in Mouse Oocytes.
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In contrast to mitotic cells, oocytes lack classic centrosomes and, therefore, bipolar spindle assembly in meiosis depends on self-organization of numerous MTOCs into two poles. The molecular mechanism regulating this self-organization in oocytes is not fully understood. We previously demonstrated that the histone H3, threonine 3 protein kinase, Haspin, regulates the chromosome arm localization of Aurora kinase C (AURKC) and the chromosomal passenger complex (CPC). When this localization was perturbed by inhibiting Haspin with 5-Iodotubercidin (5-Itu), we observed alterations in chromosome condensation and an increase in improper attachments of microtubules to kinetochores at metaphase I (Met I). We show here that inhibition of Haspin also significantly increased the percentage of oocytes with multipolar Met I spindles. These results suggest an additional function of Haspin in regulating meiotic bipolar spindle assembly. AURKA is the known Aurora kinase family member that is responsible for bipolar spindle assembly in mouse oocytes. However, we found that AURKA MTOC localization was not perturbed in 5-Itu-treated oocytes. To determine if this function is due to mislocalization of AURKC-CPC, we first asked if AURKC localizes to spindle poles. Using a fixation procedure that is sensitive for detecting proteins at MTOCs, we found that AURKC localized to spindle poles. However, we did not detect any other CPC components at the poles suggesting that AURKC has CPC-independent functions to regulate bipolar spindle formation in mammalian oocytes. Importantly, we found that AURKC spindle pole localization was ablated in 5-Itu-treated oocytes. To validate our results, we overexpressed AURKA, AURKB or AURKC in 5-Itu-treated oocytes. We found that only AURKC rescued the multipolar spindle phenotype, indicating that Haspin is essential for bipolar spindle assembly, at least partially, by regulating AURKC localized function. To further confirm our hypothesis, we perturbed AURKC function in 3 different ways and examined the spindle configurations in these systems. These systems are oocytes from Aurkc−/− mice, oocytes that express a mutant allele of Aurkc that selectively inhibits AURKC
activity, and oocytes treated with small molecule AURKB/C inhibitor. Perturbation of AURKC function resulted in a significant increase of multipolar spindles in all 3 systems confirming that AURKC regulates bipolar spindle assembly during meiosis I. Because AURKC-perturbed oocytes contained spindles with an increased length/width (L/W) ratio we asked if the Eg5-kinesin, a motor protein that drives the outward force of spindle poles, was perturbed. Perturbing AURKC function resulted in a significant up-regulation of Eg5. Importantly, inhibition of Eg5 with monastrol rescued the multipolar spindle and spindle L/W ratio phenotypes in AURKC perturbed oocytes. These data indicate that Eg5 functions in the AURKC pathway to regulate bipolar spindle assembly. These results are the first to show a new function for AURKC to regulate bipolar spindle assembly and to shed light on the underlying mechanism regulating acentriolar bipolar spindle assembly in oocytes. This research was supported by a grant from the NIH (R00HD061657) and by RU institutional start-up funding to KS.

553. EGFR inhibition prevents meiotic resumption in bovine oocytes co-cultured with follicular hemisections.
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Oocyte meiotic maturation involves signaling cascades coordinated by the epidermal growth factor receptor (EGFR) pathway in mural and cumulus granulosa cells. It has been shown that the presence of follicular wall cells (follicular hemisections) in a co-culture system with cumulus-oocyte complexes (COCs) delays oocyte meiotic progression and nuclear maturation. In this study, we evaluated the effect of EGFR inhibition during the co-culture of COCs with follicular hemisections on meiotic resumption of oocytes and gene expression in granulosa cells, cumulus cells and oocytes in culture. In the first experiment, COCs (n=52/group) were cultured for 15h in 200µl of TCM199 medium with follicular hemisections in the presence of 0.05, 0.5, 5 or 50µM of the EGFR inhibitor, AG1478. Most of the oocytes exposed to 5µM (89.28%) and 50µM (90.56%) remained at the germinal vesicle (GV) stage at the end of treatment, which was significantly higher than those treated with the lower concentrations, 0.05µM (25%) and 0.5µM (45.09%) AG1478. In the second experiment, the reversibility of the meiotic inhibition was assessed by culturing COCs that were exposed to 5µM AG1478 for 15h in standard IVF media for an additional 20 h. This confirmed that most of the oocytes (83.01%) matured and reached Telophase/Metaphase II stage. In vitro fertilization and embryo culture revealed that blastocyst formation rates were similar between oocytes matured in the control group (41.09%) and those that were matured for 15h in the presence of 5µM AG1478 followed by 20h in control medium (39.34%). To determine genes affected by EGFR inhibition, we assessed the transcript levels by qPCR in granulosa cells of follicular hemisections (Pgr, Prgmc1, Adams1l, Pgter2, Pgter4 and Ptg2s2), cumulus cells (Ptg2s2, Tnfaip6, Has2, Cx43 and Pgr) and oocytes (Pde3a, Cenbl1, Sic1, Anxa1 and Serpine1) obtained from positive control (without follicular hemisections), negative control (with follicular hemisections) and 5µM AG1478 treated groups after 15h co-culture. All data were tested for normal distribution using Shapiro- Wilk test, normalized by log transformation when necessary and analyzed by ANOVA. Differences between groups were assessed by LS MEANS Student’s T test. There was no significant difference in transcript levels between oocytes from control and AG1478 treated groups. In cumulus cells, mRNA abundance of Tnfaip6 and Has2 was lower in AG1478 treated compared to negative control group. The mRNA abundance of Cx43 in negative control group was lower when compared to positive control group and not different from AG1478 treated group. In granulosa cells, mRNA levels of Pgr and Adams1l were lower in AG1478 treated than control samples. These findings indicate that: i) addition of EGFR inhibitor in the co-culture system is an effective and reversible method to maintain bovine oocyte at GV stage; ii) exposure of bovine COCs to the EGFR inhibitor AG1478 is not detrimental for embryo development to blastocyst stage; and iii) EGFR inhibition alters gene pathways in both mural granulosa cells and cumulus cells, which might be involved in the arrest of meiotic progress. Research supported by CAPES and CNPq – Brazil (PBDG) and NSERC – Canada (VB).

554. Effect of Lysophosphatidic Acid on Porcine Oocyte Maturation and Embryo Development in vitro.
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Lysophosphatidic acid (LPA) is a natural small phospholipid and produced extracellularly in the serum and plasma, mainly from lysophosphatidylcholine. The function of LPA has been identified in various biological actions, such as cell proliferation, differentiation, migration, and morphogenesis. This study was performed to compare the developmental competence of porcine embryos created via in vitro fertilization (IVF) and parthenogenetic (PA) in culture medium supplemented with LPA, in comparison with a control group. The effects of LPA on porcine oocyte maturation and pre-implantation embryonic development were also examined. Addition of LPA to the oocyte maturation medium at a concentration 10 µM significantly increased the proportions of oocytes attaining metaphase I (MI) or metaphase II (MII) (P<0.05 for both comparisons) and enhanced embryonic developmental potential. When present during oocyte maturation, LPA significantly increased the expression levels of phosphorylated ERK1/2 in MI and MII oocytes (P<0.05 for both comparisons), showing that LPA enhanced nuclear maturation via activation of the mitogen-activated protein kinase (MAPK) pathway. In addition, cyclin B1 levels were elevated in MI- and MII-stage oocytes (P<0.05 for both comparisons), suggesting that LPA plays a role in both nuclear and cytoplasmic maturation of such oocytes. After fertilization, the frequency of polyspermy in embryos obtained using LPA-treated oocytes was less than that in the control group (23.0% vs. 14.8%, P<0.05). Further, blastocyst formation and blastocyst cell number were enhanced, and apoptosis reduced, upon LPA treatment of embryos created via IVF and PA. Upon LPA treatment of blastocysts derived via IVF and PA, the expression level of the anti-apoptotic Bcl-xl gene increased whereas those of the pro-apoptotic genes BAX and CASP3 fell. Together, our data indicate that LPA supplementation improves porcine oocyte maturation and subsequent development of pre-implantation embryos upon in vitro culture.

555. GVBD of mouse oocytes is regulated by miR-125a-3p and Fyn kinase through modulation of actin microfilaments.
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Oocytes are arrested at the prophase of the first meiosis and characterized by their prominent nucleus known as the germinal vesicle (GV). In response to LH, selected oocytes resume meiosis, a process recognized by GV breakdown (GVBD). We showed that inhibition of Fyn, a member of the Src-family kinases, markedly decreased the percentage of oocytes undergoing GVBD, demonstrating its indispensability for oocyte meiosis. In a recent study we characterized miR-125a-3p as a post-transcription regulation of Fyn in HEK-293T cells. In light of the above, we hypothesized that miR-125a-3p participates in the regulation of GVBD by modulating Fyn expression within the oocytes. To challenge this hypothesis we interfered with the expression of miR-125a-3p and Fyn by microinjecting mouse GV oocytes with either microRNA-mimics or in-vitro transcribed-RNAs. Each experiment was repeated at least three times and evaluated by ANOVA, Chi-square or two-tailed student’s t-test. Using luciferase assay, we confirmed that miR-125a-3p directly binds Fyn 3’UTR regulating its expression, post-transcriptionally. We further showed that the expression of endogenous miR-125a-3p decreases during the GV/GVBD transition. Over-expression of miR-125a-3p in GV oocytes led to a decrease in the number of oocytes that underwent GVBD, with no effect on its timing. The rate of GVBD in oocytes microinjected with miR-125a-3p, together with Fyn RNA that lacks the miR-125a-3p binding site, resembled that of control, implying that the inhibition of GVBD by miR-125a-3p is Fyn-dependent. We further found that over-expression of miR-125a-3p hampers the actin cytoskeleton organization and that inhibition of actin polymerization by cytochalasin D decreases GVBD rate in mouse oocytes. Using HEK-293T cells we showed that over-expression of miR-125a-3p reduced the interaction of actin and lamin A/C; this was reversed in cells co-transfected with miR-125a-3p and the Fyn-construct that lacks miR-125a-3p binding site. Overall, our results imply that miR-125a-3p participates in the regulation of GVBD by modulating Fyn expression. We further suggest that in mammalian oocytes actin microfilaments dynamics is necessary for GVBD and is partially regulated by miR-125a-3p through its effect on Fyn expression.

556. Effects of cAMP modulators on meiotic arrest, gap junction transfer and gene expression levels in cultured rat cumulus cell–oocyte complexes.
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Suppression of spontaneous meiotic resumption in oocytes that have been removed from their follicular environment and matured in vitro is critical for acquisition of developmental competency and improved embryological outcomes. In most mammalian oocytes, meiotic arrest is maintained through sustained intra-oocyte cyclic adenosine monophosphate (cAMP) levels. Intra-oocyte cAMP concentrations are regulated by activation of adenylyl cyclase, hydrolysis by phosphodiesterases (PDE) and levels of cyclic guanosine monophosphate (cGMP) that compete for PDE hydrolysis sites. Activation of natriuretic peptide receptor 2 (NPR2) on cumulus cells by C-type natriuretic peptide (CNP) stimulates cGMP production. The aims of this study were to investigate the effects of potential cAMP modulators on (i) timing of meiotic resumption, (ii) gap junction communication, and (iii) expression levels of key regulatory genes in rat COC cultured over time. The cAMP modulators included CNP, estradiol (E2) and appropriate PDE inhibitors (50mM dipyridamole and 50mM rolipram). The transfer rate of fluorescent dye from cumulus cells to the oocyte was used as a measure of gap junction transfer. Our results showed that PDE inhibitors did not affect the proportion of oocytes (29% vs 36%) that resumed meiosis (GVBD) within COC (n=22-30/group) after 4 hours incubation. Overall, gap junction transfer was higher (P<0.05) in COC incubated with PDE inhibitors at all incubation times (1, 5, 19 hours) however, there was an overall decline (P<0.05) with time. The addition of 100nM CNP, 200 ng/mL E2 or a combination (CNP+E2) to media containing PDE inhibitors did not affect gap junction transfer rate within COC (n=45-50/group) at any incubation time. Surprisingly, there was a significant increase (P<0.05) in the proportion of oocytes (n=11-15/group) resuming meiosis following 5h incubation with CNP+E2. Moreover, CNP+E2 increased (P<0.05) Pde4a mRNA levels whilst, CNP, E2 and CNP+E2 increased (P<0.05) Npr2 mRNA levels in cumulus cells of COC incubated with PDE inhibitors. Our results followed other reported data that in the absence of PDE inhibitors, oocytes from cultured COC (n=10/group) undergo GVBD in time-dependent manner (P<0.05). The addition of CNP, E2 and CNP+E2 decreased (P<0.05) the proportion of oocytes undergoing GVBD in a dose-dependent manner in COC (n=10/group) incubated for 6h without PDE inhibitors. There were no significant effects of the highest dose of CNP (1000nM), E2 (1000ng/mL) or a combination (CNP+E2) on oocyte-derived Pde4a mRNA levels however there was a marked decrease (P<0.001) in Pde3a mRNA levels in oocytes that had undergone GVBD, regardless of treatment. In summary, incubation of rat COC with PDE inhibitors did not delay spontaneous resumption of meiosis, despite increased gap junction transfer. Incubating rat COC with reagents reported to increase cGMP production delayed spontaneous resumption of meiosis, which was not observed using PDE inhibitors. This study highlights the importance of adding reagents to incubation media that physiologically stimulates cAMP levels, rather than inhibits cAMP hydrolysis, when culturing rat COC.

557. Developmental competence of bovine oocytes following 24h in a meiotic arrest medium containing cAMP and cGMP modulators.
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In vitro maturation (IVM) of cumulus oocyte complexes (COC) results in poor oocyte quality that negatively impacts subsequent embryo development compared to in vivo oocyte maturation. One hypothesis for the decrease in developmental competence of IVM oocytes is that once removed from the follicular niche, the communication between cumulus cells and oocytes is abruptly terminated resulting in premature resumption of meiosis. Oocyte meiotic arrest is maintained in vivo and in vitro through high concentrations of cAMP within the oocyte. cAMP activates protein kinase A, which phosphorylates multiple targets and prevent the oocyte from resuming meiosis. Recent studies in mice and bovine have shown that a short period (2h) of pre-maturation (pre-IVM) in the presence of cAMP modulators that inhibit meiotic resumption is beneficial for oocyte developmental competence. Moreover, other studies have recently demonstrated that cGMP also plays a crucial role in inhibition of meiotic resumption. cGMP flows from cumulus cells to the oocyte to inhibit cAMP
degradation, contributing to the maintenance of meiotic arrest. Our objective was to examine the effect of a cGMP modulator in combination with a cAMP modulator during a 24h period of pre-IVM on oocyte nuclear maturation and subsequent embryo development following IVF in the bovine. COCs were collected at Desoto Biosciences LLC (Seymour, TN) in the presence (pre-IVM, n=581) or absence (control, n=352) of cGMP and cAMP modulators. Pre-IVM COCs were placed in pre-IVM medium that contained cGMP and cAMP modulators; control COCs were placed into our standard defined maturation medium. COCs were then shipped to our laboratory at 37°C within 24h. Upon arrival, pre-IVM COCs (n=452) were washed and transferred into standard maturation medium; 5 COCs per 50 μl drop under oil for 22-24 h, at 37°C in 7.5% CO2 and 21% O2. Upon arrival (control) and after IVM (pre-IVM), oocytes were fixed for assessment of nuclear maturation, or fertilized and cultured in vitro. Results were analyzed by ANOVA. After 24 h, 64.7 ± 9.1 % of pre-IVM oocytes remained at the germinal vesicle stage of meiosis; following IVM 96.9 ± 0.7 % of the control oocytes were mature (MII). After IVM, 91.1 ± 5.9 % of the pre-IVM oocytes were mature, demonstrating that pre-IVM oocytes are able to resume meiosis after being arrested for 24 h. Subsequent embryo development demonstrated that pre-IVM treatment decreased cleavage per COC compared to control (56 ± 1.2 % and 74.2 ± 3.9 % respectively). Blastocyst development and hatching/hatched blastocyst per cleaved oocyte were not significantly different between control and pre-IVM (41.2 ± 2.3 % and 33.2 ± 7.8 %, respectively (blastocyst); 23.5 ± 2.7 % and 18.2 ± 4.1 %, respectively (hatching/hatched)). Moreover, advanced blastocyst development (hatching/hatched blastocyst on D7) per COC was not significantly different between pre-IVM and control (10.3 ± 2.4 % and 17.2 ± 1.5 % respectively). In conclusion, a combination of cGMP and cAMP modulators during oocyte culture and a 24h pre-IVM period inhibits meiotic resumption in the majority of treated oocytes. Although pre-IVM treated oocytes resulted in lower embryo cleavage per COC, likely due to the in vitro aged, non-arrested oocytes present, blastocyst development on D7 was not negatively affected. The ability to arrest oocyte meiotic resumption during shipment of COC between locations could be a powerful tool for the bovine industry and laboratories with limited access to bovine ovaries.

558. **KIF2B protects against chromosome segregation errors during meiosis-I in young mouse oocytes.**

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Mammalian oocytes are particularly prone to errors in chromosome segregation during their first meiotic division and, consequently, eggs often feature an aneuploid genome. Importantly, meiosis-I chromosome segregation errors increase in frequency with advancing maternal age, and are a leading cause of the age-related decline in female fertility, spontaneous abortions, and developmental disabilities such as trisomy 21. Yet, why oocytes from older women are more prone to these errors than oocytes from younger women is poorly understood. Kinesin family member 2B (KIF2B) is a specialised motor protein that has been implicated in preventing chromosome segregation errors in mitotic cells by driving microtubule depolymerisation in prometaphase. However, the role of KIF2B in oocytes has not been analysed. Given the extended period of prometaphase in oocyte meiosis-I, we hypothesised that KIF2B is required to protect against chromosome segregation errors. The specific objective of this study was to examine the impact of impairing KIF2B function during meiosis-I. To do this, we engineered a full-length dominant-negative mutant construct in which the motor domain of KIF2B is inactivated (KIF2B mutant- GFP) to specifically impair KIF2B function. Germinal vesicle stage oocytes from six week old mice were co-microinjected with mRNA encoding for KIF2B mutant-GFP and H2B-RFP, and protein localisation and chromosome movement was tracked in real-time using time-lapse 3D-projection confocal imaging during oocyte maturation. Oocytes were also fixed and stained with immunofluorescent markers to detail chromosome alignment and spindle architecture. We found that, in line with the expression pattern of KIF2B during mitosis, KIF2B-GFP localises to microtubule organising centres both in germinal vesicle stage oocytes and following germinal vesicle breakdown, and to the spindle poles and kinetochores during prometaphase and metaphase of meiosis-I. Strikingly, we find that 54% of KIF2B mutant-GFP expressing oocytes feature characteristic ‘lagging’ chromosomes during anaphase of meiosis-I (n=71), compared with 16% of GFP-only (n=32) and 19% KIF2B wild type-GFP controls (n=35) expressing comparable GFP levels (P<0.005). Interestingly, the lagging chromosomes in KIF2B mutant-GFP oocytes occur despite similar timings of meiosis, and normal chromosome alignment and spindle architecture at metaphase of meiosis-I (n=21-28 per group, P>0.05). At metaphase-II, on the other hand, chromosome alignment and spindle architecture is markedly altered, with 93% of KIF2B mutant-GFP eggs featuring chromosomes misaligned away from the metaphase-II plate, compared to 5% of GFP- only controls, and spindles displaying increased length (mean length 38 μm compared with 26 μm in GFP-only controls) (n=26-29 per group, P<0.001). Taken together, these findings implicate KIF2B as an essential spindle-regulating protein required to protect against chromosome segregation errors during meiosis-I in mouse oocytes. We speculate that defective KIF2B activity during oocyte maturation may contribute to the increase in chromosome segregation errors observed with advancing maternal age.

559. **The X-linked TEX11 gene regulates the genome-wide recombination rate and its mutations cause male infertility in humans.**


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Infertility is a worldwide reproductive health problem. As males are hemizygous for the X chromosome, mutations in the single-copy X-linked germ cell-specific genes may represent “hot spots” for infertility- causing mutations in men. TEX11, an X chromosome-encoded factor, is a constituent of meiotic recombination nodules. Our previous studies in mice have shown that TEX11 interacts with SYCP2, an integral component of the synaptonemal complex and plays a dual function in meiosis: chromosomal synopsis and crossover formation. Disruption of *Tex11* in mice leads to meiotic arrest and infertility in males. In the current study, we sequenced the TEX11 exons and their flanking intronic regions in 246 azoospermic men and 175 control men. We found one frameshift mutation, one splice site mutation, and five missense mutations in azoospermic men, but none of these were present in control men. By sequence alignment analysis, we found that three of these five residues are highly evolutionarily conserved among diverse species. To further investigate whether these mutations are causative, we developed a novel and powerful knockin strategy to generate and characterize mice bearing mutations.
analogous to these human TEX11 single amino acid substitutions. In this strategy, we engineered an autosomal Tex11 retrogene under the transcriptional and translational control of Tex19 through gene targeting in ES cells. The autosomal Tex11 retrogene rescued infertility in adult Tex11 knockout mice. By genetic modeling of human TEX11 missense mutations in mice, our results reveal that one TEX11 missense mutation found in an infertile man causes synopsis defects and reduced sperm count in mice. This mutation is most likely a genetic cause of infertility in azoospermic men. Together, TEX11 is a hot spot for causative mutations of infertility at a frequency of 1% in azoospermic men. In functional studies we identify TEX11 as a dosage-dependent regulator of meiosis and TEX11 modulates genome-wide recombination rate in both sexes in a dosage dependent manner. Our data indicate that TEX11 alleles with variable expressing levels or SNPs may contribute to variation in recombination rates between sexes, individuals, and species.

560. Fluoride Impairs Oocyte Maturation and Subsequent Embryonic Development in Mice.
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The damage caused by fluorosis is permanent, and has been recognized as a public health problem in a number of regions of the world. Although multiple studies provided evidence that sodium fluoride (NaF) elicits adverse effects on reproductive function, the effect of fluoride on female reproduction is not well understood. Therefore, the aim of the current study was to evaluate the effect of fluoride treatments on in vitro maturation and developmental potential of mouse oocytes. In the present study, female ICR mice were treated with a range of doses (0, 30, 60, and 150 mg/L) of NaF. After treatment, mice were superovulated to collect ovulated oocytes. The effects of NaF on oocyte quality, fertilization, and early embryonic development were evaluated, and the underlying mechanisms were investigated. Our results show that NaF treatment was associated with abnormal spindle configuration, actin cap formation, and cortical granule-free domain (CGFD) formation. Additionally, overexposure of mice to NaF notably reduced ATP production and mitochondrial membrane potential, further influencing in vitro fertilization (IVF) (91.5%; 82.8%; 66.3%; 62.4%; respectively) and subsequent embryonic development (54.6%; 45.2%; 28.7%; 15.2%; respectively). Additionally, significantly higher number of apoptotic cells was observed in the blastocysts of NaF-treated mice. NaF treatment increased the expressions of pro-apoptotic genes (Caspase 3 and Bax, P < 0.05) and reduced the expression of anti-apoptotic genes (Bcl-xL, P < 0.05). In conclusion, overexposure to fluoride in vivo was associated with a significant disruption of cytoskeletal dynamics and decreased oocyte quality, affecting the oocyte's subsequent fertilization and embryonic development. Results of this study provide a rationale for treating reproductive diseases such as infertility or miscarriage caused by environmental contaminants.

Keywords: Fluoride; actin; spindle; cortical granule-free domain; mitochondria; in vitro fertilization

561. Twenty-four hours of oocyte meiotic arrest using cAMP/cGMP modulators does not compromise development or quality of mouse embryos following in vitro maturation.
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In vitro maturation (IVM) of cumulus oocyte complexes (COC) results in reduced oocyte quality compared to in vivo oocyte maturation. Oocytes may lose competence after resuming meiosis too quickly when inter-cellular communication is abruptly disrupted upon removal of the oocyte from the follicle. Studies in mice and bovine have shown that a short 2 h period of pre-maturation in the presence of meiotic inhibitors, before IVM, enhances oocyte developmental competence. Our objective was to examine the effect of a prolonged pre-IVM period (24 h) in the presence of cAMP/cGMP modulators to inhibit meiotic resumption, compared to a short pre-IVM period (2 h) in mice. COC were collected from two month old outbred CF1 mice 48 h after PMSG (5 IU) in the presence (pre-IVM) or absence (control) of meiotic inhibitors (C-type natriuretic peptide, Cilostamide and Sildenafil). Pre-IVM COC were then placed in pre-maturation medium that also contained meiotic inhibitors (C-type natriuretic peptide). After 2 h or 24 h, pre-IVM COC were washed and transferred to our in house prepared, completely defined IVM medium for 18 h of maturation. Control COC were matured in the same IVM medium under identical conditions for 18 h without pre-IVM treatment. After pre-IVM and IVM, oocytes were fixed for assessment of nuclear maturation, or fertilized and cultured in vitro. Time-lapse images of embryo development were documented using the Embryoscope® (Vitrolife) and cell number and allocation of blastocysts was performed to investigate embryo quality. Results were analyzed by ANOVA. A short 2h pre-IVM period slows subsequent meiotic resumption, as 23.7 ± 6.3 % of the oocytes remain at the GV stage after 2h of IVM, whereas 100 % of control oocytes had resumed meiosis at this time point. On the contrary, meiotic resumption is not delayed after a 24 h pre-IVM followed by 2 h of IVM, as 100 % of oocytes had resumed meiosis. However, neither duration of pre-IVM had a negative impact on oocyte nuclear maturation to metaphase II at the time of fertilization (98 ± 2 % and 100%, respectively). A 2 h pre-IVM treatment improved oocyte developmental competence as demonstrated by increased embryo development. More (p<0.01) blastocysts (96 h of culture) developed in the 2 h pre-IVM treatment compared to control (71.9 ± 7.4% versus 53.3 ± 6.2% respectively). Nonetheless, there was no difference in blastocyst (96 h of culture) development in the 24 h pre-IVM treatment compared to control (65.7 ± 10 % versus 68.6 ± 4.5 % respectively). Time-lapse video imaging of embryo development shows no difference in developmental kinetics between the 2 h pre-IVM, 24 h pre-IVM and control. In addition, the proportion of Sox2 positive (inner cell mass) cells in hatching/hatched blastocysts was not different between 2 h pre-IVM and control, indicating that the quality of blastocysts produced following pre-IVM treatment is equal to that of IVM oocytes. In conclusion, a prolonged period of pre-IVM in presence of meiotic inhibitor does not compromise developmental competence of oocytes nor the quality of the resulting blastocyst, although 2 h of pre-IVM treatment improves oocyte competence, resulting in increased blastocyst yield after IVF.

Poster Topic Area 5: Sex determination, Ovarian function, Gonadal signaling, & Testis development and function
562. *Wnt4* Progenitor Cells are Essential for Mullerian Duct Formation and *Wnt4* Hypomorphic Allele Demonstrates a Key Role in Uterine Development.

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In mammals the Mullerian duct (MD) is the anlage for the development of the oviduct, uterus, and the upper part of the vagina, which are the main parts of the female reproductive tract. Several Wnt genes, including *Wnt4*, *Wnt5a*, and *Wnt7a*, are known to be important for the development of the MD and its derivatives. In particular the absence of *Wnt4* does not allow the formation of the MD at all. In this study, we demonstrate by the means of genetic tracing in mice that *Wnt4* serves as a factor required for the initiation of the MD forming cell migration. This notion is based on the finding that the protrusion forming MD tip cell is derived from the Wnt4 lineage. Application of anti-Wnt4 function blocking antibodies after initiation of the MD elongation demonstrated that Wnt4 is also necessary to maintain the MD cell migration. In the presence of the control IgGs, the MD had grown and reached the end of the Wolffian duct (10/10). Whereas the MD growth was completely or partially stopped (8/10) when the culture medium contained anti-Wnt4 antibodies. To examine the Wnt4 effect on cell migration properties we performed cell culture scratch wound healing assay. The relative value of 50% wound closure in serum-supplemented conditions was 4 hrs for the NIH3T3-Wnt4 cells and 8 hrs for the controls NIH3T3. An even more prominent difference in wound closure was seen in serum-free conditions. In NIH3T3-Wnt4 cells this process took 15 hrs, whereas in controls it took 64 hrs. The scratch wound healing assay with NIH3T3 cells expressing ectopic Wnt4 showed that Wnt4 markedly promoted cell migration in comparison to control. This data suggests that Wnt4 might be a factor that enhances and controls the migration of various cell types. In mice, carrying a hypomorphic Wnt4 allele (*Wnt4mCherryCreERT2*) with only a fraction of the Wnt4 activity left, the MD forms, but it is, however, defective. In contrast to the *Wnt4* knock-out mice, some of these mice survive to adulthood, but examination of their reproductive tract indicated that the uterine glands did not form, the myometrium is poorly differentiated, and they are prone to develop hydro-uterus. In conclusion, our study shows that Wnt4 is a pleiotropic signal that regulates several critical processes during MD and uterus development.

563. WITHDRAWN.

564. Sexually Dimorphic Establishment of Reproductive Tracts Requires COUP-TFII-Mediated Ductal Regression.

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The sexually dimorphic establishment of the reproductive tracts depends on the regression and differentiation of two primitive ducts (Müllerian and Wolffian ducts) into a single sex-specific reproductive tract. This sexual dimorphism is accomplished by the action of two testis-derived hormones: anti-Müllerian hormone (AMH) and androgen. In the male embryos, AMH induces regression of the Müllerian duct (the progenitor of the female reproductive tract) whereas androgen promotes the maintenance and differentiation of the Wolffian duct (the progenitor of the male reproductive tract). Female embryos lack these two hormones, thus the Müllerian duct escapes regression and differentiates into the female reproductive tract whereas the Wolffian duct degenerates. The critical function of AMH and androgen in the ductal regression is mediated primarily via their receptors, anti-Müllerian hormone type II receptor (*Amhr2*) in the mesenchyme of the Müllerian duct and androgen receptor in the mesenchyme of the Wolffian duct, respectively. We identified a novel transcription factor, chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII), as a factor required for the initiation of the Mullerian duct (MD) elongation. Where COUP-TFII is ablated using the tamoxifen-inducible *Wt1CreERT2* mouse model that targets gene deletion in the ductal mesenchyme. Regardless of the sex of the embryos, COUP-TFII ablation resulted in the retention of both Müllerian and Wolffian ducts. In the male *Wt1CreERT2* knockout embryos, the retention of the Müllerian duct implies that AMH action could be compromised. We found that AMH expression in the knockout testes was not altered. However, the expression of *Amhr2*, the receptor for AMH, was reduced significantly in the Müllerian duct mesenchyme. On the other hand, in the female knockout embryos, the retention of the Wolffian duct suggests an ectopic androgen action. We found no evidence of androgen production or aberrant androgen receptor expression of the Wolffian duct mesenchyme in the female *Wt1CreERT2* knockout embryos. Interestingly, the expression of sonic hedgehog (*Shh*), an epithelial morphogen in the Wolffian duct, was reduced in the *Wt1CreERT2* knockout female. Ablation of *Shh* using tamoxifen-inducible CreERT2 model also resulted in the Wolffian duct retention in the female knockout embryos, a phenotype similar to the *Wt1CreERT2* knockout. This result suggests that mesenchymal COUP-TFII modulates epithelial SHH in promoting the Wolffian duct regression in the female embryos. Based on these observations, we conclude that the ductal retention in *Wt1CreERT2* knockout mice is not caused by aberrant AMH and androgen production but is the result of local defects in the ductal mesenchyme. The mesenchymal COUP-TFII regulates AMHR2 in inducing the Müllerian duct regression in the male and modulates SHH in promoting the Wolffian duct regression in the female. This study not only uncovers COUP-TFII as a novel transcriptional factor that facilitates the sexually dimorphic establishment of the reproductive tracts, but also reveals a complex local regulatory network beyond the action of AMH and androgen. This work was supported by the National Institute of Health Intramural Research Fund.

565. Sex differences in first trimester placental gene expression.
Background: Certain adult diseases and conditions, which differ between the sexes, may have their origins in the fetal period. They can result from placental defects, which can occur from abnormal first trimester trophoblast invasion. Although term placenta can be used to understand the role of the placenta in understanding the contribution of sex, this does not provide a window into the early stages of placentaion. Since the gestational period provides its own unique influences on the development of potential disease states, it is important to understand the influence of sex early during gestation. Chorionic villous sampling (CVS) provides a unique opportunity to study gene expression during the earliest period of gestation in an ongoing pregnancy to determine if sex plays a role during early placentaion that can ultimately influence fetal origins of adult diseases.

Objective: To determine whether gene expression patterns in the first trimester placenta differ between male and female fetuses.

Methods: CVS samples were preserved in RNAlater and stored at -80°C. Total RNA was isolated from samples from 3 male and 3 female spontaneous conceptions. RNA-Seq libraries were constructed using Illumina TruSeq Stranded Total RNA LT with Ribo-zero kits, and libraries were generated using two different RNA amounts (200 ng and 800 ng respectively). 2 x 75 bp paired-end reads were generated using Illumina NextSeq 500 using High Output 150-cycle flow cells. The pooled libraries were run on three independent flow cells.

Results: The total number of fragments sequenced and percent of fragments mapping to the hg19 transcriptome between RNA-Seq libraries generated using 200 versus 800 ng of total RNA were highly similar, despite variation in sequence yield and accuracy across the three flowcell sequencing runs. The samples were found to separate primarily by sex, then by sample identity, and then by library prep. Both library preps (200 ng and 800 ng) revealed sex-specific transcript abundances, and small differences in biological coefficient of variation (BCV) did not dramatically affect ability to identify sex-specific changes with this small sample size. In total, 25 differentially expressed transcripts were identified as a function of sex in both library preps. Of those 14 transcripts were encoded by autosomes.

Conclusions: These results demonstrate that even with a very small sample size, significant differences can be detected in gene expression patterns in first trimester trophoblasts from male vs. female conceptions. Future studies with greater numbers of samples will allow us to identify a larger pool of genes that are differentially expressed during placentaion in male vs. female pregnancies. This provides a window into the influence of sex on early placentaion that could explain some of the sex differences in certain adult disease states.

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Recently, aspiration of blastocoel fluid or collection of blastomeres with subsequent sex analysis by PCR technique has been performed.

Gain of pregnant rats that receive 100 mg/kg of isoflavone after the 18th day. The administration of Isoflavone, at doses of 10 mg/kg and 100 mg/kg, significantly reduces the duration of pregnancy, when compared to the control group, and it also decreases the number of pups/litter, due to possible post-implantation loss. The treatment with 10 mg/kg of isoflavone increases the body weight on the PND1 in the male offspring. Male offspring exposed to 100 mg/kg of isoflavone shows lower initial body weight. Also, there is an increase in body mass on male offspring from mothers who receive 10 mg/kg.

The male offspring exposed to 10 mg/kg of Isoflavone during intrauterine life shows a delay in puberty onset. Conclusion: Considering the results of this work is possible to say that isoflavone, at 10 mg/kg and 100 mg/kg, shows estrogenic activity. Effects such as delayed puberty onset may be caused by a negatively feedback decreasing the circulating level of testosterone, the principal male sex hormone. Caution should be taken until more extensive studies be conducted to evaluate the possible effects of isoflavone consumption, especially at critical periods of development.

568. Live cell RNA imaging in transferrable equine embryos suitable for transfer – a tool for non-invasive embryo sexing?
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In horses, sex determination in embryos collected for transfer is possible by the use of invasive techniques. Only recently, aspiration of blastocoel fluid or collection of blastomeres with subsequent sex analysis by PCR technique has been performed successfully. However, these techniques request expensive and complex laboratory equipment. For a wide-spread use of embryo transfer in the horse, a technique allowing easy and fast sex determination in transferrable embryos in the field would offer many advantages. In cell culture experiments smart flaire probes have proven to detect specific RNA in live cells. Smart flare probes consist of a central gold particle coated starlike with RNA specific for cellular target RNA. Binding to the target leads to release of the complementary RNA of the partial double stranded part hybridized to the immobilized target specific RNA. The fluorophor bound to this complementary RNA after release from the particle starts emitting specific fluorescence. The aim of the present study was to investigate the uptake of Smart flare probes by equine embryos collected at transferrable stages. This was done by determination of specific fluorescence and by transmission electron microscopy for detection of central gold particles of the probes in embryonic tissue. Embryos were flushed on days 7 (n=3) and 8 (n=3) after ovulation (= day 0). The embryos were graded, their size was determined and subsequently embryos were incubated in 400 µl D-MEM 10% FCS covered with mineral oil. Immediately after incubation the fluorescence for Cy3 was determined. Then 20 µl of medium were exchanged against Smart flare Cy3 (Cyanin dye 3) uptake control (1:20 dilution with PBS without Ca2+ and Mg2+) and incubated for 24h at 38°C, 5% CO2. Subsequently Cy3 specific fluorescence was determined and the embryo finally fixed in 3% glutaraldehyde for transmission electron microscopy. In 2 embryos with zona pellucida, probe material was detected within blastomeres by fluorescence signal as well as by transmission electron microscopy. However, no probe material could be detected in 4 embryos that had already developed an acellular capsule. The preliminary results show that Smart Flare probes are able to pass the equine zona pellucida. This technique may be further developed for sex determination of early equine embryos by specific RNA probes. However, for analysis of embryos that already possess an acellular capsule, treatment for increasing permeability is necessary.

569. Receptor Isoform-specific Estrogen Signaling in Müllerian Duct Differentiation of the American Alligator.
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Perturbation of endocrine signaling during critical developmental windows has been implicated in adult reproductive disorders. This “developmental origins of disease” paradigm is the basis for our investigation of the role of estrogen signaling in embryonic differentiation of female reproductive tract in the American alligator. To this end, we first investigated pathways leading to sex reversal. Alligator eggs incubated at a temperature that produces 100% males (33.5°C) were treated with estradiol-17beta (E2) or 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), a specific agonist for estrogen receptor alpha (ERα), at a stage just prior to sex determination. E2 induced 100% sex reversal, indicated by Müllerian duct presence and gonadal histology. PPT treatment induced 100% gonadal sex reversal and abnormal enlargement of the Müllerian duct. Histological analysis indicated precocious glandular development in these tissues. Quantitative RT-PCR expression assays for steroid hormone receptors revealed significant downregulation of ESR1 and significant upregulation of progesterone receptor in oviductal tissue from PPT treated embryos. Receptor isoform-specific estrogen signaling was further studied by treating embryos incubated at a temperature that produces 100% females (30.5°C) with E2, PPT and ERb specific agonist WAY 200070. Only PPT treatment induced the previously characterized oviductal phenotype. Further investigation via immunohistochemistry and RT-QPCR continues to reveal significant differences in PPT-treated oviductal tissue. The results of this study provide insight into the factors critical for healthy reproductive system formation in this sentient species.

570. Comparative Mammary Gland Development in Male and Female Harlan Sprague Dawley Rats from Bud Development to Adulthood.
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2015 Abstracts – Page 224
Previous studies have shown that the male rat mammary gland (MG) is sensitive to ethinyl estradiol and genistein, yet little else is known about the effects of endocrine disrupting chemicals (EDCs) in male MGs. To enable future EDC testing, we compared the normal development of the MG in male and female Harlan Sprague Dawley (HSD) rats, which has not been previously documented. Between gestational days (GD) 15.5-21.5, cross sections of male and female fetuses were collected to capture the progression of mammary bud development. On post natal days (PND) 1, 4, 8, 12, 15, 21, 33, 46 and 70, the 4th and 5th MGs were collected for hematoxylin and eosin staining, MG whole mount evaluation, and immunohistochemical staining for the estrogen (ERα), progesterone (PR) and androgen (AR) receptors. We show normal mammary bud development between males and females are similar from GD 15.5 through GD 18.5. At GD 18.5, the skin above the female MG bud begins to invaginate, and is unchanged in the males. By GD 21.5 both sexes have branching ducts in the fat pads of the MG with the beginning of nipple formation in the female. We confirmed that the female MG undergoes similar developmental time points to other rat strains, the ducts and alveoli have a distinct lumen lined by a single layer of cuboidal epithelial cells with a scant cytoplasm. The male MG is structurally and morphologically similar to the females before the onset of puberty. After puberty, the male MGs develop large vaculated lobules with a pseudostatified or stratified epithelium and no visible lumens, similar to other sexually mature male rat strains. ERα is more highly expressed in the developing female MGs than the males and AR is more highly expressed in the males than the females. PR is only present in female MGs. Because of the early life similarities in MG development between sexes, male rat MG development may serve as suitable marker for endocrine disruption. These studies enhance our understanding of mammary developmental differences between sexes and will aid in interpreting effects of EDCs on male and female MGs in the HSD strain.

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571. The technology of the artificial ovary is a useful tool to evaluate the effect of anticancer drugs on preantral follicles. Denise D. Guerreiro1, Laritza Lima2, Adeline Carvalho1, Giovanna Rodrigues1, Cláudio Campello1, José Ricardo Figueiredo2, Vílceu Bordignon1, Ana Paula Rodrigues1.

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Despite the increase, the number of women who survive after cancer treatment is also growing. However, one of the principal consequences of chemotherapy is premature ovarian failure (POF). The aim of this study was to use the technology of the artificial ovary as an in vitro model to evaluate the toxicity of two anticancer drugs, doxorubicin (DXR) and paclitaxel (PTX), on the integrity and development of ovarian follicles. Fragments of the ovarian cortex of goats were cultured in vitro for 1 or 7 days in α-MEM supplemented with different concentrations of DXR (0.003, 0.03, or 0.3 µg/mL) and PTX (0.001, 0.01, or 0.1 µg/mL). Analyses were performed before and after culture to evaluate tissue integrity by classical histology, apoptosis by TUNEL assay, DNA laddering kit and detection of active caspase 3, and DNA damage by immunodetection of phosphorylated histone H2A.x (H2AXph139). Both DXR and PTX reduced the number of morphologically normal primordial follicles and developing follicles. Positive staining for TUNEL and active caspase 3 was detected in all the samples, but greater number of TUNEL positive follicles was observed in samples treated with 0.1 µg/mL PTX (P<0.05). Findings from this study suggest that PTX is more toxic for preantral follicles than DXR. Both PTX and XR have dose-dependent cytotoxicity effects on primordial follicles. Finally, we propose the technology of the artificial ovary as a useful experimental model for assessing toxic effects of chemotherapeutic agents on ovarian preantral follicles.


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Fertility of cattle is negatively affected by the incidence of anestrus. Duration of post partum anestrus can affect production if it resulted in calving intervals of > 365 day. In Egypt, anestrous and low conception rates were major causes of economic loss and culling in buffalo and cattle herds, leading to annual losses of 200 - 300 millions Egyptian pounds. Measurable amount of sex steroids were detected in the feces of cows. Fecal hormonal analysis would be a helpful non-invasive method for diagnosis of reproductive status in both dairy and beef cows. The aims of this study was to detect the main causes of infertility in native Egyptian cows, and to compare concentrations of sex steroid hormones in serum or fecal samples. Native Egyptian cows which calved since more than one year without showing estrous signs were considered to be infertile. A total number of seventy-two infertile native Egyptian cows, 3 - 8 years old and weighing 260 - 450 kg were used. Animals were subjected to thorough clinical examination for detection of different causes of anestrus. Case history, general and rectal examinations were done. Blood and fecal samples were collected at the same time for analysis of serum progesterone (P4), serum estradiol (E2), fecal P4, and fecal E2 using radioimmunoassay. The incidence of each cause of anestrus in infertile cows was 47.22 % as ovarian inactivity (OI), 51.39 % as prolonged luteal phase (PLP), while only 1.39 % was diagnosed as cystic ovarian degeneration (COD). About 70.60 %, 67.60 % of cows diagnosed as OI, PLP respectively were of B.C.S < 3. The levels of serum P4 were 0.20 ng/ml and 3.29 ng/ml for OI and PLP, respectively, and fecal P4 levels were 14.44 ng/g and 326.45 ng/g for OI and PLP, respectively. There was a highly significant relation between serum and fecal P4. While the levels of serum E2 were 2.48 pg/ml and 1.38pg/ml OI and PLP respectively, and fecal E2 level was 0.20 ng/g for both OI and PLP. From these results we could conclude that OI and PLP were the two main causes of anestrus in Egyptian native cows and that fecal P4 assay can be used as a non-invasive diagnostic tool for infertility in cows as fecal P4 level showed significant relation to that of serum, and changes in fecal P4 concentrations were significantly different according to the cause of infertility. Our methods were approved by the Animal Care and Use Committee of South Valley University, Faculty of Veterinary Medicine.
Aquaporins (AQPs) are a well-conserved family of small (~30 kDa) membrane channel proteins that facilitate rapid movement of fluids and have a unique tissue specific pattern of expression. These proteins pass through the lipid bilayer, allowing the passive water movement from side to side of the plasma membrane, which has a hydrophilic channel that is highly specific to the water molecule. Thirteen types of AQPs (AQP0-12) have been identified in mammals, on the basis of their permeability properties, they have been divided into three groups: (a) aquaporins—selectively permeating water consisting of AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8; (b) aquaglyceroporins—permeating water and glycerol consisting of AQP3, AQP7, AQP9, and AQP10; and (c) superaquaporins—having poorly conserved asparagine-proline-alanine (NPA) boxes consisting of AQP11 and AQP12. Recently we have observed the presence of AQP3 mRNA in antral follicles and a progressive expression of this protein in sheep ovarian follicles at different stages of development (primordial, primary, secondary, and antral), and this therefore provides a good starting point to demonstrate the correlation between protein channels and antrum formation in this species. However, the expression pattern of AQPs (3, 7, and 9) along the follicular development and its localization in different follicular compartments is unknown in the female reproductive system of sheep. Therefore, this study aimed to evaluate, by qPCR and immunohistochemistry respectively, the levels of mRNA and the immunolocalization of AQP3, AQP7, and AQP9 in large isolated ovine secondary follicles over a period of in vitro culture. Our analysis revealed that AQP3 and AQP9 were present predominately in follicles that exhibited antrum formation, suggesting a crucial role of these AQPs in the formation of the antrum. Interestingly, AQP7 was only expressed in follicles that had not formed an antrum by Day 12 of culture. In conclusion, the presence of protein channels (AQP3 and AQP9) seems to be essential for the formation of the antrum in isolated ovine secondary follicles cultured in vitro and thus plays an important role during folliculogenesis in this species.

574. Anti-Müllerian Hormone (AMH) Produced by Secondary Follicles is a Marker for Follicle Growth and Oocyte Maturation during Encapsulated 3-Dimensional (3D) Culture in Primates.
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The 3D culture of primate preantral follicles to the small antral stage (0.5-1.5 mm) provides a model to study indices of folliculogenesis in vitro. During culture, secondary follicles (125-225 μm) are heterogeneous in their capacity to produce AMH, with levels correlated positively with growth rates. Therefore, studies were conducted to evaluate AMH as a marker for follicle growth and oocyte maturation in vitro. Ovaries were obtained from rhesus macaques (4-12 yrs; n = 35). Secondary follicles were isolated, encapsulated into alginate, and cultured in supplemented medium with follicle-stimulating hormone (FSH) at 5% O2 for 5 weeks. Media from weeks 1 and 2 were analyzed for AMH. Follicles that reached the antral stage were treated with recombinant human chorionic gonadotropin for 34 hrs, and oocyte maturation was evaluated. The mixed effect model was used to compare AMH levels, with a bootstrapping method to estimate a 95% cutoff value. Ovaries from an additional 4 monkeys were used for small antral follicle isolation and secondary follicle culture. In vitro- and in vitro-developed antral follicles were pooled per animal for RNA extraction and RNA sequencing. The mRNA levels of selected genes were validated. RNA sequencing data were analyzed using DESeq software. A permutation test was used to compare validated mRNA levels. Differences were considered significant at P < 0.05. Two distinct cohorts of viable follicles were observed based on their growth in culture. Non-growing follicles remained similar in size to the initial secondary follicles without antrum formation, whereas growing follicles increased their diameters with antrum formation. Metaphase II (MII) oocytes were only produced by growing follicles. AMH levels produced by growing follicles at week 1 were higher (P < 0.01) than those of nongrowing follicles (1.5 ± 0.1 vs. 0.7 ± 0.1 ng/ml). Over 95% of secondary follicles producing > 1.4 ng/ml AMH at week 2 grew to the antral stage at week 5. With a cutoff of 1.4 ng/ml AMH, 85% of nongrowing follicles can be filtered out by sacrificing 5% of growing follicles. Growing follicles that generated MII oocytes secreted greater (P < 0.01) amounts of AMH at week 2 than did those yielding germinal vesicle oocytes (6 ± 0.9 vs. 3 ± 0.3 ng/ml). The mRNA expression profiles were comparable between in vivo- and in vitro-developed antral follicles; only 917 out of 15,236 (6%) annotated genes differed > 2-fold (P < 0.05). There were no differences in mRNA levels for genes that are critical for gonadotropin signaling (luteinizing hormone/choriogonadotropin receptor, FSH receptor), AMH signaling (AMH, AMH receptor), or oocyte quality (growth-differentiation factor 9, bone morphometric protein 15). However, compared with in vitro-developed follicles, steroid 17α-hydroxylase levels were lower (P < 0.05), while aromatase levels were higher (P < 0.05) in in vitro-developed follicles. Thus, primate secondary follicles are heterogeneous in terms of their ability to grow and mature in vitro. AMH levels produced by secondary follicles during the first 2 weeks of culture correlated positively with further follicle growth and oocyte maturation. The cutoff value of 1.4 ng/ml AMH can be used to remove non-growing from growing follicles during the initial week of culture. The culture environment alters follicular capacity to produce steroids. Research supported by NIH UL1DE019587, RL1HD058294, PL1EB008542, 2K12HD043488, 8P51OD011092, Collins Medical Trust, American Society for Reproductive Medicine.

575. Ovulation inducing factor/nerve growth factor (OIF/NGF) increases vascularization and progesterone secretion during the early Corpus Luteum development in llamas.
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Ovulation Inducing Factor is a protein molecule that has been isolated and purified from seminal plasma of llamas and recently identified as beta Nerve Growth Factor (β-NGF); referred herein as OIF/NGF. Llama β-NGF from seminal plasma origin has been
576. SFRP4 Downregulates the Expression of Gonadotropin Responsive Genes in Granulosa Cells During Follicle Development.
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The WNTs are a large family of secreted signaling molecules that regulate a variety of ovarian processes, including follicle development, granulosa cell (GC) proliferation and differentiation, steroidogenesis and ovulation. Secreted Frizzled Related Proteins (SFRPs) are antagonists of WNT signaling, but the role of SFRPs in the ovary is poorly understood, and SFRP2 in particular has not been studied. However, it is known that the expression of Sfrp4 and Sfrp2 is strongly induced in rodent GCs in response to gonadotropins, but for what purpose remain unknown. Our overall hypothesis is that SFRP4 and -2 act to modulate gonadotropin signaling in GCs during follicle growth and development in mice, and may function in a redundant manner. The objectives of the present study were 1) to determine the roles of Sfrp4 and -2 in GCs and 2) to characterize and compare the ovarian phenotypes of mice null for Sfrp4, Sfrp2 or both. The effects of SFRP4 and -2 on GCs were initially evaluated in a cell culture system in vitro. GCs from immature, eCG-primed wild-type mice were cultured in vitro and treated with recombinant SFRP4 or -2 proteins for 3h. Their effects on GC gene expression profiles were determined by microarray analyses. The results indicated that the addition of SFRP4 to GC cultured in vitro resulted significant (at least 2.0 fold) changes in expression levels of >3000 genes, including several important regulators of GC function and differentiation during follicle growth and development. Conversely, the addition of SFRP2 affected the expression of very few genes. Most interestingly, SFRP4 markedly suppressed the expression of FSH-responsive genes including Prl, Cyp19, Fshr and Lhcgr (P<0.05), while SFRP2 did not. To complement these in vitro findings, mice null for Sfrp4, Sfrp2 or both were studied. Sfrp4-/- females were found to be slightly hyperfollicular, as they produced ~1 more pup per litter on average relative to controls (P<0.05). Unlike Sfrp4-null mice, the fertility trials of Sfrp2-/- females indicated no difference in comparison to controls with regards to litter size (P>0.05). Interestingly, mice null for both Sfrp2 and 4 were also found to be slightly hyperfollicular, with no additional effect in comparison to Sfrp4-null mice on fertility. We then measured by real-time RT-PCR the mRNA abundance of important SFRP4- and -2 target genes (revealed by the microarray analyses) in GCs isolated from eCG-primed immature Sfrp4 -/- and control mice 0, 4, 8 and 12h after administration of an ovulatory dose of hCG. The expression of Prl, Cyp19, Fshr and Lhcgr was up-regulated in vivo only at time 0h in Sfrp4-/- mice in comparison to controls (P<0.05). In Sfrp2-/- mice, only mRNA abundance for Cyp19 was higher than controls, and only at time 0h (P>0.05). The expression of a number of LH- responsive genes involved in critical ovarian processes (including Areg, Ereg and Ptgs2) were found to be increased before and around the time of ovulation in GCs from Sfrp4 and -2 KO mice relative to wild-type controls (P<0.05). Taken together, our data suggest that these two SFRPs do not exert redundant roles in mouse GCs. SFRP4, functions as a negative regulator of female fertility, acting by suppressing the expression of a number of gonadotropin-regulated genes involved in follicle development, ovulation and luteinization. Ongoing experiments are testing whether SFRP4 acts to regulate gonadotropin responsiveness in granulosa cells and, if so, by which mechanism. Supported by RQR and CIHR, Canada.

577. Characterization of oviductal defects in mice deficient in miR-34b/c and miR-449.
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Female mice lacking two functionally redundant miRNA clusters, miR-34b/c and miR-449, are completely infertile. Given the normal folliculogenesis in these double microRNA knockout (dKO) females, the most likely cause of female infertility would lie in the oviductal defects because no eggs were ever observed and retrieved in the ampulla of the oviducts even after superovulation. To test this hypothesis, we examined the oviductal development, the histology and ultrastructure of the oviducts, as well as the oviductal functions, including transport of sperm from the isthmus to the ampulla and migration of the fertilized eggs towards the uterine cavity, in these mutant female mice. Our data revealed that the oviducts in dKO females displayed underdeveloped cilia, characterized by much fewer and shorter cilia, on the ciliated cells of the oviduct epithelia, as compared to wild type mice. Both sperm transport from the isthmus to the ampulla, and the migration of fertilized eggs and preimplantation embryos from the ampulla to the uterine cavity appeared to occur normally. Neither the swelling of the ampulla nor ovulated eggs were observed in the dKO females even after superovulation. Our data suggest that albeit underdeveloped cilia, the dKO oviducts can perform most of the physiological functions except egg capture. Therefore, we conclude that normal cilia are absolutely required for egg capture, but may be dispensable for sperm transport and migration of the fertilized eggs and
preimplantation embryo within the oviducts. Moreover, the two microRNA clusters control normal ciliogenesis in the oviductal epithelia.

578. The Molecular Link Between FSH and IGF-1 Signaling in Rat Granulosa Cells.
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Female fertility requires multiple hormones to regulate the maturation of ovarian follicles prior to ovulation. During maturation, follicle-stimulating hormone (FSH) is known to synergize with the insulin-like growth factor-1 (IGF-1) pathway in granulosa cells (GCs) located within follicles to regulate proliferation, steroidogenesis, differentiation, and resistance to apoptosis. However, the cross-talk that links FSH and IGF-1 signaling pathways remains unclear. We utilized serum-free primary cultures of rat GCs obtained from preantral follicles to elucidate the intersection of FSH and IGF-1 pathways and understand how FSH activates the IGF-1 pathway at subthreshold IGF-1 concentrations. Our results show that IGF-1 signaling in rat GCs follows the classical canonical pathway: the IGF-1 receptor (IGF-1R) Tyr phosphorylates insulin receptor substrate 1 (IRS1) YMXM motifs to facilitate the binding and activation of phosphoinositide 3 kinase (PI3K) which then activates AKT, a kinase central to many cell processes. Evidence of this pathway progression includes the ability of the IGF-1R selective antagonist NVP-AEW541 to block both FSH- and IGF-1-dependent IRS1 YMXM phosphorylation and activations of PI3K and AKT. Also, co-immunoprecipitation results show that IGF-1R is associated with IRS1 both in the absence and presence of FSH. Furthermore, transduction of GCs with an adenosine expressing IRS1 in which Tyr residues within YMXM motifs are mutated to Phe attenuates the activation of PI3K in response to FSH and IGF-1. In a series of media washout experiments and an IGF-1 ELISA, we have shown that rat GCs constitutively secrete approximately 1 ng/mL IGF-1 that is necessary but not sufficient to activate PI3K/AKT in response to FSH. The IGF-1R is primed by secreted IGF-1, but activation of downstream targets, including IRS1 and PI3K/AKT, remains restrained in the absence of FSH. Although human GCs express IGF-2 rather than IGF-1, we predict that IGF-2 facilitates the same IGF-1R priming phenotype in human GCs based on the ability of IGF-2 to activate IGF-1R and the conservation of IGF-1R in mammalian GCs. Interestingly, FSH does not regulate IGF-1R kinase activity as indicated by IGF-1R Tyr phosphorylation, but FSH does regulate IRS1 YMXM phosphorylation and PI3K/AKT. Protein kinase A (PKA) coordinates intracellular responses to FSH in GCs. We previously reported that the PKA selective inhibitor PKI blocks FSH-stimulated IRS1 YMXM phosphorylation and subsequent activations of PI3K and AKT. Our current results show that FSH, in a PKA-dependent manner, potentiates the stimulatory actions of IGF-1 downstream IGF-1R, even at saturating concentrations of IGF-1. Our results also indicate that PKA intersects the IGF-1 pathway in GCs to stimulate increased IRS1 YMXM phosphorylation, leading to the increased activations of PI3K and AKT in response to FSH. In this manner, PKA provides the synergistic effect of FSH on IGF-1 signaling. Taken together, our findings have revealed more of the intricacies of follicle maturation and female fertility as well as uncovered a unique signaling paradigm in the IGF-1 pathway. With IGF-1 expression nearly ubiquitous in humans and IGF-1R overexpression common in many forms of cancer, this novel concept of a primed pathway manipulation by PKA could have a profound impact on how we view IGF-1 signaling in not only female fertility, but also in non-reproductive tissues and various disease states. Supported by HD062053 and HD065859 (to MHD) from the NICHD and T32GM083864 from the NIGMS.

579. Effect of omega-3 fatty acids on lateral mobility of prostaglandin F₂α (FP) receptors on bovine luteal cell plasma membrane.
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Lipid microdomains are regions on the plasma membrane of cells, enriched with cholesterol and sphingolipids ranging in size from 10 – 200 nm in diameter. These structures may serve as platforms to facilitate co-localization of intracellular signaling proteins during agonist-induced signal transduction. Recent in vitro studies from our laboratory have shown that omega-3 fatty acids in fish oils disrupt lipid microdomain spatial distribution and subsequent prostaglandin F₂α signaling in bovine luteal cells. Therefore, we hypothesize that omega-3 fatty acids alter lipid microdomains in bovine luteal cells affecting FP receptor mobility preventing co-localization with downstream signaling proteins. This study examined the effects of omega-3 fatty acids on lateral mobility of FP receptors in bovine luteal cells. Beef cows were stratified by body weight and randomly assigned to receive a corn gluten meal supplement (n=4; 6% dry matter intake) or fish meal supplement, a rich source of omega-3 fatty acids, (n=4; 5% dry matter intake) for 60 days. Animals were individually fed a grass hay diet complemented with the assigned supplement which was isocaloric and isonitrogenous. Ovaries bearing the corpus luteum were surgically removed on day 10 – 12 post-estrus at approximately day 60 of the supplementation. A sample of luteal tissue (100 – 200 mg) was analyzed for fatty acid content using gas chromatography and remaining tissue was enzymatically digested. Mixed luteal cells (5 × 10⁷/dish) were incubated in 35 mm microscopy dishes containing Ham’s F-12 culture medium in an atmosphere of 95% air, 5% CO₂ at 37°C for approximately 12 h. Cells were then incubated with biotinylated FP receptor polyclonal antibody (2 µg/ml) for 15 min, washed, and incubated with streptavidin 605 QDots (0.5 nM) for 2 min. Individual FP receptors were recorded at 30 frames/sec for 30 sec using an epifluorescence microscope equipped with a high speed camera. Trajectories were generated using Spot Tracker software and mean square displacements were calculated to determine micro and macro diffusion coefficients. Luteal tissue collected from fish meal supplemented cows had a 150% increase in eicosapentaenoate (EPA) and 175% increase in docosahexaenoate (DHA), (major omega-3 fatty acids in fish oil; P < 0.05). Micro and macro diffusion coefficients of FP receptors were greater for luteal cells obtained from fish meal supplemented cows (P < 0.05). In addition, compartment diameter of domains was larger while residence time was shorter for receptors from luteal cells obtained from fish meal supplemented cows (P < 0.05). In conclusion, data indicate that dietary supplementation of fish meal increased omega-3 fatty acid content in luteal tissue which affected lateral mobility of FP receptors, increased compartment sizes and decreased residence times. This alteration in lipid microdomain size and residence time of FP receptors may decrease PGE₂ signaling in bovine luteal cells. Research supported by USDA 2013- 67015-20966 to PDB and NIH CA175937 to BGB.

580. The role of adenosine monophosphate activated kinase in luteal progesterone production.
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The luteolytic actions of PGF$_{2\alpha}$ are thought to be mediated through an elevation of cytosolic Ca$^{2+}$ and subsequently activation of protein kinase C (PKC). Our laboratory has examined the possibility that elevation of cytosolic Ca$^{2+}$ activates an additional intracellular effector, namely, calcium/calmodulin-dependent kinase 2 (CAMKK2). Expression of both CAMKK2 and its potential phosphorylation target, adenosine monophosphate activated protein kinase (AMPK) increased as the CL developed to a mature stage. Furthermore, activation of the PGF$_{2\alpha}$ receptor (FP) induced rapid phosphorylation of AMPK, which was blocked by a CAMKK2 inhibitor. In mature CL, the effect of PGF$_{2\alpha}$ on basal progesterone (P4) production was eliminated by addition of an AMPK-specific inhibitor, dorsomorphin dichloride (DM). Therefore, the objectives of this study were to explore alternative downstream components activated by the rise in cytosolic Ca$^{2+}$, such as AMPK, in developing and mature bovine CL and identify potential distal targets of AMPK, such as cholesterol transport mechanisms, when the FP receptor is activated during functional regression. In experiment 1, changes in basal P4 secretion in vitro were determined in response to PGF$_{2\alpha}$ activation via metformin (met) or 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) in developing (day 4) and mature (day 10) bovine CL. On d 4 or d 10 (d 4: n = 5; d 10: n = 5) CL were collected via supravaginal incision under epidural anesthesia. Luteal slices were incubated for 2 h at 37°C, shaking at 200 rpm with MEM (control), PGF$_{2\alpha}$ (1.0 μg/ml), met (10 mM), or AICAR (7.5 mM). Production of P4 in d 10 CL was not significantly affected by met or AICAR. However, P4 production in d 10 CL decreased with either met (0.39 ± 0.20; P = 0.006) or AICAR (0.40 ± 0.15; P = 0.0117) compared to control (0.99 ± 0.32). Direct activation of AMPK in mature CL resulted in decreased basal P4 production, similar to activation by PGF$_{2\alpha}$. Therefore, potential distal targets of AMPK during induced functional regression via exogenous PGF$_{2\alpha}$ were examined in the mature CL. Specifically, changes in protein expression of AMPK, phosphorylated AMPK (P-AMPK), steroidogenic acute regulatory (StAR), and cholesterol transport proteins (LDL, SRB-1, ACAT-1) were examined in experiment 2. Day 10 CL were removed supravaginally at 0 (n = 5), 2 (n = 5), or 4 (n = 5) hours after s.c. injection of PGF$_{2\alpha}$ or saline. Serum and luteal P4 concentration and content, respectively, decreased at 2 h (2.08 ng/ml vs. 2.96 ng/ml; P = 0.054) and 4 h (1.57 ng/ml vs. 2.96 ng/ml; P = 0.013) after PGF$_{2\alpha}$ administration (d10-h0 = 5.05 ng/ml; d10-h2 = 2.03 ng/ml; d10-h4 = 1.74 ng/ml) Protein expression of LDL decreased at 2 h (0.79 vs. 0.41; P = 0.09) and 4 h after PGF$_{2\alpha}$ injection (0.79 vs. 0.13; P = 0.004). Protein expression of AICAR (1.07 vs. 0.21, P = 0.010) and StAR (3.62 vs. 1.25, P = 0.01) increased 4 h after PGF$_{2\alpha}$. No difference in SRB-1, AMPK, or P-AMPK protein expression occurred 2 or 4 h after PGF$_{2\alpha}$. During induced functional regression, alterations in LDL and additional cholesterol transport proteins, accounted, at least in part for the decrease in luteal and serum P4 concentrations. In conclusion, developmental differences in signal transduction associated with FP, specifically CAMKK2 and AMPK, contribute to differences in the ability of PGF$_{2\alpha}$ to induce functional luteal regression in mature, but not developing, bovine CL. Furthermore, cholesterol transport via LDL might be the target of this luteal developmental difference.

581. **Fish oils attenuate prostaglandin F$_{2\alpha}$-induced apoptotic gene expression in bovine luteal cells.**

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Progesterone is a major steroid hormone that is secreted by the corpus luteum (CL) which establishes and maintains pregnancy in the cow. Prostaglandin (PG) F$_{2\alpha}$, is the endogenous luteolysin secreted from the uterus causing regression of the CL in the non-pregnant cow. PGF$_{2\alpha}$ binds to a G-protein coupled receptor on luteal cells and initiates an intracellular signaling cascade that increases expression of apoptotic genes resulting in involution of the CL. Often viable embryos fail to adequately inhibit PGF$_{2\alpha}$ synthesis resulting in the loss of pregnancy. Fish oils enriched with omega-3 fatty acids have been reported to attenuate agonist-induced signaling in many cell types. Omega-3 fatty acids might be a novel method to reduce PGF$_{2\alpha}$ signaling in early pregnant animals and preventing early embryonic loss. This study examined the effects of fish oils on PGF$_{2\alpha}$-induced expression of apoptotic genes using real time quantitative PCR. Bovine corpora lutea were obtained from a local slaughterhouse (n=4) and enzymatically digested using collagenase. Mixed luteal cells were incubated in T-75 culture flasks containing Ham’s F-12 culture medium supplemented with 5% fetal calf serum, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), 100 U/ml penicillin, 0.1 mg/mL streptomycin, and 0.25 mg/ml amphotericin B (pH 7.34) until confluency in an atmosphere of 95% air, 5% CO$_2$ at 37°C. Cells were then seeded and incubated in six well culture plates for 48 h in medium alone or in 0.03% fish oil to incorporate omega-3 fatty acids into the plasma membrane. Cells were washed and treated with or without 10nm PGF$_{2\alpha}$ analog in triplicate for 0, 6, 12, or 24 h to examine effects of fish oil on PGF$_{2\alpha}$ induction of apoptotic genes. Total RNA was extracted from cultured cells and cDNA was generated. Bovine specific primers were used to amplify the target genes Bax, Bcl-2, and Caspase-3 and the reference genes β-actin and GAPDH. Fold expression of target genes was determined using the 2$^{-\Delta\Delta Cq}$ method using the 0 time point as the reference point. PGF$_{2\alpha}$-induced expression of proapoptotic genes (Bax and Caspase-3) in bovine luteal cells were significantly reduced in fish oil treated cells (p < 0.05). The ratio of Bax to Bcl-2 (a lower ratio indicating healthy cells) was reduced for cells treated with fish oil as compared to controls by 0.79 vs. 0.13 (p < 0.05). Therefore, the inclusion of fish oils enriched with omega-3 fatty acids in the diet of breeding beef and dairy cows may reduce PGF$_{2\alpha}$-induced gene expression of apoptotic genes in bovine luteal cells and enhance reproductive performance. Research supported by USDA 2013-67015-20966 to PDB.

582. **Evaluation of the Stress Response of Mares after Induction of Luteolysis with Two Different PGF$_{2\alpha}$ Agonists.**

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Treatment of mares with PGF$_{2\alpha}$ agonists is routine in equine breeding management. Adverse effects of PGF$_{2\alpha}$ have been reported. Treatment with prostaglandin analogs reduces these side effects substantially. However, it remains to be elucidated whether usage of prostaglandin analogs still causes a stress response and thus affects animal welfare. In the current study, in addition to the luteolytic effect of PGF$_{2\alpha}$ agonists a potential stress response was monitored by assessment of cortisol in saliva as well as heart rate and heart rate variability (HRV). Luteal phase mares (n=8) were treated with two different prostaglandin analogs in the minimal dose recommended by the manufacturer. Mares were regularly checked for estrus and ovulation by transrectal ultrasonography. They received a single injection of either luprogestrol (3.75 mg im; Prosolvin; Virbac, Bad Oldesloe, Germany; LUP), R(+)cloprostenol (22.5 μg im; Genestran; aniMedica, Senden, Germany; CLO) or 0.9% NaCl (0.5 ml im; CON) on day 8 after ovulation in three independent estrus cycles. Treatment order was alternated (cross-over design) and always one cycle was left untreated between experimental cycles. For the experiment, a catheter was placed in one jugular vein and mares were fitted with an equine heart rate monitor (S 810i, Polar, Kempele, Finland) for continuous...
measurement of beat-to-beat (RR) intervals. Saliva for cortisol and blood for progesterone determination were collected at 30 min intervals from 60 min before to 120 min after treatment. Mares' behavior was monitored and respiratory rate and rectal temperature were recorded. Heart rate and HRV parameters SDRR (standard deviation of the RR interval) and RMSSD (root mean square of successive RR differences) were determined using Kubios HRV 2.2 Software (Biosignal Analysis Group, University of Kuopio, Finland). Statistical analysis was performed with the SPSS Statistics 21 software (IBM-SPSS, Armonck, NY) with the General Linear Model for repeated measures. Injection of either LUP or CLO, but not of CON resulted in the decline of plasma progesterone concentration to baseline concentrations within two days after injection of LUP and CLO, but not of CON (immediately before injection: CON 20.6±1.4, LUP 21.9±1.9, CLO 21.7±2.4 ng/ml; D2 after injection: CON 9.1±0.5, LUP 1.4±0.3, CLO 1.2±0.2 ng/ml; time: p<0.001, treatment: p=0.01, time x treatment: p=0.05). LUP and CLO, but not CON significantly increased salivary cortisol concentration (immediately before: CON 1.3±0.2, LUP 1.4±0.3, CLO 1.4±0.3 ng/ml; 60 min after injection: CON 1.0±0.3, LUP 8.0±1.4, CLO 4.2±0.7 ng/ml; time: p<0.01, treatment: p=0.001, time x treatment: p=0.001). Salivary cortisol almost returned to baseline concentrations within 120 min of injection. No effect of treatment on respiratory rate was detected. RR interval increased over time (p<0.05) independent of treatment (treatment: n.s., time x treatment: n.s.), but no changes in SDRR and RMSSD were detected. No behavioral changes, but liquefaction of fecal consistency in 50% of treated mares were observed. It can be concluded that injection of both PGF2α agonists resulted in reliable luteolysis in luteal phase mares. The injection caused a transient increase in cortisol release that may be related to induction of pituitary ACTH secretion caused by the PGF2α agonists because no other relevant signs of a physiologic stress response were recorded.

583. Improving RT-qPCR Normalization for the Equine Ovary by Using Novel Human Reference Genes. Dragos Scarlet1, Reinhard Ertl2, Christine Aurich1, Ralf Steinborn2.

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So far, there is a lack of reliable reference genes for the equine ovary. According to the current trend, several candidate reference genes are selected for the biological context which is being studied (e.g. n ≤ 8), their expression uniformity is assessed in samples of the experimental setup and a subset of them is finally recommended. However, this method neither considers exons being differentially expressed in a specific context, nor other changes that may occur, like polyadenylation or alternative splicing. We hypothesized that genes top-ranking in expression stability across a wide panel of human tissues according to RNA sequencing, including the ovary, could help to improve RT-qPCR normalization for the equine ovary. Ovaries (n = 14) were collected from euthanized (n = 4) or slaughtered (n = 3) adult mares. For gene expression analysis, fresh ovary biopsies were embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Alphen aan den Rijn, Netherlands), snap-frozen in liquid nitrogen and then stored at -80 °C less than six months. Eleven reference genes, namely Dragos Scarlet1, Reinhard Ertl2, Christine Aurich1, Ralf Steinborn2.

584. Secretion of progesterone by ovine granulosa cells: effects of nitric oxide (NO) and plane of nutrition. Anna T. Grazul-Bilska1, Casie S. Bass2, Samantha L. Kaminski2, Dale A. Redmer2.

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We have recently demonstrated that diet and/or in vivo arginine (Arg) supplementation (a precursor for proteins, NO and polyamines) affected selected ovarian functions such as endocrine activity, ovulation rates and luteal function. However, the effects of plane of nutrition on in vitro progesterone (P4) secretion by granulosa (G) cells cultured in the presence or absence of a NO donor, endothelial NO synthase (NOS) inhibitor, Arg and/or LH have not been studied. We hypothesized that effectors of the NO system will differentially affect P4 secretion by G cells collected from ewes fed adequate, excess or restricted diets. Ewes were stratified by weight and metabolizable energy and 13% crude protein [dry matter basis]/kg BW, overfed (O; n = 13; 200% NRC requirements) or underfed (U; n = 14; 60% NRC requirements). Ewes were individually fed twice daily with pelleted diets, weighed weekly, and diet rations were adjusted weekly for each animal. Control ewes were fed to maintain BW and offered 760 g/day/50 kg BW. Estrus was synchronized by a 14-day treatment with CIDRs. Follicular development was induced by twice daily injections of follicle stimulating hormone on days 13 and 14 of the estrous cycle. On day 15 of the estrous cycle, the number of visible follicles was determined, follicular fluid (FF) and G cells were collected from follicles ≥ 3 mm in diameter. Then, G cells were cultured overnight in serum-containing (20%) DMEM medium (250,000 cells/well/ml) followed by 8 h incubation in serum-free DMEM medium with or without DETA-NONOate (NO donor; 0.1, 0.5 and 1 mM), L-NAME (NOS inhibitor; 0.01, 0.1 and 1 mM), Arg (1, 2 and 4 mM) and/or LH (100 ng/ml). Concentrations of P4 and estradiol-17β (E2) in FF and/or medium were determined using chemiluminescence immunoassay or radioimmunoassay. During the 10 week nutritional treatments, C ewes gained 1.9 ± 0.8 kg, O ewes gained 13.2 ± 1.2 kg and U ewes lost 7.3 ± 0.7 kg. The number of visible follicles was similar in all groups (27.2 ± 2.4/ewe). Concentration of P4 in FF was 10.7 ± 4.9, 12.9 ± 5.5 and 21.0 ± 9.8 ng/ml in C, O and U.

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Corpora lutea function is influenced by numerous regulatory factors including nutrition, hormones, and growth and/or angiogenic factors such as: vascular endothelial growth factor (VEGF) and its receptors Flt and KDR, angiopoietin 1 (ANGPT1) and 2 (ANGPT2) and their receptor Tie2, basic fibroblast growth factor (FGF)-2 and receptor FGF2R2, as well as placentomal growth factor (PGF). Arginine (Arg) is an amino acid and precursor for proteins, polyamines, and nitric oxide (NO); the latter being involved in the regulation of blood vessel function and angiogenesis. We hypothesized that Arg supplementation to non-reproductive ewes of different nutritional planes would impact P4 production and angiogenic factor expression at several stages of the estrous cycle. Therefore, the aim of this study was to determine if Arg treatment of control (C), overfed (O) or underfed (U) ewes would affect luteal function measured by luteal P4 content and gene expression of selected angiogenic and/or growth factors in CL at early, mid and late luteal phases. Ewes were categorized by weight and randomly assigned to one of three nutritional groups: C (100% of NRC requirements; 2.14 Mcal/kg; n=37), O (2xC; n=37), and U (60% of C; n=37) beginning 60 days prior to Arg treatment. Estrus was synchronized, and one day after CIDR removal ewes from each nutritional group were randomly assigned to one of two treatments; Arg (L-Arg-HCl; 155 µmol Arg/kg BW) or saline (~10 ml). Treatments were administered three times daily (0700, 1400, 2100 h) via jugular catheter beginning on the first day of the estrous cycle until tissue collection at the early or mid-luteal phases of the second estrous cycle, or the late-luteal phase of the first estrous cycle. A portion of CL from each ewe was snap-frozen for determination of P4 concentration using chemiluminescence immunoassay, and mRNA expression of 9 angiogenic factors using quantitative real-time RT-PCR. Luteal concentration of P4 was greater (P<0.001) at mid- than early- or late-luteal phases (10.5±0.8 vs. 7.3±0.9 and 6.1±0.8 µg/g of tissue) and was not affected by Arg- treatment or nutritional plane. VEGF mRNA expression was greater (P=0.02) at mid- than early- or late- luteal phase, was not affected by Arg-treatment or nutritional plane, and was positively correlated (P=0.0001) with Flt, KDR, ANGPT1, ANGPT2, FGF-2, Tie2, and PGF. For KDR mRNA, nutritional plane x Arg-treatment x luteal phase interactions (P=0.05) demonstrated that in mid-luteal stage expression was greater in 1) Arg-treated compared to saline-treated C ewes, and 2) Arg-treated C and U than O ewes similarly treated. ANGPT2 mRNA expression was less (P=0.01) at early- than mid- or late- luteal phases and was not affected by Arg-treatment or nutritional plane. Expression of Flt, ANGPT1, Tie2, FGF2, FGF2R and PGF was similar in all groups. The results demonstrate that 1) luteal P4 concentration is greatest during the mid-luteal phase, 2) VEGF and ANGPT2 mRNA expression depends on the stage of luteal development, and 3) KDR mRNA expression is dependent upon stage of luteal phase, nutritional plane and Arg. Thus, luteal phase, nutritional plane and Arg-treatment affect luteal P4 concentration and mRNA expression of select angiogenic factors. The mechanisms of regulation of mRNA expression of angiogenic factors remain to be elucidated. Supported by USDA- AFRI grant 2011-67016-30174, and Hatch Projects ND01754 and ND01748 to ATGB and DAR.

586. Granulosa cell-specific Brcal loss alone or combined with Trp53 haploinsufficiency and transgenic FSH expression fails to induce ovarian tumors.

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Brcal mutations are associated with ovarian cancer. Previous studies reported that targeted murine granulosa cell (GC) Brcal loss caused ovarian/uterine tumors resembling serous cystadenomas, however tumor pathogenesis was complicated by ectopic (eg. pituitary) Brcal disruption and altered estrous cycling in this model. To selectively study ovary-specific Brcal disruption, we used transgenic (Tg) AMH.Cre mice with proven ovary and GC-specific Cre activity to target Cre/loxP-mediated Brcal disruption. Also, ovary-specific Brcal loss (denoted B) combined with global Trp53 haploinsufficiency (denoted T) and elevated FSH (Tg FSH, denoted F) expression as a multi-hit strategy to study genetic and hormonal factors associated with ovarian tumorigenesis. Twelve month old females grouped into B, BF, BT and BTF genotypes were compared to aged-matched control (C) or F females (n = 5-10/group). Ovary weights were significantly increased approximately 40-60% in all B genotype females (2-way ANOVA, B effect P < 0.001, F effect P = 0.52). Likewise, ovarian stroma was significantly increased approximately 35% in B females (2-way ANOVA, B effect P < 0.001, F effect P < 0.57). Genotype B ovaries also exhibited unhealthy follicles containing higher levels of pyknotic/irregular-shaped granulosa cells (2-way ANOVA, B effect P < 0.001, F effect P = 0.52) relative to equivalent follicles in controls. Consistent with reduced follicle quality, total corpora lutea (CL) numbers were significantly decreased by at least 75% in all B carrying groups vs C females (2-way ANOVA, B effect P < 0.001, F effect, P = 0.80). No detectable ovarian/uterine tumors were found in any B females, regardless of combined T and/or F genotypes. Estrous cycling stages (vaginal smearing 5-12 days) were highly variable in aged female groups, and were not significantly
altered in B vs C females, contrasting with the longer proestrous stage found in the previous Brca1 GC-modified model. The previous Brca1 model also exhibited an increased preovulatory (proestrus);postovulatory (metestrus) stage ratio, relative to control mice. In contrast, BT, F, BF and BTF females displayed significant reduced (56-81%) preovulatory:postovulatory ratios vs C mice (1-way ANOVA: BT, P < 0.005; F, P < 0.05; BF, P < 0.001; BTF, P < 0.001), which may reflect the decreased ovulation rate in females carrying the B mutation. Our findings reveal that specific intra-follicular Brca1 loss impaired ovarian follicle health and CL number, and increased ovarian stroma. However, follicular Brca1 modification alone or combined with cancer- promoting genetic (Tp53 loss) and endocrine changes (high serum FSH), was not sufficient to cause ovarian tumors. Absence of ovarian tumours in our TgAMH.Cre-modified Brca1 model suggests that extra-ovarian rather than GC-driven defects were responsible for cystic tumors reported in the earlier Brca1 mutant model. Our findings show that the ovarian environment per se is remarkably resistant to tumorigenic changes (elevated FSH, GC loss of Brca1 and Tp53 for up to 12 months), supporting an emerging view of an extra-gonadal origin for apparently ‘ovarian’ cancers.

587. **MCM9 mutations are associated with ovarian failure, short stature and chromosomal instability.**


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Premature ovarian failure (POF) is genetically heterogeneous and presents as hypergonadotropic hypogonadism as part of a syndrome or in isolation. We studied two unrelated consanguineous families with daughters exhibiting primary amenorrhea, short stature, and 46,XX karyotype. Genetic analyses, including whole exome sequencing (WES), were conducted to identify possible causes. WES utilized the Agilent SureSelectXT Human Exon v4+UTRs Kit (Agilent), 2x100 bp paired-end WES on an Illumina HiSeq 2500 (Illumina Inc.), and data was aligned to NCBI37/hg19. Gene variants were filtered to match an autosomal recessive inheritance model. In both families, analyses identified homozygous pathogenic variants in MCM9, implicated in homologous recombination and double strand DNA break repair. In one family, the MCM9 c.1732+2T>C variant alters the splice donor site at the end of exon 9 resulting in the production of three abnormal splicing products. Sequencing of the most abundant product resulted in exon 9 skipping. We generated an MCM9-GFP cDNA construct lacking exon 9 and transfected HEK293T cells with either wild-type MCM9-GFP or mutant MCM9-GFP (c.1732+2T>C), treated with 300 nM mitomycin C (MMC) for 6 hours, and GFP foci were counted in 15 cells per experiment. In damaged cells expressing wild-type MCM9-GFP, an average of 20±3 (SD) nuclear GFP foci/cell formed at sites of DNA damage. In MMC exposed cells expressing mutant MCM9-GFP, GFP signal was localized to the nucleus but foci could not be found at sites of DNA damage, indicating that MCM9 c.1732+2T>C, lacking exon 9, inhibits MCM9 from being recruited to sites of DNA damage. In the second family, MCM9 c.394C>T (p.Arg132*) results in a predicted loss of functional MCM9. Since MCM9 is implicated in DNA damage repair, we assayed the DNA repair capabilities of peripheral lymphocytes derived from an unrelated control, unaffected and affected individuals from both families. Cells were stimulated with phytohaemagglutinin (PHA), cultured with MMC (0 nM, 50 nM, 150 nM, or 300 nM), and harvested after 72 hours of incubation at 37°C. At least ten metaphase spreads/sample were evaluated for chromosome aberrations and breaks. Significantly more chromosomal breaks were observed in cells from affected individuals than from either family when compared to controls. This trend was more apparent as MMC concentrations increased. Treatment with 300 nM MMC revealed more chromosomal breaks in cells from affected individuals of either family when compared to control (p<1x10^-5 for all comparisons) or heterozygous family members (p<1x10^-6 for all comparisons). The c.394C>T variant appears to have a greater effect on DNA damage repair in cells treated with MMC at 50 nM MMC than those with the splice site variant (c.1732+2T>C). At higher MMC concentrations, repair appears to be equally impaired in cells from homozygous carriers of either MCM9 mutation. Thus, homozygous carriers of the MCM9 variants (c.394C>T or c.1732+2T>C) fail to repair DNA damage as effectively when compared to heterozygous carriers or wild-type individuals. Preferential sensitivity of meiosis to MCM9 functional deficiency, and compromised DNA repair in the somatic component, lead us to conclude that autosomal recessive variants in MCM9 cause a genomic instability syndrome associated with hypergonadotropic hypogonadism, ovarian failure, and short stature.

588. **Endothelial nitric oxide synthase (eNOS) protein expression in ovarian follicles during the estrous cycle in sheep: Effects of plane of nutrition and arginine.**


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Plane of nutrition has been shown to alter reproductive functions including follicular development and expression of selected genes in the ovary. Arginine (Arg) is a precursor for proteins, selected amino acids, nitric oxide (NO), and polyamines. NO is synthesized from L-Arg by the enzyme endothelial nitric oxide synthase (eNOS), and binds to its receptor resulting in vasodilation. Nitric oxide has been demonstrated to be involved in the regulation of ovarian function. We have recently shown that plane of nutrition and/or Arg supplementation affects selected endocrine and ovarian functions such as steroidogenesis, ovulation rate, and luteal function. However, effects of plane of nutrition and Arg supplementation on follicular development and eNOS protein expression in ovarian follicles have not been studied in detail. We hypothesized that plane of nutrition and Arg-treatment will affect eNOS protein expression during folliculogenesis. Therefore, the aim of this study was to investigate the role of the NO system in ovarian function by determining if Arg supplementation impacts eNOS protein expression in ovarian follicles in nutritionally compromised ewes. Ewes (n=97) were stratified by weight and randomly assigned into either maintenance (control, C; 100% NRC requirements, 2.4 Mcal of metabolizable energy and 13% crude protein [dry matter basis]/kg BW), excess (overnourished, O; 2 x C), or restricted (undernourished, U; 0.6 x C) diets 8 weeks prior to Arg-treatment. Ewes from each nutritional group were randomly assigned to one of two treatments: saline (~10 mL) or Arg (L-Arg-HCl, 155 μmol Arg/kg BW) which was initiated on day 0 of the estrous cycle and administered 3 times per day until ovary collection at the late-
luteal stage of the first estrous cycle, or early or mid-luteal stages of the second estrous cycle. Ovaries were fixed in Carnoy’s solution followed by immunohistochemical localization of eNOS, and image analysis of thecal tissue in healthy antral and atretic follicles. During nutritional treatment, C maintained BW, O gained 6 ± 1.2 kg, and U lost 14 ± 1.3 kg. eNOS was not detected in the granulosa layer, but was present in blood vessels of thecal tissue and other ovarian compartments. Expression of eNOS was greater (P<0.006) in U than C and O ewes (5.2 ± 0.3 vs. 4.2 ± 0.2 and 4.3 ± 0.3%), and was greater (P<0.0001) in healthy antral than non-healthy (atretic) follicles (4.2 ± 0.2 vs. 2.8 ± 0.2%). Arg-treatment did not affect eNOS protein expression in thecal tissue. Interactions among plane of nutrition, Arg-treatment and stage of the estrous cycle were detected (P<0.002) demonstrating lower expression of eNOS at the late than early luteal phase in C ewes, and inhibitory effects of Arg-treatment on expression of eNOS at the early luteal phase in O ewes and the mid-luteal phase in U ewes. These data show that: 1) eNOS is expressed in blood vessels in the ovary; 2) Arg inhibitory effects on eNOS expression in blood vessels of thecal tissue depends on plane of nutrition and stage of the estrous cycle; 3) eNOS expression is greater in blood vessels of thecal tissue in healthy antral than atretic follicles. Thus, plane of nutrition and Arg may affect follicular function via eNOS expression in ovarian blood vessels in sheep. Supported by U01-DA14992 and Hatch Projects NDS06254 and NDS0174 to ATGB and DAR.

589. Aberrant Folicule Growth in Cultured Ovarian Follicles From a DHT-induced Polycystic Ovary Syndrome (PCOS) Mouse Model.
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Polycystic Ovary Syndrome (PCOS) is a common cause of infertility, affecting 5-10% of women worldwide. Despite advances such as IVF and IVM, infertile women suffering from PCOS seeking fertility via assisted reproductive technologies (ART) achieve fewer pregnancies due to reduced conception and implantation and increased early miscarriage rates, reflecting suboptimal oocyte functional viability. We aimed to investigate the mechanisms of defective follicle selection and ovulation in PCOS using an in vitro follicle culture combined with our established long-term, DHT-induced mouse model of PCOS. Follicles, isolated from control or PCOS mice and cultured individually for 5 days, were divided into 4 different size ranges – late preantral (151-200µm, group 1, n=12-30), small antral (201-250 µm, group 2, n=9-18), large antral (251-350 µm, group 3, n=44-45) and preovulatory (351-450 µm, group 4, n=17-25). Following culture, group 1, 2 and 3 follicles from PCOS ovaries displayed decreased growth rates, while group 4 follicles exhibited a significant increase in growth rate compared to control follicles (all P < 0.01). Group 1 PCOS follicles displayed normal ability to form an antrum and maintain health (assessed by prevalence of pyknotic granulosa cells and oocyte health) during culture comparable with controls. Group 2-4 follicles from PCOS ovaries exhibited a decrease in the oocyte:follicle ratio (P < 0.01), indicating an altered pattern of follicle growth. Furthermore, the survival rates and health of PCOS follicles from groups 3-4 were significantly reduced (both P < 0.05). Our results show that although PCOS follicles cultured from the preantral stage (group 1 follicles) exhibit slower growth rates than control follicles, they retain normal follicle survival, oocyte health and oocyte:follicle ratio unlike those PCOS follicles cultured from later stages. This may reflect that early folliculogenesis is predominantly controlled by oocyte factors, such as GDF-9, rather than endocrine and paracrine signals as is the case with later stage follicles. Despite removal from the in vivo hyperandrogenic environment, later developmental stage follicles from DHT-induced PCOS mice display significantly stunted growth rates, indicating that chronic exposure to androgen excess may create sustained intrinsic defects in follicles which are not reliant on ongoing androgen exposure, leading to their poorer survival in culture. The significant increase in unhealthy follicles observed in the PCOS follicles may explain the decrease in successful outcomes from ART in women with PCOS. Furthermore, our findings propose that the longer the follicles remain exposed to the hyperandrogenic environment, the less likely they are to survive and be healthy and fully functional in culture. These findings imply important testable hypotheses for the future improvement of ART for patients with PCOS, with particular focus on the early culture of preantral follicles in these patients. Research supported by Australian National Health and Medical Research Council project grant (APP1022648) and Australian Research Council Discovery Early Career Research Award (DE120100796).

590. Genetic variations of the folicule stimulating hormone receptor and their impacts on intrafollicular hormone production.
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Background: Folicule stimulating hormone (FSH), is one of the main hormones controlling the ovarian hormone production, through the interaction with the FSH receptor (FSHR). The FSHR is almost exclusively expressed on granulosa cells and the effects of FSH are clearly expressed in the follicle fluid which acts as a reservoir for granulosa cells secreted hormones. Three common genetic variations of FSHR have been identified across populations: FSHR-29G>A (FSHR 29), Thr307Ala (FSHR 307) and Asn680Ser (FSHR 680). FSHR-29 is located in the promoter region of FSHR, and has been shown to affect the mRNA FSHR transcription levels, and the response to controlled ovarian stimulation (COS). FSHR 307 and FSHR 680 are located within the active protein in linkage disequilibrium, and several studies have correlated the 307Ala/680Ser (A/A) genotype to a poor response to COS. Objective: To elucidate how the genetic variations of FSHR affect normal ovarian function, this study correlates the FSHR genotypes to the ovarian gene expression and hormone production of small human antral follicles. Methods: The sample material derives from ovarian surplus tissue from the fertility preservation program at Laboratory of Reproductive Biology, Denmark. The results combines two individual studies: in the FSHR 307/680 study, 69 patients were included, and the hormone content of 179 follicle fluid (FF) samples and gene expression levels of 85 granulosa cell (GC) samples are analysed according to FSHR 307/680. In the FSHR-29 study, 77 patients were included, and the hormone content of 246 FF samples and
the gene expression levels of 132 GC samples were analysed according to FSHR -29. The following parameters were evaluated: follicle diameter, intrafollicular levels of Anti-Müllerian Hormone (AMH), progesterone, estradiol, testosterone and androstenedione, as well as granulosa cell gene expression levels of FSHR, LHR, AR, CYP19a1, AMH and AMHR2. Results: For FSHR -29, significant differences in intrafollicular androgen levels were observed between the genotypes, with increased androgen levels for the A/A (mutant) genotype. In comparison, significant differences in intrafollicular estradiol levels were observed for FSHR 307/680, with increased estradiol levels for the G/G (mutant) genotype. Furthermore, significantly higher gene expression levels of LHR and CYP19a1 gene was found for the G/G genotype, whereas AMHR2 gene expression was significantly reduced. Conclusion: Significant changes in intrafollicular milieu can be observed as effects of FSHR genotype. FSHR-29 significantly affected the androgen levels, whereas FSHR 307/680 affected the estradiol production, influencing the gene expression levels of LHR and CYP19a1, and the intrafollicular estradiol levels.

591. **PEDF regulation by hCG in granulosa cells.**

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Ovarian hyper stimulation syndrome (OHSS) is a potentially life-threatening complication of assisted reproduction. OHSS is triggered by administration of the ovulation-inducing human chorionic gonadotropin (hCG), which results in release of vascular endothelial growth factor (VEGF) from the ovary. We have previously shown that expression of pigment epithelium-derived factor (PEDF) in a granulosa cell line is regulated by hCG reciprocally to VEGF. The physiologic PEDF-VEGF counterbalance was found to be impaired in triggered by administration of the ovulation-inducing human chorionic gonadotropin (hCG), which results in release of vascular endothelial growth factor (VEGF) from the ovary. We have previously shown that expression of pigment epithelium-derived factor (PEDF) in a granulosa cell line is regulated by hCG reciprocally to VEGF. The physiologic PEDF-VEGF counterbalance was found to be impaired in mice with induced OHSS. Treatment of OHSS-induced mice with low doses of recombinant PEDF (rPEDF) significantly alleviated OHSS characteristics as edema and vascular leakage. These data led to our working hypothesis that PEDF plays a crucial role in ovarian physiology and reproductive pathologies. To meet this hypothesis, we characterized the signaling network by which hCG down-regulates PEDF expression in the ovary. We applied specific chemical inhibitors and stimuli to human primary granulosa cells and to rodent granulosa cell line and followed which of the known hCG signaling molecules causes PEDF down-regulation. We found that inhibition of PKA and PKC activity significantly abrogated hCG ability to decrease the levels of PEDF protein and mRNA; stimulation with PKC agonist led to PEDF down-regulation. In addition, we showed that an EGF-like factor also takes part in PEDF down-regulation. Finally, we showed that the hCG downstream effectors, ERK1/2 and AKT, are part of hCG mediating PEDF down-regulation. Intriguingly, screening the literature we found that hCG uses the same signaling network to up-regulate VEGF. The current OHSS therapeutic armamentarium consists of symptomatic managing; any new suggested treatment needs to be screened for fertility related side effects. Thus, our aim was to follow whether rPEDF treatment will induce any reproduction complications in our model system. Monitoring ovulation, pregnancy rates and embryo weight indicated that rPEDF treatment had no adverse effects. Our extended knowledge of the biological regulation of ovarian angiogenesis may lead to the development of a potent physiological replacement therapy for OHSS.

592. **Brown Adipose Tissue Response to Excess DHT is Mouse Strain Dependent.**

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Polycystic ovarian syndrome (PCOS) is a major cause of infertility in women of reproductive age, and its symptoms include ovarian dysfunction, increased risk for metabolic disorders and other health complications including increased BMI. One cause of PCOS is excess serum testosterone due to unknown etiology. In this study several strains of mice including C57BL/6, NOD/ShiLtJ and A/J were implanted with either placebo or 5α-dihydrotestosterone (DHT) pellets for 90 days, to mimic PCOS-like metabolic and ovarian symptoms. These strains were selected to compare genetic background differences in response to excess testosterone. The 3 strains are commonly used inbred strains. Body weights were recorded weekly; C57BL/6 and NOD strains exposed to excess DHT showed continued increase in body weight over the course of the study, A/J mice showed an increase although it was not significant. Body composition analysis performed toward the end of the study showed that A/J and NOD mice had a significant increase in total lean fat while the C57BL/6 did not. Interestingly, the C57BL/6 mice had a decrease in bone mineral content while the other strains did not have significant differences. All three strains of mice treated with DHT showed an increase in AGD compared to placebo treated groups. Histological examination of several types of white adipose tissue (WAT) including gonadal, inguinal and retroperitoneal fat had increased adipocyte size in the animals treated with DHT compared to placebo treated littersmates. Interestingly, the response of brown adipose tissue (BAT) to DHT varied depending on genetic strain of mice. The BAT in A/J mice looked similar in both placebo and DHT treated animals, however the BAT in NOD mice resembled WAT in NOD mice treated with DHT compared to placebo treated animals. Current studies are underway to investigate comparative differences of gene expression in the BAT of NOD and A/J mice to determine if DHT may cause transdifferentiation of the adipose tissue. The varied response of BAT in the different mouse strains suggests that genetic differences may contribute to altered adipose tissue responses to excess androgens in women with PCOS resulting in differential severity of metabolic syndrome.

593. **Liver receptor homolog-1 (Nr5a2) expression in the mouse ovary is key to granulosa cell proliferation.**

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Liver receptor homolog-1 (Lrh-1), also known as Nr5a2, is expressed in the mouse ovary, specifically in granulosa cells. It is essential for fertility, as granulosa-specific deletion of Nr5a2 in mice from primary follicles forward results in anovulation, deficient luteinization and infertility. As an increase in the number of granulosa cells is critical for a proper development of follicles from primary to antral stages, we hypothesized that Nr5a2 is important for granulosa cells proliferation. We generated two models of granulosa-specific knockout mice, one with Nr5a2 depletion beginning at the preantral follicle stage (genotype Nr5a2f/fAmhr2Cre/+; Amh cKO); the other
with depletion of Nr5a2 in granulosa cells in antral and later follicles (genotype Nr5a2floxed/CypCre/+; Cyp cKO). Our first objective was to determine the proliferative competence of Amh cKO and Cyp cKO granulosa cells. Immature cKO females of both genotypes and their control littermates were injected with eCG to stimulate follicular development and with bromodeoxyuridine (BrdU) 20h after eCG and 24h before euthanasia. The replicating cells were detected by immunofluorescence and counted by CellProfiler software. Results showed that the percentage of proliferating cells was dramatically reduced (p<0.0001) in the Amh cKO ovary, less than half (14.6%) relative to the controls (32.5%), while Cyp cKO and its control displayed no difference (31.3% vs 30.5%). To evaluate proliferation from antral follicle formation forward we injected two groups of Cyp control and cKO with eCG 44h before concurrent hCG and BrdU and tissues were collected at 4h or 12h later. The dividing cells were counted as previously described. We noted, as reported in a previous study, that the proliferation rate decreased with the hCG injection but there was still no difference between the controls and cKO groups (hCG 4h: 12.77% vs 12.72% and hCG 12h: 22.5% vs 21.82%). Our second objective was to determine how Nr5a2 depletion in granulosa cells affects genes involved in proliferation. As BrdU experiments showed that the number of cells entering S phase was significantly decreased in Amh cKO mice compared to controls and studies have shown that Nr5a2 promotes cell proliferation in hepatic and pancreatic cells by inducing G1 cyclins, we evaluated cyclin D and E expression. Granulosa cells from immature Amh cKO and control females superstimulated with eCG, were isolated by puncture and proliferation-related genes were evaluated by qPCR. There was a greatly reduced expression of Ccnd1, Ccnd2, and Ccne1 (p<0.05) in cKO compared to control, while Ccne2 showed a tendency to downregulation. As expected, cyclin dependent kinases Cdk2 and Cdk4 showed no significant difference between control and cKO mice. We also tested E2f1 and Rb1, two of the key mediators of cell cycle progression through pathways such as p16/RB/E2F which controls transit through the G1 restriction point, as they are downstream targets of cyclins D and E. While Rb1 showed a tendency to downregulation, E2f1 was significantly reduced (p<0.05). We conclude that Nr5a2 is essential for proper proliferation of granulosa cells during follicular development. Further, the lack of Nr5a2, impacts the expression of downstream targets such as cyclins and transcription factors involved in the G1/S phase transition of cell cycle. Supported by a CHIR operating grant to BDM.

594. TGF Beta-1 Induces an Epithelial-to-Mesenchymal Transition and Increases Ptgs2 Expression to Increase Ovarian Surface Epithelial Stem Cell Characteristics. Lauren E. Carter, Lisa F. Gammell, Olga Collins, David P. Cook, Curtis W. McClosey, Barbara C. Vanderhyden. 1University of Ottawa, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada; 2University of Ottawa, Ottawa, Ontario, Canada; 3Ottawa Hospital Research Institute, Ottawa, Ontario, Canada.

The ovarian surface epithelium (OSE) is the monolayer of epithelial cells surrounding the ovary. During ovulation, the OSE layer surrounding the apex of the ovulating follicle is ruptured, creating an ovulatory wound. After ovulation, this wound is repaired, but the processes involved are poorly understood, as are the consequences of improper healing. Like other tissues with repetitive wounding and healing, the OSE may contain a stem cell population responsible for wound repair. We have previously identified Stem Cell Antigen 1 (Scal1) expressing OSE cells that exhibit stem cell characteristics such as increased sphere forming capacity. These characteristics and the percentage of Scal1-positive OSE cells were increased upon treatment with Transforming Growth Factor Beta-1 (TGFβ1), a factor found in the follicular fluid at the time of ovulation. These data suggest there is a stem cell population in the OSE that may be regulated during ovulation to aid in ovulatory wound repair. To study how TGFβ1 is increasing OSE stem cell characteristics, mouse OSE (mOSE) were treated with TGFβ1 recombinant protein. TGFβ1 treatment increased the stem cell marker CD44 (3.6-fold) and induced an epithelial-to-mesenchymal transition (EMT), as seen morphologically, through an increase in Snai1 (8.3-fold) and a decrease in KRT19 (93%). These changes then reverted back to untreated conditions when TGFβ1 was removed from culture. Snai1 overexpression in mOSE cells resulted in a morphological EMT and a 56% decrease in CDH1. Furthermore, Snai1 overexpression increased CD44 (1.5-fold) and sphere forming capacity, as seen with TGFβ1 treatment. A TGFβ1 Signaling Targets PCR array was used to identify additional gene targets of TGFβ1 treatment. Ptgs2 was increased 9.7-fold in TGFβ1-treated mOSE, compared to control mOSE. An ELISA showed that under the influence of TGFβ1 treatment, the Ptgs2 increase was accompanied by an increase in Prostaglandin Endoperoxide Synthase 2 (PG2) protein (1.9-fold), a down-stream product of PTGS2. Analysis of the Snai1-overexpressing cells also showed an increase in Ptgs2 (4-fold). Treating mOSE cells with PG2 protein did not increase Snai1, but increased CD44 (4.1-fold) suggesting Ptgs2 is downstream of Snai1 in this pathway. Activation of the Ptgs2 receptor, EP4, increased CD44 and Scal1 (22- and 6.0-fold, respectively), suggesting PG2 is signaling through the EP4 receptor to increase mOSE stemness. EP4 activation also increased Snai1 and Ptgs2 (5.0 and 8.9-fold, respectively), suggesting positive feedback loops. TGFβ1 signals through the canonical Smad2/3 pathway to exert these effects. Inhibition of SMAD2/3 phosphorylation completely eliminated the effects of TGFβ1 on mOSE stemness, suggesting this proposed pathway is Smad2/3 dependent. The effects of TGFβ1 on EMT induction and increasing stemness in mOSE cells is also seen in human OSE cells, suggesting this proposed pathway is translational into humans. In summary, these data suggest that TGFβ1 acts through the canonical Smad2/3 pathway to induce an EMT and increase Ptgs2 expression as a possible mechanism for mobilizing OSE stem cells to promote ovulatory wound repair. Since ovulation is the primary non-hereditary risk factor for ovarian cancer, understanding the mechanisms underlying ovulatory wound repair may help to identify dysregulations that may contribute to OSE transformation.

595. Mouse oocytes maintain FOXL2 expression in granulosa cells in coordination with estrogen. Chihiro Emori, Wataru Fuji, Kunihiko Naito, Koji Sugiuara. 1The University of Tokyo, Bunkyo-ku, Tokyo, Japan.

Forkhead box L2 (FOXL2) transcription factor plays a critical role in the maintenance of the ovarian phenotype in adult mice. This was evidenced by the conditional deletion of Foxl2 in the adult ovary, which results in an increased expression of Sox9 in granulosa cells and the transdifferentiation of granulosa cells into Sertoli-like cells in vivo. We previously reported that mouse oocytes regulate the expression of FOXL2 in granulosa cells in vitro. Moreover, we also reported that coordination of oocyte signals with estrogen is critical for regulating development and function of granulosa cells. Therefore, the aim of the present study was to examine the coordinated effects of oocytes and estrogen on the regulation of FOXL2 expression in mouse granulosa cells. In addition to this, we tested whether Sox9 expression could be induced to granulosa cells by paracrine signals in vitro. First, we examined the effects of co-culturing granulosa cells, isolated from eCG-primed mice, with oocytes and/or estrogen (17β-estradiol, E2) on the mRNA and protein levels of FOXL2. When granulosa cells
were treated with either oocytes or E2, there were no significant effects on Foxl2 mRNA levels; however, when treated with both, Foxl2 mRNA expression was significantly increased. Furthermore, protein expression of FOXL2 was promoted by oocytes, and this effect of oocytes was augmented by the presence of E2, which itself had no effects on the FOXL2 protein level. Moreover, although the FOXL2 expression was decreased in granulosa cells cultured without oocytes or E2, Sox9 expression was not increased. Since the deletion of Foxl2 gene results in elevated expression of Sox9 in granulosa cells in vivo, we next examined the effects of supplying medium with a follicular fluid on the Sox9 expression. Due to the small size of mouse follicles, we used follicular fluid of pigs. As a result, porcine follicular fluid (pFF) promoted the expression of Sox9 in granulosa cells in a dose-dependent manner. However, induction of Sox9 expression by pFF was not observed in the presence of oocytes and E2. These results suggest that oocytes and estrogen are required for maintaining the expression of FOXL2 in granulosa cells, and this regulation appears to be required for suppressing the expression of Sox9 in granulosa cells in vitro.

596. Prostaglandin-induced luteolysis in cattle alters the levels of transcripts encoding anti-mullerian hormone (AMH) and bone morphogenetic proteins (BMP1, 2, 4 and 6).


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It has been shown that transforming growth factor beta (TGFβ) superfamily members play multiple roles on folliculogenesis, including follicle growth, atresia, steroidogenesis, oocyte maturation, ovulation and corpus luteum (CL) formation. However, the role of TGFβ superfamily members during luteolysis has not been systematically investigated. The objective of this study was to evaluate if the transcript levels of AMH and BMPs (1, 2, 4 and 6) are altered in CL cells after the induction of luteolysis in cattle. CLs and blood samples were collected at 0 (control group), 2, 12, 24, and 48h after prostaglandin F2 alpha (PGF) treatment, which was given on Day 10 of the estrous cycle (n = 4-5 cows/time-point). Concentrations of progesterone in serum declined within 2h after PGF injection and reached basal concentrations by 24h after treatment, which confirmed functional luteolysis. Tissue examination revealed loss of plasma membrane integrity, reduced cytoplasmic volume and nuclear pyknosis in luteal cells from CLs collected at 24 and 48h after PGF treatment, which confirmed structural regression of the CL. BMP1 transcripts were increased at 12h but decreased at 48h after PGF. BMP2 transcripts increased at 12h but declined to the levels of control group (0h) by 24h after PGF. Transcripts for BMP4, BMP6 and AMH were all increased at 2h after PGF. While BMP4 transcripts remained higher than control group at 12, 24 and 48h, BMP6 and AMH transcript levels declined to the levels of control group by 12h after PGF. These findings suggest that AMH, BMP4 and BMP6 may play a role during early luteolysis (functional luteolysis), while BMP1, 2 and 4 may be involved in the later stages of luteolysis (structural luteolysis) and CL regression eventually. Functional studies are necessary to determine the role of these TGFβ superfamily members during luteolysis. Supported by CAPES and CNPq (Brazil) and NSERC (Canada).

597. Expression and localization of angiogenic and vasoactive factors in canine corpus luteum during pregnancy and non-pregnant cycles.

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Vascularogenesis and angiogenesis are required for proper formation and maintenance of the corpus luteum (CL). They are regulated by orchestration of many locally-produced mediators such as prostaglandins (PG), growth and angiogenic factors. Recently, our group characterized the luteotrophic activity of PGE2 in canine CL. Direct, STAR-mediated and indirect, prolactin receptor (PRLR)-mediated, effects were indicated. However, there is still very little information available concerning the role of angiogenic factors in CL of dogs. Therefore, in order to better understand auto/paracrine regulation of luteal vascularogenesis and angiogenesis, here we investigated expression and localization of the vascular endothelial growth factor (VEGF) system (VEGFA and its receptors, VEGFR1 and VEGFR2), and the endothelin (ET) system (ET-1 and -2, ET receptors ET-A and -B and ET converting enzyme (ECE-1)) during different stages of gestation, i.e., pre-implantation, post-implantation and mid-gestation, and at prepartum luteolysis (n=3-5 per group). qPCR and immunohistochemistry were applied. Additionally, expression of the ET system was investigated throughout the luteal lifespan in non-pregnant dogs (days 5, 15, 25, 35, 45 and 65 after ovulation; n=5 per group). Moreover, canine lutein cells collected from early CL (up to 21 days after ovulation) were stimulated with PGE2 and the possible interplay with ET system expression was investigated.

In contrast to VEGFR2, which was relatively stably expressed throughout gestation, VEGFA and VEGFR1 showed the highest expression in early CL, and decreased significantly towards the mid-luteal phase. Further downregulation of VEGFR1 was observed during prepartum luteolysis. VEGFA was predominantly localized in the luteal cells, VEGFR1 stained additionally within the tunica media of blood vessels, and VEGFR2 signals were found predominantly in capillary endothelial cells.

ETB (a strong vasodilator) was elevated during the early CL phase and decreased gradually by mid and late dioestrus. This differed from ETA (a strong vasoconstrictor), which did not change until late dioestrus both in pregnant and non-pregnant bitches. Its expression increased, however, significantly during prepartum luteolysis. ETA was targeted to capillary pericytes, contrasting with the more ubiquitous expression of ETB in luteal and interstitial cells, and the tunica media of arteries. In contrast to ET1, which remained more or less constant during dioestrus, ECE1 (enzyme activating ETs) and ET2 were upregulated at the beginning of dioestrus and progressively decreased by mid-dioestrus. A new increase of ET2 expression was then observed simultaneously with upregulated ETA levels during prepartum luteolysis. Steroidogenic cells stained positively for ET1 and ET2 was targeted solely to capillary endothelial cells. ECE1 was found both in endothelial and luteal cells. The most interesting aspect of the in vitro study was the finding that PGE2 is capable of stimulating ETB and ET2 expression in canine luteal cells.

In conclusion, in addition to the expected high expression of vasoactive and angiogenic factors at early dioestrus, associated with the increased blood supply and vascularity rate, the upregulated expression of ETA and ET2 at prepartum luteolysis, both localized
predominantly in endothelial cells, indicates that vascular functionality might be involved in structural and functional luteolysis in dogs at term, but not in cyclic CL.

598. Large luteal cells purified by flow cytometric sorting from bovine corpora lutea show very distinct DNA methylation and mRNA expression pattern.

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As a result of the preovulatory LH surge follicular granulosa and theca layers undergo dramatic morphological and physiological transformations thus culminating in ovulation and the formation of the progesterone producing corpus luteum. These processes are preceded and accompanied by fundamental changes of gene expression profiles. The proper regulation of these luteinization processes is essential for cycling and maintenance of pregnancy in cattle. The aim of the present study was the genome-wide expression profiling of different types of steroidogenic luteal cells and DNA methylation analysis of CYP19A1, the key gene of estrogen biosynthesis, as an experimental paradigm for the cell type-specific epigenome. Cells were isolated from corpora lutea of non-pregnant Holstein cows at days 10 to 11 of the estrous cycle by collagenase digestion. Mixed luteal cell populations were counted by conductometry in a cell counter (Multisizer II, Beckmann Coulter, USA). Different cell types were then purified by fluorescence-activated cell sorting using a MoFlo XDP high-speed flow cytometry sorter (Beckman Coulter, USA). Purified luteal cell populations were collected in PBS and re-analyzed by a flow cytometry analyzer (Galilios, Beckman Coulter, USA). Gene-specific methylation analysis was done by bisulfite direct sequencing with ESME software, and mRNA expression profiling with Bovine Gene 1.1 ST microarrays (Affymetrix). Statistical and Bioinformatic evaluation of microarray data were done with the Expression and Transcriptional Analysis Consoles (Affymetrix) and the Ingenuity Pathway Analysis (IPA) tool. Diameters of cells ranged between 5.2 µm - 46.0 µm. From these, we could purify on average 0.46% large luteal cells (LLC) with 28.5 µm – 46.0 µm size and 2.5% medium sized luteal cells (MLC) with 21.0 µm - 28.5 µm size. Methylation analysis of 4 different CpG dinucleotides adjacent to the granulosa-specific start site of transcription of the CYP19A1 gene showed remarkably lower levels of DNA methylation, especially in the purified LLC fraction compared to MLC and unsorted luteal cells. Purified LLC also showed a distinct gene expression profile, which was very different from that of unsorted luteal cells, but also from MLC. Comparing LLC with MLC or unsorted luteal cells we found about 2000 and 3000 differentially expressed genes, respectively. Only 300 genes were found specifically expressed in LLC compared with unsorted cells. In conclusion, by flow cytometric sorting a highly specific population of LLC, which is characterized by a distinct DNA methylation pattern and gene expression profiles could be purified for the first time from bovine corpora lutea. Specific marker genes and pathways could be identified for LLC thus suggesting their granulosal origin.

599. Impact of Long-term Treatment with Acyline, a GnRH Receptor Antagonist, on Gonadotropin Secretion, Follicular Waves and Dominant Follicle Development in Cattle.

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GnRH antagonists such as Acyline inhibit endogenous gonadotropin secretion and ovarian steroid production and are useful to improve ovarian stimulation protocols during assisted reproductive technologies in women or superovulation in cattle. Short-term Acyline treatments decrease LH secretion in cattle, gilts and horses but produce mixed results in suppressing dominant follicle (DF) growth and FSH secretion. The present study tested the hypothesis that long-term administration of Acyline inhibits FSH and LH secretion and blocks occurrence of follicular waves and DF development during estrous cycles, which increase the number and size of follicles ≥ 3 mm in diameter and corpora lutea (CL) growth. Data were analyzed by ANOVA. Results showed that after 10 days of treatment both Acyline doses blocked (P<0.01) emergence of follicular waves and DF development, decreased size of CL, and reduced LH secretion compared with controls. While controls exhibited three separate peaks of FSH secretion coinciding with each follicular wave, these FSH peaks were blocked (P<0.01) in Acyline-treated cattle. Based on these results, we conclude that long-term Acyline treatment blocks gonadotropin secretion sufficiently to hinder follicular waves and DF development during estrous cycles, which may prove useful in design of new methods to improve response to superovulation in cattle. (Project supported by funds from the Michigan State University AgBioResearch to JI).

600. Anti-Müllerian Hormone in the Serum and Ovaries of Domestic Dogs.

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The reproductive cycle of the domestic dog is characterized by periods of inactivity, or anestrus. Elucidation of the mechanisms of anestrus termination / estrus resumption would allow for the improvement of estrus synchronization or ovarian hyperstimulation protocols. In this study we evaluated anti-Müllerian hormone (AMH) in the transition from late anestrus through estrus. Serum samples from five beagles (age = 2 - 4.5 yr) were taken 3 times per week at least 30 d prior to the onset of estrus and assessed for AMH, progesterone, and 17 beta-estradiol. Ovaries from females of other breeds (aged 8 mo - 5 yr, n = 4) undergoing routine ovariohysterectomy were evaluated for reproductive stage based on visual evaluation of the dog (vulvar swelling, discharge) and gross evaluations of follicle populations of the ovaries (presence of large follicles or corpora lutea). Cortical sections of ovaries were either fixed and paraffin embedded for AMH immunohistochemistry (IHC), or used to mechanically isolate antral (330 µm to 3 mm) and preovulatory stage
follies (> 3 mm diameter) for RNA extraction. Amh and Smad mRNA expression was assessed by qRT-PCR, using Gusb as the reference gene. Serum AMH concentration increased significantly during proestrus (0.30 ± 0.01 to 0.64 ± 0.03 ng/ml, P < 0.05) and preceded the rise in serum estradiol concentration. AMH declined sharply back to baseline values beginning day -4 prior to the LH surge. Immunoreactivity for AMH appeared to be more pronounced in antral follicles than in preovulatory follicles, whereas mRNA expression of Amh and Smads were comparable for the two follicle stages. Taken together, our data suggest that the rise in serum AMH concentration is coincident with the increase in the numbers of antral follicles during late anestrus / early proestrus in the domestic dog ovary. Increased AMH in serum may be useful in predicting the onset of estrus. In four of five dogs, the LH surge occurred 8-9 days after AMH first increased two-fold above baseline. Future work should evaluate the cellular targets of elevated AMH and determine if it plays an active role in the events leading up to a successful ovulation or in the establishment of the subsequent anestrous period of the domestic dog. This research was supported by NIH DP-OD-006431 (A.J.T.), Cornell University’s Diagnostic Endocrinology Laboratory, the Baker Institute for Animal Health, the Atkinson Center for a Sustainable Future, and the Smithsonian Institution Predoctoral Fellowship.

601. Abnormal Luteal Development During Early Diestrus in the Goat.
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The corpus luteum is a transient endocrine organ formed from cells of the ovulating follicle. Corpora lutea (CL) produce progesterone (P4), which is required throughout pregnancy in the goat. In the absence of a normal fertilized embryo, which initiates maternal recognition, CL regress in two distinct phases: deterioration of P4 production (functional luteolysis) followed by the disappearance of luteal tissue (structural luteolysis). Functional and structural luteolysis have been researched extensively in the cow and sheep, but not characterized widely in the goat. Cyclic female goats undergoing estrus synchronization are also susceptible to reduced luteal function 5 days after estrus and ovulation. The goal of this study was to compare the endocrine, morphological and histological characteristics of abnormal and normal CL, collected on Day (D) 5 after a synchronized estrus, with CL undergoing normal functional and structural luteolysis. Our hypothesis is mechanisms responsible for sub-normal luteal function early in diestrus are distinct from those governing luteolysis in late diestrus. Alpine does were synchronized (n=21) using CIDR inserts. After 9 days CIDRs were removed and 500 mg of PGF2α and 50 mg of GnRH were administered at 0 and 48 h, respectively. Does were checked for estrus daily (D0) and mated with a vasectomized buck. Blood samples were collected daily from D0 to D5 (synchronized) and from D0 to D17 (n=3) or D18 (n=2) of a normal cycle. Serum concentrations of P4 were used as an indicator of abnormal luteal function. Ovaries were removed from goats on D5 (n=11), D17 and D18. CL were excised from the ovary, weighed and fixed in 4% paraformaldehyde. Serum P4 concentrations were greater (p<0.01) in normal cycling does on Days 3-5 than does exhibiting abnormal luteal function. Mean CL weights were similar (p=0.2) when normal D5 (0.35± 0.08 g) and abnormal D5 CL (0.43± 0.03 g) were compared, likely due to one large, fluid-filled luteal structure (0.66 g) within the normal group. Normal D5 CL were significantly smaller (p=0.01) than those collected on D17 (1.33± 0.13 g) and D18 (0.76± 0.1 g). D17 CL were also larger (p<0.05) than D18 CL. Paraffin embedded sections, stained with hematoxylin and eosin (H&E) and Masson’s Trichrome, revealed a poorly organized cellular architecture in abnormal D5 CL. Additional sections were processed for immunohistochemistry to assess markers for progesterone synthesis (anti-HSD3B7), autophagy (anti- Erk 1/2), and the distribution of M1 (anti-TRAF3) and M2 (anti-CD163/M130) macrophages. Distinct differences were detected when abnormal D5 CL were compared to normal D5, D17 and D18 CL. Staining intensity for HSD3B7 was stronger in normal D5 and D17 CL when compared to normal D18 CL. Staining in abnormal D5 CL was weak and diffuse. Erk 1/2 staining suggests that autophagy was low in normal D5 CL, more intense in D17 and D18 CL and strongest in abnormal CL obtained on D5. Staining for M1 macrophages was strong on D17 and in abnormal D5 CL, while staining for M2 macrophages was most intense in the normal Day 5 CL. Therefore, abnormal Day 5 CL have reduced steroidogenesis and contain macrophages that are associated with luteolysis (M1), rather than a luteotropic environment (M2). Whether these changes are due to improper follicle maturation prior to ovulation or an improper uterine environment conducing to normal CL development is unknown. Supported by Evans-Allen funds.

602. The chemerin effect on granulosa cell transcriptome study showed its impact on the expression of three molecules associated with PCOS, the adrenergic receptor alpha 1, propioliolanocortin and adiponectin in rat.
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Background Chemerin (also named Retinoic Acid Receptor Responder 2) negatively regulates FSH-induced rat follicular growth and granulosa cell steroidogenesis. It is an adipokine but also highly expressed in the ovarian follicles, it has been implicated in the regulation of several biological processes, including immune responses, adipocyte differentiation, glucose uptake and insulin resistance. Polycystic Ovarian Syndrome (PCOS) is a heterogeneous syndrome affecting 10% of women in reproductive age and account for 75% of anovulatory fertility. It is associated with obesity, metabolic disorder, cardiovascular diseases, increased insulin resistance, type 2 diabetes. In the ovary, it is associated with small antral follicle growth arrest, minimal granulosa cell proliferation and hyperandrogenemia. The etiology of the PCOS is complex, and genetic and environment factors are believed to be involved. Objectives The overall objective is to determine if and how chemerin regulates follicular growth and functions, and participates in the PCOS condition. The specific objectives are to examine granulosa cell gene expressions in response to chemerin particularly in the context of PCOS. Furthermore the goal is to identify the path leading chemerin to regulate expressions associated with PCOS phenotype and how it induces part of the PCOS phenotype in granulosa cells. Hypothesis Chemerin overexpression in PCOS results in decreased oocyte growth and developmental competence, disturbed follicular growth, steroidogenesis and ovulation. Methods (1) The extension of the characterized 3 months DHT treated PCOS rat model to 1 and 2 month(s) DHT was performed (n=7). (2) The effect of chemerin on granulosa cell gene expression profile was done using granulosa cell primary cultures in absence or presence of 100 ng of rm-chemerin/mL during 24h from 21-22 days old SD rats, n=8, 10-11 rats per group. RNA was extracted, analysed, hybridized on Affymetrix based microarrays (n=4) and reverse transcribed, real-time PCR was used to validate differential expressions (n=4). (3) The effects of chemerin and those of 3 months DHT PCOS model on gene expression profile were compared with other PCOS transcriptome studies. Results (1) The extension of the DHT model showed that the hyperandrogenemia induced PCOS phenotype in rat
starts in the ovary (ovarian width, cysts presence, irregular cycle, oocyte heath) before than at the metabolic/systemic (body weight, insulin resistance) level. (2) Analysis of the microarrays data revealed 1202 differentially regulated transcripts and selected candidates were validated at the transcriptomic level by real-time QPCR. (3) Literature review identified the PCOS related microarrays studies. Our microarrays data analysis and cross comparisons with existing studies, permitted to define the distinct effect of chemerin on granulosa gene expression profile and to distinguish it from those of hyperandrogenemia and other tissues or cell types. (4) Adrenergic receptor alpha-1 (ADRA1), propiomelanocortin (POMC) and adiponectin (ADIPOQ) transcripts known to be associated with PCOS were associated, for the first time, in granulosa cells and as differentially expressed by chemerin. Perspectives The next steps ongoing are the evaluation of dose and time effect of chemerin on the expression profile of the identified genes, and to perform gain and loss of functions studies to identify the path leading chemerin to regulate them and how they induce PCOS phenotypes.

603. Follicular Fluid Extracellular Vesicles (EVs) Regulate Cumulus-Oocyte-Complex (COC) Expansion and Gene Expression in Cumulus Cells.
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Cumulus oocyte complex (COC) expansion is critical for ovulation of a fertilizable egg. Expansion of the COC is accomplished by the cumulus cells creating a hyaluronan (HA)-rich extracellular matrix, which is under the regulation of a number of genes, including prostaglandin-endoperoxide synthase 2 (Ptgs2), pentraxin-related protein 3 (Ptx3), tumor necrosis factor, alpha-induced protein 6 (Tnfip6), and hyaluronan synthase 2 (Has2). Genetic knockouts of Ptgs2, Ptx3, or Tnfip6 or knockdown of Has2 by RNAi in vitro blocks normal COC expansion. Factors released by mural granulosa cells into the follicular fluid are known to affect the COC, and similarly COC released factors are critical for mural granulosa cell function. The follicular fluid that separates these distinct granulosa cell types is a rich and complex fluid containing numerous proteins, nucleic acids, and other macromolecules. Recent studies have shown that extracellular vesicles (EVs) are abundant within antral fluid, yet to date, no functions of EVs have been demonstrated within the ovary. In our study, the effect of follicular fluid EVs on COC expansion was assayed. EVs were isolated using ultracentrifugation from follicular fluid of early follicles. The exosomal marker, CD81 was highly enriched in EV preparations from both early and late follicles. Uptake of EVs was evident within the cytoplasm of cumulus cells as numerous green punctate spots. Functional analysis indicated that EGF induced COC expansion and expression of marker genes with the exception of Has2. EVs from early or late antral follicles stimulated COC expansion, by 20% or 15% compared with controls cultured without EVs (p<0.05). Expression of Ptgs2 and Ptx3 increased significantly upon treatment of EVs from early follicles but not late follicles. Tnfip6 expression followed the same trend while Has2 expression did not change in response to EVs. In conclusion, EVs isolated from follicular fluid positively regulated COC expansion and marker genes expression, with a paradoxically greater effect of small follicle derived EVs than large follicle derived EVs. This study is the first to demonstrate a biological effect of follicular fluid EVs on COC expansion and gene expression, critical events in the process of ovulation.

604. Gene expression of FSH, LH and prolactin receptors in corpus luteum of Iberian lynx (Lynx pardinus) and domestic cat (Felis catus) – similarities and differences.
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The female reproductive cycle of lynxes is characterized by a special feature – after ovulation corpora lutea (CLs) persist for more than two years. Until now, neither their function nor the mechanism that maintains the persistence are fully understood. In other species, prolactin (PRL) and luteinizing hormone (LH), both products of the adenohypophysis, are known as luteotropic factors; therefore, we analyzed the gene expression of prolactin receptor (PRLR) and luteinizing hormone/choriogonadotropin receptor (LHCGR). In addition, the receptor for follicle-stimulating hormone (FSHR) was examined. For comparison within feline species, we studied the expression of these receptors in domestic cat CLs of pseudopregnancy (PP) and pregnancy (PR) as well. Ovariohysterectomy in two Iberian lynx (IL) females was performed seven days after natural mating and both freshly ovulated (IL1: n = 3, IL2: n = 5) and persistent (IL1: n = 8, IL2: n = 6) CLs were collected. Cat samples, obtained after castration, were classified according to their histomorphology as follows: for PP: formation (n = 9), development/maintenance (n = 13), early regression (n = 13), late regression (n = 9); for PR: pre-implantation (d2-d10 of PR, n = 6), post-implantation (d14-d36, n = 11) and regression (from d38 onwards, n = 5). Furthermore, we had a group of corpus albicans samples (n = 4). Gene expression was analyzed by quantitative PCR. LHCGR was significantly higher expressed in fresh compared to persistent CLs of Iberian lynx. For PRLR, but also for FSHR, we observed the opposite – the expression in persistent CLs was significantly higher. Likewise, the expression of FSHR was significantly lower during formation and development/maintenance in cat PP samples, whereas the expression of LHCGR was significantly higher, compared to the regression stages. The FSHR gene expression significantly increased with on-going luteal cycle during PP. Comparing the CLs of PR, we could not identify statistically significant differences for analyzed receptor expression. However, in PR we observed the same tendency like in PP for LHCGR (decreasing levels during PR) and FSHR (increasing levels), with the exception of freshly formed CLs (d2 for FSHR and d2/4 for LHCGR), where the values were high for FSHR and low for LHCGR. The expression of PRLR during PR seems to be more or less constant. In lynxes, the higher expression of PRLR in persistent CLs may contribute to survival and functional maintenance of these CLs via luteotropic action of prolactin. Based on the receptor expression profile, FSH seems to have a so far unknown luteal effect in persistent lynx CLs, maybe similar to prolactin. In contrast to lynx, both receptors are highly expressed during regression stages of PP in cat CLs. Thus, we suggest that the
luteal effect of FSH and prolactin in felids is diverse. The high expression of LHCGR in early CL stages of cat and lynx might indicate a luteotropic effect of LH in a functional CL. The study was funded by DFG (Je 163/11-1) and DAAD (A/10/86242). Iberian lynx samples were provided by Environmental Council of the Government of Andalusia and ILCBP.

605. The expression of Smad 3 and Smad 4 in human granulosa cells of women with and without endometriosis.
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Introduction: Members of the TGF-beta family, which includes TGF-beta, Nodal, Activins, Inhibins, GDFs, BMPs, start their actions through receptors type II and I. As consequence activates intracellular Smads proteins that interact with a common Smad, which is translocated to nucleus combining with DNA -binding proteins to promoter regions of target genes. These members are expressed in the reproductive organs, especially in the ovary, and are involved in the control of folliculogenesis and ovulation. In this sense, understanding the pathways of these events is crucial for understanding the causes of infertility. Endometriosis is a gynecological disease characterized by endometrial tissue (glands and stroma) outside the uterine cavity and it is associated with pelvic pain and infertility that, among other factors, has also been attributed to an endocrine and ovolatory disorders. The aim of the present study was to evaluate the expression of Smad 3 and Smad 4 in granulosa cells (GC) of women with and without endometriosis. Material and Methods: The GC were obtained from 53 consecutive patients (8 with and 45 without endometriosis) undergoing in vitro fertilization. Total RNA was isolated and cDNA synthesized, followed by quantitative real-time RT-PCR to evaluate the gene expression of Smad 3 and Smad 4. Results: We found a significant decrease of gene expression of Smad 4 in GC of patients with endometriosis (p < 0.001). There was no difference in gene expression of Smad 3. Conclusion: In conclusion, our results showed that the Smads pathway could play an important role in folliculogenesis and ovulation process, especially in patients with endometriosis. Financial Support: National Institute of Hormones and Women’s Health – INCT, CNPq, FUNDEP.

606. Role of the aryl hydrocarbon receptor in the endocrine function of the prepubertal ovary in mice.
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In mice, the endocrine maturation of the ovary occurs mostly after birth, when follicular growth is initiated. Several hormones, including estradiol (E2) are synthesized from an early stage of the prepubertal period. E2 production is critical for the development of both reproductive system and sexual behavior. Whereas most studies so far have focused on the regulation of E2 synthesis in the cycling female, there is still little information available in the prepubertal period. The objective of this study was to investigate the possible role of a key regulator of E2 synthesis identified in cycling females, the aryl hydrocarbon receptor (AHR). This orphan receptor was initially discovered as a mediator of xenobiotics such as dioxins. Generation of recombinant mouse models wherein the Ahr gene has been deleted (Ahr−/− mice) has demonstrated the key role of AHR in physiological processes, including ovarian function. Indeed, Ahr−/− mice are subfertile and display impaired E2 synthesis, in part as a result of the dramatic decrease in the expression of the enzyme converting androgens into estrogens, i.e., P450- aromatase (cyp19a1), and of the receptors for FSH (fshr) and LH (lhcg). However, to our knowledge there is no information regarding AHR contribution to the endocrine function in immature mouse ovaries. We undertook a number of studies to assess in vivo the role of AHR in the ovarian endocrine function of prepubertal females at 7, 14, 21 and 28 days postnatal (dpn) (puberty occurs around 30 dpn). By real-time RT-PCR, we found Ahr to be expressed in the ovary throughout the prepubertal period, with a maximal expression level at 14 dpn. We then analyzed the abundance of mRNAs from several key steroidogenic enzymes involved in E2 biosynthesis (cyp11a1 [P450-SCC], cyp17a1 [17α-hydroxylase] and cyp19a1 [P450-aromatase]), and from the receptors for FSH (fshr) and LH (lhcg) in the ovaries of Ahr+/+ and Ahr−/− mice (n=4-16 females/group). In Ahr−/− mice, age-related alterations in transcript abundance occurred during the prepubertal period, with the highest expression levels being observed at 14 dpn for most of these genes, except for those of fshr that increased constantly until 28 dpn. In Ahr−/− mice, a similar ontogenesis was observed for these transcripts. Importantly, transcripts for lhcg were significantly less abundant in Ahr−/− mice than in Ahr+/+ mice, but exclusively at 14 dpn (p<0.001). A four-fold decrease in cyp17a1 mRNA levels was observed at 28 dpn in Ahr−/− mice (p<0.001). However, Ahr ablation did not induce substantial changes in cyp19a1 and fshr expression levels at any studied ages, in contrast with what was reported for adults. In addition, no alteration in cyp11a1 transcript levels was observed. Based on these results, we propose that AHR could regulate ovarian function by modulating LH receptivity and steroidogenesis in immature ovaries, but at specific stages of the prepubertal period. Additional studies will have to analyze the functional decrease of Ahr on the endocrine function of the immature ovary. Moreover, subsequent studies will have to establish the mechanism underlying the different regulation of cyp19a1 and fshr expression by AHR between prepubertal and cycling females.

607. Ontogeny of estradiol synthesis and its regulation by gonadotropins in prepubertal female mice.
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In mice, follicles start growing immediately after birth. These follicles, which rapidly develop up to the antral follicle stage, constitute the first follicular waves. They undergo a massive depletion by atresia after 14 days post-natal (dpn) and very few will ovulate at the time of puberty (occurring around 30 dpn). Previous works from our group have suggested that the first follicular waves may ensure the endocrine capacity of the ovary during the prepubertal period by producing several hormones, including estradiol (E2). Such an early E2 synthesis appears essential for the establishment of the reproductive function and sexual behavior. Little is known, however, about E2 concentration in the prepubertal female mouse and its possible regulation by the gonadotropin hormones FSH and LH. The aim of this study was to conduct a thorough analysis of the ontogenesis of E2 synthesis and to determine whether it could be regulated by gonadotropins as in cycling females. Blood and ovaries were collected at different ages (7-27 dpn) before puberty. The determination of E2 concentration by gas chromatography/mass spectrometry after solid-phase extraction showed that intra-ovarian E2 was detectable as early as 2015 Abstracts - Page 240
as 7 dpn (3.3 pg/ovary) and that its concentration progressively increased until 27 dpn (18.3 pg/ovary). This range is comparable to the ovarian concentrations in cycling females (3.2-53 pg/ovary). The profile of circulating E2 did not follow that of the ovary, its maximal concentrations being observed around 14 dpn. Analysis by real-time RT-PCR of steroidogenic enzymes involved in E2 synthesis (cyp11a1 [P450-SCC], cypl7a1 [P450-17α-hydroxylase], cypl9a1 [P450- aromatase], hsd3b [3 β-HSD]), and of the receptors for FSH (fshr) and LH (lhcg) revealed that their relative expression levels increased progressively in the ovary until 14 dpn to decrease thereafter, except for those of hsd3b and fshr that remained elevated. Interestingly, plasma LH and FSH levels were high as early as 7 dpn, peaked at 12-14 dpn, and then dropped abruptly after 17 dpn. The fact that the levels of circulating E2 and ovarian steroidogenesis markers becomes maximal around 12-14 dpn when plasma LH and FSH displayed their highest concentrations strongly suggest that gonadotropins are already involved in E2 synthesis during this period. Preliminary studies on organotypic cultures of 12 dpn ovaries further supported this hypothesis since co-treatment with ovine pituitary FSH and LH led to a 2-3 fold induction of the expression of cyp11a1, cypl7a1, cypl9a1 in addition to that of fshr and lhcg. Noteworthy, in situ hybridization studies showed that up to 14 dpn, growing follicles displayed some characteristics of preovulatory follicles, as revealed by the expression of cypl9a1 and lhcg in the granulosa cells of preantral and antral follicles. In older prepubertal mice, cypl9a1 expression became restricted to healthy antral follicles and that of lhcg to thecal and interstitial cells, similarly as in cycling females. In conclusion, this comprehensive study of the prepubertal ovary reveals for the first time that the ovarian endocrine capacity develops rapidly after birth to become significant around 12-14 dpn, when circulating gonadotropins are high. We propose that the unique endocrine features of the initial follicular waves, as revealed by their characteristics of preovulatory follicles, together with the hypergonadotropic environment may contribute to such a rapid ovarian maturation in prepubertal mice.

608. Active phosphodiesterases from the phosphodiesterase 8 and 11 families are present in detergent-resistant membranes of porcine granulosa cells.
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Lipids rafts are specialized membrane microdomains involved in numerous cell functions such as membrane signalling and trafficking. Due to their relative insolubility in cold-ionic detergent, lipid rafts can be isolated as detergent-resistant membranes (DRMs) in a cell homogenate. The second messenger cyclic AMP (cAMP) has a central role in ovarian physiology and its intracellular level is modulated via the equilibrium between synthesis and degradation. The degradation is carried out by the members of the phosphodiesterase (PDE) enzyme family. We hypothesized that proteins of the PDEs family are present in the lipid rafts of granulosa cells membrane. The objectives of the study were: 1) isolation of DRMs from granulosa cells and 2) detection and characterization of the PDEs present in the DRMs. DRMs present in porcine granulosa cells have been isolated by differential detergent extraction and PDEs were detected in the fraction obtained during DRMs isolation by Dot Blot and Western Blot using specific antibodies. The distribution of the PDEs in granulosa cells leaflet was analyzed by immunocytochemistry. In addition, total cAMP-PDE activity was measured in fractions and specific activity was assessed using specific PDE inhibitors (N=5). As demonstrated by Dot Blot, GM1, a lipid raft marker, was detected in the DRMs and absent from the cytosolic and the Triton-soluble fractions obtained during DRMs isolation from granulosa cells. Thus, differential detergent extraction method allowed the isolation of DRMs from granulosa cells. In addition, PDE3A, PDE4D, PDE5A, PDE6C, PDE8A, and PDE11A were detected by Dot Blot in the DRMs isolated from granulosa cells and all the PDEs tested were showing a clear immunostaining in the granulosa cells membrane as shown by immunocytochemistry. cAMP-PDE activity measured as fMoles of cAMP hydrolyzed/min/µg of protein was 2 times higher in DRMs (49.43 ± 6.27) as compared to the cytosolic fractions (22.82 ± 2.81) while minimal activity has been measured in the Triton-soluble fraction (3.50 ± 1.90). In addition, cAMP-PDE activity in the cytosolic fraction and the DRMs was mainly contributed by the PDE8 (66.8 and 65.5% respectively) and PDE11 (25.8 and 41.3% respectively) family. Several PDE8A isoforms have been detected by western blot in whole granulosa cells extract at a molecular weight of 64, 66, 75, and 93 kDa. Interestingly, those 4 isoforms were not uniformly present in the fractions obtained during DRMs isolation. In fact, the 93 kDa isoform was absent from the cytosolic fraction and present in the Triton-soluble fraction and the DRMs. The 75, 66 and 64 kDa isoforms were present in all the fractions. However, the intensity of the band corresponding to the 75kDa isoform was two times higher in the cytosolic fraction and the DRMs as compared to the Triton-soluble fraction and the intensity of the band corresponding to the 64 and 66 kDa isoforms was 3 and 7.6 times higher in the DRMs as compared to the cytosolic and Triton-soluble fractions respectively. We showed for the first time that active PDEs from the PDE8 and PDE11 families are present in lipid rafts isolated from granulosa cells suggesting a specific role of lipid rafts as a platform for cell signalling in the ovarian follicle.

609. Overexpression of the orphan nuclear receptor liver receptor homolog-1 (Nr5a2) in mouse granulosa cells alters luteal function.
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The nuclear receptor Nr5a2, a.k.a. liver receptor homolog 1 (Lrh-1), is an essential regulator of the ovarian function and female fertility. We have previously employed tissue-specific paradigms of Nr5a2 depletion in mouse granulosa cells and the exploration of the phenotype revealed failure in cumulus expansion, ovulation and luteinization. Our objective was to establish the consequences of overexpressing Nr5a2 in ovarian granulosa cells in vivo. A mouse model bearing a lox-STOP-lox (Nr5a2fl/fl) construct driven by a constitutive promoter along with Flag and myc-tags inserted in the Nr5a2 coding sequence was employed. This mouse line was bred to granulosa specific Amhr2-Cre mice to produce Nr5a2-overexpressing (Nr5a2-OE) females. We confirmed the occurrence of Cre recombinase induced overexpression in granulosa cells by Myc immunohistochemistry in the ovaries. Moreover, the abundance of Nr5a2 mRNA in granulosa cells is upregulated in the OE group compared to the littermates (p<0.05). Immature Nr5a2-OE and control females (n=5/group) were superovulated using PMSG and hCG and the oviducts were collected 16h post-hCG. Surprisingly, the average number of ovulated cumulus-oocyte complexes was not different comparing the two groups (p>0.1). Adult Nr5a2- OE and control females (n=7/group) were housed with reproductively proven C57BL/6 males for 1 year. Cages were inspected daily, and parturition dates and litter sizes were recorded. No significant difference was observed in the number of pups per female (p>0.1). Further analyses of ovarian gross morphology revealed no apparent phenotype after Nr5a2 overexpression. However, histological examination of adult ovaries revealed...
luteal disruption and precocious antrum formation in the Nr5a2-OE mouse ovary. The gene expression of Fshr, which is necessary for follicular development, was upregulated in the Nr5a2-OE granulosa cells (p<0.05) compared to the controls after hormonal stimulation. Given the known effects of Nr5a2 on proliferation, we then evaluated the expression of genes related to this pathway. Although the expression of the cell cycle genes Ccnd1, Ccnd2 and Ccnel appears to be normal in the OE (p>0.1), retinoblastoma (Rb1), an important regulator of cell division, is overexpressed in the Nr5a2-OE ovaries (p<0.05). To determine whether the overexpression of Nr5a2 affects the pattern of the estrous cyclicity, a group of Nr5a2-OE and control adult females (n=10/genotype) were daily evaluated by vaginal smears during 21 days and the vaginal cytology was classified. Interestingly, the estrous cycle in the Nr5a2-OE is significantly prolonged (p<0.05), due to an extended metestrus phase. In the mouse, during metestrus and diestrus, progesterone inhibits the secretion of LH and prevents further ovulation. We therefore evaluated the expression of genes related to steroidogenic hormones synthesis. The expression of steroidogenic acute regulatory protein, StAR, is not affected by the overexpression of Nr5a2 (p=0.1), whereas the key factors in steroidogenesis, aromatase, Cyp19a1 (P<0.05), and the scavenger receptor B1, Scarb1 (p<0.1), are upregulated in the OE group. In conclusion, the conditional overexpression of Nr5a2 in ovarian granulosa cells lengthens the luteal phase and thus the estrous cycle, and the normal pattern of expression of genes related to follicular development, cell division and steroidogenesis is disrupted. Supported by CIHR grant to BDM.

610. TRANSCRIPT AND LIPID CONTENT OF EXTRACELLULAR VESICLES ISOLATED FROM BOVINE OVARIAN FOLLICLES.


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Ovarian follicles are isolated by a basement membrane and contain granulosa, cumulus and an oocyte. Intercellular communication is crucial to induce cell proliferation and differentiation during follicle growth. The consequence of the follicular growth is production of hormones and a viable oocyte capable to generate a pregnancy. Part of the crosstalk occurs in cavity filled by follicular fluid called antrum. Recently, extracellular vesicles such as exosomes and microvesicles were identified within the follicular fluid and suggested as mediators of cell communication. Extracellular-vesicles can transfer bioactive molecules such as lipids, proteins, mRNA and miRNAs. Our hypothesis is that exosomes from bovine follicular fluid present coding and non-coding RNA molecules and different lipid composition associated with oocyte competence. In order to test this hypothesis bovine ovaries were collected from slaughterhouses and follicles between 3-6 mm were individually isolated. Follicle contents were separated under a stereomicroscope to allow the collection of follicular fluid and the COC. Follicular fluid was submitted to differential centrifugation for removal of cellular components and debris before freezing at -80C. COCs were in vitro matured for 18h and then evaluated for the presence of the 1st polar body. Activation of the denuded oocytes to generate parthenogenetic embryos started 26h after the beginning of maturation. After seven days in culture we accessed the oocyte competence and grouped the follicular fluid according to developmental competence. Developing groups were based on the ability or inability of the oocytes to mature and generate a blastocyst. Based on the competence groups we isolated extracellular vesicles from pools of 10 follicular fluids. Total RNA and lipids were extracted from the pools of extracellular vesicles and analyzed by Next Generation Sequencing and tandem mass spectrometry. Transcriptome analysis demonstrated the presence of coding and non-coding RNA species. Functional annotation analysis of exosomal RNA content demonstrated to be enriched for RNA molecules involved in the regulation of chromatin remodeling or transcription activation. Based on lipids analysis we identified different lipids enriched according to the development competence of the oocytes. We identified six lipids associated with poor oocyte competence; monoalkenyl diacylglycerol (MADAG 52:8+NH4 (-FA 18:1 (NH4))) and MADAG 48:8+NH4 (-FA 16:1 (NH4))) are examples of the lipids found in extracellular vesicles. Three lipids were associated with oocyte capability to generate a blastocyst, for example Digalactosyldiacylglycerol (DGDG 36:2+NH4 (-DGDG (NH4))), which was identified present in extracellular vesicles. Thus, our results demonstrated that extracellular vesicles carry coding and non-coding RNA molecules. Also, lipids identified are differently expressed in extracellular vesicles and are associated with oocyte competence. Further experiments are necessary to explore the different lipid molecules present in the extracellular vesicles and their role during follicle growth and oocyte maturation. Supported by FAPESP GIFT-2012/50533-2; CEPID-CTC-2013/08135-2; BPD-2013/10473-3.


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The importance of metabolic signals in regulation of ovarian function has long been recognized and metabolic stress in lactating dairy cows contributes to infertility. Recent studies revealed that there is major secretion of bile acids by the liver during the onset of lactation in dairy cows, and that they act as endocrine signals through specific membrane receptors. We previously showed that bile acids are twofold or more abundant in serum and in the follicular fluid of the dominant follicle of lactating cows relative to cycling heifers. Our objective was to determine whether one of the bile acids, cholic acid (CA), had an effect on the transcriptional regulation of steroidogenic genes, and, consequently, estrogen accumulation in a primary culture of bovine granulosa cells. Cells were cultured in 24-well plates, in Minimal Essential Media containing sodium bicarbonate (10 mmol/l) supplemented with 20 mM HEPES, sodium selenite (4 ng/ml), bovine serum albumin (BSA 0.1%), penicillin (100 U/ml), streptomycin (100 mg/ml), transferrin (2.5 mg/ml), nonessential amino acid mix (1.1 mmol/l), bovine insulin (10 ng/ml), androstenedione (10^-7 M), and bovine FSH (1 ng/ml) in the complete absence of serum. The cell density was 5.0x10^5 / ml, established to optimize both proliferation and the estradiol production of the granulosa cells. The cells were
incubated in a humidified atmosphere with 5.0% CO2 at 37 °C and cultured, with 70% of the spent medium replaced with fresh medium every 48 h. Cholic acid was added at 48 h after initiation of culture and the cells were then incubated for a further 2-72 h, in four wells per treatment. Estradiol was determined in medium by radioimmunoassay. The abundance of mRNA for steroidogenic factors STAR, CYP11A1, CYP17A1, CYP19A1 and HSD3B was determined in medium by qPCR. Doses of 5-100 nM CA impaired the accumulation of estradiol in culture. Time course trials indicated reduced accumulation could be recognized at 48 (>50 %) and 72 h (>75 %) following addition of 3nM CA. CYP19A1 mRNA abundance was significantly reduced at 12, 24 and 48 h, while CYP11A1 had a tendency to reduction at 12h and was reduced at 24–48h. STAR, CYP17A1 and HSD3B were unaffected by CA treatment. Preliminary investigation of effects of CA on intracellular signaling indicated that forskolin caused a fourfold increased in the accumulation of CYP19A1 at 2 to 6 hours of incubation. CA in the presence of forskolin diminished this stimulatory response by more than 75 %. The protein kinase A inhibitor, H89, did not prevent the CA induced impairment of CYP19A1 accumulation. We conclude that CA at concentration consistent with that found in follicular fluid of dairy cows inhibits estrogen synthesis. This suggests that the prevalence of bile acid in the follicular fluid in lactating dairy cows is implicated in their compromised fertility. Funded by a FRQ-NT grant to N. Gévy.

612. Identification of IL-17 as a Potential Mediator of Luteolysis: Inhibition of Progesterone Secretion.
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Recent studies have implicated cytokines and immune cells in the processes of luteolysis. Our objective was to identify cytokines that play a role in the regression of the bovine corpus luteum (CL). Our approach was to examine cytokine pathways and upstream regulators following acute (< 4 hr) treatment with a luteolytic dose of prostaglandin F2α (PGF). We then determined whether luteal cells were responsive to the identified cytokines by examining cell signaling effectors, progesterone synthesis, and cell viability in vitro. Postpubertal female cattle were treated at midcycle with saline or PGF (Lutalyse) and CL were collected at five time points (0, 0.5, 1, 2, and 4 h); n = 3 cows/time point. RNA from each CL was used for gene expression analysis using Affymetrix bovine whole transcript microarrays. Data were preprocessed using RMA then LIMMA was applied to compare the log ratio between each of the time points and the controls (false discovery rate < 0.05). Differentially expressed genes were analyzed by Ingenuity Pathway Analysis (IPA) to examine canonical pathway activation and upstream regulators. IL-17 signaling was identified as one of the major canonical pathways activated at each timepoint. Furthermore, NFκB signaling was identified as a major upstream regulator. Signaling by IL-17 was prominent at early time points; 30 min (P = 1.38E-05) and 1 h (P = 2.89E-05). Additionally the 2 and 4 h time-points indicated direct activation of downstream targets of IL-17. Enriched bovine small and large luteal cells (SLC and LLC, respectively) were prepared by centrifugal elutriation and used to examine the actions of IL-17, PGF, and tumor necrosis factor (TNF) either alone or in combination. Western blot analysis revealed that treatment with PGF for up to 4 hr resulted in activation of MAPK signaling but not NFκB signaling. Treatment of SLC and LLC for 15 min with increasing concentrations of IL-17 stimulated phosphorylation of NFκB P65 in a concentration-dependent manner; maximum stimulation at 10 ng/mL (SLC 2.4-fold, P < 0.001, n = 4; LLC 2.2-fold, P < 0.01, n = 3). Temporal studies revealed that the response to IL-17 (10 ng/mL) on phosphorylation of P65 was rapid (5 min: SLC 2-fold, P < 0.01; LLC 2.3-fold, P < 0.001) and sustained for an hour. To evaluate the effect of IL-17 on progesterone synthesis, SLC and LLC were pretreated for 24 hr with IL-17 alone or in combination with TNF and PGF, and then stimulated with or without LH (10 ng/mL). Treatment of SLC with IL-17, PGF or TNF alone had no effect on basal or LH-stimulated progesterone secretion. However, IL-17 in combination with TNF and PGF significantly reduced (93% inhibition, P < 0.05, n = 3) LH-induced progesterone secretion in SLC. In LLC neither IL-17 alone nor in combination had an effect on basal or LH-stimulated progesterone secretion. Furthermore, neither SLC nor LLC treated with IL-17 alone or in combination with TNF and PGF showed significant changes in cell viability as measured by MTT (SLC n = 4; LLC n = 3) and determined by a two-way ANOVA. PGF induces expression of immune regulatory genes in vivo, noteworthy was the IPA prediction of IL-17 signaling and NFκB activation. We determined that IL-17, but not PGF, directly activate NFκB signaling in luteal cells and IL-17 in combination with TNF and PGF reduced LH-stimulated progesterone secretion in SLC. Our study suggests that PGF induced IL-17 and cytokine activation of NFκB signaling contributes to the dampening of progesterone synthesis by inhibiting LH-stimulated progesterone secretion. Research supported by: USDA AFRI, the VA Medical Center, and Olson Center for Women's Health.

613. The effects of obesity in female Ossabaw mini-pigs on the microbiome of the urogenital tract.
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Two thirds of Americans are overweight or obese, resulting in a significant financial burden on the healthcare system in the United States. Obesity negatively impacts both the immune and reproductive systems, resulting in increased systemic inflammation and abnormal ovarian function. Utilizing next generation sequencing technology, research has shown that the dynamic of the microbial community in mucosal tissues correlates with different states of physiological “health”. The goal of our research program is to examine how triggers of inflammation – obesity as an example – alter reproductive function as assessed by investigations of the urogenital microbiome. Specifically, we hypothesize that obesity negatively impacts the immune protection of the microbial community profile within the urogenital tract and alters serum progesterone and estradiol concentrations throughout the estrous cycle. To test this hypothesis we utilized the Ossabaw pig as an animal model of a “thrifty” metabolic phenotype. Ossabaw pigs have a loss of function mutation in the Val199Ala allele of the PRKAG3 gene (the γ3 isofrom of AMP-activated protein kinase) that is associated with accumulation of increased intramuscular fat. Five nulliparous, sexually mature Ossabaw pigs were fed an excess calorie, high fat/cholesterol/fructose diet (n=3) or a control diet (n=2) for seven months. After a six cycle (~ three-month) diet induction period, pigs remained on their respective diets for an additional four months and had collection of vaginal swabs, cervical flushings, ovarian ultrasound, and paired serum samples on cycle days

2015 Abstracts – Page 243
1 (estrus), 4, 8, 12, 16, 18, 20 and 22 for two estrous cycles. Vaginal swabs and cervical flushings were paired with serum samples to measure microbial changes within the urogenital tract in relationship to ovarian hormone profiles. Ovarian ultrasound was used to help assess ovarian function. Sample days 4, 8, 12 corresponded with the luteal phase and sampling days 1, 16, 18, 20, and 22 corresponded with the follicular phase of the estrous cycle. Bacterial phylotype profiles were derived from the uterine tract (vaginal swabs and cervical flushings) from control and obese animals. Briefly, microbial community structures were generated using deep rDNA sequencing of the hyper-variable V3-V5 region of the 16S ribosomal RNA (rRNA) gene and then sequenced using the MiSeq platform. We were able to isolate bacterial DNA in all samples collected longitudinally from all animals. Bacterial DNA has been sent for sequencing, and clustering of sequence data into specific operational taxonomic units (OTUs) will determine ecological dynamics through the inference of taxonomy utilizing Qiime. Mother and LeFseq software programs. With these data, we will identify uterine bacterial microbiome profiles induced by obesity as well as the influence of cycling ovarian hormones on urogenital microbial dynamics. The data from this pilot study may indicate important pieces of information about previously unrecognized bacterial species, genera, families, or phyla that may cause or exacerbate reproductive and immune disorders in obesity or may, in fact, maintain “health” in an obese environment. This research was supported by research funds of Dr. Braundmeier-Fleming (SIUSOM) and NIH K01 RR031274-01A21 (Newell-Fugate).

614. Direct inhibitory effects of progesterone on porcine luteal macrophages; a role for genomic and/or membrane-associated progesterone receptors?
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Introduction: Resident macrophages have been identified within the corpus luteum (CL) of multiple species. Although the number of macrophages in the CL increases towards the end of the luteal phase, at which time they appear to play a role in luteal regression, they are also present in the early stages of the estrous cycle, when they play a critical role in the development of the luteal vasculature. Luteal macrophages are also present during the mid-luteal phase, and although their role at this stage of the estrous cycle is not yet entirely clear, we have shown that TNF-a is capable of sensitizing porcine luteal cells to the luteolytic actions of PGF-2α. Resident macrophages exist within the corpus luteum at a time when luteal progesterone secretion is maximal and data from other species has shown that progesterone has marked effects on macrophage function. Thus in this study we planned to 1) examine the direct effects of progesterone on porcine CL-Derived (i.e. CL resident) Macrophages (CLDM) in vitro (Experiment 1), and 2) examine CLDM for the presence of genomic (PGR) and membrane-associated (mPRs) progesterone receptor mRNAs (Experiment 2). Methods: For both experiments, mid-cycle porcine corpora lutea were subjected to collagenase dispersion and plated on collagen-coated culture plates in M199 + 10% FCS overnight to collect CLDM. In Expt. 1, CLDM (~0.5 x 10^6 per ml) were cultured for 24h with progesterone (0, 0.005, 0.05, 0.5 and 5 mg/ml), after which CLDM were collected for RNA extraction, and analysis of cell surface marker (CD40, CD68 and CD163) and cytokine (TNF-a, IL-1β, IL-6, IFN-g, TGF-b and IL-10) mRNAs by Q-PCR. In Expt. 2, CLDM isolated by plating were subjected to RNA extraction and quantification of genomic (PGR) and membrane-associated (mPRs; PAQR5, PAQR7, PAQR8, PGRMC1 & PGRMC2) mRNAs by Q-PCR. Results: In Expt. 1, progesterone treatment resulted in a 2-5 fold decrease in cell surface marker, and a 2-3 fold decrease in cytokine mRNA expression. In Expt. 2, CLDM were shown to express PGR and mPR mRNAs, in the following order of abundance; PGRMC1 (100 arbitrary relative units) > PGRMC2 (28) > PGR (1.6) > PAQR5 (0.8) > PAQR8 (0.7) > PAQR7 (0.4). Conclusions: Porcine CLDM express both genomic and membrane-associated progesterone receptors, and respond directly to exogenous progesterone with an inhibition of mRNA expression of cell surface markers and cytokines. These data suggest that progesterone, at levels comparable to those seen in the mid-cycle CL, may directly inhibit the function of resident luteal macrophages in vivo. This project was supported by AFRI Grant no. 2012-67015-19349 from the USDA/NIAP (JEG, JC), a grant from NIH/ORIP T35OD11070 (AF), a grant from NCSU Office of Undergraduate Research (AO), and by funds from the State of North Carolina (JEG, JC).

615. Early Pregnancy Induces Cell Survival Pathways and Inhibits Cell Apoptosis Pathways in the Corpus Luteum in Sheep.
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In sheep, days 14-16 are the critical period for regression and rescue of corpus luteum (CL) during which exposure to a variety of cell survival or death singling. During luteolysis, pulsatile release of PGF2α by the endometrium is locally transported to CL and causes functional and structural luteolysis. If healthy conceptus present in the uterus, the conceptus and/or endometrium derived factor may directly inhibit the function of resident luteal macrophages in vivo. This project was supported by AFRI Grant no. 2012-67015-19349 from the USDA/NIAP (JEG, JC), a grant from NIH/ORIP T35OD11070 (AF), a grant from NCSU Office of Undergraduate Research (AO), and by funds from the State of North Carolina (JEG, JC).
endometrial and/or conceptuses derived molecules, yet to be identified mechanistically, are locally transported to ovary through utero-ovarian plexus and activate several cell survival pathways and inhibit apoptosis pathways and thus protect the CL from apoptosis/death at establishment of pregnancy. This study was supported by USDA-NIFA awards 2008-35203-19101 and 2013-67015-20967.

616. Ovarian ring finger protein 6 (RNF6) regulates granulosa cells growth via a modulation of androgen receptor (AR) activity. Jung Jin Lim1, Ji Eun Han2, Hannah Mazier1, Dong Ryul Lee2, Benjamin K. Tsang1.
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Background: Follicular cell proliferation and survival are tightly regulated by androgen. Androgen receptor (AR) is the key transcription factor mediating androgen signaling, which plays important regulatory roles in follicular development. In addition to AR expression, the degradation of AR also plays an important role in regulating the intracellular steady state level of AR and the responsiveness of the cells to androgen. Ring finger protein 6 (RNF6), a member of the small nuclear Ring finger protein family, induces AR ubiquitination although its role in AR degradation is still unclear. The relative expression of RNF6 and its role in the regulation of ovarian folliculogenesis by androgen is not known. Objective: The objective is to examine the expression and role of RNF6 in cell fate determination during the regulation of ovarian follicular cells development by androgen. Methods: To examine the expression level of AR and RNF6 in the rat theca cells, granulosa cells (GCs) and oocyte at different stages of follicular development, ovarian sections in 21 days old rats were assessed by immunofluorescence. To examine if AR and RNF6 expression in the GCs is regulated by androgen (DHT; 5α-dihydrotestosterone) and if this regulation is dependent on the stage of follicular development, GCs from pre/early antral follicles (3 days injection of DES) and from late antral follicles (1 day injection of PMSG) were cultured in the presence of DHT (0-10 µM) for 24 hours and AR and RNF6 content was examined by Western blot and immunofluorescence. In addition, GCs proliferation (S-G2 phase) and apoptosis (sub-G1) were examined using cell cycle analysis. Results: Immunohistochemistry studies indicate that AR immunostaining in rat ovary tissue section was strongly observed in the granulosa cells but not in the oocyte and theca cells of all follicular stages. Similarly, RNF6 was strongly expressed in granulosa cells at all follicular stage in rat ovary, and its expression was low in the theca cells. In vitro studies showed that, AR and RNF6 expression were detected in cultured GCs from pre/early antral follicles is regardless of the concentration of DHT (0-10µM), increased. Also, cultured GC from late antral follicles showed similar expression pattern, but there is no significant difference in each DHT treated cells. Cell cycle analysis indicates that DHT increased cell proliferation and decreased apoptosis in GCs from pre/early antral follicles, but the opposite responses (particularly in the cell proliferation) were observed in the GCs from the more mature follicles. Conclusion: This study demonstrates, for the first time, that the expression of RNF6 during ovarian follicular cell growth and that this response is regulated by the actions of androgen in a follicular stage-dependent manner. Our findings significantly contribute to the current understanding of the cellular basis of androgen action in ovarian follicular growth. This work was supported by a grant from the Canadian Institutes of Health Research (CIHR to KBT) and the HDCYH-Training Program in Reproduction, Early Development, and the Impact on Health (REDIH to JJL and HM).

617. Granulosa Cell Cycle Regulation and Steroidogenesis in a High Androstenedione Follicular Microenvironment.
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Anovulatory infertility (either chronic or sporadic anovulation) affects up to 40% of infertile women. In fact, sporadic anovulation in humans may often go unnoticed since recent literature has reported that 8-13% of normally menstruating women (250 total, two reproductive cycles) exhibit sporadic anovulation. To gain a greater understanding of anovulation, our lab has identified a naturally-occurring bovine model system which includes a subpopulation of cows with a 17% reduction in calving rate (P < 0.07) as well as endocrine profiles and ultrasound-tracked follicular development profiles that resemble those of women with chronic or sporadic anovulation. These cows exhibit excess androstenedione (A4) accumulation in their follicular fluid (10%; >30 fold higher) and lack an increase in estrogen production of similar magnitude (only 2 to 4 fold higher). Our objective was to gain a mechanistic understanding of the causes and consequences of this high-androgen follicular microenvironment.

In pursuit of this objective, our lab performed microarray analysis using Affymetrix Bovine GeneChip 1.0 ST on the granulosa cells from control Low Androstenedione (Low A4; n=4, follicular A4 <20 ng/mL) and High A4 (n=5, follicular A4 > 40 ng/mL) cows. Overall, based on ANOVA array statistical testing (P<0.005 and FDR<0.05) there were 1229 genes upregulated and 255 downregulated in granulosa cells from High A4 cows compared to Low A4 cows. Using Ingenuity Pathway Analysis, we found that granulosa cells from the High A4 population exhibit a strong inhibition of the cell cycle, especially G1/S checkpoint proteins (e.g. cyclins and cyclin-dependent kinases), regulators of chromosome alignment and segregation (e.g. kinesins and related molecules), and other cell cycle regulators. Validation of the microarray results with qRT-PCR has confirmed the differential regulation of several genes. For example, CCNA2 (Cyclin A2) regulates cell cycle checkpoint progression and was downregulated in High A4 granulosa, suggesting that those cells may be undergoing cell cycle arrest. An additional gene, ECT2, is required for signal transduction pathways that control cytokinesis and is expressed in a cell cycle-dependent manner (with expression during DNA synthesis through cytokinesis). ECT2 is downregulated in High A4 granulosa, which suggests that fewer cells in the sampled population are in S Phase, G2, or mitosis and/or that cytokinesis may be impaired. Moderate increases in genes associated with lipid intake (membrane transporters) and lipid metabolite breakdown (UGT family) in High A4 cows suggest increased steroidogenesis in granulosa cells from High A4 vs Low A4 cows.

This cow population with excess intrafollicular androgen and sporadic anovulation is an excellent model to study mechanisms involved in female anovulatory infertility and ultimately develop therapeutics and procedures to enhance reproductive success in women. Furthermore, this bovine population can also be used to develop infertility markers in agriculturally relevant species which may be used in selection programs to ensure better reproductive success within the livestock industry.

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progression to stages 2 and 3 suggesting that VEGFA165b can antagonize the actions of angiogenic isoform VEGFA165. Therefore, these cortex which supports previous data in our lab. Interestingly, VEGFA165b inhibited VEGFA165-dependent stimulation of follicular (0.037±0.001). Thus, we can conclude from these results that VEGFA isoform, VEGFA165, can stimulate follicle progression in bovine with VEGFA165 (0.112±0.001; P < 0.0001) vs the other treatments: VEGFA165b (0.019±0.001), Combination (0.010 ±0.001), and PBS (0.20±0.02). Furthermore, there was also an increase in the number of secondary follicles (stage 3) that were in the bovine cortex treated with VEGFA165 (0.112±0.001; P < 0.0001) vs the other treatments: VEGFA165b (0.019±0.001), Combination (0.010 ±0.001), and PBS (0.37±0.001). Thus, we can conclude from these results that VEGFA isoform, VEGFA165, can stimulate follicle progression in bovine cortex which supports previous data in our lab. Interestingly, VEGFA165b inhibited VEGFA165-dependent stimulation of follicular progression to stages 2 and 3 suggesting that VEGFA165b can antagonize the actions of angiogenic isoform VEGFA165. Therefore, these
bovine cortex cultures are an excellent model to understand the antagonistic mechanisms of VEGFA165b, the antiangiogenic isoform, on the ability of VEGFA165 to stimulate follicle progression. It is possible that VEGFA165b contributes to the block in follicle development in the ovary to maintain the ovarian reserve and guard against premature ovarian failure.

620. Crosstalk Between The Retinoic Acid and Calcium Signaling Pathways in Mouse Ovarian Granulosa Cell Function. Michael Demczuk¹, Carl White², Huiya Huang⁰, Jingjing Kipp¹. ¹Biological Sciences/DePaul University, Chicago, IL, USA; ²Physiology and Biophysics/Rosalind Franklin University of Medicine and Science, North Chicago, IL, USA

Normal development of ovarian follicles is critical for female reproduction and endocrine function. We have identified retinoic acid (RA) and the RA-degrading enzyme CYP26B1 as regulators of ovarian follicle development. We have shown that RA and a CYP26 inhibitor stimulate ovarian granulosa cell growth. Tuning of the calcium signal is also known to modulate cell proliferation and apoptosis, suggesting that the calcium signal may orchestrate multiple aspects of granulosa cell function. The current study was designed to examine the role of intracellular Ca²⁺ signaling in mediating the effects of RA/CYP26B1 on primary mouse granulosa cell proliferation. In single-cell Ca²⁺ imaging experiments, treatment of cultured granulosa cells with RA was found to increase the steady-state Ca²⁺ content of the endoplasmic reticulum (ER) stores. This correlated with increased store-operated Ca²⁺ entry (SOCE) and enhanced IP₃-dependent Ca²⁺ release in response to purinergic stimulation. In proliferation assays, RA treatment or Cyp26b1 knockout stimulated proliferation while Cyp26b1 overexpression inhibited proliferation. When cells were treated with 2-Aminooxydiphenylborane (2-APB), a blocker of ER Ca²⁺ release and SOCE, or treated with Xestospongicin C, a selective IP3 receptor antagonist that specifically blocks ER Ca²⁺ release, cell growth was inhibited. When RA was given together with 2-APB or Xestospongicin C, the stimulatory effect of RA on cell proliferation was abolished. Further investigation showed that 2-APB or a specific SOCE blocker, 3,5-bis(trifluoromethyl)pyrazole (BTP-2), inhibited RA induction of RA response element (RARE) activation, confirming an important role for calcium signaling in mediating RA actions. Overall, these data support a model in which RA regulates ovarian follicle development by stimulating granulosa cell proliferation and this regulation is at least partly driven by the modulation of Ca²⁺ signals mediated by increased ER Ca²⁺ store filling. Support for this work was provided by a DePaul-RFUMS Collaborative Research Grant (JK and CW).

621. Investigating influence of gestational hyperleptinemia on ovarian microsomal epoxide hydrolase 1. Ahmad A. Al-Shabib¹, Laura C. Schulz², Kelly E. Pollock³, Aileen F. Keating⁴. ¹Iowa State University, Department of Animal Science, Ames, IA, USA; ²Dept of Obstetrics, Gynecology, and Women’s Health, University of Missouri, Columbia, MO, USA; ³Department of Animal Science, Iowa State University, Ames, IA, USA

Altered energy homeostasis during obesity has been linked to changes in ovarian physiology. Leptin, an adipose tissue-produced hormone, regulates appetite via hypothalamic signaling, and is frequently elevated in obese females. We have found that high maternal lep in during pregnancy protects female offspring from obesity, resulting in lower body weights, lower serum insulin, and reduced leptin mRNA. We previously observed obesity-induced increased protein abundance of ovarian microsomal epoxide hydrolase 1 (EPHX1) – a xenobiotic biotransformation enzyme. Therefore, we hypothesized that offspring of hyperleptinemic mothers would have lowered abundance of ovarian EPHX1. Ovaries were obtained from wild type females exposed in utero to either control conditions; wild type genotype (WT) or saline pump (SP), or to two paradigms of gestational hyperleptinemia; dams that were db⁻/⁻ (DB), which were severely hyperleptinemic, and dams fitted with a leptin pump (LP), which were moderately hyperleptinemic. Total ovarian RNA or protein were isolated and quantitative RT-PCR or western blotting performed to evaluated changes to mRNA or protein, respectively, encoded by Ephx1. Decreased (P < 0.05) Ephx1 mRNA was observed in offspring of DB relative to WT dams. Despite there being no changes in Ephx1 mRNA abundance between the LP relative to SP offspring, Ephx1 protein abundance was reduced (P < 0.05) by 11.2%. These data suggest that ovarian EPHX1 levels may be impacted by maternal body condition during gestation. These data add concern regarding gestational progamming of ovarian capacity to respond to environmental ovotoxicants.

622. The ovary is an alternative site of origin for high-grade serous ovarian cancer in mice. Jaejeon Kim¹, Lang Ma², Donna M. Coffey³, Martin M. Matzuk⁴. ¹Pathology & Immunology / Baylor College of Medicine, Houston, TX, USA; ²Pathology and Laboratory Medicine / The Houston Methodist Hospital, Houston, TX, USA

Though named “ovarian cancer,” it has been unclear whether the cancer actually arises from the ovary, especially for high-grade serous carcinoma (HGSC), also known as high-grade serous ovarian cancer, the most common and deadliest ovarian cancer. Besides, the tumor suppressor p53 is the most frequently mutated gene in HGSC (96-100%). Yet it remains unknown whether a mutated p53 can cause HGSC. In this study, we bred a p53 mutation, p53R172H, into conditional Dicer-Pten double-knockout (DKO) mice, a mouse model duplicating human HGSC, to generate triple-mutant (TKO) mice. Like DKO mice, these TKO mice develop metastatic HGSCs originating from the fallopian tube. Unlike DKO mice, however, even after fallopian tubes are removed in TKO mice, ovaries alone are capable of developing metastatic HGSCs, indicating that a p53 mutation can drive HGSC arising from the ovary. To confirm this, we generated p53R172H-Pten double-mutant (DMu) mice, one of the genetic control lines for TKO mice. These DMu mice, as anticipated, also develop metastatic HGSCs from the ovary, verifying the HGSC-forming ability of the ovary with a p53 mutation. Our study therefore demonstrates that ovaries harboring a p53 mutation, as well as fallopian tubes, can be a source of high-grade serous carcinoma in mice.

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623. Evaluation of glucose and lactate production by canine luteal cells in early cyclic and gestational diestrus. Ana Augusta P. Derussi¹, Flavia C. Destro¹, Carlos Renato F. Guaitolini¹, Rodrigo Volpato¹, Natalia Rodriguez¹, Angie Lagos-Carvajal¹, Mateus Sudano², Maria Denise Lopes². ¹Biological Sciences/DePaul University, Chicago, IL, USA; ²Physiology and Biophysics/Rosalind Franklin University of Medicine and Science, North Chicago, IL, USA
There is great similarity in several aspects between the cyclic and pregnant canine luteal phase. In order to investigate the metabolic pathways of luteal cells in early cyclic and gestational diestrus. We aimed to analyze glucose and lactate production in early cyclic and gestational diestrus luteal cells in vitro culture. Ovariectomy of 6 females was performed (3 on early gestational diestrus and 3 cyclic diestrus). The corpora lutea (CL) were enzymatically digested in solution containing DMEM and collagenase. The contents were filtered, centrifuged and resuspended in DMEM supplemented. These culture containing luteal cells were distributed on 24 well plate and incubated. A plate containing only culture medium served as control. Experimental and control solution containing luteal cells were filtered, centrifuged and resuspended in DMEM supplemented. These culture containing luteal cells were analyzed for glucose and lactate production using Metabolic Flux Analysis (MFA) and qPCR analysis of ovarian genes Dppa3, Pou5f1 and metabolic markers in the ovary, Tnfa, Il6, Il1b, Tlr4, and Tnfr1. The statistical analysis was performed using ANOVA (p <0.05) in SAS PROC GLM. Glucose consumption and lactate production increased during in vitro culture in both gestational luteal cells and cyclic diestrus, but glucose consumption and lactate production in gestational CL was greater than cyclic CL in initial (glucose in stage 1, p=0.0328 and lactate in stage 2, p=0.0221) and final (glucose and lactate in stage 3, p=0.0085- and p= 0.0009, respectively) culture stage. According to these results, we believe that there is no difference in the metabolic pathways used by pregnancy and cyclic luteal cells, however energy metabolism appears to be greater in pregnant CL than cyclic CL, since glucose consumption and lactate production was higher in gestational CL. This research was Financial supported by FAPESP.

624. Increased Firmicutes in the Cecum of Obese Female Mice is correlated with Increased Pou5f1 and Dppa3 mRNAs in Growing Oocytes.

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Our previous studies showed that embryo development is reduced and oocyte mRNA abundance is increased in a mouse model of obesity. Specifically, Dppa3 and Pou5f1 were increased 2-fold (P<0.02) in growing oocytes and 6- (P=0.024) and 12-fold (P<0.033), respectively, in ovulated oocytes collected from obese compared to lean mice. Chromatin immunoprecipitation data also indicated obesity-dependent increases in Dppa3 and Pou5f1 promoter activity in growing oocytes. However, obesity-dependent mechanisms of increased Dppa3 and Pou5f1 transcription have not been defined. Recently the relationship between gut microbial communities and obesity has been demonstrated. Indeed, the ratio of Bacteroidetes to Firmicutes, which represent the major phyla of gut microbes, is typically decreased in obese individuals and is associated with the development of chronic inflammation and increased lipid deposition. Despite the explosion of studies documenting the impact of the gut microbiota on the function of metabolically important tissues, studies linking the community structure of gut microbes and markers of ovarian function are lacking. Thus, the objective of the current study was to determine if obesity-dependent increases in oocyte mRNA abundances is correlated to differences in the gut microbiome. For this study, C57BL/6J (B6) or DBA2J (D2) mice were maintained on normal rodent chow (ND; 13% kcal fat) or a high fat diet containing either 45% (45HFD) or 60% (60HFD) of kcal from fat for 12 weeks. At 17 weeks of age, ovulation was induced using exogenous gonadotropins. Ovulated oocytes, whole ovary, adipose tissue, and cecum contents, were collected from each animal 16h post-hCG. There were no differences in ovulation rate or ovarian weight between experimental groups and all oocytes were only populated with corpora lutea and similar numbers of growing, transcriptionally active oocytes. As expected, abdominal adipose tissue weight was increased in B6 and D2 mice fed a 45HFD or 60 HFD indicative of an obese phenotype. The microbial community structure of each mouse was evaluated by amplifying and sequencing the V3 region of the 16S rRNA gene in cecum contents using an Ion Torrent PGM to a depth of ~13,000 reads per sample. The resulting quality passed reads were clustered using UPARSE and were further analyzed using Quantitative Insights Into Microbial Ecology (QIIME). Multivariate correlation analysis was performed using Multivariate Association with Linear Models (MaAsLin). The MaAsLin showed that ovarian Dppa3 mRNA is positively correlated with OTU1418 (P<0.0016) while ovarian Pou5f1 mRNA was positively correlated with, OTU328 (P=0.0009) and OTU1433 (P=0.0032). These 3 species of bacteria were classified as belonging to the phyla Firmicutes and family Lachnospiraceae at 80 % confidence based on the RDP “classifier” algorithm. To determine if the diet induced obesity also altered inflammatory markers in the ovary, qPCR analysis of Tnfa, Tlr4, Il6, and Il1b was carried out. Tnfa and Tlr4 mRNA was increased 2-fold (P<0.05) in whole ovary of 60HFD B6 females; however, no difference in Il6 or Il1b transcripts were detected. Together, these data suggest a potentially novel mechanism by which gut microbes influence the expression of ovarian genes and thereby contribute to the quality of oocyte development. Furthermore, manipulation of the gut microbiome may represent a therapeutic strategy to improve pre-conception care in overweight and obese women.

625. Androgen Action within the Preantral Follicle; A Role for the Epidermal Growth Factor Receptor (EGFR)?

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Polycystic ovary syndrome (PCOS) is the most common cause of anovulatory infertility, and affects 5 to 10% of the female population. The polycystic ovary is characterized by aberrant early follicle development in which hyperandrogenism is thought to play a key role. Although the molecular mechanisms of androgen action within the ovary remain largely unknown, recent evidence suggests androgens may be acting, in part, through modulation of key growth factor signalling pathways. We have recently shown that members of the epidermal growth factor (EGF) family play an important role in promoting preantral follicle development in the mouse, and have identified receptor subtypes EGFR (ErbB1), ErbB2 and ErbB3 in primary and secondary follicles. The aim of this study was to investigate evidence for androgen-EGF interaction within preantral follicles in the mouse. Preantral follicles obtained from postnatal day (PND) 16 mouse ovaries (C57BL/6) were mechanically isolated and cultured in the presence of EGF (10ng/ml) or dihydrotestosterone (DHT) (10nM) with or without the specific EGFR inhibitor AG1478 (10mM). Follicle growth was measured over 72 hours, with samples processed for qPCR and immunohistochemistry at 24 and 72 hours. Granulosa cells were isolated from PND26 mice (C57BL/6) and cultured for 48 hours before DHT (10nM) treatment. Following treatment, protein was extracted and analysed using immunohistochemistry and western blotting. Two-way ANOVA was used for statistical analysis unless otherwise stated. DHT significantly increased preantral follicle growth
from 24 hours (n = 6, p < 0.05). Additionally, EGF treatment increased follicle growth within the first 24 hours (n = 6, p < 0.001), with little further effect after this time point. Combined incubation with EGF and DHT resulted in elevated growth above that of individual treatments (n = 6, p < 0.05), indicating an additive effect of the two ligands. DHT treated follicles displayed no change in EGFR, ErbB2 or ErbB3 at the mRNA level, however, ErbB2 protein expression was down-regulated at 24 hours (n = 12, one-way ANOVA, p < 0.001), indicating a role for androgens in the regulation of ErbB abundance independent of transcription. Treatment with DHT increased androgen receptor (AR) protein expression in follicles at 24 hours, with elevation sustained for 72 hours (n = 8, one-way ANOVA, p < 0.001), confirming DHT as a positive regulator of the AR. The addition of AG1478, a specific EGFR tyrosine kinase inhibitor, not only reversed EGF stimulated follicle growth but also attenuated the effect of DHT on growth at 48 hours (n = 6, p < 0.01), and showed no inhibitory effects when added to culture alone. The suppression of the DHT stimulatory effect suggests that the actions of DHT on follicle development are mediated, at least in part, through the EGFR. Granulosa cells treated with DHT, showed a significant increase in ERK phosphorylation at 2 and 5 minutes, indicating the presence of non-genomic androgen signalling within mouse granulosa cells. Whether this non-classical pathway involves the EGFR is unclear and currently under investigation. In conclusion, our studies have shown that DHT stimulated growth is mediated partly through the EGFR. Rapid phosphorylation of ERK in the presence of DHT further suggests that androgen action is both genomic and non-genomic in the preantral follicle. Research supported by MRC (UK) PhD Studentship.

626. In Vivo Impact Of Vitamin A Deficiency or Retinoic Acid Synthesis Inhibition On Ovulation And Oocyte Maturation In Mice.
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Our previous studies have indicated that retinoic acid (RA), an active metabolite of vitamin A (VA), plays a role in regulating ovary development. Using an in vivo dietary VA-deprivation animal model, we have demonstrated that VA deficiency (VA-) causes a variety of ovarian pathologies, including reduced numbers of total follicles and corpus luteum, formation of hemorrhagic and atretic follicles, and formation of bursa and follicular cysts. The decreased number of corpus luteum in the VA- mice may result from ovulation defects. To test this hypothesis, current study examined both longterm and acute impact of VA- or RA-deficiency on ovulation and oocyte maturation in mice. To examine the longterm impact of VA-, VA- or control female mice were superovulated at D19 or Wk7 via a subcutaneous injection of pregnant mare’s serum gonadotropin (PMSG) followed by human chorionic gonadotropin (hCG). Ovulated oocytes were collected, counted, and classified. Oocyte count showed that the number of ovulated oocytes was significantly reduced in the VA- mice as compared to the controls at Wk7 but not at D19. Oocyte classification showed a reduced percentage of GV-oocytes at D19 and reduced percentages of GV-, GVBD-, and MII-oocytes at Wk7 in the VA- mice, suggesting abnormal oocyte maturation. To investigate whether acute RA-deficiency may affect ovulation event, mice on a regular diet were injected with the RA synthesis blocker WIN18446 at D19 or Wk7 for three days during superovulation induction. The results showed that a lower dose of WIN18446 suppressed ovulation and hindered oocyte maturation in Wk7 mice, as the number of ovulated oocytes and the percentages of GVBD- and MII-oocytes were reduced. At a higher dose, WIN18446 also suppressed ovulation and reduced the percentages of GV- and MII- oocytes in D19 mice. Overall, our results suggest that RA signaling plays a critical role in vivo in regulating ovulation and oocyte maturation and older animals are more susceptible to the impact of VA- and RA-deficiency. Supported by DePaul URAP (RU), Summer Research Grant (JK), and URC Grant (JK).

627. Altered tissue remodeling requisite for ovulation in a rat model and women with endometriosis is associated with subfertility.
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Ovulatory dysfunction occurs in women with endometriosis. We hypothesize that TIMP1 from endometriotic lesions alters ovarian proteinase activity and blocks tissue remodeling requisite for ovulation and hence is associated with subfertility in endometriosis. The objective of this study was to quantify ovarian expression of specific matrix metalloproteinase enzymes and their extracellular matrix substrates in a well-established rat model of endometriosis and in women, to provide insights into possible mechanisms of subfertility. Ovaries were collected from rats with surgically-induced endometriosis (Endo) and surgical controls (Sham) approximately 8 hours post-ovulation, and from women with and without endometriosis. mRNA levels of matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9), collagen IV (Col IV), fibronectin (FN) and laminin (LN) were measured by Q-RT-PCR and MMP-2 and MMP-9 enzyme activity assessed by zymography in rats. The spatial localization Col IV protein was studied by immunofluorescent histochemistry in ovaries from women with and without endometriosis. In Endo ovaries compared to Sham ovaries, MMP2 mRNA levels were down-regulated (-2.1-fold, P=0.002), Col IV mRNA was up-regulated (2.5-fold, P=0.019) and the MMP-9, LN, and FN mRNA levels were unchanged. MMP-2 (-4.6-fold, P=0.031) and MMP-9 (-1.9-fold, P=0.03) activities were both lower in Endo rats compared to Sham. More Col IV localized in the ovarian follicles of women with endometriosis compared to controls (2.36, P=0.005) while LN and FN localization were similar. Reduced MMP-2 and MMP-9 enzyme activity as well as MMP-2 mRNA combined with elevated mRNA Col IV in the ovaries of rats with endometriosis causes an imbalance in the proteinase / substrate milieu critical for the ovulation and is likely part of a mechanism impeding ovulation in endometriosis. These support or prior data showing TIMP1 from endometriotic lesions localizes in the ovarian theca, which correlates with reduced ovulation. Identification of increased Col IV basement membrane substrate in ovaries from women with endometriosis, and our prior data showing increased localization of TIMP1, suggests a similar mechanism may be present. (Supported by NICHD HD057445 to KST)

628. C/EBPα and C/EBPβ are Master Regulators of Vascular Remodeling in the Ovarian Follicle.
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Ovulation and luteinization are initiated by the ovulatory surge of luteinizing hormone (LH) and mediated by the induced EGF-like factors and their activation of MAPK3/1 (ERK1/2) that are essential for these processes. Two transcription factors, CCAAT enhancer binding protein alpha and beta (C/EBPα and C/EBPβ), are key targets of LH/MAPK that impact the terminal differentiation of granulosa cells and the vascularization of the ovulated follicle. Dysregulation of these events is involved in pathological conditions of the ovary, such as polycystic ovarian syndrome (PCOS), ovarian hyperstimulation syndrome (OHSS), and luteal insufficiency. We report herein that vascular remodeling in ovulatory follicles fails to occur in mice with granulosa cell-specific deletion of Cebpα and Cebpβ (Cebpα/-KO). Histological and immunofluorescent examination using multiple vasculature markers (Collagen IV, PECAM-1, KDR and Laminin-alpha) demonstrate that while extensive neovascularization occurs in corpora lutea (CL) of wild-type mice shortly after ovulation, this process is completely blocked in Cebpα/-KO mice. Light sheet fluorescence microscopy imaging of preovulatory follicle vasculature labeled with rhodamine-dextran further reveals that clearing of the capillary bed at the follicle rupture apex occurs in wild-type but not in Cebpα/-KO mice. A hormonal regimen that induces OHSS in wild- type mice with increased vascular density and macrophage infiltration in the CL does not induce these changes in the Cebpα/-KO mice, further supporting the indispensable role of C/EBPα/β in controlling vascular activity. To elucidate the molecular mechanisms by which C/EBP α/β regulate the ovarian follicular vasculature, we performed microarray analyses at 8 and 24 hours after human chorionic gonadotropin (hCG) induced ovulation. We show that known and novel genes involved in vascular remodeling in the ovary are dysregulated in the Cebpα/-KO mice compared to controls. Genes with decreased expression in the Cebpα/-KO mice include Vegfa and several of its isoforms, Kdr, Plxn1, Nrp1, Cdh5, Flt1, Cxcr4 and Sdc1; genes with increased expression include Thbs1, Thbs4, Sema3a, Sema3e, Hif1a, Angpt2 and Vegfb. Some of these genes, such as Vegfa, Plxn1, Thbs1, and Angpt2, have been shown to be direct targets of C/EBPβ in ChIP-seq analyses in adipose tissue. The markedly decreased expression of proangiogenic factors and increased expression of anti-angiogenic factors highlight C/EBPα/β as master regulators of vascular remodeling in ovulating follicles. We further characterized the expression of Sdc1 and Nrp1 and show that SDC-1 is highly expressed in newly formed luteal cells whereas NRP1 is elevated in follicular and luteal endothelial cells. Of clinical relevance several vascular genes downstream of C/EBPα/β are dysregulated in luteinized granulosa cells of PCOS patients, such as increased level of Thbs1 and decreased levels of Nrp1 and Cxcr4. Taken together, our results support the concept that dynamic vascular remodeling is an integrated process during ovulation and luteinization, and that C/EBPα/β are master regulators of this process.

629. **Cows with intrafollicular androgen excess have lower sex hormone binding globulin and appear to be chronic or sporadic anovulatory.**

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We have identified a population of cows with excess intrafollicular concentrations of androstenedione (A4; >30 fold) compared to their control counterparts with no similar fold increase in estrogen levels. Furthermore, these cows have reduced calving rates (17%; P < 0.07) suggestive of female subfertility. Therefore, our objective with the current study was to investigate endocrine and follicular profiles in cows with Low intrafollicular A4, (Low A4; < 20 ng/ml, control) and High A4 (>20ng/ml) during a reproductive cycle. After classification of cows as High A4 (n=6) and Low A4 (n=5), their estrous cycles were synchronized with an injection of prostaglandin in cows with Low intrafollicular A4, (Low A4; < 20 ng/ml; control) and High A4 (>20ng/ml) during a reproductive cycle. After their control counterparts with no similar fold increase in estrogen levels. Furthermore, these cows have reduced calving rates (17%; P < 0.07) suggestive of female subfertility. Therefore, our objective with the current study was to investigate endocrine and follicular profiles in cows with Low intrafollicular A4, (Low A4; < 20 ng/ml, control) and High A4 (>20ng/ml) during a reproductive cycle. After classification of cows as High A4 (n=6) and Low A4 (n=5), their estrous cycles were synchronized with an injection of prostaglandin (2015 Abstracts – Page 250

630. **Canine ovary-derived stem cells express mesenchymal and pluripotency marker genes and can differentiate into mesodermal and neuronal-like lineages.**

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The aim of this study was to compare the expression of pluripotent genes in canine ovarian and adipose stem cells and their neuronal differentiation potential. Ovarian and adipose tissue samples were collected after ovariectomy from four females aged from 6 to 12 months. After collagenase digestion and cell culture in DMEM with 10% FBS, ovarian- (oMSC) and adipose-derived (aMSC) mesenchymal-like stromal cells were analyzed for the expression of pluripotent markers and evaluation of their neural differentiation potential, after incubation with specific induction media. oMSC and aMSC were stable for over 15 passages and maintained positive expression of the MSC markers CD90, CD44 and CD105 while remaining negative to the hematopoietic markers CD34 and CD45. In addition, although pluripotent marker gene was present in early and advanced passages in both tissues, oMSC showed higher expression of OCT4 when compared to aMSC. Nonetheless, both oMSC and aMSC were able to differentiate not only to mesodermal lineages, i.e. adipocyte, osteocyte and condrocyte, but also the neural-like cells as characterized by neuronal morphology and immunocytochemistry for the neuronal markers β-tubulin III and nestin. However, only oMSC showed GDNF and BDNF gene expression by semi quantitative RT-PCR, suggesting enhanced differentiation when compared to aMSC. Therefore, the present study demonstrates that MSC-like lines can be obtained from canine ovarian tissue. Moreover, oMCS express pluripotent genes and can differentiate into meso- and ectodermal lineages in vitro, indicating an enhanced potential for use in canine therapeutic applications.

631. Differential effects of transforming growth factor beta 2 (TGFβ2) in two different models of preantral follicle growth.

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The onset of mammalian follicle development is marked by granulosa cell (GC) proliferation, GC shape change and oocyte growth. Despite their critical role in female fertility the factors and mechanisms underlying these cellular changes remain largely unknown. Members of the transforming growth factor beta (TGFβ) superfamily have been strongly implicated in early follicle growth. The SMAD 2/3 proteins are intracellular mediators of TGFβ signalling and are present in the GCs of early preantral follicles. Recent unpublished data from our lab has demonstrated the absence of SMAD 2/3 in the nuclei of proliferating GCs of growing follicles. This suggests SMAD 2/3 export from the nuclei may be necessary for GC proliferation and therefore follicle growth. We propose a role for the SMAD 2/3 pathway in regulating early follicle development. This study therefore aimed to examine the role of the TGFβ superfamily members, which act in regulating the SMAD 2/3 pathway, on follicle activation and preantral follicle growth. Preantral follicles were mechanically isolated from postnatal day (PND) 21 mouse ovaries (C57BL/6) and qRT-PCR performed to compare transcript abundance of key ligands, receptors and antagonists of the SMAD2/3 pathway. The most abundantly expressed ligand in both isolated preantral follicles and oocytes was TGFβ2. To relate protein expression with transcript data PND 12 ovary sections were immunolabelled for TGFβ2 which was localized in the oocytes of preantral follicles and the ovarian surface epithelium. To investigate follicle activation PND 4 mouse ovaries were treated with TGFβ 2 (1, 10, 100 ng/ml) in a whole-ovary culture system for 6 days. Cultured ovaries were fixed for immunohistochemical localization of vasa and laminin (to define oocyte and basement membrane for morphological classification) and caspase-3 (apoptosis). Image analysis was performed to quantify follicle proportions and abundance of caspase-3 staining. TGFβ2 suppressed follicle activation in the whole ovary as demonstrated by a smaller proportion of growing follicles in TGFβ2 treated ovaries compared to vehicle treated (n = 3 - 6 ovaries, logistic regression; 1ng/ml (p < 0.05) and 100 ng/ml (p < 0.01)). No qualitative differences in caspase-3 staining were demonstrated between treatment groups (i.e. no evidence of TGFβ2 toxicity). Preantral follicles were isolated from PND 15 mouse ovaries and cultured with TGFβ2 (0.01, 0.1, 1 ng/ml), SB505124 (a selective inhibitor of TGF-β type 1 receptor ALK5) or TGFβ2 neutralizing antibody (100 ng/ml and 10 ng/ml). Follicle area was measured at 0, 24, 48 and 72 hours (ImageJ). TGFβ2 promoted increased growth of isolated follicles, relative to vehicle, at all concentrations after 24, 48 and 72 hours (n = 6 ovaries, 2-way ANOVA; p < 0.0001). Preliminary work demonstrated that the ALK5 inhibitor blocked the growth promoting effects of TGFβ2 and when added alone reduced follicle size at 48 and 72 hours, however the effect may not be specific as the inhibitor can potentially block all ligands that signal through ALK5. To ensure a more targeted investigation we employed a TGFβ2 neutralizing antibody. The TGFβ2 antibody suppressed the effect of exogenously applied TGFβ2. However, at the doses used, the antibody did not supress follicle growth below that of the vehicle suggesting it is not affecting any endogenous TGFβ2. In conclusion, our results highlight differential effects of TGFβ2 in two different models of preantral follicle growth. Research supported by MRC (UK) PhD Studentship.

632. FIBROBLAST GROWTH FACTOR 10 STIMULATES CUMULUS EXPANSION IN BOVINE OOCYTECTOMIZED CUMULUS-OOCYTE COMPLEXES.

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We have previously observed that the presence of the oocyte is required for full cumulus expansion and glycolytic activity in bovine cumulus-oocyte complexes (COC) submitted to IVM. Fibroblast growth factor 10 (FGF10) is an oocyte derived factor capable of enhancing expansion and embryo development when added to the in vitro maturation (IVM) medium. In this study, we tested whether FGF10 would compensate for the absence of the oocyte and promote full expansion and glycolytic activity in oocytectomized cumulus-oocytes complexes (OOX) submitted to IVM. Since FGF10 restored cumulus expansion but not glycolytic activity, we tested the effects of oocytectomy and FGF10 on mRNA levels of rate-limiting genes of the hexosamine pathway that leads to hyaluronic acid production, the major component of the expanded extracellular matrix [glutamine-fructose-6-phosphate transaminases 1 and 2 (GFPT1 and GFPT2), and hyaluronan synthase 2 (HAS2)]. Follicles at 3-8mm in diameter were aspirated from abattoir bovine ovaries, and grades 1 and 2 COCs were selected. Intact COCs, OOXs and OOXs with FGF10 at 1ng/ml and 10ng/ml were submitted to IVM in groups of 20 complexes. Oocytectomy was performed by aspiration of the ooplasm with a micromanipulator. IVM was performed at 38.5°C and 5.5%CO2 in 96 well plates containing 100μl of TC medium supplemented with pyruvate (22μg/ml), amikacin (75μg/ml), FSH (1μg/ml) and BSA (0.4mg/ml) during 22 hours (to assess cumulus expansion, glucose uptake, lactate production and gene expression) or 4 hours (to assess earlier changes in gene expression). Expansion degree was visually assessed (grades 1 to 3) and glucose and lactate concentrations were measured by dry
chemistry. Cumulus cells were mechanically separated from oocytes, total RNA was extracted and abundance of mRNA encoding HAS2, GFPT1 and GFPT2 was assessed by real-time RT-PCR using Power SybrGreen (LifeTech®) and CYC-A as the housekeeping gene. Cumulus expansion and lactate production were decreased in OOXs compared with intact COCs. FGF10 at 10ng/ml restored cumulus expansion but not lactate production in OOXs. Levels of HAS2 mRNA were lower in OOXs after 4 or 22 hours of IVM, and treatment with FGF10 did not restore HAS2 mRNA expression. The removal of the oocyte decreased abundance of GFPT1 mRNA, but not of GFPT2, in cumulus cells at 4 hours of IVM. FGF10 did not alter GFPT1 or GFPT2 mRNA expression in cumulus cells from OOX at 4 hours of IVM, but increased GFPT1 mRNA abundance at 22 hours of IVM. These data suggest that FGF10 can stimulate expansion of OOX by increasing GFPT1 mRNA expression and thus driving glucose to the production of hyaluronic acid.

633. PRENATAL NUTRIENT RESTRICTION INDUCES ACCELERATED PRIMORDIAL FOLLICLE RECRUITMENT AND REDUCED OVARIAN FOLLICLE RESERVE.

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The intrauterine environment induces developmental adaptations that impact health and disease risk later in life. We have shown using a rat model that offspring born to mothers that were nutrient restricted during pregnancy are growth restricted, enter puberty early, and as adults, display follicle loss and ovarian oxidative stress; characteristics of early ovarian aging. The underlying mechanisms remain unclear. We hypothesize that early life nutritional adversity impacts key proteins involved in ovarian follicle recruitment and growth including the PI3K/Akt pathway. Pregnant Wistar rats were randomized into one of 2 groups: mothers fed control diet during pregnancy and lactation (CON), and mothers fed 50% of control intake during pregnancy (UN) and then fed a control diet ad libitum during lactation. At 21 days of postnatal life (P21), offspring were weaned to a control diet and reproductive cyclicity was evaluated at 60 days of age. At P4, P27, and P60, follicles were counted, processed, sectioned at 5µm, and follicle numbers assessed. Sections were stained for immunopositive phospho-Akt (pAkt) and melatonin receptors type 1 & 2 (MT1, MT2). Serum levels of gonadotropins, estradiol, progesterone, and anti-müllerian hormone (AMH) were measured at P27 and P60 using ELISA. Prenatal nutrient restriction resulted in irregular estrous cyclicity and persistent estrus (p<0.02) in UN offspring compared to control. In young adulthood (P60), prenatal UN offspring demonstrated a decrease in ovarian antral (p<0.01), corpora lutea numbers (p=0.03), and tended to have lower primordial follicle numbers (p=0.095). This loss was accompanied by an increase in atretic secondary follicles (p=0.02). Neonatal (P4) and pre-pubertal (P27) follicle numbers were similar between groups. Immunopositive pAkt in P4 and P27 ovaries was localized to the oocyte, and semi-quantitative analysis demonstrated a modest pAkt increase at P4 (p=0.08) and P27 (p=0.07) in UN offspring ovaries. Immunostaining for MT1 and MT2 was localized in the granulosa cells, theca cells, and oocyte in P27 and P60 offspring ovaries. Semi-quantitative analysis showed no change in MT2 in P27 and P60 UN offspring ovaries. However, at P27 MT1 is decreased in primordial (p=0.02), and secondary (p=0.04) follicles in UN offspring ovaries. At P60 there is a significant increase in MT1 in secondary follicles of UN offspring (p<0.01). Serum estradiol concentrations were decreased (p=0.04) in P27 UN offspring compared to CON. Serum AMH levels were slightly lower (p=0.08) in P60 UN offspring. Serum FSH, LH, and progesterone levels were similar between groups. Our results show that prenatal UN impaired follicle recruitment and resulted in a loss of antral follicles already in young adulthood. Although follicle numbers are not different early in neonatal and prepubertal life, it appears that early in neonates, pAkt-mediated primordial follicle recruitment could be accelerated, contributing to a loss later in life. We hypothesize that this loss may be associated with a reduction in melatonin’s actions on folliculogenesis, mediated through MT1 in a age dependent manner. Studies investigating accelerated recruitment of primordial follicles as a mechanism to explain a loss of follicle reserve are ongoing.

634. Role of Progesterone Receptor Membrane Component-1 in regulating bovine granulosa cells mitosis: a preliminary study.

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Progesterone Receptor Membrane Component-1 (PGRMC1) is expressed in bovine granulosa cells (bGC) during all folliculogenesis stages. Many experimental evidences in rat and human ovarian cell lines indicate that the mechanisms of action through which PGRMC1 regulates cell proliferation include a direct function during the mitotic (M) phase of the cell cycle. Therefore, the present studies were designed to assess PGRMC1’s role in regulating mitosis in bGC. First, PGRMC1’s cellular localization was evaluated by immunofluorescence at each stage of the cell cycle in bGC collected from antral follicles and cultured in vitro in the presence of serum added to stimulate mitotic activity. These studies revealed that PGRMC1 is localized in both nuclear and cytoplasmic compartments during interphase, while it concentrates in the area of the spindle apparatus during M-phase, showing a specific localization in the midzone and the midbody during Ana/Telophase and Cytokinesis, respectively. In order to assess the effect of perturbing PGRMC1 function on bGC cell proliferation, bovine PGRMC1 expression was silenced using small interfering RNA (RNAi). Cells were transfected with PGRMC1 or CTRL RNAi using lipofectamine and cultured for 24, 48 or 72h. Transfection efficiency was tested using fluorescent-labeled RNAi and was estimated to be 80%. Quantitative RT-PCR and western blotting analysis confirmed that RNAi treatment significantly reduced PGRMC1 expression up to 72 h, when compared to the CTRL- RNAi treated group (t-test, P<0.05). Importantly, cell counting at each time point revealed that PGRMC1 depletion significantly reduced cell proliferation by 30.5 and 41.7% after 48 and 72 h of treatment respectively (t-test, P<0.05). Furthermore, flow cytometry showed that the lower growth rate of PGRMC1 depleted cells associated to a higher percentage of cells arrested at G2/M phase compared to CTRL RNAi treated group at 72h (2.3±0.9% and 9.7±1.2%, t-test, P<0.05). Altogether, these data suggest that PGRMC1 depletion delays mitotic progression in bGC, confirming previous studies in rat granulosa cells. Ongoing live imaging experiments will clarify the precise stage(s) of the M-phase that is mostly affected by PGRMC1 depletion. Finally, in order to explore possible PGRMC1’s mechanisms of action in mitotic cells, we have started to hypothesize that PGRMC1 could play a role in vesicular trafficking during M-phase, which in turn is emerging as a fundamental process that ensures proper cell division, since PGRMC1 is a membrane protein found associated to cellular vesicles. Therefore, we conducted immunofluorescence and in situ-Proximity Ligation Assay studies that indicated that PGRMC1 co-localizes and directly binds to clathrin during interphase and all stages of

2015 Abstracts – Page 252
the M-phase. This is important in that clathrin not only participates in endosomes formation but also has an important function during cell division; during mitosis, indeed, clathrin concentrates at the spindle apparatus, where it stabilizes fibers of the mitotic spindle aiding chromosomes congression (Royle et al, Nature 2005). Moreover, clathrin mediated endocytosis is important for cytokinesis and final abscission (Warner et al, Traffic 2006). Taken together, our study set the stage for further investigations that will elucidate the role of PGRMC1 in granulosa cell mitosis. Funding: ‘FP7-PEOPLE-2011-CIG, 303640, Pro-Ovum’ and ‘FONDO di RICERCA GIOVANI RICERCATORI (15-6-3027000-54) University of Milan’.

635. Quantification of C-type natriuretic peptide (NPPC) and immunolocalization of type 2 receptor (NPR2) in bovine antral follicles.

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The NPPC-NPR2 signaling pathway is essential for the maintenance of meiotic arrest in murine oocytes, however, this system has not been characterized in bovine species. The aim of this study is to detect and quantify NPPC protein in granulosa cells (GC), follicular fluid (FF) and extracellular vesicles (ECV) of antral follicles, and localize its receptor (NPR2) in the bovine ovary. For that purpose, antral follicles were dissected and separated into three groups according to the follicular diameter (3-6 mm, 6-8 mm and > 8 mm). FF was aspirated from 10 follicles then, GC were retrieved by centrifugation (10,000 × g for 3 min) and ECV by ultracentrifugation (two steps 100,000 × g for 70 min) of the follicular fluid. GC and ECV samples were homogenized for 3 minutes with Polytron® (UltraTurrax) and vortex, respectively, and immersed in Trizol® (Invitrogen) for total protein extraction according to the manufacturer's protocol. The NPPC protein was investigated in pure FF, in GC and ECV by immunosorbent assay (ELISA) using C- Type Natriuretic Peptide (CNP) ELISA Kit (Blue Gene - E02C0339 Cat.). For immunodetection of NPR2, deparaffinized sections of bovine ovaries (3 µm) were placed into citrate solution 10 nM (pH 6) for 30 min in the pressure cooker Pascal® (DakoCytomation) for antigen retrieval. Then the sections were incubated with anti-human NPR2 antibody (1: 200, Novus Biologicals, Cat H00004882-M01) for 18 hours in humidified chamber at 2 to 8 °C. After incubation, slides were washed and incubated with the secondary antibody Envision Dual-Link System® (DakoCytomation) for one hour at room temperature. Immunostaining was detected with liquid DAB® (DakoCytomation). The NPR2 was detected by ELISA in all three samples assessed and higher levels were detected in ECV, regardless follicular diameter. In GC, smaller follicles (3 to 6 mm) showed higher concentrations of NPPC compared to large follicles (> 8 mm). No differences among the three groups of follicular diameter were observed in FF and ECV. NPR2 was predominantly detected in GC of bovine ovaries and in a lesser intensity in follicles (3 to 6 mm) showed higher concentrations of NPPC compared to large follicles (> 8 mm). No differences among the three groups. In conclusion, our results indicate that NPPC-NPR2 system is present in the bovine antral follicles and suggests a regulation of NPPC on follicular maturation. This research was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

636. Flaxseed Reduces The Pro-Carcinogenic Microenvironment In The Ovaries Of Normal Hens By Altering The Estrogen Pathway.

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We have previously established that flaxseed diet decreases the incidence and severity of ovarian cancer, in the laying hen. Anti-oncogenic properties of flaxseed are predominantly due to the omega-3- fatty acids and the lignan, Secoisolaricirescinol diglucoside (SDG). The omega-3-fatty acids cause a decrease in inflammatory prostaglandins. SDG is converted to weak estrogenic compounds, (Enterodiol) ED and (Enterolactone) EL in the gut. There has been growing evidence indicating that Estradiol (E2) may contribute to ovarian cancer initiation and progression. Estradiol is metabolized by a series of CYP enzymes in the ovary and liver to form specific hydroxy-E2 metabolites. 2- methoxyestradiol (2-MeOHE2), a weak estrogen, is known to have anti-angiogenic and pro-apoptotic properties. The objective was to yield a better understanding of the mechanisms by which the components of flaxseed influence the estradiol signaling and metabolism. In this study, 3-year-old chickens were fed diets supplemented with different components of flaxseed (control, whole flax (WFX), defatted flax meal (DFM) and flax oil) for 3 months. Blood was collected at different times points and tissues were harvested on sacrificing the birds at the end of the study. ED and EL were only detected in chickens that were fed the WFX and DFM diets, using LC MS/MS analysis. Western blotting and immunohistochemistry analysis revealed that the expression of estrogen receptor alpha (ERα) protein decreased in the WFX group. Levels of the ‘good’ E2 metabolite, 2- methoxyestradiol, were higher in the serum from DFM and WFX fed groups with a corresponding increase in CYP1A1 enzyme in the liver of DFM fed birds, as analyzed by ELISA and qPCR. Expression of CYP1B1 and CYP3A4 was not significantly altered among the different diet groups. Chicken specific sabiosciences RT² Profiler PCR array analysis for estrogen target genes revealed that flaxseed diet significantly upregulated transcript levels of c-Fos, BRCA1 and PPAR- γ and downregulated levels of MMP-9 transcript. c-Fos overexpression is associated with a less invasive phenotype in ovarian cancer while MMP9 is commonly involved in metastasis, invasion and angiogenesis. Theses data indicate that flaxseed diet results in a more pro-apoptotic anti-proliferative ovarian microenvironment. In vitro studies using HEK 293 cells demonstrated that Enterolactone significantly inhibited activation of ERα in turn shedding light on the possible pathway through which flaxseed might be influencing estrogen signaling. Flaxseed components influence the estradiol pathway by altering estrogen target gene expression and levels of estrogen metabolites. Taken together, our data strongly supports early dietary intervention using flaxseed, as a preventative approach for ovarian cancer. NIH RO1 AT00408

637. Resumption of folliculogenic factor mRNA expression varies during photostimulated recrudescence in Siberian Hamsters (Phodopus sungorus).

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In many seasonally breeding species, folliculogenesis does not occur consistently across different seasons, and is instead regulated by photoperiodic effects on the hypothalamic-pituitary-gonadal axis. Exposure of Siberian hamsters to short day inhibitory photoperiods (SD; 8 hours of light: 16 hours of dark per day) decreases gonadotropin production. This central decline in follicle stimulating hormone (FSH) and luteinizing hormone (LH) subsequently results in ovarian regression; decreases in ovulation, antral follicle development, and plasma estradiol concentrations. If photoregressed hamsters are transferred to long day stimulatory photoperiods (LD; 16L:8D), FSH and LH levels increase, effectively restoring both folliculogenesis and steroidogenesis. Although gonadotropins mediate photoperiod-induced changes in ovarian function centrally, the intra-ovarian changes that promote the return of folliculogenesis upon photostimulation are not well understood. We hypothesized that: 1) the mRNA expression of early folliculogenic factors would be differentially expressed with different photoperiod regimes, and 2) specific follicle types (primordial, primary, secondary, and tertiary) would be differentially affected. As a first step to test this hypothesis, we exposed adult, female hamsters to one of five photoperiod groups: LD for 14 weeks (cycling ovaries, control), SD for 14 weeks (non cycling ovaries, regressed), or SD for 14 weeks followed by transfer to LD for 2, 4, or 8 weeks (PT, post-transfer recrudescing ovaries). Following tissue collection, qPCR was used to determine mRNA expression of family genes involved in early follicle development: c-Kit, Kit ligand (KITL), and wingless-type MMTV integration site family, member 4 (wnt-4). Expression of wnt-4 mRNA was present in all groups, and increased 5.5-fold following SD exposure as compared to both LD and PT groups (p< 0.05). In contrast, while mRNA expression for c-KIT and KITL mRNA was detected, no significant differences were noted between photoperiod exposure groups (p>0.05). Numbers of antral follicles and corpora lutea declined with SD exposure as compared to LD and PT groups (p<0.05), as expected; however, numbers of primordial, primary, and secondary follicles did not differ between photoperiod exposure groups (p>0.05). These findings suggest that the photoinhibited ovary may not be fully quiescent. It may be that some genes, for example, wnt-4 are upregulated during photoinhibition to potentially maintain primary and secondary oocyte viability in preparation for rapid return to folliculogenesis. Others, notably genes involved in the primordial to primary follicle transition such as c-Kit and KITL may continue to function in the FSH-independent portions of folliculogenesis in regressed ovaries. It is likely that, in addition to central regulation by gonadotropins, additional intra-ovarian signals regulate the resumption of folliculogenesis in the photostimulated ovary. Research was supported by NIH grant 1SC3GM089611 (KAY) & NIH MARC T34GM008074 (AKS).

638. Transmembrane protein 135 (TMEM135) is potentially a novel Liver X Receptor (LXR) target gene with a divergent response to LXR agonists in luteal and non-luteal cells.

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LXR receptors (LXRs) are cholesterol sensors belonging to the nuclear receptor superfamily. Upon agonist stimulation they induce the expression of genes involved in cholesterol efflux such as the ATP-binding cassette transporter A1 (ABCA1). LXRs heterodimerize with the retinoid X receptor (RXR) and bind to the LXR response element (LXRE) in the promoters of target genes. The LXRE motif consists of two hexanucleotide half sites separated by a four nucleotide spacer known as a direct repeat 4 (DR4). In the basal state, LXR/RXR heterodimers are bound to the LXRE and are associated with co-repressors that inhibit transcription. Agonist binding to the LXRs increases the expression of target genes by causing dissociation of co-repressors and recruitment of co-activators to the LXR/RXR heterodimer resulting in initiation of transcription. We have previously implicated the LXRs as potential mediators of luteolysis in the corpus luteum (CL). Increased cholesterol efflux due to enhanced LXR target gene expression could reduce intracellular cholesterol concentrations thus inhibiting steroidogenesis. As the LXRs are nuclear transcription factors, additional target genes may not have been identified yet. The identification of novel target genes will further facilitate the study of the LXR’s role in luteolysis. Therefore, our objective was to identify novel LXR target genes in the CL. To address this, we previously performed microarray analysis of primate luteal cells treated in the presence or absence of a synthetic LXR agonist (T0901317 or T09) to quantify LXR-mediated changes on the macaque luteal transcriptome. One gene that was significantly (>2.5 fold, p< 0.05) increased by T09 was transmembrane protein 135 (TMEM135), which has not previously been identified as an LXR target gene. Mouse and C. elegan studies have shown that TMEM135 is found in mitochondria and is involved with fat storage and metabolism, and mitochondrial membrane potential. Analysis of the TMEM135 promoter with MatInspector software revealed three potential LXREs further supporting the hypothesis that it is an LXR target gene. It is known that the α isoform of the LXRs (NR1H3) is highly expressed in hepatocytes and macrophages and these cells are also important in lipid storage and metabolism. Consequently, due to the limited availability of primary cells we selected human hepatocyte (HepG2) and monocyte (THP-1) immortalized cells for our further studies of TMEM135 transcription. The THP-1 monocytes were differentiated into macrophages prior to experiments. As expected, the known LXR target gene ABCA1 was significantly (p< 0.05) increased in both cell types in response to LXR agonists as determined by real-time PCR (QPCR). Surprisingly, in contrast to primate luteal cells TMEM135 was significantly (p< 0.05) decreased in a time-dependent manner in both HepG2 and THP-1 cells (up to 70% decrease) in response to T09, and was also significantly (p< 0.05) decreased by natural LXR agonists (22R and 27-hydroxycholesterol) in differentiated THP-1 macrophages. In conclusion, our data indicate that TMEM135 might be a LXR target gene; however, it has a divergent pattern of expression in luteal and non-luteal cell types following LXR agonist stimulation. This pattern of LXR agonist responsiveness is unique from any known LXR target genes. This research was supported by NICHD R00 HD067678 (RLB).

639. Hormonal profiles and follicular dynamics in cattle that are carriers of a high fecundity allele.

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A high fecundity bovine genotype has been discovered. Carriers of this allele have multiple ovulations (MO) and half-sibling, non-carriers generally have single ovulations (SO). Based on fine-mapping to a region of chromosome 10 containing SMAD genes, we hypothesized that carriers of the MO genotype would have reduced follicle growth rate and earlier follicle differentiation than SO animals. Two experiments were performed to characterize the profiles of circulating hormones and follicular dynamics for MO (n=9) compared to SO (n=5) cattle. In experiment 1, a synchronized follicular wave was induced by follicular ablation with follicle growth in a controlled...
progestrone (P4) environment (no CL, one intravaginal P4 implant for 5 d, P4 removal to allow ovulation). In experiment 2, a complete interovulatory interval was evaluated. Circulating FSH, P4, and estradiol (E2) were evaluated by RIA and size of follicles and CL were determined by ultrasound every 12 h (Expt 1; ablation to ovulation) or every 24 h (Expt 2; complete interovulatory interval). In experiment 1, number of ovulations was greater (P=0.0003) for MO (4.0±0.4) than SO (1.6±0.2) as expected. Antral follicle count at wave emergence was not different (21.6±2.3 vs 22.8±2.2, P=0.705; MO vs. SO). Consistent with previous experiments in high fecundity ovine genotypes, mean ovulatory follicle size was greater (P=0.0004) for SO (15.7±0.8 mm) than MO (9.5±0.6 mm). Of particular interest, mean follicle growth rate was greater (P=0.0021) for SO (1.47±0.11 mm/day) than MO (0.97±0.07 mm/day) cows. Peak FSH concentrations were similar (0.66±0.04 vs 0.70±0.06 ng/ml, P=0.65; for MO and SO) with declining but similar FSH during the next 2 d for MO and SO. However, nadir FSH concentrations (72 h after final follicle aspiration until CIDR removal) were greater (P=0.023) for MO (0.25±0.02 ng/ml) than SO (0.17±0.02 ng/ml) cows. Mean E2 concentrations during the first 48 h after wave emergence were greater (P=0.003) in MO than SO but were not different after this time (P=0.197). In experiment 2, length of estrous cycle was not different between genotypes (22.1±0.9 vs 24.0±1.2 d, P=0.258, MO vs. SO). Number of ovulations for first (4.0±0.5 vs 1.2±0.2, P=0.002) and second (3.8±0.4 vs 1.2±0.2, P=0.004) ova latory events were greater for MO than SO animals. Following ovulation, there was no difference between genotypes (MO vs. SO) in luteal volume (day 10 = 4505.7±524 vs 6458±1380 mm3; P=0.243), circulating P4 concentrations during the first 14 d of ovulatory events were greater for MO than SO animals, however circulating P4 during luteolysis (P=0.976) and peak circulating E2 (P=0.004) were greater for MO than SO animals, however circulating P4 during luteolysis (P=0.976) and peak circulating E2 (P=0.004) did not differ between genotypes. Interval from onset of estrus until ovulation did not differ between genotypes. In the first parovulic wave, peak FSH was similar (P=0.285), although FSH was greater in MO than SO during the FSH decline (P=0.022) and FSH Nadir (P=0.009). Thus, MO cows have reduced rate of follicle growth in spite of similar or sometimes greater FSH concentrations, consistent with reduced rate of FSH-induced granulosa cell proliferation in individual follicles. Increased E2 from smaller follicles is consistent with differentiation of granulosa cells to a dominant phenotype at a smaller follicle size in MO than SO genotype.

640. Small Molecule Negative Allosteric Modulators of Follicitropin Receptor Inhibit FSH Induced cAMP and Progesterone Production in Human Granulosa Cell Line KGN With Paradoxical Increase in Estrogen Production.

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We have been studying negative allosteric modulators of the Follicle Stimulating Hormone (FSH) receptor (FSHR). These small molecules inhibit FSH-induced cAMP and progesterone production in rat granulosa cells. In addition, when human FSHR is expressed in HEK293 cells, these small molecule FSHR NAMs inhibit cAMP production. Interestingly we have found that some FSHR NAMs block both FSH-induced progesterone and estradiol production while other FSHR NAMs block only progesterone production and not estradiol production. Furthermore, we have in independent studies demonstrated that FSHR NAMs which block both progesterone and estradiol production have contraceptive effect in vivo in rats, whereas FSHR NAMs which only block progesterone do not have a contraceptive effect. In order to further assess the paradoxical effect of FSHR NAMs on estradiol production in the context of a human granulosa cell genetic background we utilized the human granulosa cell line KGN. Not surprisingly all FSHR NAMs tested blocked FSH-induced cAMP production in KGN cells expressing human FSHR. Moreover, FSHR NAMs that blocked both FSH-induced progesterone and estradiol production in rat granulosa cells, did so also in the KGN cells. Furthermore, we observed similar paradoxical effects in KGN cells, where FSHR NAMs that blocked FSH-induced cAMP and progesterone production in rat granulosa cells but did not block estradiol production, also failed to block estradiol production in KGN cells. We reasoned that the paradoxical effect of FSHR NAMs on estradiol production was due to a bifurcation of canonical signaling pathways. As a first test of this hypothesis we examined FSH-induced CREB and ERK phosphorylation in HEK293 cells expressing hFSHR. The cells were challenged with FSHR NAMs that block or do not block FSH-induced estradiol signaling and then stimulated with FSH. We observed that both CREB and ERK phosphorylation were suppressed by FSHR NAMs regardless of their effect on estradiol production. These observations have lead us to conclude that contraceptive effects of FSHR NAMs in rats will potentially translate to humans based on activity in vitro. Additionally, the paradoxical effect of some FSHR NAMs, which inhibit FSH-induced cAMP and progesterone production but do not block estradiol production suggest that a minimal amount of cAMP is required for estradiol production and that sensitive kinase pathways downstream of cAMP are necessary and sufficient for estradiol production. Moreover, this particular class of FSHR NAMs that are ineffective in blocking estradiol production may be useful as boosters of endogenous estradiol production potentially obviating traditional hormone replacement therapy in the perimenopausal period.

641. A Novel Ex Vivo Ovulation Identifies An Essential and Conserved Role of Follicular Adrenergic Signal in Ovulation.

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The stress hormone norepinephrine (NE) plays a critical role in mammalian ovulation; however, the underlying cellular and molecular mechanisms remain controversial. It has been postulated that NE regulates ovulation by activating adrenergic receptors directly in the ovary to control smooth muscle contraction or indirectly by regulating the secretion of luteinizing hormone. In contrast, the Drosophila equivalent of NE, octopamine (OA), is thought to control ovulation by regulating oviduct contraction and secretion. Therefore, it is not clear whether NE/OA regulates ovulation through a conserved mechanism. Recent work in our lab has shown that Drosophila ovulation resembles mammalian ovulation at both the cellular and molecular levels. During ovulation, posterior follicle cells (FCs) surrounding a mature oocyte are selectively degraded and the residual FCs remain in the ovary to form a corpus luteum after the mature oocyte is ruptured into the oviduct. Like in mammals, this rupturing process also depends on Mmp2 activity localized at the posterior end of the mature follicle, where oocytes exit. In the present study, we investigated the role of adrenergic signal in ovulation using our Drosophila model system. We discovered that the entire follicle rupturing process can be recapitulated through OA stimulation in an ex
vivo ovulation system, which has no oviduct or ovarian muscle sheath. OA’s role in follicle rupture can be replaced by NE, although less potently. In addition, OA/NE-induced follicle rupture depends on intact follicular Oamb (Octopamine receptor in mammalian bodies) and Mmp2, as knocking down Oamb or Mmp2 specifically in mature follicle cells using RNA interference completely abolished OA/NE-induced follicle rupture. The role of follicular Oamb in ovulation is further verified in vivo. Our data strongly suggest that OA/NE activates follicular adrenergic signal to control Mmp2 activity and ovulation and that this mechanism is likely conserved in mammals.

642. Human Granulosa Cells Initiate An Innate Immune Response To Bacterial Pathogens.
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The microenvironment of the ovarian follicle is key to the developmental success of the oocyte. Minor changes within the follicular microenvironment can significantly disrupt oocyte development, compromising the formation of developmentally competent embryos and resulting in reduced fertility. Previously described as a sterile environment, the ovarian follicle of women has been shown to contain colonizing bacterial strains, distinct from those in the vagina. Pregnancy rates of assisted reproduction patients are reduced when endotoxin is present in follicular fluid or menstrual effluent. The ovarian follicle is replete of hematopoietic immune cells; in contrast, follicular dynamics and ovulation have been described as inflammatory like events. Work in the bovine describes the innate immune function of granulosa cells in response to bacterial components, potentially altering the microenvironment of the follicle. Here for the first time we investigate the innate immune capabilities of human granulosa cells in response to bacterial components common in a range of pathogens. We hypothesize that human granulosa cells will initiate an innate immune response to various bacterial pathogens via the Toll-like receptor (TLR) complex. Briefly, human granulosa cells were collected from patients undergoing assisted reproduction at the University of Florida’s Reproduction, Endocrinology & Infertility Unit. Following follicle aspiration, granulosa cells were purified by Percoll gradient centrifugation and following an equilibrium period were treated with TLR agonists lipopolysaccharide (LPS; TLR4 ligand), FSL-1 (TLR2/6 ligand), Pam3CSK4 (TLR1/2 ligand) and a TLR4 antagonist, Rhodobacter sphaeroides derived LPS. Following a 24 h exposure to 10-fold increasing concentrations of individual TLR ligands qPCR was performed to assess the relative expression of the inflammatory mediators IL1B, IL6 and IL8, in addition to the endocrine regulators STAR and CYP19A1 (aromatase). Exposure to LPS increased granulosa cell expression of IL1B, IL6 and IL8 in a dose dependent manner (7.49, 5.97, 4.04-fold, respectively), while FSL-1 exposure also increased IL1B, IL6 and IL8 expression (2.9, 9.09, 10.3-fold, respectively). Surprisingly, CYP19A1 expression in granulosa cells was not altered following exposure to LPS, while Pam3CSK4 decreased its expression by 68%, similar to bovine granulosa cells. Conversely, the cholesterol transport mediator important in progesterone synthesis, STAR, was increased 4.6-fold in granulosa cells exposed to FSL-1. Rhodobacter sphaeroides derived LPS is a TLR4 antagonist that blocks the co-receptor MD2 from initiating LPS/TLR4 signaling. Here, 100-fold excess of Rhodobacter LPS did not inhibit LPS induced expression of IL1B or IL8 but had a tendency to reduce IL6 expression. Combined these data suggest that human granulosa cells can initiate an innate immune response to the TLR ligands LPS and FSL-1. Interestingly, LPS did not affect granulosa cell CYP19A1 expression as in bovine granulosa cells. These data confirm human granulosa cells initiate an innate immune response to TLR ligands and modulate endocrine machinery. While further investigation is needed, these data may aid in clarifying how follicular microbes contribute to infertility in assisted reproduction patients.

643. Aging-related Premature Luteinization of Human Granulosa Cells is Avoided by Early Oocyte Retrieval.
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Pregnancy rates gradually decline with age in association with in vitro fertilization (IVF). Why it, however, declines more precipitously after age 43 is unknown. We compared granulosa cell (GC) function in young oocyte donors (n=31, ages 21-29), middle-aged (n=64, ages 30-37) and older infertile patients (n=41, ages 43-47). Gene expression patterns related to gonadotropin activity, steroidogenesis, apoptosis and luteinization were examined by real-time PCR and western blot in GCs collected from follicular fluid after oocyte retrievals for IVF purpose. FSH receptor, aromatase (Cyp19a1) and 17β-hydroxysteroid dehydrogenase (17β-HSD) expressions were found down regulated with advancing age (P<0.05), while LH receptor, P450scc and progesterone receptor expressions were up regulated (P<0.05). The expressions of estrogen receptor, androgen receptor, STAR, Bcl-2, Bax, Survivin were not different significantly (P>0.05). After in vitro cultures for 5 days, GCs were found to exhibit lower proliferation and increased apoptosis in age-dependent manner (older infertile group had lowest cell proliferation and highest apoptosis, P<0.05). While FSH supplementation in culture medium stimulated GCs growth, prevented apoptosis and luteinization in vitro in younger women, the effect disappeared with increased maternal age. These observations demonstrate age-related functional declines in GCs, characterized by changes in gene expression, proliferation, apoptosis and sensitivity to FSH, consistent with premature luteinization. To avoid premature luteinization in women above age 43 (n=39), we advanced oocyte retrieval by administering human chorionic gonadotropin (hCG) at maximal leading follicle size of 16mm (routine 19-21mm), thus shortening time between hCG administration and oocyte retrieval from 36 to 30 hours. Compared to 91 control cycles in women of similar age retrieved in routine fashion, earlier retrieved patients demonstrated only a marginal increase in oocyte premature (p=0.05), yet exhibited improved oocyte and embryo quality and respectable clinical pregnancy rates (16.7 vs. 8.9) by avoiding oocyte atretic (P<0.05). To conclude our study, premature follicular luteinization appears to contribute to the rapidly declining IVF pregnancy chances after age 43, and can be avoided by earlier oocyte retrieval from smaller follicles. This study was supported by The Center for Human Reproduction and grants from The Foundation for Reproductive Medicine.

644. A Novel Role of the steroidogenic acute regulatory (StAR) Protein in Non-steroidogenic Cardiac Cells: Induction of a Survival Factor Following Myocardial Infarction.
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2015 Abstracts – Page 256
StAR is a vital mitochondrial protein essential for high output steroid hormone synthesis in specialized cells of the gonads and the adrenal cortex. In the steroidogenic cells of these organs, StAR activity facilitates the translocation of cholesterol from the outer to the inner mitochondrial membrane where catalysis by CYP11A1 converts the sterol to the first steroid, pregnenolone. The latter diffuses out of the mitochondria to be further converted to progesterone by the endoplasmic reticulum 3beta-HSD. We have recently reported that StAR is also highly expressed in the non-steroidogenic tissue of the left ventricle in mouse heart recovering from experimental myocardial infarction (MI) (Amaka et al., Mol Endocrinol 27:1502, 2013). Temporal analysis of StAR expression shows a peak pattern of the protein expression at days 1-3 after MI, a period that constitutes a typical inflammatory phase known to occur in the damaged heart tissue suffering necrotic and apoptotic cell death. Immuno-histochemical studies suggest that StAR is not expressed in the cardiomyocytes. Instead, high content of mitochondrial StAR is expressed in resident cardiac fibroblasts, in endothelial cells (CD31+) and blood-born progenitor cells (CD34+) exclusively observed in blood vessels of the infarct area. Altogether, this pattern suggests that StAR is expressed in pre- or proto-myofibroblasts known to optionally differentiate in vivo from these progenitor cell types. What then is the new role of StAR in the heart fibroblasts totally deprived of CYP11A1/3beta-HSD? Complementary studies using cardiac fibroblasts isolated from normal adult rat heart and put to culture, show that within 4-12 days such cells spontaneously differentiate to myofibroblasts endowed with typical cellular markers such as alpha-smooth muscle actin, high content of talin and vinculin, both observed in super-mature focal adhesion complexes, and secretion of myofibroblast-specific ED-A-fibronectin and type 1 collagen. In addition, throughout the process of cellular differentiation such cells can be induced to express high StAR content when exposed to a strong pro-apoptotic inducer, staurosporine. In doing so, cells expressing StAR acquire anti-apoptotic robustness that solely depends on StAR presence and activity since StAR knockdown by siRNA, or expression of loss-of-function StAR mutants failed to protect the cells from apoptosis. Further studies suggested that the anti-apoptotic impact of StAR function includes the inhibition of pro-apoptotic BAX translocation from the cytosol to the mitochondrial outer membrane, BAX proteolytic processing and activation, cytochrome c release, mitochondrial fission and binding of annexin V to the cells. We therefore hypothesize that cardiac StAR protein functions in the capacity of anti-apoptotic survival factor protecting the fibroblast progenitor cells from cell death when recruited to the detrimental infarct environment. The spared cells can thus proliferate and undergo differentiation to active myofibroblasts responsible for the post-MI life saving wound healing and tissue repair process. Research supported by the Israel Science Foundation grant 677/12 to JO.

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The corpus luteum (CL) is a temporary endocrine organ, which undergoes the stages of formation, maintenance and regression, reaching full functional activity when formation is completed. The mechanisms regulating CL function and life span in non-pregnant bitches are not completely understood, although many inputs have been added in the last 4 decades. Under these circumstances new generation high-performance sequencing technologies appear as an effective tool for studying large-scale transcriptome and scanning different molecular pathways at the same time. Eighteen non-pregnant bitches were submitted to ovarioalpingohysterectomy every 10 days between days 10 and 60 (n = 3/group) after ovulation (po). The collected corpora lutea were submitted to RNA sequencing strategy (RNA-seq). Illumina HiScanSeq generated a total of 771.288.718 reads (approximately 42 million per sample), which showed an average length of 100bp. The software Cufflinks revealed that the reads corresponded to 34.408 genes, from which 9000 were not annotated to the canine genome and 29.011 showed some level of expression in at least one of the studied time points. Additionally, the analyses showed that 5116 genes were differentially expressed, although 1106 among them were not annotated yet to the canine genome. Three annotated genes (PAPPA, CAPN6 and NKD2) were differentially expressed among all compared time points. The gene PAPPA encodes a metalloproteinase responsible to cleave IGFBP4 and its expression was higher at the beginning of diestrus . The NKD2 gene encodes a negative regulator of canonical WNT signaling, whereas CAPN6 encodes an intracellular cysteine protein, whose activity is highly dependent upon Ca2+ ions. Both NK2D and CAPN6 were more expressed at the end of diestrus (60 p.o.). The analysis detected 1215 differentially expressed isoforms during diestrus and from these, 499 do not match a corresponding described gene. SOTA program clustered the differentially expressed isoforms into 12 sets of similar expression profiles and revealed that 854 genes were up-regulated in the first half, 264 genes in the middle and 634 genes in the end of diestrus (50 p.o.), when luteal morphological regression occurs. A DAVID Functional Annotation Clustering analysis was performed after SOTA clustering pointing towards an enrichment of Epidermal Growth Factor (EGF) related functions in the first half of diestrus; genes related to cell signaling, as for example, GnRH and VGEF signaling were up-regulated during luteal regression. Using day 10 as reference, the Venn diagram showed the possible intersections among differentially expressed genes: 395 genes were expressed in all studied time points and the number of the genes differentially expressed was increased when comparison time points were more distant from each other. In summary, preliminary results provide an overview of differentially expressed genes along canine diestrus, revealing different not explored molecular pathways up or down regulated depending on the studied period.

646. Significance of the association of PRAP/HSD17B-7 with the intracellular domain of PRL-RS in estradiol production by the corpus luteum.

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Prolactin (PRL) is a polypeptide hormone with multifaceted actions in the corpus luteum (CL) - formation, function and demise. It exerts its action by binding to two membrane bound receptor isoforms classified as the long form (PRL-RL) and the short form (PRL-
Contrary to previous beliefs that PRL signals through only PRL-RL, we have demonstrated, by generating mice expressing only PRL-RL, that PRL-RS is essential and that the concerted cooperative actions of both the receptor types are required. We have also shown that PRL-binding to PRL-RS induces the activation of transcription factors and also leads to the association of PRAP/HSD17B-7 with the intracellular domain of PRL-RS. HSD17B-7 is an enzyme essential for luteal synthesis of estradiol, a steroid shown to play a key role in CL vascularity, hyper trophy and steroidogenesis. In this study, we found that the absence of PRL-RS in vivo in mice led to a great reduction in the level of HSD17B-7 enzyme. Using a PRL-RS expressing luteal cell line (GG-CL) we examined how PRL activation of PRL-RS affects the level of HSD17B-7 expression. Interestingly, we found that PRL stabilizes HSD17B-7 protein, prevents its degradation, and induces a high level of this enzyme in luteal cells. Further, we found that this stabilization of HSD17B-7 is not at the level of transcription, but depends on a post translational mechanism. To examine whether this PRL action involves HSD17B-7 association with the intracellular domain of PRL-RS and its phosphorylation by Jak2; tyrosine 296 in this enzyme, located in the conserved domain of Jak2 target site, was mutated or cells were treated with Jak2 inhibitor (Jak Inhibitor I). This HSD17B-7 mutation prevented PRL-mediated phosphorylation of HSD17B-7, whereas the inhibitor prevented PRL-mediated stabilization of HSD17B-7 protein, suggesting that Jak2 activation is essential for HSD17B-7 phosphorylation and stabilization. Taken together, our results reveal the significance of the association of a steroidogenic enzyme involved in estradiol biosynthesis with the intracellular domain of the PRL-RS and suggest strongly that estradiol production in the CL depends on this mechanism.

647. **Human Luteinized Granulosa Cells Express Multiple STARD4 and STARD6 Transcripts.**

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Members of the StAR-related lipid transfer (START) domain family of proteins can facilitate steroidogenesis by increasing the transfer of cholesterol to the mitochondrion in cells with steroidogenic capacity. The prototypical member of this family, STARD1 (sterogenic acute regulatory protein), plays a critical role in hormone stimulated cholesterol transport for steroidogenesis. STARD1 targets the mitochondrion via its N-terminal sequence, yet cholesterol entry into the mitochondrion utilizes its C-terminal START domain sequence. STARD4 and STARD6 protein, lack a mitochondrial targeting sequence, are mainly comprised of a START domain, can bind cholesterol, and in model systems can increase steroidogenesis. Both STARD4 and STARD6 are localized to the human ovary and could potentially play a role in steroidogenesis by supplying mitochondria with cholesterol. We found both STARD4 and STARD6 protein expression in all steroidogenic compartments of the human ovary. We have found STARD4 mRNA levels, but not STARD6 mRNA levels to be regulated by cyclic AMP stimuli in cultured human luteinized granulosa cells (hLGC). Western blots have demonstrated STARD4 and STARD6 immunoreactive proteins from hLGC at larger than predicted molecular mass. In order to determine the correct protein masses for the mRNAs from hLGC, we aimed to clone all the major transcripts of STARD4 and STARD6 and express their resulting proteins. Poly-A RNA was isolated from pooled cultured hLGC and a cDNA library was produced for PCR. For STARD4, three major transcripts were isolated. Two STARD4 transcripts varied by 6 nucleotides in the untranslated exon 1, but were predicted to yield full-length STARD4 protein. A third STARD4 transcript possessed an exon 4 deletion that introduced a premature stop codon, and was predicted to yield a 55 amino acid protein matching the first 52 amino acids (aa) of the 205 aa full-length protein. Using expression vectors, both the full-length and exon 4 deleted transcript protein function were compared using the COS cell F2-steroidogenic assay. Full-length STARD4 was able to significantly (P < 0.05) increase pregnenolone (P5) production (ng P5/ng protein) a mean 3.8-fold compared to empty vector, whereas the 2.1-fold change produced by the truncated (exon 4 deletion) was not significant (n=3). Three major STARD6 transcripts included the predicted full-length wildtype protein and two alternative transcripts with an insertion of 285 nucleotides of genomic sequence (corresponding to 285/380 nucleotides of intron 1). In addition, the two STARD6 alternative transcripts exhibited exon deletions. One STARD6 alternative transcript had a deletion of exon 3 and the second had deletions of exons 3 and 5. The predicted translation of the two STARD6 alternative transcripts indicated that the extra sequence inserted after exon 1 resulted in a premature stop codon that if translated would yield a very short protein lacking most of the START domain. It is unlikely that the alternative transcripts of STARD6 yield a functional protein. In summary, the alternative transcripts of STARD4 and STARD6 from hLGC would yield proteins lacking most of the START domain and likely have little or no ability to promote steroidogenesis by transferring cholesterol. It may be possible that these transcripts code for competitive inhibitors of the full-length STARD4 and STARD6 proteins, which will need to be tested in future studies. Supported by NIH R03 HD070126.

648. **The effect of human ovulatory follicular fluid on fallopian epithelial cell proliferation and DNA damage accumulation.**

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The process of ovulation results in the release of a cumulus-oocyte complex along with a composite mixture of follicular fluid (FF). These materials are taken up by the fallopian tube, effectively exposing the epithelium to the contents of FF (i.e., hormones, lipids, proteins, inflammatory cytokines, ions, extracellular vesicles). High levels of lipid accumulation have detrimental effects on the cumulus-oocyte complex, but the role of lipotoxicity in the oviduct has not yet been explored. During the reproductive lifespan of a woman, repeated exposures of the fallopian epithelium to FF may increase the likelihood of neoplastic alterations contributing to ovarian and fallopian carcinogenesis. Recent studies have shown that high grade serous ovarian carcinomas originate in the fallopian epithelium, and risks of ovarian cancer are significantly reduced by inhibiting ovulation. We hypothesize that FF may contribute to the malignant transformation of the fallopian epithelium. To examine this, and to assess the role of compounds in FF that may have lipotoxic effects on the fallopian epithelium, we obtained FF and plasma from 8 patients (4 lean and 4 obese) that were undergoing infertility treatment due to male factors. We examined the effect of FF on cell proliferation and DNA damage accumulation in normal immortalized human fallopian tube epithelium. Cellular proliferation was measured by BrdU incorporation, and DNA damage accumulation was measured by DNA damage foci analysis as well as comet assays. Cells were treated with patient FF or plasma (2.5%, 5.0%, or 10.0%) for 24 hours before proliferation and DNA damage was assessed. FF treatment (5%) induced cell proliferation by 2.0 fold in lean patients, and 1.8 fold in obese patients. Importantly, plasma from the same patients increased cell proliferation by only 1.2 and 1.3 fold, respectively, indicating that FF has a
greater effect. We also observed increased DNA damage accumulation in cells treated with FF. In conclusion, treatment of cells with FF can recapitulate some of the early markings of cancer progression when examined in vitro, however the factors within the FF responsible for this effect are unknown. There is a great need to diagnose ovarian cancer in early progression to improve patient survival rates. We are currently working to identify the major constituents of FF that induce these changes, to shed light on the pathways and molecular alterations that occur in early ovarian carcinogenesis. This research was supported by R01 HD061580 (LKC).

649. Mechanisms driving tumorigenesis and histotype differentiation of high-grade serous ovarian cancer in Stk11/Pten-deleted mouse ovarian surface epithelium.
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Epithelial ovarian cancer (OvCa) is the most lethal gynecological cancer in the United States, and the serous histotype is the most common and most deadly histotype of ovarian cancers. The mechanisms driving high-grade serous OvCa (HGSOC) development and progression have not yet been clearly determined. Our lab has developed a HGSOC mouse model in which Lkb1 and Pten are conditionally deleted in the OSE leading to development of HGSOC at approximately 12 weeks of age (WOA) with 100% penetrance. These transgenic mice provide a unique view into the role of Lkb1 deletions in HGSOC in the context of whole animal physiology. By 10-12 WOA, pathophysiology and immunohistochemistry clearly show HGSOC in these ovaries that are immunopositive for the human HGSOC markers. However, it remained unclear how and when tumorigenesis occurred. Through hematoxylin and eosin (H&E) staining, this disease displays a steady, but aggressive progression from normal OSE to borderline tumors to HGSOC. At 4 weeks of age (WOA), 40% of ovaries appear normal while 60% show papillae formation or cell shedding, signs of borderline or low-grade serous OvCa (LGSOC). At 6 WOA, only 12.5% of ovaries appear normal while 87.5% display LGSOC. At 8 WOA 100% are LGSOC and by 10 WOA, 87.5% of ovaries display HGSOC while only 12.5% remain LGSOC. Mouse OSE (MOSE) cells isolated from 4 WOA double mutant (DM) transgenic mice that mostly appear normal through histological evaluation show transformative properties: preliminary evidence from culturing these cells and performing allograft experiments suggests that DM MOSE cells grow faster in vitro and in vivo than control MOSE cells. At this early time point, DM MOSE cells also show differential expression compared with control. For example, SLC38A1, a solute carrier protein, is shown to be amplified or upregulated in 11% of HGSOC human patients and is also upregulated in our DM MOSE cells by RNAseq and RTPCR. PAX8 expression is observed in 99% of HGSOC tumors, making it the gold standard diagnostic marker for HGSOC. To investigate the role of PAX8 in the tumorigenesis, we looked at its expression throughout our time course. Immunohistochemical analysis of PAX8 over time shows that PAX8 immunopositivity does not occur until HGSOC has developed at 10-12 WOA. Together this data implicates that PAX8 1) is not an early driver of differentiation and 2) is acquired in the transformation process of OSE cells implicating the OSE as one site of origin for HGSOC. Unlike the DM mice, Lkb1 knockout alone in our single mutant (SM) mice only produces borderline or LGSOC. IHC reveals elevated levels of PTEN in the MOSE of SM mice compared with control MOSE indicating that PTEN may be a compensation method for suppressing tumorigenesis in our SM mice through suppression of the mTOR pathway. Through further characterization and validation of our model, novel insight can be made into the pathogenesis of this disease from early stages to late stages. This can lead to better histotype-specific treatment for HGSOC since most patients, regardless of histotype, are currently treated uniformly, with the same standard of care that has been used for decades, platinum and taxol-based therapies.

GONADAL SIGNALING: Program Numbers 650–657

650. Differentiation into steroid-producing cell and EMT regulation in SF1-mouse embryonic stem cells.
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Introduction: Steroidogenic factor 1 (SF-1) is essential for the development and function of steroidogenic tissues. Stable incorporation of SF-1 into embryonic stem cells has been reported to prime the cells for steroidogenesis. Methods: In this study, we established SF1 transgenic mouse embryonic stem cell (SF1 -mES cells) and analyzed expression of steroidogenesis-related genes and gonadal lineage-markers. We measured the secreted progesterone in the cell medium because progesterone is the first metabolite of sex steroid hormone. As well as, we differentiated mES cells into steroid-producing cells using various culture condition including growth factors or hormones. Results: As well as the transforming factor for granulosa-like cells, we confirmed transcripts of specific forkhead transcription factor FOXL2 and the follicle stimulating hormone receptor (FSHHR). In the other hand, we monitored some specific genes related with differentiation into testicular tissue. We observed the progress to primitive streak–mesendoderm by gene expression analyses. In addition, we observed that differentiated SF1-mES cells expressing the steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase, cytochrome P450-containing enzyme (CYP)-11A1, and CYP19A1. We induced functional granulosa-like cells. Also, we explored EMT-related genes such as N-Cadherin, SNAIL, TWIST, and Vimentin. Conclusions: We established the effective protocol to generate functional steroid-producing cells. The derivation of these cells explores new avenues for the further study and potential application of these cells in steroidogenesis. This study was supported by a grant (15182KFDA460) from the Korea Food and Drug Administration.

651. DCAF17 homozygous mutations cause hypergonadotropic hypogonadism in humans and mice.
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Premature ovarian failure (POF) occurs in 1-4% of women under age 40. POF prior to age 20 is rare, and likely due to genetic pathology that derails gonadal development. In order to identify a range of genetic pathologies that cause POF, we identified and sequenced families likely to have genetic etiology of POF. Four sisters with primary amenorrhea were born to a couple that was first cousins. Clinical endocrine and genetic evaluation of the affected sisters revealed elevated follicle stimulating hormone, undetectable anti-Mullerian hormone, low estradiol, and a normal karyotype. This is consistent with a diagnosis of ovarian insufficiency and hypergonadotropic hypogonadism. However, the cause of this diagnosis, like most cases of ovarian insufficiency, remained unknown. We used whole-exome sequencing on two out of the four affected sisters and their parents to identify a novel homoygous splicing mutation in the DCAF17 (exon 2: c. 127+1 G>C). These mutations were also present in the other two affected sisters. The parents were heterozygous for the mutation, as expected for autosomal recessive disorder manifesting in homozygous daughters. There were no male children in this family. The mutation is predicted to cause abnormal splicing and abnormal DCAF17 protein product. DCAF17 has been implicated in hypogonadism in both boys and girls as part of the Woodhouse-Sakati syndrome. Human mutations have not been functionally studied, and mouse knockout has not been previously reported. We used CRISPR/Cas9 strategy to mimic human mutation in mice. After successfully validating the function of DCAF17 sgRNA in Neuro2A mouse cell line, we injected Cas9-sgRNA into one cell stage C57BL/6 mouse embryo. Out of 27 F0 mice, 7 male and 3 female mice had frameshift mutation in exon 2. Male and female mice with splicing mutation (dcaff17 ΔΔ) were infertile. Immunohistochemistry of mutant male mice showed widespread defective spermatogenesis with absence of mature spermatozoa in most of the seminiferous tubules when compared with the wild type. Mutant males had no mature spermatozoa in the lumen of the epididymis. Our results show that DCAF17 plays an important developmental role in gonadogenesis, in both mice and humans.

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Identity of the gonads depends upon the fate decision process that transforms somatic cell precursors into ovarian granulosa cells or their male counterpart Sertoli cells. In the ovary, establishment and maintenance of granulosa cell identity is a coordinate event driven by multiple factors. Among these factors, the transcription factor FOXL2 seems to play a critical role based on goat and human mutation cases and mouse genetic models. In the mouse, inactivation of Fox2 leads to transdifferentiation of ovarian granulosa cells into testis-specific Sertoli cells, indicating that FOXL2 is required for maintenance of granulosa cells identity. Surprisingly, this sex reversal only occurs postnatally despite that Fox2 is highly expressed in granulosa cells in the fetal ovary. We hypothesized that in the fetal ovary, granulosa cell identity may be acquired and maintained by the complementary action of other pro-ovarian genes in addition to Fox2. We sought to identify these new players involved in the establishment of granulosa cells identity during ovary organogenesis. Transcriptome analyses of fetal gonads have uncovered many sexually dimorphic genes enriched in fetal ovaries. Runx1 gene caught our attention because it is an ortholog of runt, a gene in Drosophila that plays a central role in ovarian determination. RUNX1 belongs to a family of transcription factors involved in cell lineage determination and is specifically expressed in the granulosa cells throughout ovarian differentiation. Such characteristics led us to speculate that RUNX1 may be involved in granulosa cell differentiation. To address this hypothesis, Runx1 was ablated specifically from the somatic cell lineage of the fetal ovaries using the Sfi-Cre mouse model. We compared ovarian differentiation in wild type, Runx1 and Fox2 single knockout, and Runx1/Fox2 double knockout ovaries. While granulosa cell identity was maintained during fetal life in Runx1 or Fox2 single knockout ovaries, the somatic cells in the Runx1/Fox2 double knockout ovaries were sex-reversed to Sertoli cells. This observation demonstrates that RUNX1 and FOXL2 play redundant roles in maintaining fetal granulosa cell identity, possibly through the repression of Sertoli cell program. If indeed this is the case, a forced expression of FOXL2 or RUNX1 in Sertoli cells in the fetal testis should transform Sertoli cells into granulosa cells. We therefore created a Rosa26-STOP-Fox2 mouse model that allows us to induce ectopic FOXL2 expression in the somatic cell lineage of the fetal testis (the Runx1 ectopic expression model is under development). Upon ectopic induction of FOXL2 expression in the fetal testis, Sertoli cells lost their identity and transdifferentiate into granulosa cells, resulting in testis-to-ovary sex reversal. Altogether, these results led to the identification of a new factor, Runx1, in the maintenance of granulosa cells identity and bring to light the ability of Fox2 to drive and maintain fetal granulosa cell identity by suppressing the program for Sertoli cell differentiation. This work was supported by NIEHS Intramural Research Fund.

653. Irx3 and Irx5 Promote Healthy Contacts Between Somatic and Germ Cells to Ensure Oocyte Survival and Proper Follicle Maturation.
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Follicle development and maturation within the ovary, which is critical for female fertility, depend on intimate communications between the germ cell and its surrounding somatic cells. Our previous results using the Fused Toes (Ft) mutant mouse model showed disrupted oocyte – granulosa cell contacts leading to oocyte and follicle death. Among the genes deleted in the Ft locus, only Irx3 and Irx5 (Irx3/5) exhibited ovary specific expression upon comparison of male versus female transcripts during gonad development. Thus, we hypothesized that irx3/5 are critical for coordinating germ cell – somatic cell communications underlying oocyte and follicle survival. The main objective of our current study was to characterize the location of Irx3/5 transcripts and proteins over time and determine their functions within the developing ovary. Real-time qPCR results showed that Irx3/5 had similar expression patterns during ovary development as their transcripts increased during germline nest formation and peaked around birth when nests broke down to form primordial follicles. Shortly thereafter, their expression diminished. Immunohistochemistry analyses on ovaries at embryonic days (E) 13.5, 15.5 and postnatal days (P) 0 and 2 showed that IRX3 and IRX5 were co-localized to somatic cells during development and then were detected in both germ and somatic cells around birth. Their expression decreased first in somatic cells of established primordial follicles, but was maintained for a few more days in germ cells. Next, we characterized irx3/5 double knockout, irx3/5/irx3/5 (irx3/5 DKO), ovaries. This mutation is embryonic lethal at E13.5; therefore, we used kidney capsule transplantation (KCT) of ovaries to analyze
time points equivalent to P0, 3, 7 and 14. Histology and transmission electron micrograph of KCT ovary grafts showed that Irx3/5 DKO follicles developed abnormal granulosa cell morphology, gaps between germ and somatic cells, and oocyte death similar to that seen in the Ft mutant model at P7 and 14. Next, we generated a somatic cell specific double knockout mouse model using SF1Cre, SF1Cre<sup>−/−</sup>, Irx3<sup>−/−</sup>/Irx5<sup>−/−</sup> (Irx3/5 sFΔ), to evaluate the role of Irx3/5 in somatic cells in the developing ovary. Histological analysis of adult Irx3/5 sFΔ mutant ovaries displayed an overall smaller size with more zona pel lucida remnants and rare corpora lutea. Because the Irx3/5 sFΔ mice were small and too weak to perform breeding studies, we examined fertility using superovulation followed by in vitro fertilization. Our current results indicated that Irx3/5 sFΔ mutant females ovulated fewer oocytes, had a higher incidence of egg fragmentation, and fewer 2-cell embryos (3: 66.6%; 33.33%; n = 1) compared to no Cre (: 11: 15.13%; 41%; n = 3) and wild-type controls (: 21: 3.57%; 67.25%; n = 3). To investigate female fertility through natural breeding, we generated another somatic cell specific double knockout model, SF1Cre<sup>−/−</sup>, Irx3<sup>−/−</sup>/Irx5<sup>−/−</sup> (Irx3/5 sFF). Irx3/5 sFF mutant mice were robust, and preliminary breeding study results indicated that mutant females could reproduce, but were subfertile. Together, our results indicate that Irx3/5 work together during follicle development in the ovary to promote effective communication between the oocyte and nascent granulosa cells to ensure oocyte survival and proper follicle maturation. These functions may depend on Irx3/5 expression specific to ovarian somatic cells. Supported by NIH-R01HD075079 (JSJ).

654. Cysteine-rich Secretory Protein-3 (CRISP-3) Expression in the Reproductive Tract of Prepubertal and Mature Stallions.

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Seminal plasma proteins play an important role in many aspects of reproduction including sperm maturation and transport, fertilization, as well as the inflammatory response to breeding. Cysteine-rich Secretory Protein-3 (CRISP-3) has been correlated with increased fertility and first cycle conception rates, and has been suggested to be involved in the regulation of PMN-phagocytosis of spermatozoa during the inflammatory response to breeding. Previous research demonstrated that equine CRISP-3 is primarily localized in the ampulla of the vas deferens with a more moderate amount expressed in the seminal vesicles, but no comparisons were made between prepubertal and mature males. In humans and rodents, CRISP-3 has been described as an androgen-dependent protein, but androgen dependency has not been identified in the horse. The objectives of this study were to a) confirm the localization of equine CRISP-3 in the stallion reproductive tract, and b) determine if expression of CRISP-3 increases after puberty. We hypothesized that expression of CRISP-3 would be localized primarily to the ampulla of the ductus deferens with a more moderate expression in the seminal vesicles, and that expression of CRISP-3 would increase after puberty. Reproductive tissues were collected postmortem from three prepubertal colts (<6 months) and six mature stallions (>3 years). Fixed tissues and tissues in RNAlater were collected from the ampulla of vas deferens, seminal vesicles, bulbourethral gland, prostate gland, tests, as well as the cauda, corpus, and caput aspects of the epididymis. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using an equine specific CRISP-3 probe. The data were confirmed with immunohistochemistry (IHC) using an equine CRISP-3 antibody raised in rabbits. A mixed linear additive model was used to compare mRNA expression between age groups, and significance was set to P<0.05. There was a significant interaction between maturity and tissue type (P<0.0001). mRNA expression of CRISP-3 was found primarily in the ampulla of the ductus deferens with more moderate expression in the seminal vesicles, and expression of CRISP-3 was higher in the mature stallion when compared to the prepubertal colt for the ampulla (P=0.0027) and seminal vesicles (P=0.0028). IHC confirmed that CRISP-3 was expressed in the ampulla of the vas deferens of mature stallions, primarily located in the outer muscular layer, with gradually decreasing expression as the muscularis approached the epithelium of the lumen. CRISP-3 was also expressed in the epithelium of the lamina of seminal vesicles in the mature stallion, but this was variable amongst stallions. Further studies are required to confirm if equine CRISP-3 is either androgen or estrogen dependent in the equine reproductive tract. In conclusion, equine CRISP-3 is primarily expressed in the muscularis of the ampulla of the ductus deferens and to a lesser degree, expressed in the seminal vesicles. Mature stallions have significantly higher expression in the ampulla suggesting that it may be regulated by steroid hormones whose synthesis is increased after puberty.

655. Irx3 and Irx5 are Regulated by Canonical Wnt Signaling in the Somatic Cells of the Developing Ovary.

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Canonical Wnt/b-catenin signaling is one pathway that is required for ovarian development. Oocyte death is a prominent feature of Wnt<sup>d</sup> null ovaries and is thought to be caused by disrupted interactions between oocytes and supporting somatic cells. Somatic cell-specific disruption of b-catenin also causes oocyte death. Previously, our laboratory discovered that members of the IrxB cluster, specifically Irx3 and Irx5 (Irx3/5), were required to maintain oocyte-somatic cell interactions in the developing follicle. Significant gaps separate the oocytes and supporting somatic cells in Irx3/5 double knockout ovaries (Irx3<sup>−/−</sup>; Irx5<sup>−/−</sup>) that ultimately lead to oocyte death and suggest a link to the Wnt/b-catenin pathway. Irx3 has been used as a marker for active Wnt signaling in several tissues, including the developing ovary; however, the mechanism by which Wnt regulates this factor is unclear. We hypothesize that Irx3 and Irx5 are directly regulated by the canonical Wnt/b-catenin signaling pathway to manage oocyte-somatic cell communication during ovarian development. Our goals were to manipulate gonadal b-catenin activity in vitro and in vivo to investigate changes in Irx3 and Irx5 expression and to define TCF/LEF binding sites within the Irx3/5 locus. Embryonic day (E) E11.5 ovary explants were cultured with iCRT14, a potent inhibitor of b-catenin stimulated transcription. As expected, positive control Axin2 was significantly decreased (0.23x, p<0.001) and negative control Rps29 was unaffected. Expression of both Irx3 and Irx5 were significantly decreased by 0.27 and 0.24 fold, respectively (n=4, p<0.003 for both). In a converse experiment, E11.5 testis explants were cultured with LiCl, a GSK3b inhibitor that results in stabilized and activated b-catenin. Results showed little change in Rps29 and significantly increased expression of Axin2 (4x, p<0.03), Irx3 (8x) and Irx5 (5x) (n=5, p<0.01 for both). Next, b-catenin activity was manipulated in vivo by breeding SF1-Cre (somatic cell specific) to Ctnnb1<sup>AlexΔ</sup> or Ctnnb1<sup>AlexΔ/μ</sup> mice. Control (SF1-Cre<sup>−/−</sup>; Ctnnb1<sup>−/−</sup>) and knockout (SF1-Cre<sup>−/−</sup>; Ctnnb1<sup>−/−</sup>) ovaries were harvested from E14.5 embryos and subjected to qPCR analysis to evaluate loss of b-catenin function. Rps29 was unaffected while Axin2 (0.24x, p<0.01), Irx3 (0.35x, p<0.001) and Irx5 (0.40x, p<0.01) expression was significantly decreased in mutant versus control ovaries. The converse study investigated transcripts from testes with stabilized b-catenin (SF1-Cre<sup>−/−</sup>; Ctnnb1<sup>AlexΔ/μ</sup>). Preliminary results are similar to data from testes explant culture experiments.

2015 Abstracts – Page 261
In conclusion, our results from complementary in vitro and in vivo experiments suggest canonical Wnt/b-catenin signaling is responsible for Irx3 and Irx5 regulation. Based on these findings, we next used in silico analysis to identify putative TCF/LEF binding sites within the Irx3/5 locus. Chromatin immunoprecipitation experiments are ongoing to confirm these sites. Taken together, these data suggest Irx3/5 respond to canonical Wnt/b-catenin signaling in ovarian somatic cells to set up the proper foundation for essential interactions between somatic cells and oocytes. This relationship begins within germline nests during development and translates to their intimate connections within primordial follicles that ensure oocyte survival. Supported by NIH-R01HD075079 (JSJ).

656. **Expression of activin A and activin receptors in bovine fetal ovaries and potential interactions between estradiol and activin A**: activin A stimulates bovine follicle formation and activation in vitro.

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Despite the importance of primordial follicles (PFs) to female fertility, the regulation of follicle formation and activation (initiation of growth) is not well understood. Previous studies with mice suggested that activin A stimulates follicle formation, which occurs shortly after birth in rodents. In cattle and women, follicle formation and activation begin during fetal development and the role of activin in these critical processes is not clear. Therefore, in Exp. #1, we first measured mRNA for activin A and activin receptors and localized activin A in bovine fetal ovaries from 88 to 217 days of gestation (length of bovine gestation ~ 279 days; n = 6-10 fetuses). Semiquantitative RT-PCR detected mRNA for activin A and its receptors ActRIIA and ActRIIB in all fetal ovaries examined. Immunohistochemical analysis further revealed activin A in oogonia, oocytes in ovigerous cords and oocytes in follicles at all stages. Weak immunostaining was also detected in some granulosa cells of primordial and primary follicles. These results suggest a role for activin in regulating early follicular development in cattle. Therefore, in Exp. #2, we investigated the effects of activin A on follicle formation and activation by culturing ovarian cortical pieces from 92 to 102-day-old bovine fetuses (6 pieces/treatment; 2 from each of 3 fetuses) in medium supplemented with TS+ (transferrin-selenium+) in the absence or presence of insulin (6.25 µg/ml; positive control) or activin A (100 ng/ml) for 10 days. The age of fetuses was chosen based on our previous studies showing that bovine PFs first appear around day 90 of gestation. Freshly isolated (day 0) and cultured cortical pieces were fixed and embedded in plastic for serial sectioning and morphometric analysis. On day 0, cortical pieces contained mostly oogonia and oocytes, but only a few PFs. Total follicles (primordial + primary) in day 10 controls and all treated cultures were higher than on day 0 (P < 0.05), suggesting that follicle formation occurred during culture. Furthermore, pieces cultured with insulin or activin A contained more total (2.3- and 1.6-fold increase, respectively), primordial (1.9- and 1.5-fold increase, respectively) and primary (16- and 6.3-fold increase, respectively) follicles than day 10 controls (P < 0.05). These results show that activin A, like the positive control insulin, stimulates follicle formation and activation in vitro. Since estradiol (E2) inhibits bovine follicle formation and E2 suppresses activin expression and signaling in the early mouse ovary, in Exp. #3 we examined whether E2 alters the abundance of mRNA for activin A and its receptors in cortical pieces dissected from 100 to 120-day-old bovine fetal ovaries and cultured with E2 at 1 µM for 10 days (n=3 fetuses). Preliminary semiquantitative RT-PCR analyses showed that E2 tended to decrease activin A mRNA (P < 0.1), but had no effect on the abundance of mRNA for ActRIIA and ActRIIB. In summary, activin A stimulated bovine follicle formation and activation in vitro and bovine fetal ovaries expressed protein and/or mRNA for activin A and its receptors, suggesting a role for activin in early folliculogenesis in cattle. E2 tended to decrease activin A mRNA and potential interactions between E2 and activin A in follicle formation and activation in cattle remain to be elucidated.

657. **INFLUENCE OF BASAL CELLS AND PROSTAGLANDINS ON CONNEXIN 43 IN THE RAT EPIDIDYMIS.**

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Gap junctions are comprised of hexameric connexons that are themselves composed of integral proteins termed connexins. In the adult mammalian epididymis, connexin 43 (GJA1) is localized between basal cells and either principal or clear cells. The targeting of GJA1 is regulated in part by testosterone. The objective of the study was to determine the role of basal cells and prostaglandin E2 (PGE2), which is secreted by basal cells, on GJA1 in the rat epididymis. Immunolocalization of GJA1 in the epididymis at different ages of development indicated that at day 14, prior to the differentiation of basal cells, GJA1 is localized at the apical lateral margins between adjacent epithelial cells. By day 21, when basal cells are present at the base of the epithelium, GJA1 becomes associated between basal and principal cells, where they remain immunolocalized until adulthood. Basal cells, as determined by the presence of p63, appear to differentiate from the epithelial cells and express prostaglandin-endoperoxide synthase 1 (PTGS1) by 21 days of age, when GJA1 localization is altered. To assess the effects of PGE2 on GJA1, a rat epididymal cell line (RCE) developed in our laboratory, was used. RCE cells were exposed to PGE2 (50umol) for 3 hrs. Controls were exposed to vehicle (EtOH) alone. Total cellular proteins were extracted and subjected to western blot analysis. Treatment with PGE2 resulted in a significant two-fold increase in the levels of GJA1 in RCE cells. Furthermore, to understand the mechanism by which PGE2 induced levels of GJA1, levels of β-Catenin, phospho-β-Catenin, and phospho-Akt were measured. The results indicate that all three proteins were increased by PGE2 treatment. Levels of other junctional proteins, such as cadherin1 (CADH1; also known as E-cadherin) and claudin1 (CLDN1) were not significantly altered by PGE2. Together the data suggest that the development of epididymal epithelium and the differentiation of epididymal basal cells regulate the targeting of GJA1 intercellular communication and this effect appears to be mediated by PGE2. Supported by NSERC.

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658. **Genetic Ablation of Androgen Receptor Signaling in Fetal Leydig Cell Lineage.**

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In conclusion, our results from complementary in vitro and in vivo experiments suggest canonical Wnt/b-catenin signaling is responsible for Irx3 and Irx5 regulation. Based on these findings, we next used in silico analysis to identify putative TCF/LEF binding sites within the Irx3/5 locus. Chromatin immunoprecipitation experiments are ongoing to confirm these sites. Taken together, these data suggest Irx3/5 respond to canonical Wnt/b-catenin signaling in ovarian somatic cells to set up the proper foundation for essential interactions between somatic cells and oocytes. This relationship begins within germline nests during development and translates to their intimate connections within primordial follicles that ensure oocyte survival. Supported by NIH-R01HD075079 (JSJ).
It is commonly accepted that the androgen-producing cells Fetal Leydig Cells (FLCs) are substituted by Adult Leydig Cells (ALCs) during perinatal testis development. The mechanisms influencing this process are unclear. We used mice with the Rarb-cre transgene expressed in embryonic FLC precursors, but not in postnatal testis, and a dual fluorescent Cre reporter to label FLCs and ALCs in vivo. All FLCs in newborn testes had the recombinant, whereas the majority of LCs in adult testis had the non-recombinant reporter. Primary LC cultures from adult testes had either recombinant (20%) or non-recombinant (80%) cells, demonstrating that the FLCs survive in adult testis and their ontogeny is distinct from ALCs. We showed that the proportion of recombinant LCs in testicular interstitium remained the same from 30 to 100 days after birth. Conditional inactivation of androgen receptor (AR) allele using Rarb-cre transgene resulted in a 50% increase of AR-negative LCs in adult testis. The mutant males became infertile with age with all LCs in older testes showing signs of incomplete differentiation, such as a large number of large lipid droplets, an increase of finger-like protrusions, and a misexpression of steroiogenic or FLC and ALC-specific genes. Collectively, these results suggest that FLCs have different ontology than ALCs, FLCs remain in adult testis, FLC precursors survival is regulated by AR, and that the disruption of androgen signaling in FLC lineage could lead to abnormal differentiation of all LCs and infertility in adults. We propose that the anti-androgenic exposure during early development may similarly result is the increase of FLCs in adult testis leading to the abnormal LC differentiation and infertility. Thus, normal androgen signaling in differentiating FLCs might have broad implications for normal functional status of adult testis.

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Introduction: Spermatozoa mature as they transit through the epididymal duct, and acquire motility and the ability to fertilize the egg. The epithelial cells of the epididymis are essential to the maturation process by the endocytosis of substances from the lumen and synthesis and secretion of proteins into the epididymal lumen. Heparan sulfate is a component of the extracellular matrix, basement membranes and the apical cell surface, and it is abundant in the testis and epididymis. After endocytosis, heparan sulfate is degraded in a stepwise fashion in lysosomes by the action of three glycosidases, three sulfatases, and heparin-alpha-glucosaminid N-acetyltransferase (HGSNAT). Although HGSNAT is not a hydrolase, it catalyzes the transmembrane acetylation of the terminal glucosamine residues of heparan sulfate prior to its hydrolysis by α-N-acetylgalcosaminidase. A deficiency in HGSNAT results in a variant type of Sanfilippo syndrome (MPS IIIA), a human genetic disorder, which is characterized clinically by morphological and functional disorders of the brain. In the mouse, inactivation of the Hgsnat gene leads to a mild form of MPS IIIC, and animals at late ages show an apparent reduced size of liters. Objectives: To determine the morphological and biochemical effects of Hgsnat inactivation on epithelial cells of the testes and epididymis by routine LM and immunocytochemical analyses and by EM observations. Materials and Methods: The testes and epididymides of both wild type and Hgsnat−/− adult mice at different ages, i.e., 7 and 11 (n=3 for each age) and 14 months (n=1 for each) were fixed by cardiac perfusion with 2.5% glutaraldehyde buffered in sodium cacodylate and processed for EM analysis. Prior to perfusion, the testes and epididymides of one side were removed and immersed in Bouin’s fixative for LM immunocytochemical studies with different antibodies. Results: In Hgsnat deficient mice, some seminiferous tubules revealed highly vacuolated areas, while other tubules demonstrated a major depletion of the germ cell population. Furthermore, approximately 20% of the tubules decreased in size as compared to wild type mice. These abnormalities increased with age of the mice. In the wild type epididymis, principal cells contained distinct dense spherical lysosomes, which contrasted the numerous empty looking vacuoles of different shapes and sizes that appeared both supranuclearly and infranuclearly in knockout mouse. LM immunocytochemical data revealed a specific expression of cathepsin D and prosaposin in these vacuoles suggesting that they were lysosomal in nature. Clear cells became highly vacuolated and lacked their characteristic numerous dense lysosomes. These cells appeared to lose contact with the lumen and be greatly increased in size as they spanned a wider area of the epithelium. Moreover, halo cells were prominent as they were enlarged in size. Numerous spermatozoa were present in the lumen of the epididymal duct, but in addition, small spherical cells and debris were noted. These abnormalities were especially prominent in the caput to caudal regions with the initial segment being less affected. Discussion: Our results show that Hgsnat inactivation affects spermatogenesis and the normal appearance of epididymal epithelial cells, with a phenotype similar to well characterized lysosomal storage disorders. This provides the first evidence that glycans catabolism is important for normal reproductive functions. Supported by NSERC and CIHR.

660. Maternal Exposure to Butylparaben Impairs Testicular Structure and Function on Adult Male Wistar Rats.
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Parabens (alkyl esters of p-hydroxybenzoic acid) are commonly used as preservative in pharmaceuticals, personal care products, cosmetics formulations, and food and beverage processing. These substances have been described as antiandrogenic agents and also may bind to estradiol receptors and mimic estrogen. Previous data from our laboratory have shown that this chemical may cause loss in sperm quality. The objective of this study was to assess the effects of butylparaben (BP) exposure during the critical period of reproductive system development on testicular parameters of male offspring. Pregnant Wistar rats received corn oil (control group, C, n=9), or BP at doses of 10 mg/kg (daily intake limit, n=9), 100 mg/kg (lowest-observed-adverse-effect level - LOAEL, n=9) or 200 mg/kg (n=7), injected subcutaneously daily, from gestational day 12 until postnatal day (PND) 21. The testicular function from the offspring was evaluated at adulthood (PND 110) via: spermatogenesis kinetics, number of Leydig cells, and immunohistochemistry for estrogen receptor alpha (ERα) and androgen receptor (AR); the level of circulating testosterone was also evaluated. For comparison of inter-group data, Anova with an a posteriori Dunnett’s test or non-parametric Kruskal-Wallis with an a posteriori Dunn test were utilized. Exposure to BP during initial periods of life adversely affected spermatogenesis with significant reduction in the number of seminiferous tubules on stages I-VI at 10mg/kg and 200mg/kg and on stages VII-VIII at 200mg/kg. The higher dose of BP also provoked a decrease in the cytoplasmatic
immonostaining of ERα on elongated spermatids on stages I-VI and round spermatids on stages VII-VIII. Moreover, the animals exposed to 200mg/kg of BP presented diminished AR immunostaining in the nuclei from Sertoli cells, significative increase in the number of Leydig cells and higher serum testosterone concentrations. We suggest that BP, at concentrations representative of human exposure, impairs testicular structure and function in the rat. Research supported by grant # 2013/00314-5, São Paulo Research Foundation (FAPESP) and Canadian Institute of Health Research MOP 81178.

661. ALTERATIONS IN MALE REPRODUCTIVE PARAMETERS FOLLOWING IN UTERO EXPOSURE TO BETAMETHASONE.


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Glucocorticoids are potent anti-inflammatory, anti-inflammatory and immunosuppressive drugs. As such, they are the drugs of choice for antenatal treatment to promote fetal lung maturation, thus decreasing the incidence of respiratory distress syndrome, neonatal mortality and morbidity. Previous studies in our laboratory have shown that prenatal exposure to betamethasone altered testosteron levels and sperm parameters in adult male offspring. The aim of this study was to evaluate changes in male reproductive parameters following in utero betamethasone exposure. Pregnant Wistar rats were treated with vehicle or 0.1 mg/kg betamethasone on gestational days 12, 13, 18 and 19. Maternal weight gain, anogenital distance and male offspring weights at postnatal days (PND) 1, PND 21 (weaning), and after preputial separation (after PND30) were measured. At PND 45, a subset of animals from each mother and treatment group was killed and the following parameters evaluated: body and reproductive organ weights, hormone levels (FSH, LH and testosterone), testicular morphology and immunohistochemistry for connexins 43 (Cx43) and PCNA. Maternal weight gain was reduced in the betamethasone group (p<0.05). A significant reduction in the weight of male pups (n=12) in the betamethasone group at PND 1 (p<0.05) was also observed. However, this reduction in body weight was transient, and by PND21, there were no significant differences. There was a significant delay in the start of puberty in the in utero betamethasone treated group according to the age of initial and final preputial separation. Furthermore, at PND 45, testosterone levels were decreased as were seminal vesicle weights. Interestingly, testicular weights were increased. Histological analysis of testes from control and treated rats indicated a significant decrease (p<0.05) in morphologically normal tubules (97.9% versus 95.6% in the betamethasone group). In the treated group, many tubules displayed a disruption in germ cell organization and distribution. These tubules showed abnormal migration of Sertoli and germ cells towards to the lumen of the seminiferous tubule. Immunostaining of the gap junction protein Cx43 was less intense in the betamethasone group; however, in the tubules with altered cell migration, Cx43 immunostaining was present apically but remained associated with mis-localized Sertoli cells. Furthermore, PCNA immunolocalization was observed in spermatogonia near the base of the tubules in both groups. However, in the betamethasone group, it was also detected on spermatogonia located near the middle of the tubule. Our results demonstrate that in utero exposure to betamethasone results in dramatic alterations in reproductive parameters during the development of male offspring. These results support previous reports of sperm alterations in adults treated in utero with betamethasone. Thus, in utero betamethasone exposure can impact peri-pubertal development of males and may result in testicular lesions that are manifested in adulthood. This study was supported by grant #2012/25350-1 of the São Paulo Research Foundation (FAPESP), the Fonds de recherche du Québec - Nature et technologies, and the Natural Sciences Research Council of Canada

662. Muscle glycogen synthase isoform is responsible for testicular glycogen synthesis: glycogen overproduction induces selectively apoptosis in male germ cells.

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Glycogen is the main source of glucose for many biological events. However, this molecule may have other functions, including those that have deleterious effects on cells. The rate-limiting enzyme in glycogen synthesis is glycogen synthase (GS). To date, it is not clear how glycogen metabolism is regulated and the role of glycogen in the testis. The aim of this study was to evaluate the general mechanism of synthesis and regulation of testicular glycogen, and its possible role in testicular development. Using RT-PCR, Western blot and immunofluorescence, we have detected MGS (muscle glycogen synthase) expression but not LGS (liver glycogen synthase) in mice testis during development. We have also evaluated GS activity and glycogen storage at different days after birth and we showed that both GS activity and levels of glycogen are higher during the first days of development. Using RT-PCR and immunoblotting, we have also shown that malin and laforin are expressed in testis, key enzymes for the regulation of GS activity. These proteins form an active complex that regulates MGS by poly-ubiquitination in both Sertoli and male germ cell lines. In addition, we reported that the accumulation of glycogen in testis of transgenic animals overexpressing a constitutively active form of glycogen synthase (KIN-GS) enhances apoptosis of premeiotic cells in seminiferous tubules. Similarly, the activation of endogenous glycogen synthase (GS) in a germ cell line (GC-1) stimulates the deposition of glycogen and triggers the activation of caspase 3. By spectrophotometric analysis, we found that glycogen synthesized in Sertoli (42GPA9) and GC-1 cell lines—by expression of a superactive form of GS or by activation of endogenous GS by PTG (Protein Targeting to Glycogen)—is poorly branched. In addition, the immunodetection of cleaved caspase 3/9 suggests that cellular death induced by polyglucosan molecules affects GC-1 but not 42GPA9 cells. GC-1 cells showed changes in intracellular ATP and cytochrome C content after polyglucosan accumulation, thereby suggesting mitochondrial impairment and activation of an intrinsic apoptotic pathway. Furthermore, we analyzed the effects of glycogen deposition during the establishment of an in vitro blood-testis barrier. The results using a non-permeable fluorescent molecule (Evans blue) showed that, in conditions of over-synthesis of glycogen, 42GPA9 cells do not lose their capacity to generate an impermeable barrier. In the same cell line, immunodetection showed that the levels of connexin43 (Cx43), occludin (Occl), and ZO1 proteins were not affected by glycogen accumulation. Similarly, in KIN-GS mice, the
distribution and intensity of signals for Cnx43, Occl, and ZO1 point to a Sertoli cell-only syndrome in this model, affecting the viability of male germ cells but not the viability or stability of Sertoli cells, as assessed by confocal microscopy analysis. These findings suggest that GS activity and glycogen synthesis in testis can be highly regulated and a disruption of this process may be responsible for the apoptosis and degeneration of seminiferous tubules and possible cause of infertility.

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663. Impairment of Sperm Quality in Rats Exposed to Bupropion, an Antidepressant and Smoking Cessation Drug.

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Bupropion is a dopamine and norepinephrine reuptake inhibitor used as smoking cessation and antidepressant drug with low incidence of male sexual dysfunction. We showed previously that sibutramine, a norepinephrine/serotonin reuptake inhibitor, reduced male rat fertility. This effect was ascribed to the acceleration of sperm transit time through epididymis due to an increased contractility of epididymal smooth muscle. Due to the importance of sympathetic nervous system on the male reproductive function and since bupropion blocks norepinephrine transporter in vitro and increases extracellular levels of this catecholamine in vivo, the aim of the present study was to investigate the effects of bupropion administration on sexual behavior, spermatic parameters and fertility of male Wistar rats as well as its influence on epididymal duct in vitro contractility. For this, male rats (90-day-old) were randomly allocated into three experimental groups of 9 animals each, that received bupropion 15 mg/kg, 30 mg/kg or distilled water, the vehicle (control group) daily during 30 days orally. At the end of the treatment the animals were euthanized and serum was collected for hormone dosage; testis and epididymides were used for sperm counts; sperm motility and morphology determined in material collected from the cauda epididymidis (CE); reproductive organ weights were recorded. Another set of animals (n=10 per group) was used to test sexual behavior and fertility. As the epididymal transit time is dependent on the epididymal duct contraction we also evaluated the in vitro CE duct contractility, as follows: (i) the effects of bupropion exposure on the contractility of the isolated CE duct from untreated rats; (ii) the contractility of the isolated CE duct from rats treated with vehicle, bupropion 15mg/kg or 30mg/kg during 30 days.

Bupropion 15 mg/kg increased serum luteinizing hormone level and the epididymal duct contractility, but the sperm quality was not affected. At 30 mg/kg bupropion impaired sperm quality increasing the incidence of non-progressive sperm. The male sexual behavior and fertility were not modified at both bupropion doses, as well as other reproductive parameters. The reduced sperm quality of bupropion-treated rats did not result from modification of the epididymal sperm transit time suggesting a modified epididymal microenvironment. Studies investigating the bupropion effects on human sperm quality are urged as bupropion use has been suggested as an adjuvant treatment to selective serotonin re-uptake inhibitors (SSRI)-induced sexual dysfunctions. This work has been funded by CAPES and CNPq.

664. Initial segment differentiation begins during a critical window and is dependent upon lumicrine factors and SRC.

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Without a fully developed and functioning initial segment, the most proximal region of the epididymis, male infertility results. Therefore, it is important to understand development of the initial segment. During postnatal development of the epididymis, many cellular processes of the initial segment are regulated by lumicrine factors. Prior to postnatal day 15 (P15), the proliferation and survival of the initial segment epithelial cells are lumicrine factor-independent. However, from P19 onwards, deprivation of lumicrine factors by efferent duct ligation resulted in declined proliferation and increased apoptosis. Therefore, P15 to P19 is a critical window that establishes the dependency of lumicrine factors in the initial segment epithelium. The initial-segment-specific kinase activity profile, a marker of initial segment differentiation, was also established during this window. The SRC (SRC proto-oncogene, non-receptor tyrosine kinase) family of kinases, ERK pathway components, and AMPK (AMP-activated protein kinase) pathway components had increased activities from P15 to P19, suggesting lumicrine factors regulated SRC/ERK/AMPK signaling to initiate differentiation of initial segment from P15 to P19. In addition, compared with the latter mate controls, juvenile Sce null mice displayed lower level of MAPK3/1 activities and reduced height of epithelium in the initial segment. Thus, providing further evidence that SRC signaling through MAPK3/1 was important for initial segment differentiation. It is clear that initial segment differentiation is important for male fertility as shown by our previous study (Xu et al., PNAS, 52:18643-48, 2014). Therefore, our future studies will examine SRC and its role in male fertility regulation. Supported by NIH-NICHD HD 068365.

665. E2F1 a Master Regulator of Testicular Descent and Function.

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Introduction: Well recognized causes of infertility include endocrine disorders, cryptorchidism, karyotype abnormalities, Y-chromosome microdeletions, and gene mutations. However, in ~40% of cases of infertility and cryptorchidism the causes are unknown and the defect is believed to result from a genetic or genomic defect. Recently, we showed that gene dosage changes resulting from microdeletions and microduplications encompassing E2F1 are present in a subset of non-obstructive azoospermic (NOA) men. Importantly, targeted deletion or overexpression of E2F1 in mouse models by transgenesis results in spermatogenic failure, recapitulating the NOA phenotype. The mechanism by which E2F1 causes testicular failure is not understood. Objectives: To define the functional consequences of E2F1 gene-dosage changes in testicular descent and function. Methods: Wild-type, E2f1-KO, and E2f1-Wnt4-double-KO mice were mated to control females over a seven-month period to define their fertility. Testicular location and organ weights were assessed at different time points. RNA was extracted from testis and gubernaculum for gene expression studies by qPCR. Tissues were fixed and processed for histologic analysis. Sperm isolated from the cauda epididymis were used to estimate density and motility. Male mice were housed separately and blood drawn for measurement of circulating hormone levels. Results: Five cryptorchid boys with E2F1...
Sertoli cells are reproductive cells that span the whole seminiferous tubule epithelium. They play important roles in spermatogenesis by providing spatial and nutritional support to developing germ cells as well as degrading remnants of residual bodies and apoptotic germ cells that have been endocytosed/phagocytosed. A primary culture system of Sertoli cells is therefore desirable for studies of their biochemical/physiological properties. However, in adult rats/mice, Sertoli cells constitute only 5% of total cells in the seminiferous tubules, making it difficult to obtain their pure population following enzymatic digestions of the tubules. Hence, Sertoli cells are usually isolated from 20-day old rats/mice when the first round of spermatogenesis has just started and not many germ cells are yet present. However, Sertoli cells obtained from rats/mice of this age are very unlikely to have the same properties as those from adult animals, since testosterone levels at the two ages are significantly different. A primary culture of a pure population of Sertoli cells from adult rats/mice is therefore needed for physiological studies ex vivo. This is particularly relevant when male infertility/subfertility, which stems from Sertoli cell dysfunctions, manifests in adulthood. Our objective was to establish a method of this primary culture in adult mice (>6 weeks old). In the first part of our method, a mixture of loose Sertoli cells and testicular germ cells were prepared following the previously described procedures for 20-day old rats. Briefly, seminiferous tubules from decapsulated testes were digested with enzymes (collagenase, trypsin, hyaluronidase and DNase I) in a stepwise manner and Sertoli cells were selectively collected due to their higher sedimentation rate. Sertoli cells with remaining co-isolated germ cells were then plated onto a laminin coated Petri dish in DMEM/F12 medium supplemented with EGF, insulin and transferrin (Day 1). The same medium was used to wash off germ cells on successive days from the plate. On Day 8, Sertoli cells constituted 90-95% of total cell population. Specifically, we modified two steps in the previously described methods for Sertoli cell primary culture from adult rats in our protocol. First, we omitted the hypotonic treatment of the cells on the plate. Although germ cells were selectively ruptured by hypotonic treatment, injury to Sertoli cells was also observed by the transient presence of vacuoles. This omission is relevant to Sertoli cells of certain knockout mice, which already have abnormal functions in vivo. Second, we used laminin instead of Matrigel as the extracellular matrix. Since germ cells are less adherent to laminin than Matrigel, they can be effectively removed from the laminin coated plate. We demonstrated that Sertoli cells isolated from adult mice by our protocol exhibited tight junctions between each other as revealed by the presence of claudin11 and ZO1. These Sertoli cells also contained prosaposin and clusterin. Lipidomic analyses revealed that Sertoli cells from adult mice had significantly higher levels of triacylglycerols, cholesterol esters, and seminolipid, as compared with those from 20-day old mice, findings which are in agreement with higher numbers of lipid droplets in adult Sertoli cells. In summary, we have provided a method for a primary culture of Sertoli cells with physiological properties from adult mice. Supported by CIHR.

Sertoli cell-specific elimination of Vegfa impairs spermatogonial stem cell (SSC) maintenance and reduces fertility in male mice.

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We have previously demonstrated that vascular endothelial growth factor A (VEGFA) isoforms are critical for homeostasis of spermatogonial stem cells (SSCs). Treatment of perinatal mice with the antiangiogenic isoform, VEGFA165b, at the time of SSC formation reduced SSC colonization in germ cell-depleted recipients. Elimination of both angiogenic and antiangiogenic VEGFA isoforms in Sertoli and germ cells using pDmr1-Cre mice reduced fertility, epididymal sperm numbers and the number of PLZF-positive spermatagonia per seminiferous tubule whereas conversely causing a compensatory increase in the expression of genes that regulate SSC homeostasis (Ret, Sin3a, Neurog3) in 6 month- old male mice. From these two studies, we hypothesized that angiogenic isoforms of VEGFA promote SSC self-renewal while the antiangiogenic isoforms reduce SSC number, possibly by driving differentiation and/or cell death. We have generated a new transgenic mouse line to further investigate the effects of cell-specific VEGFA loss on male fertility. Cre recombinase-mediated deletion of Vegfa was driven by the Sry promoter. Either control or Sry-Cre;Vegfa−/− (KO) mice were mated to control females of proven fertility when they were 2 months-of-age and allowed to mate for up to 8 months. KO males took longer to get females pregnant.
667. WITHDRAWN.

670. WITHDRAWN.

672. Lectin binding of human sperm associates with DEFB126 mutation and serves as a potential biomarker for subfertility.

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Coating on the sperm surface, glycocalyx, plays a key role in sperm motility, maturation and fertilization. A comprehensive profile of sperm surface glycans will greatly facilitate both basic researches and clinical studies. Because of the capability of recognizing different glycan moieties with a variety of specificities, lectins are widely used in glycochemistry and related researches. However, lacking high-throughput technology, limited lectins have been reported for analyzing the glycan of human sperm. In the current study, we employed a lectin microarray for profiling the surface glycans of human sperm, on which 54 out of 91 lectins showed positive binding. Based on this technique, we compared lectin binding profilings of sperm with homozygous DEFB126 rs11467417 mutation, a 2-nucleotide frame-shift mutation, with that of wild type. DEFB126 was reported to contribute to the sialylation on sperm surface and male subfertility. Six lectins (Jcadin/A1A, GHA, ACL, MPL, VVL and ABA) were found to develop lower binding affinity to sperm with homozygous \textit{del/del}. Further validation with flow cytometry showed that these lectins, especially ABA and MPL, can be single or combined biomarkers for clinical diagnosis of homozygous \textit{del/del} of DEFB126. Our research sheds insight on the detection of unexplained male subfertility, and the lectin microarray strategy is generally applicable for infertility/subfertility sperm biomarker discovery.

673. Tubular morphometry in the testes of rats with Walker 256 Tumor treated with L-glutamine.

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Quantitative loss of germinal epithelium is frequent in patients with testicular cancer, and may be associated with oxidative stress. Thus, in order to alleviate or treat injuries related to oxidative stress, glutamine analogous compounds have been used in therapy against tumors in experimental models. However, studies about the effect of glutamine in spermatogenesis in models of rats with the Walker 256 tumor cells are not described in the literature. Thus, this study aimed to evaluate the effect of supplementation with L-glutamine on the morphometry of the seminiferous tubules of rats transplanted with Walker 256 tumor cells. This study was approved by the Ethics Committee of the State University of Maringá (Protocol nº 099/2012). There were used 40 Wistar rats with 52 days, divided into four groups with 10 animals in each: without L-glutamine control (C), supplemented control with L-glutamine (CG), Walker 256 tumor without supplementation with L-glutamine (TW) and Walker 256 tumor supplemented with L-glutamine (TWG). For supplementation, 2 % L-glutamine was incorporated in their meals for 14 days. After this period, the animals were sacrificed for the collection of the testes, which were fixed in Bouin solution for 24 hours and subjected to routine histological processing for embedding in paraffin and analysis by light microscopy. Photomicrographs were taken under Motic\textsuperscript{®} microscope and morphometry was made using the software Image-Pro Plus\textsuperscript{®}. The results were submitted to ANOVA followed by the Student-Newman-Keuls test with significance level of P < 0.05 and expressed as mean ± standard deviation. The body weight of the individuals in group C (299.60±14.88g) was significantly higher compared to the TW group (257.60±15.85g), while the TWG group (284.40±10.43g) had significantly higher body weight compared to the TW group, which may be related to a beneficial effect of supplementation on this parameter. Significant differences in gonadal weight and gonadosomatic and tubulossomatic indexes were not observed, which respectively quantify the percentage of body weight in relation to the gonads and the seminiferous tubules (average of 2.7g, 0.97% and 0.69%, respectively). The percentage of seminiferous tubules represented both at the first parturition (P = 0.0003) and over all parturition intervals (P < 0.0001). While there was no difference in the number of pups born from the first litter, KO males sired fewer pups in all litters measured compared to control males (P < 0.03). Male KO and control offspring were collected at various ages: postnatal day 0 (P), P5, P30, P60, P120, and P200. Hematoxylin-eosin staining of testis cross-sections demonstrated a progressive loss of germ cells within some seminiferous tubules in KO males. By P200, there were many Sertoli cell-only tubules visible and in some cases no visible germ cells in any section analyzed. Transplantation assays were conducted using P30 control and KO males as donors. Busulfan-treated C57B6 mice were used as germ cell- depleted recipients, and SSCs were allowed 6 weeks to colonize recipient tubules. Histological analysis of recipient testes revealed approximately 30-40% of seminiferous tubules devoid of germ cells in males that were microinjected with cells from KO donors compared to controls at P30. Finally, quantitative real-time PCR analysis was performed using whole testis mRNA. Abundance of \textit{Id4} was increased almost 2-fold (P < 0.009) along with an almost 3-fold increase in \textit{Bcl6b} (P < 0.003) in testes from KO males compared to controls at P60. Both \textit{ID4} and \textit{BCL6B} are important for the self-renewal of undifferentiated spermatagonia, and \textit{ID4-positive} staining is relegated to a subpopulation of undifferentiated spermatogonia, possibly the SSCs. The increases in genes that promote SSC maintenance is a similar trend seen previously when all VEGFA isoforms were eliminated in Sertoli and germ cells. The current study demonstrates that VEGFA isoforms produced by Sertoli cells are critical for normal SSC homeostasis as well as overall male fertility. This research has been supported by NIH HD051979 and NDHHS – Stem cell grant 2009, 2012.
by lumen was significantly lower in the TW group (24.30±1.74%) compared to C (29.47±2.84%), as well CG (25.74±1.76%) compared to C. No statistical differences were observed in relation to the percentage of seminiferous epithelium and tunica propia. The tubular diameter in TWG was lower when compared to CG (116.14±4.08μm and 125.54±4.10μm, respectively). However, no changes were observed in the height of the epithelium, which presented an average for all groups of 27.18μm. Similarly, there were no differences in length of the seminiferous tubules, and the average tubular length per gram of testis was 60.95mm, considering all groups. Reductions in the percentage of lumen and the tubular diameter in the animals with the tumor, when compared to the controls, may be related to injuries related to the tumor. However, supplementation with L-glutamine, in these experimental conditions, was not sufficient to promote significant improvements on testicular morphometry. This study has a scholarship provided for PIBIC/UFRN.

674. Prepubertal Exposure to Low Doses of Associated Methylmercury and Aroclor: Evaluation of Reproductive Parameters in Peripubertal and Adult Male Rats.
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Methylmercury (MeHg) and polychlorinated bisphenols (PCBs) are persistent environmental toxicants and suffering biomagnification along the food chain. The main exposure source of the population is through the intake of contaminated fish and sea food. Many epidemiological and experimental studies have shown that these chemicals are neurotoxic and can cause adverse effects on reproductive parameters and endocrine system. However, there is a scarcity of studies investigating endocrine and reproductive parameters after exposure to MeHg and PCBs associated, especially in models of pre- and peri-pubertal exposure. In reproductive toxicology, the childhood/peripuberty deserves special attention, because individuals in this phase of development may be more susceptible to endocrine disruptors than older animals, possibly due to hormonal imprinting, resulting in permanent changes in hormonal status when these animals become adults. The present study aimed to investigate whether the prepubertal exposure to MeHg and Aroclor (commercial mixture of PCBs) associated at low doses, interferes in reproductive parameters of male rats at peripuberty (immediate evaluation) and adulthood (late evaluation). Wistar male rats, 21 days old, were allocated into 5 groups: G1 (n=18, negative control, received corn oil), G2 (n=18, MeHg control, received MeHg at 0.5mg/Kg/day), G3 (n=18, Aroclor control, received Aroclor at 1.0mg/Kg/day), G4 (n=18, Mixture 1, received MeHg at 0.05mg/Kg/day and Aroclor at 0.1mg/Kg/day), G5 (n=18, Mixture 2, received MeHg at 0.5mg/Kg/day and Aroclor at 1.0mg/Kg/day). Dose levels were chosen based on previous studies in the literature. The animals were treated daily, from postnatal day (PND) 23 to 53, by gavage. The rats were examined daily from PND 30 for investigation of preputial separation. The animals were euthanized at the end of treatment on PND 53 or after an interval of 62 days without exposure to chemicals, on PND115. Experimental design was approved by local ethical committee (CEUA-USC 30-13). Body weight, reproductive organs weight, liver, kidney and adrenal weight, and testes histology were evaluated at the two ages. Sperm motility was recorded in adult animals. Statistics were performed using ANOVA test or Kruskal Wallis test (p<0.05). There was no significant difference among the groups regarding preputial separation day, an indicative of puberty onset. During and after the treatment, rats from the five groups kept the same pattern of body weight gain. In the same way, no differences were observed in the absolute and relative organs weight evaluated on PND53 or 115. Quantitative analysis of testes histology did not reveal any alteration related to treatment in peripubertal and adult animals. However, there was a reduction in the number of sperm with progressive movement in G3 compared to G1 (p=0.04), with a consequent increase in sperm with non-progressive movement in G3 compared to G1 (p=0.056). Conclusion: These partial results suggest that toxicants exposure, in these experimental conditions, did not impair the development of evaluated organs. However, the treatment with Aroclor seems to compromise sperm quality in adult animals. Other parameters will be investigated to better understand possible mechanisms involved in this process. Research supported by FAPESP (2013/14477-3).

675. Identification of CRISP Proteins in the Dog for Their Potential Use in Immunocontraception.
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There is a growing need to reduce the dog population levels as a means to control diseases transmitted to human (zoonosis). Based on previous results from our group supporting epididymal CRISP1 (Cysteine -Rich Secretory Protein 1) as a good immunocontraceptive target, the aim of this work has been the identification of a homologue CRISP protein in the dog that could be used for immunocontraceptive development in this species. Using degenerated primers against conserved CRISP motives, we obtained a RT-PCR band of the expected size (approx. 120 bp) from epididymal but not testicular samples. DNA sequencing of this band showed a high (>80%) homology to CRISP proteins. Western blotting results using antibodies against different CRISP proteins revealed the presence of 25 kDa band in epididymal but not testicular extracts when an anti-mouse CRISP3 antibody was used. As expected for a CRISP molecule, the epididymal band shifted its electrophoretic mobility under reducing conditions. Moreover, the protein precipitates by exposure to a 50% saturated ammonium sulfate solution as previously observed for rodent CRISP1. Altogether, these observations support the identification of a CRISP-like molecule in the dog epididymis which is at present being further characterized for future purification and functional studies. *The first and second author are co-first authors.

676. A 3’ distal element implicated in cell-specific expression of the follicle-stimulating hormone receptor.
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The follicle stimulating hormone receptor, FSHR, is required for FSH signaling and thus is essential to normal gonad function, gametogenesis, and fertility. The mechanisms that regulate FSHR expression are poorly understood, particularly those that control cell specificity and receptor levels, but are of considerable interest because they dictate what cells respond to FSH and help establish the degree of response. FSHR expression is restricted largely to granulosa cells of the ovary and Sertoli cells of the testis. Despite its remarkable cell-
specificity and physiological importance, the transcriptional mechanisms that regulate specificity remain elusive. One likely reason for this is that the relevant regulatory sequences are located far from the gene’s transcriptional start site, making them difficult to locate. To help reveal distal regulatory sequences, we employed comparative sequence analysis to identify evolutionarily conserved non-coding regions (ECRs) and chromatin immunoprecipitation with deep sequencing (ChIP-Seq) to locate sequences bound by histones with modifications implicated in transcriptional regulation. Comparative sequence analysis identified a highly conserved region, ECR1f, located in the 3′ intergenic region between Fshr and Lhcgr. ChIP-Seq data from Sertoli cells showed that the region was associated with high levels of H3K4me3, a histone modification associated with activation. In contrast, ChIP-Seq data from myoid cells, which do not express Fshr, showed low levels of ECR1f-associated H3K4me3. ECR1f was further evaluated in Sertoli cells by ChIP-qPCR, which confirmed the enrichment of H3K4me3 and showed enhanced levels of acetylated H4 (another mark of activation). Enrichment was not observed for two modifications (H3K9me2 or H3K27me3) associated with transcriptional silencing. The data suggest that ECR1f functions as an enhancer in Sertoli cells but not myoid cells and are consistent with it having a role in Fshr cell-specific expression. Transient transfection analysis in Sertoli and myoid cells and DNase I footprinting were used to help identify the active ECR1f sequences. Transient transfection identified several transcriptionally active regions in ECR1f. However, in contrast to the in vivo data, naked ECR1f in proximity to the Fshr promoter acted as a repressor and the activity was not limited to Sertoli cells. The DNAse I footprinting revealed multiple sequences bound by nuclear proteins from Sertoli cells and myoid cells; some of which were similar while others showed differential binding. Additional binding and transfection analyses indicated that GATA4 and an unknown protein contributed to ECR1f activity.

To conclude, studies to date indicate distal sequences control cell-specific expression of Fshr and the current study suggests that at least some of these sequences reside within ECR1f. This research was supported by NIH HD053549 (LLH).

677. Production and Regulation of Activins A and B in Rat Spermatogenic Cells In Vitro: a Role for Toll-like Receptor 4 Signaling in Regulating Spermatogenic Cell Activity.
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The activins (activin A and B) are potent regulators of cell differentiation, apoptosis, inflammation and immunity. Production of the activins and their homologous inhibitors, inhibin A and B, by the Sertoli cell is reciprocally regulated at the transcriptional level. This regulation involves follicle-stimulating hormone, which acts via cAMP-protein kinase A to stimulate the inhibin α-subunit and βB-subunit expression, and inflammatory mediators, such as bacterial lipopolysaccharide, interleukin-1 and tumor necrosis factor, which stimulate βA-subunit expression (thereby producing activin A). Production of activin B appears to be indirectly regulated, principally through control of α-subunit levels. It has long been known that spermatogenic cells also express the activin/inhibin β-subunits genes, but not the α-subunit, suggesting that these cells also may be a source of activins in the seminiferous epithelium; however, production and regulation of the mature proteins have not been studied previously in spermatogenic cells. Consequently, activin A and activin B were measured in adult rat pachytenic spermatocytes and round spermatids isolated by centrifugal elutriation, in comparison with Sertoli cells isolated from 20 day-old rats, using quantitative polymerase chain reaction assays and two-site enzyme-linked immunosassays. Spermatocytes and spermatids (>80% purity) both expressed extremely low levels of βA-subunit mRNA and βB-subunit mRNA, compared with purified Sertoli cells (<5% relative expression based on Ct values). They also produced correspondingly low levels of activin A and activin B, over 16 h in culture. Spermatocytes and spermatids expressed the lipopolysaccharide receptor, Toll-like receptor 4, and its co-receptors (MD2 and CD14), and Toll-like receptor 4 was expressed on the cell surface of both cell types, as determined by flow cytometry. Nonetheless, treatment with lipopolysaccharide had no effect on the basal expression of the βA- or βB-subunit or several other inflammatory genes (tumor necrosis factor, interleukin-6 and prostaglandin-endoperoxide synthase 2) by either cell type, over 1-3 h in culture. In cultured spermatids, but not in spermatocytes, treatment with lipopolysaccharide increased Toll-like receptor 4 expression on the cell surface, marginally stimulated production of activin A, and dramatically increased the rate of apoptosis. Neither interleukin-1 nor pam3cys, which activate the same intracellular signaling pathways as Toll-like receptor 4, albeit via different cell surface receptors, had any significant effect on activin production by either cell type. These data indicate that developing spermatogenic cells are unlikely to make a substantial contribution to activin production in the testis under either basal or inflammation conditions. The data also indicate that spermatogenic cells express Toll-like receptor 4 and its essential co-receptors, and that round spermatids, but not pachytenic spermatocytes, respond to Toll-like receptor 4 activation in vitro by undergoing apoptosis without displaying a pro-inflammatory gene response. As there is increasing evidence for endogenous Toll-like receptor 4 ligand activity in the seminiferous epithelium, these data suggest a role for Toll-like receptor 4 signaling in directly regulating post-meiotic cell activity and survival.

678. STAT3 mRNA Expression in the Caprine Testis.
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Signal transducer and activators of transcription (STAT3) is one of seven STAT proteins found in mammals. This family of proteins has been shown to have many functions, and STAT3 has been shown to play an important role in cell growth and cell survival. Not only was STAT3 localized to gonocytes in mice testes, it is expressed in the testis during acrosome development in mice. Additional findings suggest that STAT3 is necessary for the self-renewal of germline stem cells in Drosophila. Collectively, the data suggest a role for STAT3 in spermatogenesis. It is well documented that estrogen plays a significant role in testicular function and recent findings suggest that STAT3 is regulated by estrogen. Although STAT3 has been studied in several species, limited information is available in goats. The objective of this study was to determine mRNA levels of STAT3 in testis at three developmental stages of goats. Total RNA was extracted from the testes of fifteen Kiko X Boer goats - neonates (n=5; >30d), juveniles (n=5; ~120d), and adults (n=5; ~180d) and mRNA expression levels quantified by using quantitative RT-PCR. The results reveals that STAT3 was expressed at each of the developmental stages (adults = 3.35 ± 0.18; juveniles = 7.57 ± 0.26; and neonates = 5.61 ± 0.33; however no significant differences were
679. Effects of phytosterols as food additive on the endocrine and reproductive function in the Japanese quail.
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It is known that phytosterol (PS) disrupts the intestinal cholesterol absorption and metabolism and decrease the low-density lipoprotein cholesterol (LDL-C) and atherosclerosis. PS has disrupting effects on the normal endocrine function. In the Japanese quail, PS affects the adrenal steroidogenesis in a dose dependent manner. We hypothesized that overloading with PS may change the normal gonads and adrenal functions in the Japanese quail. Two studies were conducted to this hypothesis: in the first study, 100 male and female quails were randomly assigned to 5 groups [2 controls and 3 PS treatments (0.008, 0.08 and 0.8mg/g BW)] after 7 day post hatching. For treatment groups, PS was gavaged into the crop sac. After 44 days PS feeding. 50% of all the birds from each groups were selected and subjected to a 6-day ACTH challenge and 50% of the birds were kept as ACTH control. Every second days of ACTH injection, blood was collected from the jugular vein. In the second study, 50 adult male and female were divided to 5 groups (2 control and 3 doses treatment groups) and faced to one day subcutaneous (SC) PS injection. After injection, blood samples were collected after 3, 6 and 24 hours. E2 was injected as positive control for estrogenic effect of PS. For the statistical analysis we used One- Way ANOVA for body weight and organs weight and for hormones analysis we used Two- Way ANOVA (Dun nett test, Graphpad prism version 5). The P<0.05 was considered to be significant. The results indicated that PS feeding significantly decreased (p<0.05) the weekly bodyweight in the PS low and high dose in male and female. Moreover, both testicular weights were lower (p<0.05) in compare to the control. Liver and adrenal weight was lower in the male and female treatment animals but not significant. Plasma corticosterone (CORT) level was significantly high (p<0.001) in the male treatment groups on the 4th and 6th d ACTH challenge. Intriguingly, CORT levels were also high (p<0.001) in the male (after 3h) and female (p<0.05 and p<0.001) after 6 and 24 respectively in the short time PS injected animals. Unlike to the CORT, male and female were fed PS the testosterone (T) level was significant low after 4th and 6th d ACTH challenge (p<0.01) and after 6d in the control (p<0.01) comparing to the intact animals. But in the PS SC injected birds, no significant changes were observed except of male E2 control (p<0.001). Progesterone level was high (p<0.05) only in the high dose PS in the female gavaged and SC injected female animals. LH level was not significant change at all. In conclusion, our results demonstrate that overfeed of PS additives can disrupt the endocrine and reproductive functions in the Japanese quail.

680. Seasonal expression of androgen receptor, aromatase, estrogen receptor alpha and beta in testes of wild ground squirrels (Citellus dauricus Brandt) during the breeding, nonbreeding seasons and pre-hibernation.
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Background: Testosterone is converted in target cells to estradiol by the enzyme cytochrome P450 aromatase. Estrogen action is displayed by means of two different estrogen receptors (ERs), estrogen receptor-alpha (ERα) and estrogen receptor-beta (ERβ), localized in the different testicular cells types. The wild ground squirrel (Citellus dauricus Brandt) is a typical seasonal breeder which has a strict and extremely compressed breeding period from April to May, a long period of sexual dormancy from June to the following March, and a 6-month hibernation (from October to March). Aim: In this study, we investigated the immunohistochemical localization of AR, P450arom, ERα and ERβ as well as their mRNA expression in the breeding, nonbreeding seasons and pre-hibernation, to gain insight of the relation between steroid hormones and testicular function throughout the reproductive cycle of the wild ground squirrel. Materials and methods: The wild male ground squirrels were captured by box traps in September 24 (n=12) of 2012 and in April 13 (n=15) and June 27 (n=10) of 2013 in Hebei Province, PR China. Testicular tissues were excised quickly after necropsy. On necropsy, the testes were excised, weighed, measured and tissues fixed in 4% paraformaldehyde in 0.05 M PBS (pH 7.4) for histological and immunohistochemical observations, and the others were immediately frozen in liquid nitrogen and stored at -80 C until used for RNA isolation. Results: The largest values of testicular weight, size and seminiferous tubules diameter were found in the breeding season (April), and the smallest values in the nonbreeding season (June). Meanwhile, HE staining showed that all stages of sperm cells could be identified in the seminiferous epithelium in the breeding season, only spermatogonium and primary spermatocyte in the nonbreeding season, but there was no secondary spermatocyte in the nonbreeding season, which could be found in the seminiferous epithelium of pre-hibernation. The staining of AR was observed in the Leydig cell, Sertoli cell and peritubular myoid cells during the breeding season and pre-hibernation. In the nonbreeding season, only weak immunostaining was shown in the Leydig cells. Immunoreactivity of ERα was detected in the Leydig cells and Sertoli cells during the breeding season and in the Sertoli cells during pre-hibernation, whereas no staining was detected in the nonbreeding season. With regard to ERβ, positive immunostaining was shown in the Leydig cells and week signal was found in germ cells during breeding season, yet no signal in the nonbreeding season and pre-hibernation. Stronger immunohistochemical signal for P450arom was present in Sertoli cells, Leydig cells and germ cells during the breeding season; P450arom was also presented in Sertoli cells and Leydig cells during pre-hibernation, while there was no positive P450arom signal in the nonbreeding season. The AR gene was started at a high level during the breeding season, and there was no remarkable difference in expression during the rest of the reproductive cycle. For ERα mRNA, the expression peaked in the breeding season, rapidly reduced during the nonbreeding season, and returned to a relatively high level during pre-hibernation. With regard to ERβ gene, the level decreased drastically from the breeding to the rest of the nonreproductive cycle. For P450arom mRNA, the expression in the breeding season, significantly higher in the breeding season. After the obvious reduction of the nonbreeding season, the level of P450 mRNA showed a marked increase in pre-hibernation. Conclusion: The present results demonstrated that seasonal changes in testicular weight, size and seminiferous tubule diameters in the wild ground squirrels are correlated with changes in spermatogenesis and testicular distribution of AR, P450arom and ERs during the breeding and nonbreeding seasons and pre-hibernation. This study is supported by a Grant-in-Aid from National Natural Science Foundation of China (NSFC, No.J103516; No.J1310005)
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Capacitation of the mammalian sperm is a series of maturation events that confer the fertilization potential by inducing sperm hyperactivation, characterized by an increase in flagellum beating frequency and amplitude, and sperm capacity to perform the acrosomal reaction prior to the interaction with the oocyte. During these processes substantial changes occur both at the plasma membrane (modification of lipids composition, hyperpolarization) and the cytoplasmic level (increase in cAMP contents, activation of Protein kinase A followed by an increase in tyrosine phosphorylation and intracellular alkalinization). Ion fluxes, more precisely Ca++, Cl- and HCO3- fluxes trigger part of these modifications and in consequence several ion transporters have been described and shown to be required for sperm motility, although their specific contribution and their mode of cooperation to these processes remain sometimes unclear. We have previously described the SLC26a8 (Solute Like carrier 26 A8; Testis Anion Transporter 1), as a testis specific member of the SLC26 family of anion exchangers and shown that the deletion of Slc26a8 in the mouse induces male sterility due to the lack of sperm motility, impaired capacitation and severe structural defects of the flagellum. In addition we have shown that SLC26A8, like other SLC26 members, physically interacts with the Cystic Fibrosis Transmembrane conductance Regulator channel (CFTR) and stimulates CFTR function, indicating that both anion transporters cooperate to control sperm motility. SLC26A3 and SLC26A6, which have been well studied in regard to their Cl-/HCO3- exchange functions in the gastro-intestinal epithelia, have also been reported to be expressed in the testis and in the epididymis. Interestingly, the use of SLC26A3 and SLC26A6 inhibitors, suggest their implication in the changes of alkalinisation and membrane hyperpolarization occurring during sperm capacitation. In this study, we aim to determine the specific contribution of each of these Slc26 proteins in sperm function and capacitation. For this purpose, we have characterized mice models in which the genes coding for Slc26a3 and Slc26a6 are knocked-out and compared them to the Slc26a8 mutant mouse model, with respect to testicular and epididymal structure together with sperm count, structure, motility and capacitation.

682. Combined LH and FSH stimulation of the testis of the juvenile monkey (Macaca mulatta) for 48 hours results in upregulation of miRNAs in association with initiation of spermatogonial differentiation. 
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We recently showed that treatment of juvenile male rhesus monkeys for 48h with LH and FSH, in combination, results in the decision by undifferentiated spermatogonia (A pale, Ap) to commit to a pathway of differentiation. miRNAs are emerging as an important class of regulators of differentiation, proliferation and apoptosis, and a few of these non-coding transcripts have already been assigned roles in regulating undifferentiated spermatogonia in the mouse by others. In an earlier study, RNA was extracted from the entire testis of juvenile monkeys (14-24 months of age) that had been treated for 48 h with either LH and FSH, in combination, or with vehicle (N=3 for each group) to identify the testicular transcriptome (coding) associated with initiation of spermatogonial differentiation. Here, we report the concomitant changes in the miRNA landscape as revealed by miRNASeq using the Ion Torrent platform and conducted by the Genomics Research Core, University of Pittsburgh. To identify known and novel miRNAs, bioinformatic analysis was performed using miRDeep2 (v 2.0.0.5) with miRNA sequence files of the rheMac2 genome as reference. In addition, a miRNA sequence file from a related species (human hg19) was provided to improve the performance of miRDeep2 in miRNA detection. miRNA sequence files were obtained from database miRBase v20, and differential expression of miRNA was examined using edgeR. More than 50% of the known 914 mature miRNAs in rheMac2 database were detected. The predominant effect of gonadotropin stimulation was to enhance transcription of selected miRNAs. Ten miRNAs (147b, 431, 484, 503, 665, 671, 675, 1185-1, 1185-2 and 1260b) were found to be consistently up-regulated (fold change >1.5; false detection rate, fdr, <20%) by combined LH and FSH treatment. miRNA-221 and 222, previously shown by others to indicate that expression of the genes coding for DROSHA and DICER was influenced by 48 h of gonadotropin stimulation. The identities of the testicular cells in which the miRNA landscape changes in response to gonadotropin stimulation remains to be determined, as do the cellular processes governed by these transcripts. Supported by NIH grant R01HD072189.

TOPIC AREA 6: Pituitary

PITUITARY: Program Numbers 683–693

683. Cross-Talk between Ghrelin and Serotonin in the Regulation of GH And PRL Secretion in Ewes. 
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In temperate latitudes, sheep are seasonal breeders whose reproductive activity is regulated mainly by photoperiod. However, it is believed that other factors connected with metabolism or food intake may play a role in regulating seasonal processes. Ghrelin is a hormone that regulates appetite and energetic homeostasis and it also interacts to regulate secretion of melatonin. It is presumed that ghrelin, directly or indirectly, modulates serotonin (melatonin precursor). Furthermore, it has been proposed, based mainly on studies in rodents, that ghrelin and serotonin may jointly govern the secretion of somatotropinotropic hormones. Whether or not such interactions between the two factors exist and how they are modulated by photoperiodic influences and nutritional status in seasonally polyestrous animals remain unknown. To test this hypothesis in vivo, experiments were conducted using a 5-HT2C serotonin receptor agonist, m-CPP [1-(3-Chlorophenyl) piperazine hydrochloride]. The experiments were carried out during both natural short (SD) and long days (LD). Twelve 2

2015 Abstracts – Page 271
to 3 yr-old ovariectomized ewes of the Polish Longwool sheep breed, each bearing subcutaneous estradiol implants, were used and fed ad libitum. Within season and replicate, ewes were assigned randomly to 1 of 4 groups (4 ewes/treatment) and infused intravenously beginning at sunset. Groups consisted of: 1) Control (saline); 2) Ghrelin (2.5 µg/kg BW); 3) mCPP (2.5 mg/kg BW); 4) Ghrelin and mCPP as in 2 and 3. Jugular blood samples were collected at 15-min intervals beginning immediately before the start of infusions and continued for 3 h. At the conclusion of blood sampling, a washout period of at least 3 d elapsed before ewes were re-randomized and treated with one of the treatments described above. Prolactin and growth hormone concentrations were determined using RIA. Ghrelin and mCPP enhanced (P<0.05) GH secretion in fasted ewes during LD and in normally fed sheep throughout the year, respectively. The mCPP treatment significantly decreased GH concentrations in fasted ewes during SD. Circulating PRL concentrations were lower (P<0.01) in normally fed ewes after ghrelin + mCPP than after treatment with mCPP alone. There are interactions among ghrelin, serotonin, photoperiod and metabolic status that impinge on GH and PRL secretion in ewes. This provides the basis for future studies of the pathogenesis of metabolic disorders associated with the alterations in nutritional status and day length, using an ovine experimental model. Research supported by NCN 2013/05/B/NZ4/02408.

684. Estrogenicity of octamethylcyclotetrasiloxane via induction of CaBP-9K on GH3 cell.
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Introduction: Interrupting the organism’s hormonal balance by intervening with the hormone and their target receptor arise various problems such as developmental disorder or cancer related to reproduction. Though some chemicals contained in natural creature like Red clover are inducing these problems, most chemicals not found in natural compound partake in disrupting endocrine system. We call these chemicals altogether as an endocrine disrupting chemicals (EDCs). Some chemicals included in EDCs influence to estrogenic, androgenic or thyroid specifically or more than one of these. Silicone is widely used in medical devices, cleaning products and cosmetic products. Cyclic volatile methylsiloxanes (cVMSs) are group of silicone that including octamethylcyclotetrasiloxane (D4), decamethylcyclopentasiloxane (D5) and dodecamethyl-cyclohexasiloxane (D6). These chemicals have a property that high vapor pressure, high octanol/water partition coefficient and low molecular weight. Due to its properties, they can be easily found in air and well absorbed in lipid. In human, because of higher exposure probability other than creature and its characteristics, human can significantly be affected. In recent study, hazardousness of D4 was evaluated and its estrogenicity was noticed. Method: GH3 cells, rat pituitary cell, were exposed to vehicle, 17β-estradiol (E2; 1.0x10^-9M) or D4 (1.0x10^-7M). For approving the pathway of D4, ICI 182 780 (ICI; 1.0x10^-7M) was exposed before those chemicals. Transcription level of CaBP-9K, estrogen receptor alpha (ER α) and progesterone receptor (PR) was quantified by qRT-PCR. And those protein quantities were measured by western blot. Result: CaBP-9K and PR were up-regulated by E2 and D4. Elevated levels of those were down-regulated by ICI. ER α is decreased by E2 and D4. A decreased level of it was increased by ICI. Conclusion: We sustained estrogenicity of D4 on in vitro assay using calcium binding protein 9K (CaBP-9K) as a biomarker for estrogen. This work was supported by the National Research Foundation of Korea (NRF) grant of Korean government (MEST) (No. 2013-010514).

685. Effects of Leptin and Leptin Antagonist on Expression of SOCS-3 mRNA in the Ovine Pituitary.
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Leptin, a hormone produced primarily by white adipocytes, exerts influence not only on the energetic homeostasis, but it is also an important neuroendocrinal factor, which regulates metabolic processes and hormonal status, involving reproductive processes. The JAK/STAT pathway is the most important pathway of the signal transduction from leptin to target cells. This signaling pathway could be modulate by various factors, including SOCS (Suppressor of Cytokine Signaling) proteins. In sheep, which are the short day breeders, secretion of leptin is dependent on the length of the day. Moreover, hypothalamus of seasonal-breeding sheep is programmed biannually to become leptin resistant in long-day (LD) and leptin sensitive in short-day (SD) season. The aim of this study was to investigate the expression of SOCS-3 mRNA in the ovine pituitary gland, depending on the photoperiod, leptin and its antagonist. Pituitaries isolated from 8 ewes (four per month) decapitated in May (LD) and November (SD) were used. Tissues were cut into approximately 50 mg explants, which were treated with: 1) control medium or medium containing: 2) leptin (0.1 µg/ml; RayBiotech), 3) leptin receptor antagonist (1.0 µg/ml; SLAN-3 - Specific Ovine Super-Active Leptin Antagonist; Protein Laboratories, Rehovot, Izrael) or 4) leptin and SLAN-3 (0.1 µg/ml and 1.0 µg/ml, respectively). SLAN-3 was administered to the respective experimental groups after 45 minutes of equilibration, and leptin was added 15 minutes later (at 60 minutes of experiment). Incubation was maintained for different time intervals: 0, 30, 60 or 120 minutes, from the time of administration of exogenous leptin. The level of expression was analysed in at least three explants for each experimental group and for each time interval. Real-time PCR was used to measure SOCS-3 mRNA levels. Total RNA was isolated using TriReagent (Invitrogen). RNA (0.5 µg) was reverse transcribed to cDNA using QuantiTect Reverse Transcription Kit (Qiagen). Amplification of cDNA was performed using TaqMan Gene Expression Master Mix (Life Technologies). The results confirmed the hypothesis that expression of SOCS-3 mRNA is dependent on the time of year - higher expression was observed in the pituitary gland collected during the short day, than during long day season (p < 0.05). It was also showed that exogenous leptin reduced (P <0.01) the expression of SOCS-3 in sheep pituitary gland during the LD, while during SD tendency to stimulation of this process by leptin was noticed. In the tissues incubated with SLAN-3, level of SOCS-3 transcripts was comparable in relation to the control during SD, and was reduced (P <0.05) in long-day season. During LD, it was also observed that when the tissues were incubated in the presence of both leptin and SLAN-3 at the same time, their inhibiting effect on the expression of SOCS-3 was blocked. During SD, SOCS-3 transcripts levels in explants treated with leptin and its antagonist was significantly lower (P <0.01) than in the control group and highly significantly lower (P <0.001) than in explants treated with leptin alone. It has been shown that leptin can both stimulate and inhibit the expression of SOCS-3, and the effect of its action remains largely depends on additional factors for instance photoperiod or endocrine status of the animal. Leptin antagonist (SLAN-3) blocked leptin effects, regardless of its action on SOCS-3 expression.
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686. Study and Melanocyte Adrenocorticotropic Effects on Sugar Metabolism and Immune Response in Rabbits Oryctolagus cuniculus.
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The functioning of the pineal gland, the transducer body of environmental information to the neuroendocrine system is subject to a circadian rhythm. Melatonin is the main neuro-hormone expressing this operation. It is synthesized in the pinealocytes after conversion serotonin via N-acetyl-transferase enzyme, the same subject to a photoperiodic modulation (activation dark inhibition by light). Some scientists have suggested that melatonin is involved in diabetic disease and which expresses have a diabetogenic effect. To this study the effect of this hormone on glucose metabolism has long been subject to controversy. Agreeing in effect and hyperinsulinemic hypoglycemic effect. In order to illustrate the level of interaction of melatonin with neuro-immune-corticotropin axis and its impact on carbohydrate metabolism, we studied the impact homeostatic (glucose) through the solicitation of two control systems (gliad pineal and corticotropin axis). We and found that melatonin could have an indirect influence on insulin control (glucose metabolism) to the levels of the growth hormone axis (somatostatin) and adrenocorticotropic (corticotropin). In addition, we have suggested that melatonin might limit the hyperglycemic action of corticosteroids by direct action at peripheral level.

687. Female mice lacking SMAD3 DNA binding activity and SMAD4 in gonadotropes are FSH deficient and infertile.
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Pituitary follicle-stimulating hormone (FSH) is an essential regulator of gonadal function and fertility in females and of quantitatively normal spermatogenesis in males. Pituitary-derived activins directly stimulate FSH synthesis by regulating transcription of the FSHβ subunit gene (FsHB) in gonadotrope cells. According to in vitro models, activins stimulate the formation of complexes of the signaling molecule SMAD3, its obligate co-factor SMAD4, and the forkhead transcription factor FOXL2. SMAD4 and FOXL2 bind adjacent cis-elements in the proximal FSHβ promoter and the two proteins are linked through their mutual association with the C-terminal Mad homology 2 (MH2) domain of SMAD3. Consistent with this model, mice harboring loss of function mutations in Smad4 or Foxl2 specifically in gonadotropes are FSH deficient and infertile. SMAD3’s role in vivo is less clear, however, as gonadotrope-specific Smad3 ‘knockout’ mice exhibit quantitatively normal FSH synthesis and fertility. Importantly, these mice may express a truncated form of the SMAD3 protein that lacks the N-terminal DNA binding MH1 domain but possesses the entirety of the MH2 domain. The latter represents the domain of the protein that is activated by activin receptors and mediates protein-protein interactions. Unfortunately, there are no extant mouse models that enable the complete and selective abrogation of SMAD3 function in gonadotropes or other cell types. However, based on the current in vitro model of activin induction of FSHβ transcription, we reasoned that mice lacking both SMAD3 DNA binding activity and SMAD4 in gonadotropes (hereafter, S3/4 conditional knockout or cKO) should be profoundly FSH deficient and sterile. Indeed, both basal and activin-stimulated FSHβ mRNA expression is significantly impaired in primary pituitary cultures from male S3/4cKO mice. Furthermore, S3/4cKO females are hypogonadal with thread-like uteri. At 12 weeks, their ovaries lack pre-ovulatory follicles and corpora lutea, suggesting severe FSH deficiency and anovulation. Indeed, after more than 2 months in breeding trials, S3/4cKO females (n=5) appear to be sterile. Though not yet complete, our analyses suggest that: 1) SMAD3 is required for FSH synthesis in vivo and 2) residual FSH synthesis in Smad4 deficient mice likely reflects compensatory actions of SMAD3. This research was funded by CIHR MOP-133394 to D.J.B.

688. The Pituitary, an Overlooked Potential Site of RHOX Transcription Factor Action.
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The Reproductive Homeobox X-linked, Rhox, genes encode transcription factors whose expression and regulation have been extensively characterized in the ovary, testis, epididymis, and placenta. Prior panel screens of reproduction-associated tissues have omitted the pituitary. In mice, there are 13 principle Rhox genes. However, 3 genes in the alpha subcluster; Rhox2, Rhox3, and Rhox4, have duplicated multiple times resulting in a total of 33 distinct genes. We determined the relative expression levels of the Rhox cluster in adult pituitary (n = 10) by qRT-PCR using probes designed to span 1 or 2 exon/intron junctions in order to identify properly spliced mRNA (as compared to testis controls, n = 5) transcripts. Most Rhox genes are expressed in germ cells of the embryonic and postnatal testes. Rhox6, Rhox9, and Rhox12, that are predominately expressed in trophoblast, but have been postulated to contribute to embryonic germ cell development, were not detected in adult pituitary. Similarly, Rhox7, Rhox10, and Rhox11, which are significant products of adult germ cells, were not reproducibly detected in adult pituitary. Rhox1 exhibited equivalent expression in adult pituitary and testes, but these levels were only 2-fold above (-) RT control, suggesting its expression may not be physiologically relevant. Rhox2 was 5-fold higher than Rhox1, but its relative expression was 100-fold lower in pituitary than testes. Interestingly, mRNA levels for Rhox3, Rhox4, and Rhox13 were ~10-fold higher in pituitary than testes. The RT-PCR products from Rhox2, Rhox3, and Rhox4 were a mixture of the expected (~200 bp) products and larger species which could not result from amplification across unspliced introns or mispriming from any non-Rhox gene in the NCBI database. This suggests there may be pituitary- specific exon or promoter usage between the paralogous copies of these duplicated genes, and that the paralogous copies may be differentially regulated. RHOX5 and RHOX8 are produced by Sertoli cells and are the highest expressed Rhox genes in the testis. Mature transcripts for Rhox3 and Rhox8 were expressed in the pituitary, but both were present at ~5% of their adult testis levels. We do not yet know what may control Rhox gene expression in the pituitary. However, we determined that Rhox3 transcripts were initiated specifically from Rhox3’s distal promoter, which we have previously shown to drive expression in ovarian granulosa cells, whereas Rhox3’s androgen-dependent proximal promoter was inactive. At present, we can only speculate whether the RHOX factors are active as transcription factors in the pituitary. Colocalization of RHOX proteins and LHB in
gonadotropes yielded specific, but predominantly cytoplasmic, staining in adult pituitary sections. However, earlier ages have yet to be examined. We currently have no putative function for RHOX homeobox factors in the pituitary. Rhox3- and Sertoli-specific Rhox8-knockdown male mice are sub fertile due to excessive germ-cell apoptosis and poor sperm motility. Normal androgen levels in Rhox5-null and Rhox8-knockdown mice suggests the HPG axis is intact or that redundancy exists with other RHOX factors in the pituitary. However, formal investigation of pituitary function in our transgenic mice has not yet been pursued. While the expression of most Rhox genes in the adult pituitary is relatively low (ranging from 3-fold to 30-fold below that of Pgr), it will be interesting to determine whether they are differentially expressed and putatively function during pituitary development.


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Due to the heterogeneity of the pituitary cell population, application of approaches such as high-throughput RNA sequencing to a specific pituitary cell type is problematic. In the case of gonadotropes, this is particularly challenging as these represent only 5-10% of the total population of endocrine cells in the anterior pituitary. Therefore, the effect of any treatment on virtually any parameter of gonadotrope biology, whether global alterations in gene expression, association of transcription factors with promoter sequences, epigenetic changes, or phosphorylation of proteins is difficult to detect and parse from the background “noise” created by the other 90% of cells in the pituitary. Thus, in non-rodent species, the simple reality is that without enriched populations of gonadotropes, advances in our understanding of the molecular regulation of gonadotropin synthesis and secretion will be very slow. Herein we have utilized a nadenoviral vector containing 1500bp proximal promoter from the human a glycoprotein hormone subunit (hαGSU) directing expression of GFP. For experiment 1, dispersed ovine pituitary cells were infected at 1, 10, 150, 250, and 500 multiplicity of infection (MOI). Four days after infection cells were harvested, filtered (50 mm) and subjected to fluorescence activated cell sorting (FACS) with a MoFlo Legacy flow cytometer using a 488 nm laser, a 530/40 nm bandpass filter, 400V, and a 100 nm flow cell tip. Flow cytometer conditions included large forward scatter vs. side scatter and “enrich” vs. “purity” sort modes. The enrich mode will render a higher yield of GFP expressing cells but with reduced selectivity (more false positives) whereas the purity mode yield but increased selectivity (few false positives). After sorting, both selected (GFP positive) and non-selected (GFP negative) cells were lysed and LH and TSH content was quantified by RIA. Regardless of sort mode, a MOI of 250 or 500 yielded the greatest number of positive, sorted cells. In the purity mode these conditions isolated a GFP expressing cell population that ranged from 0.3 to 1% of the total cell population. In the less selective mode (enrich), the selected population increased ranged from 3 to 10% of the total cell population. After adjustment for cell number, there was a 170-fold enrichment for LH and a 0.33-fold reduction in TSH in positive cells selected in the purity mode whereas the enrich mode resulted in a 27 and a 2.3-fold enrichment for LH and TSH, respectively. When hormone concentrations were calculated as LH fold change divided by TSH fold change, 50X more LH than TSH was detected in positive cells vs. negative cells sorted in the purity mode and 17X more in the enrich mode. In experiment 2, pituitary cells were infected as described above but then treated with 50 nM E2 or ethanol for 12 h prior to FACS using the purity mode. After FACS, the selected cells were assayed for cell surface GnRH receptor numbers by radioceptor assay. In non-E2 treated cells, GnRH binding was detected in GFP positive sorted cells, but not in the GFP negative population. As expected, E2 treatment increased the number of GnRH receptors in the purified population. We suggest that adenoviral targeting of GFP represents a robust and reliable approach for producing a highly enriched population of gonadotropes from primary cultures of ovine pituitary cells. This project was supported by NIH, grant No 5R01HD065943-05 to CMC.

690. Hormone Signal Modulators Among Protein Hormone Pro-peptides and Hormone Fragments.

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As part of our exploration for biologically active peptides produced during target cell degradation of protein hormones we have recently constructed a database of all known human protein and peptide hormones using PeptideDB, www.peptides.be, as a starting point. Augmenting this with UniProt, NCBI, EROP-Moscow (erop.inbi.ras.ru/), PepBank (pepbank.mgh.harvard.edu/) and published review data we have assembled sequence information on the products of over 757 gene loci including over 1633 protein or peptide products, pro-protein sequences, and known proteolytic products of the protein hormones cataloged. These account for roughly 2.4% of the human proteome. All entries have been searched and annotated for known proteolytic cleavage sites and the enzymes responsible. BLASTp (one dimensional, 1D) and LabelHash (three dimensional, 3D) have been used on hormones, hormonal pro-peptides, and known and predicted hormonal proteolytic fragments to identify proteins other than parent proteins or known closely related proteins (peptide matching motif proteins, PMMPs) containing 1D motif matches with E<2, coverage, similarities, and identities exceeding 70%, and 3D motif matches with least root mean square deviation, LRMSD, between structures of <3.2 Å and p<0.01. Motif matches are common with many located on PMMP surfaces. The proteins containing these matched motifs were used as central nodes in network neighborhood searches using STRING, BioGrid, IntAct or DIP. Interactions identified by binding, yeast two-hybrid studies, complex formation, or previous participation in biochemical pathways were considered physically demonstrated. Searches of the Protein Data Base, PDB, for 3D structures of the PMMPs allowed location of the matched residues. 3D structures for complexes of the network neighborhood partners of the PMMPs in combination with the PMMPs were found in a few cases. These locate the matched residues relative to the binding interaction between the PMMP and its network neighbor. Most 3D structures were of the isolated network neighbor proteins and the isolated PMMPs; these will be used with docking software to identify the location of matched residues relative to protein-protein interaction sites. Protein hormone pro-peptides not previously ascribed with a function have been found to contain motifs that match sequential motifs in: hormone receptors of non-related hormones (e.g., GHRelin pro-peptide 52-75 matches K+ voltage-gated channel subfamily E member 4 139-148 which interacts with other channel proteins and protein kinase C); non-related hormones themselves (e.g., VIP pro-peptide 156-170 matches FAM83H 50-62 which helps develop and calcify enamel in part by associating with HSP90AA1 a molecular chaperone); transduction pathway enzymes (e.g., glucagon pro-protein 131-145 matches Abelison tyrosine-protein kinase 2 514-
522 which interacts with oncogenic activators of MAPK, microtubule tracking proteins, and a ras modulator); and transcription-related factors (e.g., GHrelin pro-peptide 99-117 matches DDX39A 240-248 an ATP-dependent RNA helicase that interacts with proteins of the nucleolar protein complex, ubiquitin C, a subunit of the BRCA1-A complex, part of the DNA replication complex, and part of the RNA spliceosome complex). The results suggest that pro-peptides or their proteolytic fragments as well as protein hormone proteolytic fragments perform previously unappreciated modulatory actions in hormone control and feedback systems.

691. L2 Loop Region of Marmoset Follicle Stimulating Hormone β-Subunit Harbors Species Specific Epitope.
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Follicle stimulating hormone (FSH) is required for folliculogenesis in females and maintenance of spermatogenesis in males. Common marmoset (Callithrix jacchus) is a New World primate monkey, used as animal model in the area of reproductive biology. These New World primates are believed to fall between the order Rodentia and Catarrhini primates (apes and humans) in the mammalian phylogenetic tree. Consequently, FSH-FSHR (FSH receptor) system in marmosets might be different than humans. This possibility is supported by the fact that exogenous human FSH is administered in extremely high dosages in these monkeys for superovulation compared to humans and other non-human primates. Furthermore, the animals develop antibodies to the heterologous gonadotropin indicating structural differences between the two proteins. In this study we attempted to understand some of these differences using anti-peptide antibody approach. Based on predicted immunogenicity, surface accessibility and sequence non-homology, peptides corresponding to region 32-50 and 95-111 of human and marmoset FSH β-subunit were synthesized and polyclonal antibodies were generated against the peptides in rabbits. The antibodies were characterised for specificity and sensitivity for the respective peptides and proteins. Interesting results, in terms of degree of cross-reactivity of anti-human FSH β peptide antibody for marmoset FSH and of anti-marmoset FSH β peptide antibody for human FSH, were observed in enzyme-linked immunosorbent assay (ELISA) and Western blotting, indicating structural dissimilarity between human and marmoset FSHβ, especially with respect to the 32-50 region which forms a part of the L2 loop of β-subunit. The binding of the antibodies to cross-species protein was different under reducing and non-reducing conditions. These observations were corroborated by in silico studies on FSH-FSHR models. Apart from providing opportunities for studying marmoset specific FSH- FSHR interactions, marmoset FSH specific antibody could be used to develop a homologous immunoassay for measurement of FSH in marmosets and other New world primates. This research was supported by grants received from the Indian Council of Medical Research (BIC/12(10)/2013) and Department of Biotechnology, Government of India (BT/PR4086/AAQ/1/490/2011).

692. Superparamagnetic iron oxide nanoparticles for delivery of DNA-based contraceptive vaccines for feral cats.
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Overpopulation of feral cats has a negative impact on cat welfare, public health, and the environment. Ablation of pituitary gonadotropes via DNA immunization directed against gonadotropin releasing hormone receptor was proposed by our research group as a method of nonsurgical and nonlethal control of feral cat populations. However, efficient delivery systems are required to ensure potency of DNA-based vaccines and nanocarriers, such as superparamagnetic iron oxide nanoparticles (SPIONs), appear promising in this regard. The objectives of this work were to synthesize SPIONs modified with positively charged amine groups (A-SPIONs), to bind plasmid DNA (pDNA), and evaluate their potential application as model DNA-contraceptive vaccine delivery systems. To generate A-SPIONS, Chemicell starch coated SPION platforms were cross-linked followed by extensive dialysis. Then, the purified product was incubated with ammonium hydroxide and final aminated particles were purified and concentrated using a magnetic separator. Synthesized A-SPIONs were found to have a hydrodynamic diameter and zeta potential of 146.9±4.62 nm and 49.2±12 mV, respectively. DNA was adsorbed on A-SPIONS in a phosphate buffer (pH 7.4) at 1:7 (w/w) ratio. Adsorption of pDNA provided a loading capacity of 140.72 µg per 1 mg of A-SPIONs. Post-DNA loading, A-SPION diameter increased to 199.4±6.41 nm and zeta potential decreased to -30.6±4.96 mV, indicating successful DNA loading on the particles. Transmission electron micrographs (TEM) indicated that particle morphology was preserved post pDNA adsorption. Studies of cumulative in vitro release rate of pDNA from A-SPIONS determined that approximately 2.6% DNA was released over a 30-day period. This low pDNA release rate may be due to formation of irreversible A-SPION aggregates that potentially trapped DNA inside the complexes and, thus, interfered with its release. In order to enhance the pDNA release rate, A-SPIONS were modified with polyethylene glycol (P-SPI0N) to mask some of the surface charge and improve particle stability. The average diameter and zeta potential of P-SPIONs were measured as 146.4±28.19 nm and 19.7±7.22 mV, respectively. The pDNA loading capacity of P-SPION preparation was determined as 329.95 µg per 1 mg of P-SPIONs. Post-DNA adsorption, P-SPION average size and zeta potential changed to 277.6±14.7 nm and -36.2±5.12 mV, respectively. TEM confirmed that morphology of the particles did not change post DNA adsorption. In vitro release rate studies determined that 24.2% of pDNA was released from P-SPIONS within a 30-day period. The higher pDNA release rate from P-SPIONS is possibly due to their feature to not form permanent aggregates, but revert back to homogenous suspensions. MTT colorimetric assay results indicated that exposure of CHO-K1 cells to A-SPIONS or P-SPIONS did not negatively impact viable cell numbers. In conclusion, A- and P-SPIONS were generated and evaluated for their potential use as delivery systems for DNA-based vaccines. Characterization of the SPIONS suggests that P-SPIONS are preferred candidates for pDNA delivery. Future studies on contraceptive potentials of the proposed DNA-based vaccines include evaluation of P-SPIONS for DNA delivery in vivo. This study was funded by the Found Animals Foundation (grant number D1213-F13).

693. Seasonal Expression of Prolactin Receptor in the Scented Gland of Male Muskrat (Ondatra zibethicus).
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Background: Prolactin (PRL) is mainly synthesized and secreted by the lactotrop cells of the pituitary. More than 300 separate actions have been reported in various vertebrates, including effects on growth and development, endocrinology and metabolism, behavior, and reproduction. In males, PRL also could influence reproductive functions. PRL mediates its physiologic functions through the
engagement of prolactin receptor (PRLR). PRLR expression has been reported in a wide variety of cells and tissues. The muskrat (Ondatra zibethicus) is a seasonal breeder with sexually active period of about 8 months from March to October. Increasing daylength of the breeding period is accompanied by increased release of pituitary gonadotrophins and marked testicular recrudescence, leading to enhanced testosterone production, spermatogenesis and pronounced testicular growth. Our previous studies showed that as the target organ of androgens and estrogen, scented glands of the muskrats are capable of synthesizing androgens, estrogens as well as inhibins during the breeding season. Aim: In this study, we investigated PRLR expression and distribution patterns as well as expression profile of miRNAs of the scented gland of muskrats during the breeding and nonbreeding seasons, to gain insight of the relationship of PRL and PRLR with the scented gland function of muskrats. Materials and methods: Twelve adult male muskrats were obtained in January (the nonbreeding season) and April (the breeding season) 2012. Each pair of scented glands and testes was excised from the male muskrats after sacrifice. One side of scented glands and testes were fixed immediately for 12h in Bouin’s solution; the others were immediately stored at -80°C for western blotting and RT-PCR detections. Blood samples were collected and stored at -20°C for hormonal analysis. Results: The scented gland was sliced into half after fixation, and a few musk sinuses and the relatively independent lobes in scented gland tissues were observed. Morphologically, weight and size of the scented glands and testes were both higher in breeding season than non-breeding season. Glandular cells, interstitial cells and epithelial cells of the excretory duct were observed in the scented glands of male muskrats both during the breeding and non-breeding seasons. Glandular cells were the main cell type in the scented glands of male muskrat. During the non-breeding season, epithelial cells were sparser and interstitial cells among glandular cells turned thicker than the breeding season. Immunoreactivity for PRLR was present in glandular and epithelial cells during the breeding season, while only in glandular cells in the nonbreeding season. A PRLR-positive band of 75 kDa was identified in protein extracted from scented gland during the breeding and non-breeding seasons. The intensity of PRLR in the breeding season was significantly higher than the nonbreeding season. Densitometric analysis revealed a significant increase in mRNA level during the breeding season as compared to the nonbreeding season. MiRNAs sequencing in the scented gland revealed seasonal changes in the expression profile of miRNAs. Among the sixteen differential expressions of miRNAs, five miRNAs was significantly down-regulated during the breeding season compared to the non-breeding season, while expression of eleven miRNAs was significantly up-regulated. Some miRNA-targeted gene candidates of these differential expressed miRNAs were enriched in related circadian rhythm and reproduction pathways, such as melanogenesis, MAPK, Notch, NF-kappa B, Wnt, Calcium signaling pathways. The ELISA result showed there was a significant decrease of testosterone concentration from the breeding season to the nonbreeding season. Conclusion: The present study provided new evidences, that muskrat scented gland is the target organ of PRL. Stronger expression of PRLR in scented glands during the breeding season suggested that PRL may be involved in the regulation of seasonal changes in the scented gland function of muskrat.

**TOPIC AREA 7: Education and Research Resources**

**EDUCATION AND RESEARCH RESOURCES: Program Numbers 694–705**

**694. Effect of human uterine leiomyoma in a mouse xenograft model with 2-methoxyestradiol (2-ME).**

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2-Methoxyestradiol (2-ME) is an endogenous metabolite of 17β-estradiol (E2), and has affinity for estrogen receptors. It was reported as a promising antitumor drug due to antiproliferative activity on a wide range of tumor cell types with antiangiogenic actions. It has also been used in a number of preclinical and clinical studies for treatment of solid tumors. Thus, several studies have been conducted to investigate the cytotoxic effect of 2-ME on tumor cell lines in which it induced G2/M cell cycle arrest and subsequent apoptosis. Uterine leiomyomas (fibroids) are the most frequently occurring tumor of the female reproductive tract. This tumor is also influenced by estrogen, which acts as a promoter. The purpose of this study was to examine the anti-proliferative effect of 2-ME in *vitro* and *in vivo*, in a mouse xenograft model using human leiomyosarcoma SK-LMS-1 cell line. To performed *in vitro* experiment, we evaluated anti-proliferative effect of 2-ME on SK-LMS-1 cells using MTT assay, TUNEL assay and western blot. *In vivo* experiment also investigated effect of 2-ME on uterine leiomyoma by measured tumor size and western blot. *In vitro* condition, we confirm that 2-ME at high concentration (10⁻⁵M) and flavopiridol have anti-proliferative influence on SK-LMS-1 cell line, but 10⁻⁷M, 10⁻⁸M dose of 2-ME were not detected, rather these two doses showed little proliferative response through the MTT assay. Also, BAX/Bcl-2 and LC3 expression was increased by the 2-ME (10⁻⁵M)-treatment in western blot analysis. In a previous study, we found that 2-ME has two faces in an *in vitro* and an *in vivo* model. The 2-ME, which is used as a therapeutic agent for solid cancers, has not only apoptotic but also has proliferative effect, depends on its doses. We expect that 2-ME may be a potential therapeutic reagent for human uterine leiomyoma, but the appropriate dose of 2-ME should be used for estrogen-response tumor treatment. This work was supported by the National Research Foundation of Korea (NRF) grant of Korean government (MEST) (No. 2013-010514)

**695. Human placenta cell line (BeWo cells): Effect of EDCs on the expression of placental cation transporter.**

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²Oxygen, carbon dioxide, calcium, copper, iron, and glucose are essential factors in fetal growth. These essential factors are transferred by specific receptors located on cell membrane or cytoplasm in placenta. Cation (ex: calcium, copper, iron, etc.) transfer genes are regulated by estrogen, vitamin D, and human placental lactogen. During pregnancy, expression of specific receptors is controlled by the nutritional status of the mother and fetal. Some synthetic plastics has a structure similar to the endogenous hormone related to steroid hormone or reproducing and contains endocrine disrupting chemicals (EDCs). These substances disturb action of reproduction-related hormones (ex: estrogen, progesterone) by interacting with their receptors, or affecting the expression of transporting genes for cations. We
used a human trophoblast and choriocarcinoma cell line (BeWo cells) to test the impact of EDCs during pregnancy. We used well-known EDCs and applied different doses of octyl-phenol (OP; 10^{-7}, 10^{-6}, and 10^{-5} M), nonyl-phenol (NP; 10^{-7}, 10^{-6}, and 10^{-5} M), and bisphenol A (BPA; 10^{-7}, 10^{-6}, and 10^{-5} M) in BeWo cells for 48 h. Ethynyl estradiol (EE), which activates estrogen receptors, was used as a positive control. EDCs have been known to interfere with the endocrine system through various mechanisms. Treatment with OP, NP, or BPA in a human trophoblast cell line (BeWo cells) affected expression of calcium transporting genes (PMCA1, TRPV6), copper transporting genes (CTR1, ATP7A), and iron transporting genes (IREG1, HEPH) like positive control, EE. When EDCs were treated by concentration, expression of the gene could be determined. To clarify the effects of EDCs mediated by estrogen receptor, ICI 182 780 was treated as an estrogen receptor antagonist. A control group was treated with EE 10^{-7} M, OP, NP, and BPA 10^{-5} M, and an experimental group was treated with addition of EE 10^{-5} M, OP, NP, and BPA 10^{-5} M after treatment with ICI 182 780 10^{-5} M. Expression of the gene was confirmed in the control group and the experimental group. Therefore, we proposed that tested EDCs act as estrogen-like chemicals and regulate essential cation transporters in placenta. This work was supported by the National Research Foundation of Korea (NRF) grant of Korean government (MEST) (No. 2013-010514).

**696. Overproduction of 11β-HSD1 induces the cell death by the dysfunction in energy balance through AMPK signaling.**

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Introduction: 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) is converted from inactive 11-keto form to glucocorticoid (GC or cortisol). Glucocorticoids (GCs) are a steroid hormone found in the body and produced by the adrenal cortex, the outer layer of the adrenal glands. GCs regulate carbohydrate, fat, and protein metabolism. Method: In a previous study, we established porcine fibroblasts overexpressing 11β-HSD1. Based on these, transgenic piglets overexpressing 11β-HSD1 were born without obesity through somatic cell nuclear transfer (SCNT) and re-cloning method, which use somatic cells derived from stillborn TG piglets. Transgenic piglets were identified by PCR methods using specific primers for the targeting cassettes from the genomic DNA of piglets. Result: Six live piglets, one stillborn piglet, and three mummies were born. Integration of target gene into the genomic DNA was confirmed from all of them. However, all six live piglets died within one month, and showed the hypoglycemia. Excessive expression of 11β-HSD1 in metabolic tissues induced up-regulation of gluconeogenesis related genes (G6PT, G6Pase, PEPCk, HNF4a, FOXO1) in liver and kidney, and up-regulation of lipogenesis related genes (SREBP1c, FASN, DGAT, ACC, SCD) in muscle. To compensate for energy loss by anabolism, it stimulates AMPK and SIRT signaling, which controls energy balance and mitochondrial biogenesis. Conclusion: We proposed that the constitutive expression of 11β-HSD1 might cause continuous activation of complementary energy gaining processes and these problems would lead to development of more fatal diseases causing death even in piglets.

**697. Examination of the role of ghrelin in the regulation of TPH1 and AA-NAT protein expression in the ovine pineal gland.**

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Ghrelin (GHRL) is one of the key peptides which participate in circadian ingestion rhythm. In vivo and in vitro studies confirmed that GHRL also adjusts annual secretion of melatonin (MEL) from pineal glands in seasonally polyestrous ewes. To investigate the mechanism via which GHRL regulated the secretion of MEL we determined the effects of GHRL on the level of tryptophan hydroxylase 1 (TPH1) and arylalkylamine N-acetyltransferase (AA-NAT) protein expression measured in the pineal gland (PG) explants. Glands were incubated in medium containing either 0 or 10 ng/ml GHRL. The PG explants were harvested every 60-min, frozen in liquid nitrogen and stored at -70°C until ELISA for TPH1 and AA-NAT. Samples of medium were stored at -20°C until RIA for MEL. Treatments of GHRL decreased (P<0.05) TPH1 concentrations in the cultures of the PG explants collected in SD and LD compared with controls. There was no effect of GHRL on AA-NAT protein expression during LD. However, the treatment with GHRL resulted in lower (P<0.05) AA-NAT protein expression during LD and SD seasons. Research supported by NCN 2013/05/B/NZ4/02408.

**698. Differences in the Hypothalamic Content of Kisspeptin and GnRH During the Breeding and Non Breeding Season (NBS) in Sheep.**

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The breeding season (BS) is characterized by a higher frequency of GnRH pulses compared with NBS in sheep. We hypothesized that the higher frequency of GnRH pulses is associated with a higher content of kisspeptin (kp) in the hypothalamus during the BS, reflecting an increase in kp input to the preoptic area (POA) and mediobasal hypothalamus (MBH). Ovx ewes were administered estradiol (E2) via implants, to achieve luteal phase levels (2-3 pg/ml). Ewes were euthanized during BS (n=4) and NBS (n=2). Forebrains were collected, sagittally sectioned, and POA, anterior hypothalamic area (AHA), MBH and median eminence (ME) were isolated. Control sections adjacent to the regions of interest (POA and MBH), as well as cortex, cerebellum and brain stem were also processed. Peptides were extracted from tissue sections with acidified methanol and the concentrations of kp and GnRH quantified by RIA. Independent of season, concentrations of kp and GnRH were higher in the POA, AHA and MBH compared to cortex, cerebellum and brain stem (P<0.05). Hypothalamic concentrations of kp and GnRH in regions adjacent to the POA and MBH were not affected by season. Concentrations of kp and GnRH were higher in the POA and MBH when compared to adjacent hypothalamic regions during the BS but not during the NBS (P<0.05). The lowest concentrations of kp and GnRH were detected in the cortex, brain stem and cerebellum. GnRH concentrations were similar between seasons in the POA, AHA, MBH and ME but when the POA, AHA, MBH were analyzed as a pool, the total content of
GnRH was higher during BS than NBS (P<0.05). We have confirmed our hypothesis since, kp concentrations were higher during the BS compared with the NBS in the POA and MBH (P<0.05), but not in the AHA and ME. Regardless of the season, a positive correlation between kp and GnRH was found in the POA (r=0.83 P<0.05) and MBH (r=0.82 P<0.05), but not in other hypothalamic regions. We are processing additional samples to increase the number of animals in both the BS and NBS. The RIA appears to be a valuable tool for quantifying small changes in the hypothalamic content of kp. These studies were founded by the Colorado State University Agricultural Experimental Station and National Institute of Health, grant 5R01HD065943-05 to CMC.

699. Simulator-based training in equine gynecology - animal welfare versus learning outcome.
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Transrectal palpation and ultrasonography of the mares’ genital tract are first-day-skills for equine veterinarians and students should be able to acquire practical skills in equine gynecology during their curriculum. However, animal welfare and ethical considerations increasingly question the use of live animals irrespective of species for teaching purposes. Recently, simulators for equine gynecology teaching have become available. Before animal-based teaching, at least in part, is replaced by simulator training, it needs to be determined to what extent learning objectives are reached with simulator-based teaching. To compare the learning outcome of different teaching protocols for transrectal palpation of the mares’ genital tract, third and fourth year veterinary students were either trained once on horses (H1), 4 times on horses (H4) or 4 times on a simulator (SIM). One and 14 days after the last training session, the students’ acquired skills in performing a gynaecological examination were tested on live horses. In addition, the time needed for the examination was recorded and the students answered a self-assessment questionnaire after each test. In group-H4 students the skills of rectal palpation increased continuously from training session 1 to 4 (p<0.05) whereas the learning curve of SIM students on the simulator reached a plateau already during the third simulator training (p<0.001 over time). At test days on which all students examined live horses, H4 students scored (maximum score: 10) significantly better (score: 9.2±0.2) than SIM students (7.9±0.6; p<0.05). But SIM students scored better than H1 students (7.1±0.5). H4 students also needed the shortest time for completing the examination (Test 1: 170±15 sec) versus groups SIM (251±17 sec) and H1 (266±14 sec; p<0.001). Skills of group SIM student during test 1 were comparable to skills of group H4 students during their fourth training session. Students of all groups reached better results of palpating the left versus the right ovary (p<0.001) but H1 students were least successful in obtaining correct ovarian findings (p<0.05 versus H4 and SIM). Students’ self-assessment after the tests reflected test results with palpation of the right ovary more frequently for H1 students (p<0.01 versus H4 and SIM). Although the gynaecological examination of mares belongs to first-day-skills in equine practice it is a challenging task for veterinary students. For an acceptable learning outcome repeated training is required and a single training session is no sufficient veterinary education. Repeated simulator-based training is a useful tool to prepare veterinary students for transrectal palpation of the genital tract in horse mares. After three simulator-based training sessions students were able to transfer their acquired skills to the examination on live horses and performed on the same level as students after repeated training on horses. In conclusion, supplanting animal-based by simulator-based teaching in equine reproduction allows a reduction in the number of training sessions with live animals without compromising learning outcomes. Supported by KELDAT grant, an initiative of Volkswagen Stiftung and Mecator Stiftung.

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Follicular fluid (FF) provides part of the micro-environment that regulates oocyte development and may play a critical role in oocyte fertilization and embryo development. Intercellular signaling between granulosa luteinized cells (GLCs), cumulus cells (CM), and the oocyte is required for proper folliculogenesis, ovulation, and hormonal secretion. Exosomes are small (typically 50-150nm) membranous vesicles that contain a unique repertoire of proteins and non-coding RNAs (ncRNAs) and are found in body fluids and tissues. We hypothesize that exosomes mediate intercellular signaling within ovarian follicles. Our aim was to optimize existing techniques to isolate and characterize both human FF- and serum-derived exosomes, as well as exosomes secreted by GLCs in vitro, using a variety of techniques. We also optimized the process for a clinically applicable set-up and high-throughput process. This study was approved by the University of Toronto Research Ethics board. Samples were obtained from 30 consenting in vitro fertilization patients, FF and GLC from individual mature-sized follicles (≥18mm) from the left and right ovaries were collected, isolated, and stored at -80°C. Serum was obtained from consenting patients on the day of oocyte retrieval. Exosomes were isolated from FF, serum, and GLC conditioned media using ultracentrifugation (UC) and commercial exosome enrichment reagents. This was followed by immunoprecipitation with magnetic or latex beads targeting known exosome-specific markers CD9, CD63 and CD81. Protein analyses using western blotting and liquid chromatography–tandem mass spectrometry (LC-MS/MS), were conducted to evaluate and compare the exosomal contents of the samples. The enriched exosome samples were further analyzed using nanoparticle tracking analysis with CD9 labeling, transmission electron microscopy (TEM), scanning electron microscopy (SEM), fluorescence microscopy (FM), and immunogold labeling. We present a comparison between the different techniques and confirm the presence of CD9-positive exosomes in FF and serum, as well as their release by GLCs in culture. In conclusion, human FF and serum contain exosomes, some of which may originate from GLCs. UC, the gold-standard exosome isolation method, and various commercial kits have all shown similar results and are suitable for studying exosomes. However, it is recommended to use magnetic bead-based immunoprecipitation to isolate exosomes for downstream proteomic analyses.

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We use an extensive vocabulary of words and symbols everyday that allow us to communicate about objects and events in the world around us. Similarly, scientific discourse relies on our using the same words and symbols to describe the same objects. Without this common framework, our ability to learn and build upon existing knowledge is severely compromised.

The standardization of terminology, including nomenclature for genes, mutants, and strains is critical for integrating biological data and for supporting effective comparative analyses across disciplines and genomes. In this era of big data, our attention to uniquely and correctly identifying the objects studied is evermore important as bioinformatics analyses and data aggregation efforts cannot produce meaningful results without it. Discipline-specific jargon and local laboratory jargon are becoming anachronistic. The international authoritative resources for nomenclature reside largely with the species’ major database resources. Some examples include: for human (Human Gene Nomenclature Committee, HGNC, www.genenames.org), for mouse (Mouse Genome Database, MGI, www.informatics.jax.org), for rat (Rat Genome Database, RGD, http://rgd.mcw.edu), for zebrafish (Zebrafish Model Organism Database, ZFIN, http://zfindb.org), for birds (Chicken Gene Nomenclature Consortium, http://birdgenenames.org). Species with small research bases, particularly species whose main “data” are from whole genome sequencing, generally adopt nomenclature from a closely related species (e.g. ZFIN for other fish species).

For mammals, human, mouse, and rat strive to have parallel naming conventions and co-name orthologous genes to make translations between species as simple as possible, although this is not always possible, for example, where a gene family expansion has occurred in one of the species. Not using standard nomenclature in a publication makes your data less accessible to searches, and therefore less impactful. This, in turn, can have downstream results including fewer citations, diminished grant success, etc. Moreover, using incorrect nomenclature can cause improper inference and interpretation of data, at an extreme with misattributing results to the wrong gene or mutation, for example. The Mouse Genome Informatics (MGI, www.informatics.jax.org) resource maintains the international authoritative resource for the identity and names of mouse genes, genetic markers and genome features, mutations and alleles, and strains. Each of these objects in MGI has a unique ID, symbol, name, and an associated curated set of synonyms (including published alternate names and previous official names). MGI implements policies developed by the International Committee on Standardized Genetic Nomenclature for Mice. Guidelines are available at www.informatics.jax.org/nomen. Authors can contact MGI nomenclature staff at nomen@jax.org to reserve new nomenclature pre-publication or for help with determining correct nomenclature. Acknowledgement: The authors thank John J. Eppig for the catchy title. Supported by NIH grant HG000330.

702. Sex hormone levels in rats – A preliminary meta-analysis of the literature.
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Rats are commonly used as models in reproductive physiopathology and toxicology. Yet, there are no available official normal ranges for sex hormones in rats that would allow to strengthen data interpretation or compare studies. Our goal therefore was to compile baseline and negative control levels of three major sex hormones—testosterone, progesterone, and estradiol—across the biomedical literature to generate some normal ranges based on various factors: sex, age, breed, diet, quantization method and kit type. A systematic search for related literature using the terms “sex hormone levels,” “rats,” “serum,” “plasma,” “testosterone,” “progesterone,” and “estradiol” was performed through Pubmed database in order to collect baseline and negative control values for hormone concentrations. The compiled data were then statistically analyzed through various finite mixture models, which were modified to accommodate the wide variability found in the literature. All sexes and their respective hormone levels showed bimodal distributions, with the exception of unimodal female and male testosterone. Additionally, ANOVAs for kit type (radioimmunoassay, enzyme-linked immunoassay, etc.) were all statistically significant except for a marginal (0.05 < P < 0.07) female testosterone p-value of 0.0615, providing statistical support for the bimodal distribution. The multimodality of the distributions show that the kit type is likely to have a significant effect on measurements for all three hormones. Following hormone levels up to PND 70 also reveals a fit similar to the bottom half of a logistic curve in both females and males. Multiple reference interval analyses run on one of the data sets, female testosterone, identified several suspect/outlier data points; therefore, greater insight needs to be taken into examining the quality of data collected from the references. A few criteria that have already been established as a “scale” for evaluating the quality of references include excluding studies with coefficient of variation greater than 50% of reported values. However, additional criteria still need to be agreed upon. Moreover, our literature review and data compilation is on-going to strengthen statistical power, and for further factorial analysis into potential subgroups such as breed.

703. Molecular Membrane Modeling of Sperm Capacitation and Acrosome Reaction in Mammalian Spermatozoa: Education and Research Resource to Visualize the Biochemistry of Lipid Metabolism in Health and Disease.
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A molecular membrane model of proteins and lipids drawn at an atomistic level on a computer software application, illustrates the sperm plasmalemma membrane overlying the outer acrosomal membrane of mammalian spermatozoa and allows visualization of various pathways in lipid metabolism experienced by the spermatozoon in its quest of an ovum. The Molecular Membrane Modeling of Sperm Capacitation and Acrosome Reaction in Mammalian Spermatozoa (the “Model”) is presented as an Education and Research Resource to visualize, and to introduce students and researchers to, the biochemistry of lipids acting as structural and functional components of biological membranes and also acting as powerful mediators of signal transduction and cell fusion events in physiological phenomena of sperm-egg interactions, including docking of spermatozoa to the zona pellucida, sperm capacitation, hyperactivation of sperm motility, exocytosis of the acrosome reaction, sperm-egg fusion and embryo implantation. The Model also illustrates how similar pathways in lipid metabolism leading to the genesis of life, are also involved, paradoxically, in pathological pathways leading to immunosuppression, cardiovascular disease, neurological disorders and cancer. The present Model is derived from "A Molecular Membrane Model of Sperm Capacitation and the Acrosome Reaction of Mammalian Spermatozoa", published by the author in Gamete Research 12:183-224 (1985), depicting a lipid bilayer assembly of space-filling molecular models of sterols and phospholipids in dynamic equilibrium with peripheral and integral membrane proteins, drawn at an atomistic level, for the comprehensive visualization of the biochemical events involved in sperm capacitation and the acrosome reaction. The present Model is an upgraded and animated computerized version of the earlier
molecular membrane model, and now incorporates all species of lipids at the atomistic level (including without limitation sphingomyelin, phosphatidylserine, phosphatidylinositol, cardiolipin and plasmalogens, and their metabolites, such as platelet-activating factor). The Model aims to integrate experimental results reported in the scientific literature during the last thirty years on the subject matter in various mammalian species as well as to illustrate current and novel concepts underlying sperm-egg interactions, capacitation and acrosome reaction. The projected evolution of the Model as an Education and Research Resource ultimately aims for a virtual and visual animated molecular atlas presenting essential topics in lipidology as they relate to fertilization in mammals, such as: the structure and function of biological membranes in gametes; cholesterol transport mediated by lipoproteins in reproductive fluids; alteration of the cholesterol/phospholipid ratio and lysophospholipid accumulation as factors conducive to exocytosis (acrosome reaction); zona pellucida components linking to sperm receptors on cholesterol-sphingolipid rich lipid rafts anchoring the male gamete in close vicinity to the zona pellucida; signal transduction leading to modulation of ion channels, protein phosphorylation, G-protein coupled ion transport and activation of calcium channels, as such signal transduction may be initiated by oviductal and follicular fluid components (steroid hormones, peptides, etc.) accompanying the ovum following ovulation as well as by cumulus cells and by the zona pellucida anchoring itself onto sperm membrane receptors embedded on lipid rafts, in synchronicity with the release of acrosin and other acrosomal proteolytic enzymes in close vicinity to the zona pellucida. Correspondence may be addressed by mail to Jean Langlais, Scientific Director, Institute of Molecular Membrane Modeling of Montreal, 5-2610 Moreau St., Montreal (Quebec) Canada H1W 2M8 or by email to jean.langlais.immmm@gmail.com.

704. Opportunities to Diversity the Reproductive Biology Workforce: All Hands On Deck.
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The number of PhDs in the life sciences, including reproductive biology, awarded to African-Americans and other underrepresented minorities in the United States remains low, despite efforts over past decades to occasion a more positive outcome. Because of the Tuskegee University’s demonstrated ability to successfully recruit, train and effect the career placement of minority scientists, it has been recognized as the top producers of African American graduates who receive degrees in agricultural sciences as well as veterinary medicine, according to Diverse Issues in Higher Education. Based on this success as well as that of the Tuskegee University School of Veterinary Medicine as the producer of over 70% of African American veterinarians in the United States, numerous pre-college underrepresented minorities gravitate to Tuskegee University to take part in a variety of outreach programs geared toward broadening their awareness of career and educational opportunities in veterinary medicine and animal sciences. The current project takes advantage of these programs by incorporating hands-on activities related to the field of animal sciences/veterinary medicine with an emphasis on reproductive biology in the form of wet labs that focus on male reproductive anatomy, female reproductive anatomy (open and gravid), fetal development, and neurology associated with reproductive function. Undergraduate and high school students participated wet labs as described above and were polled to determine their increased awareness of educational and career opportunities associated with animal reproductive biology. Students showed gains in their general knowledge of reproductive biology and indicated that the exercises increased their awareness of educational and career opportunities in the field. In the future, additional activities in reproductive biology will be incorporated events that allow hands on opportunities and participants indicated the desire for additional information and opportunities in the field will receive follow up information accordingly.

705. Gene Ontology and Pathway Analysis of Spontaneous Endometriosis in a Rhesus Macaque Model.
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The Caribbean Primate Research Center (CPRC) in Puerto Rico is a unique center recognized nationally and internationally for studying non-human primates (NHPs). Humans and NHPs share many common characteristics in their physiology, behavior, and genetics; with the rhesus macaques (Macaca mulatta) being the most widely used NHP for experimental studies. The primary goal in this study was to establish the rhesus macaque as an animal model for the study of endometriosis. Endometriosis affects 1 in 10 women of reproductive age and is associated with chronic pelvic pain and infertility. Presently, there is no cure, and the available treatments including hormones and surgery only suppress the disease to a limited extent. The rhesus macaque is an appropriate model since similar to women, they menstruate and develop spontaneous endometriosis. The purpose of this study was to analyze endometriat gene expression and identify altered molecular pathways in female monkeys who had a family history of endometriosis and as a consequence developed spontaneous disease, and compare the expression patterns to healthy, disease-free controls. We used the Affymetrix Rhesus Macaque Genome Chip for transcriptome analysis of endometriotic lesions (n=3) and for comparing the gene expression pattern in the eutopic endometrium of animals with (n=3) and without disease (n=2). Our analysis identified 2,508 annotated genes that showed significant differences in transcript expression (p<0.05 with FDR<0.07) in endometriotic lesions compared to the endometrium from controls. Further classification of these genes using the Ingenuity Pathway Analysis Program revealed the disease pathways that were most significantly represented, including cancer (1,404 genes), gastrointestinal disease (655 genes), infectious diseases (264 genes), organismal injury and abnormalities (784 genes), and reproductive system diseases (642 genes). Some of the top upstream regulators identified were MAPK1, PTEN and TP53. Lesions from the rhesus macaques showed dysregulated expression of ARID1A, BRCAl, CCL2, ESRI, LPAR5, and OR10W1, in support of the recent evidence for an association of endometriosis and ovarian cancer. Gene ontology classification of biological function identified clusters that were over-represented in endometriosis, including gene expression, cellular growth and proliferation, cell death and survival, cellular development and RNA post-transcriptional modification. Given the close phylogenetic relationship between NHP and humans these types of analyses could potentially translate into new ways to diagnose and treat this disease in women. Furthermore, the Caribbean Primate Research Center will be able to provide access to one of the few non-human primate models that are used worldwide to study endometriosis.