Oregon State University's Department of Biomedical Sciences in the College of Veterinary Medicine,

Department of Animal and Rangeland Sciences in the College of Agricultural Sciences, and

The Center for Reproductive Biology at Washington State University present:

Northwest Reproductive Sciences Symposium 2016

Thursday June 2nd and **Friday June 3rd** at the **Philomath Scout Lodge**

660 Clemens Mill Road, Philomath, Oregon

2016 NWRSS Schedule

Thursday, June 2, 2016 **Great Hall, Philomath Scout Lodge**

10:30 AM	Registration opens and continues all day
11:30 AM-12:45 PM	**Lunch**
1:00 PM-1:15 PM	Welcome

Session 1: Female Reproduction- Uterine Function **Chair: Fred Stormshak**

1:15 PM – 1:45 PM	Keynote Speaker: **George Haluska**, Founding Father of the NWRSS
1:45 PM – 2:15 PM	**James Pru**, Washington State University, "TCF3 and TCF12 are essential for female fertility and bestow non-erythroid hemoglobin biosynthetic activity on the gravid uterus"
2:15 PM – 2:45 PM	**Hernan Montilla**, Oregon State University, "Diestrual intrauterine peanut oil infusion effect on the mare estrous cycle"
2:45 PM – 3:15 PM	**Wipawee Winuthayanon**, Washington State University, "Estrogen receptor α is required for the embryo transport function of the oviduct"
3:15 PM – 3:30 PM	**Shuai Li**, Washington State University, "Kallikreins in mouse uterus: the final essential step for semen liquefaction?"
3:30 PM – 3:45 PM	**Break**

Session 2: Mechanisms of Cellular Development in the Reproductive Axis **Chair: TBD**

3:45 PM – 4:15 PM	**Jon Oatley**, Washington State University, "Determinants of the stem cell state in mammalian spermatogonia"
4:15 PM – 4:30 PM	**Estela Jauregui**, Washington State University, "Leydig cell gene expression changes with the cycle of the seminiferous epithelium in the mouse"
4:30 PM – 4:45 PM	**Tessa Lord**, Washington State University, "Development of a high throughput siRNA screen to identify novel transcription factors involved in spermatogonial stem cell renewal"
4:45 PM – 5:15 PM	**Shawn Chavez**, Oregon National Primate Research Center, "Investigating the potential causes and consequences of chromosomal instability during primate pre-implantation development"
5:15 PM – 6:30 PM	**Poster Session**

6:30 PM – 8:00 PM **Dinner** **Great Hall, Philomath Scout Lodge**

8:00 PM – 11:00 PM **Social**

Friday, June 3, 2016 **Great Hall, Philomath Scout Lodge**

8:30 AM **Continental Breakfast**

Session 3: Gonadotropins- Synthesis, Secretion, and Signaling **Chair: TBD**

9:00 AM – 9:30 AM **Cecily Bishop**, Oregon National Primate Research Center, "Effects of CG and VEGF on vascular function of the primate ovary"

9:30 AM – 10:00 AM **Henryk Urbanski**, Oregon National Primate Research Center, "Neuroendocrine control of ovulation in primates: a role for two distinct populations of GnRH neurons?"

10:00 AM – 10:30 AM **Michelle Kutzle**r, Oregon State University, "Investigating the role of luteinizing hormone in non-reproductive tissues in dogs"

10:30 AM – 10:45 AM **Khawla Zwida**, Oregon State University, "Canine hemangiosarcoma expresses luteinizing hormone (LH) receptors"

10:45 AM – 11:00 AM **Break**

Session 4: Alterations in Steroid Hormone Signaling by Exogenous Factors **Chair: TBD**

11:00 AM – 11:15 AM **Mary Meaker-Smallman**, Oregon State University, "Impact of RU486 on phosphatidylinositol hydrolysis in the ovine corpus luteum"

11:15 AM – 11:30 AM **Tegan Horan**, Washington State University, "The kids are (not) alright: multigenerational estrogen exposure disrupts meiosis and reproductive tract morphology in male mice"

11:30 AM – 11:45 AM **Dakota Jacobs**, Oregon State University, "*Kiss1* expression is modulated by estrogen and endocrine disruptors in immortalized female AVPV- and arcuate-specific neuronal kisspeptin cell lines"

11:45 AM – 12:00 PM **Rebecca Veitch**, Oregon State University, "Circadian dysregulation is associated with a loss of estrogen receptor expression in mammary tissue: implications for an etiology of breast cancer"

12:00 PM – 1:30 PM **Lunch/Poster Session**

Session 5: Effects of Nutritional interventions on Reproduction **Chair: TBD**

1:30 PM – 2:00 PM **Massimo Bionaz**, Oregon State University, "Nutrigenomic intervention to prevent mastitis in dairy animals"

2:00 PM – 2:15 PM **Meredith Kelleher**, Oregon National Primate Research Center, "Maternal creatine supplementation for protection against perinatal brain injury in the non-human primate"

2:15 PM – 2:30 PM **Alexandria Snider**, Oregon State University, "Effects of OmniGen-AF© on superovulation response and embryo quality in donor beef cows"

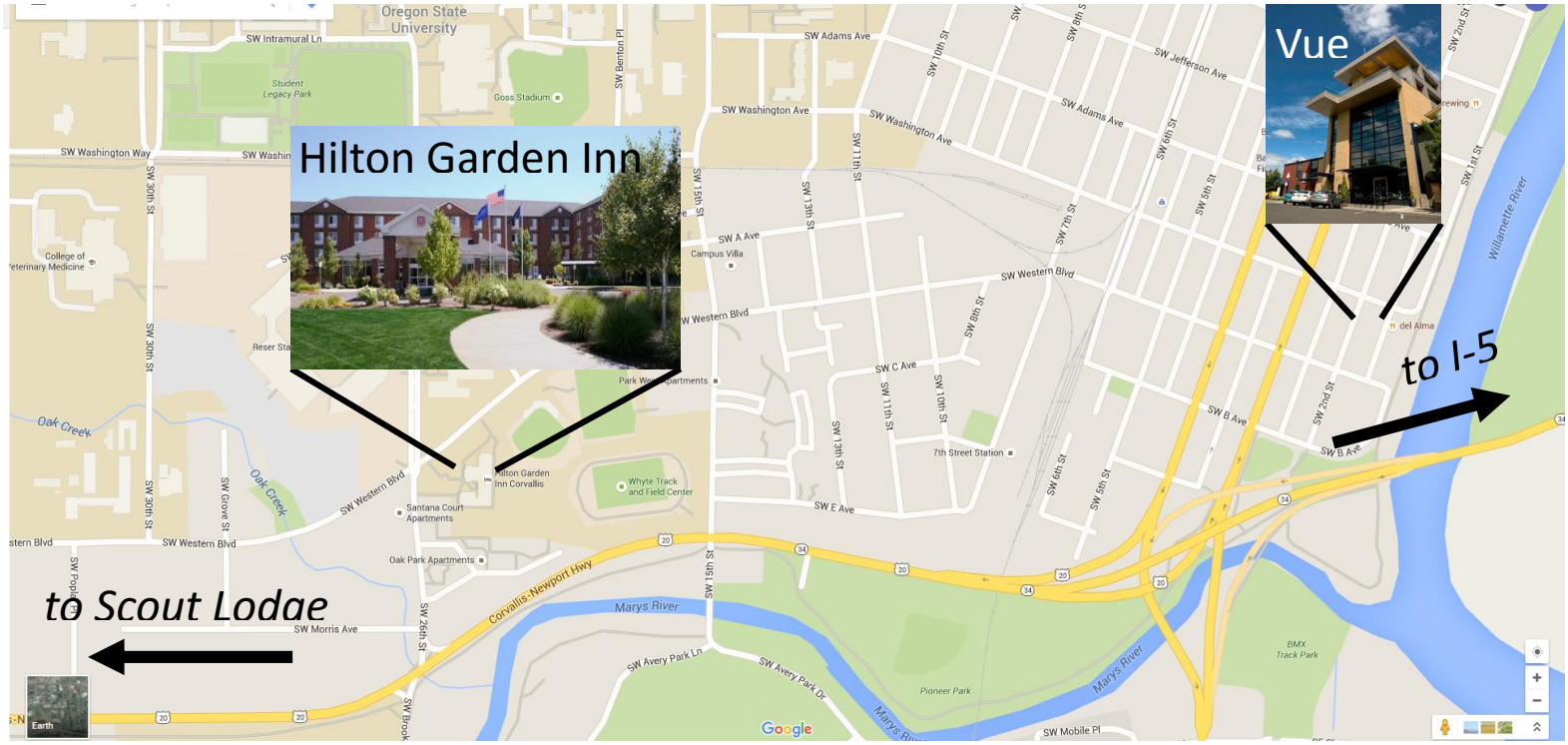
2:30 PM – 2:45 PM **Fernanda Trinidade da Rosa**, Oregon State University, "Percentage of milk fat, lactose, and protein is affected by diurnal variation in dairy goats"

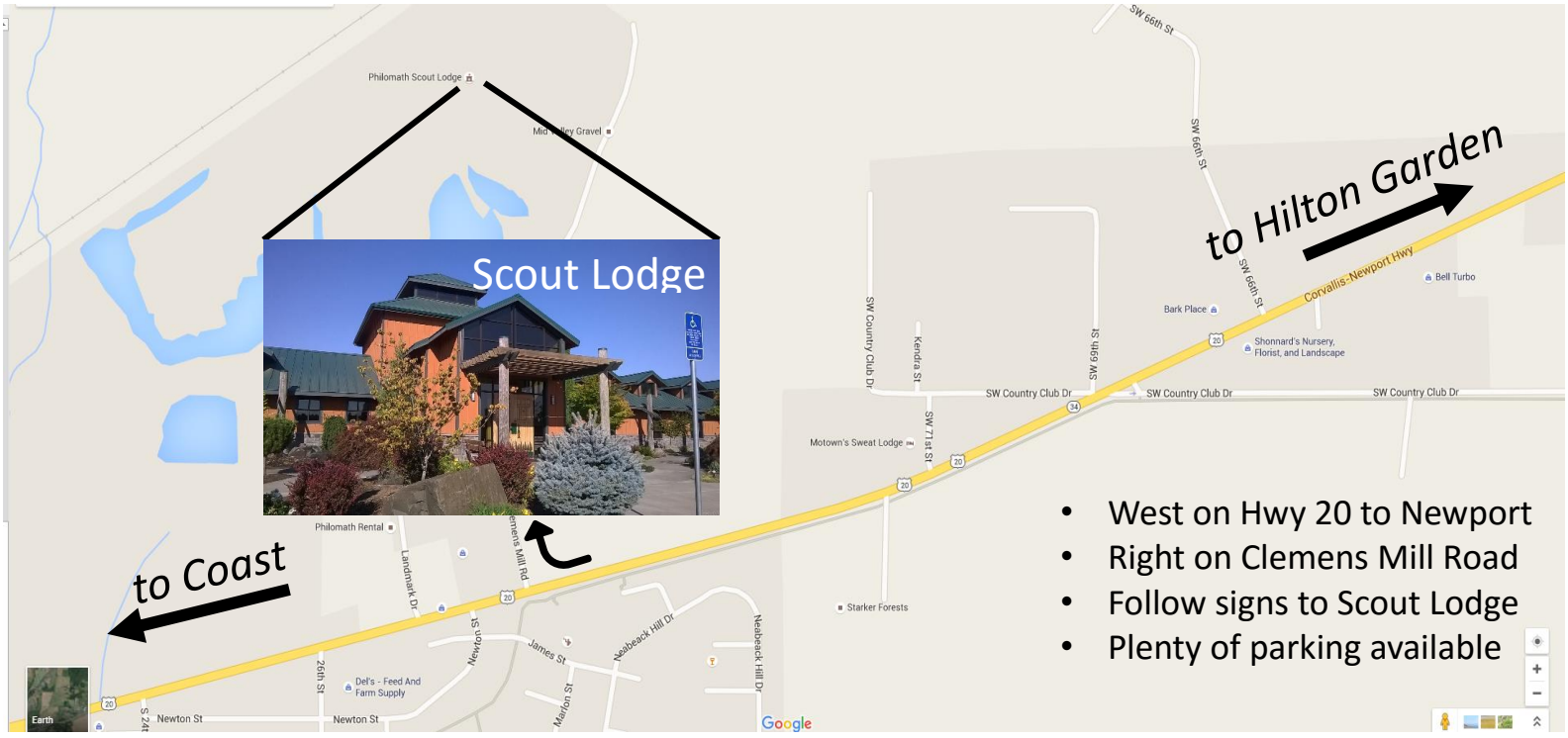
2:45 PM – 3:00 PM **Cadence True**, Oregon National Primate Research Center, "Pubertal exposure to western style diet and androgens does not alter reproductive neuroendocrine sensitivities"

3:30 PM – 6:30 PM **Hiking (with wine & cheese) at Mary's Peak *(RSVP required)***

7:00 PM – 10:00 PM **Dinner/live music** **The Vue (517 SW 2nd St. in downtown Corvallis)**

MAPS





- West on Hwy 20 to Newport
- Right on Clemens Mill Road
- Follow signs to Scout Lodge
- Plenty of parking available

Featured Speakers:

Session I: Female Reproduction- Uterine Function

TCF3 and TCF12 are essential for female fertility and bestow non-erythroid hemoglobin biosynthetic activity on the gravid uterus

James K. Pru, Cindy A. Pru, Brooke K. Compton, Michele C.L. Reinelt, Nicole C. Clark, Andrea R. Smith, Jon M. Oatley, Michele Chan

[1]Department of Animal Sciences and [2]School Molecular Biosciences, Center for Reproductive Biology, Washington State University, Pullman, WA 99164

Members of the E-protein helix-loop-helix family of transcription factors regulate developmental processes in vertebrates such as myogenesis, neurogenesis, pancreatic development and lymphopoiesis. E-proteins encoded by *Tcf3* and *Tcf12* form homodimers, as well as heterodimers with each other and other E-proteins. Given the role of these proteins in regulating differentiation, the objectives of this study were to: 1) evaluate expression of TCF3/12 in female reproductive tissues by qPCR and immunohistochemistry (IHC); 2) determine if TCF3/12 are required for female reproduction with particular focus on uterine decidualization; and 3) begin to identify and evaluate TCF3/12 target genes during pregnancy. In the endometrium, TCF3/12 transitioned from cytoplasmic/nuclear staining to exclusively nuclear staining in murine uterine stromal cells undergoing decidualization. Importantly, TCF3/12 expression was dynamically regulated in response to gonadotropins (ovary) and steroid hormones (uterus). To study the function of *Tcf3* and *Tcf12* in female fertility, double conditional knockout (dcKO) mice were generated by crossing with *Pgr-cre* mice. Control (*Pgr*$^{+/+}$*;Tcf3*$^{fl/fl}$*;Tcf12*$^{fl/fl}$) female mice gave birth to an expected numbers of pups while dcKO (*Pgr*$^{cre/+}$*;Tcf3*$^{fl/fl}$*;Tcf12*$^{fl/fl}$, n=5) female mice failed to deliver any pups over a 5 month period. Similar results were obtained when using *Amhr2-cre* driver mice. A detailed evaluation of TCF3/12 function in the pituitary and ovaries revealed that these transcription factors are necessary for LH secretion, but they are dispensable for ovarian functions (*i.e.*, folliculogenesis and ovulation). Unlike control mice that responded expectedly to artificial decidualization following ovariectomy and steroid hormone supplementation, the same protocol resulted in faulty decidualization in dcKO mice in which uterine wet weight was decreased ($p<0.05$) and stromal tissue failed to completely decidualize. RNA-seq analysis determined that conditional ablation of *Tcf3/12* from the uterus resulted in differential expression (>1.5-fold) of 474 genes (135 down-regulated and 335 up-regulated genes) in dcKO uterine tissue on day 3.5 of pseudopregnancy. Among the down-regulated genes, those associated with hemoglobin biosynthesis were most abundant and most differentially regulated. These include the hemoglobin *alpha* and *beta* genes, as well as small ribosomal RNAs linked to various anemias, heme metabolizing enzymes, and haptoglobin .These RNA-seq data were validated by some combination of qPCR, western blotting and IHC. Ongoing studies center on determining if the infertility phenotype and lack of a decidualization response stem from failure to generate non-erythroid hemoglobin and thus adequately regulate local oxygen concentration. These cumulative findings indicate that TCF3/12 are necessary for both pituitary and uterine physiology. The TCF3/12 transcription factors appear to convey a hematopoietic-like function to the endometrium during the earliest stages of pregnancy through up-regulation of genes necessary for *de novo* hemoglobin biosynthesis.

Estrogen receptor α is required for the embryo transport function of the oviduct

Wipawee Winuthayanon

School of Molecular Biosciences, College of Veterinary Medicine, Washington State University, Pullman, WA 99164

Ectopic pregnancy, a condition where embryos implant and grow outside the uterus, is a leading cause of maternal death during the first trimester. The highest risk factor for ectopic pregnancy is defective fallopian tubes that cause a disruption of the embryo transport to the uterus. The previous findings indicate that female steroid hormones, especially estrogen, are required for the transport process by regulating the function of the cilia in the oviduct. Estrogens act through estrogen receptor alpha and beta (ESR1 and ESR2). Our preliminary findings indicated that ESR1 in the oviductal epithelial cells is required for normal embryo transport function (*n*=4-5 mice/genotype). Therefore, we hypothesized that ESR1 in the oviductal epithelium is necessary for the ciliated cell function in the oviduct during embryo transport. To determine the molecular mechanism of ESR1 during embryo transport, we assessed the ciliary beating frequency and the genomic transcripts in the presence or absence of the ESR1 in the oviductal epithelium. Our result indicated that female mice lacking epithelial ESR1 had significantly reduced ciliary beating frequency compared to control littermates (*n*=3 mice/genotype). Moreover, microarray analysis of the oviducts showed an aberrant expression of transcripts in WNT/β-catenin signaling pathway in the absence of epithelial ESR1 (*n*=4 mice/genotype). WNT/β-catenin signal is one of the pathways involved in the motile ciliary function in the respiratory tract. Therefore, we further evaluated whether WNT/β-catenin is functionally required for the ciliary motility in the oviduct during embryo transport. We generated a conditional deletion mouse line of β-catenin in oviductal ciliated cells, however, the deletion did not affect the embryo transport in mice (*n*=5 mice/group). In conclusion, we report that ESR1 is required for the embryo transport function of the oviduct, potentially due to a defective ciliary motility. Our findings could improve understanding of the cause of ectopic pregnancy. However, the downstream mechanisms of estrogen regulating the cilia function during embryo transport require further investigation and are set to be tested in our future studies.

Financial support: Faculty Seed Grant, Start-up fund from WSU College of Veterinary Medicine, and National Institute of Environmental Health Sciences Intramural Research Program.

Kallikreins in Mouse Uterus: The Final Essential Step for Semen Liquefaction?

Shuai Li *and Wipawee Winuthayanon*

School of Molecular Biosciences, College of Veterinary Medicine, Washington State University, Pullman, WA 99164

Back ground: Semen hyperviscosity (non-liquefied semen) contributes to 11.8 to 32.3% of male infertility. The liquefaction of seminal coagulation liberates sperm and increases their mobility. Liquefaction is thought to be modulated by the proteolytic activity of the kallikreins (KLKs), a serine protease family secreted from seminal vesicles and prostates.
Objective: In this study we hypothesized that KLKs derived from the uterus are functionally required for semen liquefaction and female reproduction. We reported previously that conditional ablation of *Esr1* (encoding estrogen receptor α) from mouse uterine epithelium (*Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$) causes infertility due in part to a dramatic reduction in the number of sperm entering the oviduct.
Methods: WT and *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ female mice reproductive tracks were studied after mating with healthy fertile male. Histological analysis and microarray analysis were performed to test physiological abnormalities and gene expression pattern of the tissues.
Results: At 0.5 days post coitus (dpc), a completely solidified semen was observed in the absence of epithelial ESR1 in the uterus compared to a watery liquefied semen in control uteri (*n*=4-5 mice/genotype). Moreover, *Klk1b5* and *Klk1* transcripts were significantly less in *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ compared to control littermates at 0.5 dpc (*n*=4-7 mice/genotype). In addition, the collagen (one of the KLK substrates in the semen) content was detected at a higher level in the lumen of *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ compared to controls at 0.5 dpc (*n*=4-5 mice/genotype). Although matrix metalloproteinases (MMPs) in the uterus could potentially contribute to semen liquefaction in female reproductive tract, we found that this is unlikely as *Mmp2* was expressed at significantly higher level and *Mmp9* was not differentially expressed in *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ compared to *Esr1*$^{f/f}$ uteri (*n*=4-7 mice/genotype).
Conclusion: our findings suggest that 1) deletion of *Esr1* in the uterine epithelial cells causes a lack of uterine KLKs expression; 2) KLKs derived from the semen alone are not sufficient to liquefy the semen in female reproductive tract; and 3) uterine KLKs are potentially required for complete liquefaction in the female reproductive tract and successful pregnancy. Potential impact of this proposed work would be toward development of novel therapeutic approaches for contraception or for treating semen hyperviscosity to improve fertility.

Financial support: Start-up fund from WSU College of Veterinary Medicine.

Featured Speakers:

Session II: Mechanisms of Cellular Development in the Reproductive Axis

Determinants of the Stem Cell State in Mammalian Spermatogonia

Jon M. Oatley

Center for Reproductive Biology, Washington State University, Pullman, WA

Continual spermatogenesis relies on self-renewal of spermatogonial stem cells (SSCs) to sustain a foundational pool that arises in neonatal development from prospermatogonial precursors. Progenitor spermatogonia arise periodically from the SSC pool, transiently amplify in number, and then transition to a differentiating pathway. At present, the molecular mechanisms underpinning the stem cell state and thus interface of the SSC to progenitor transition are undefined. Recently, we established that the SSC pool in mice is marked by expression of inhibitor of DNA binding 4 (ID4) and generated and *Id4-Gfp* reporter mouse line to study the population in more detail. Using this resource, we have found that the levels of ID4 expression are correlated to regenerative capacity and have been able to purify an SSC population based on intensity of *Id4-Gfp* transgene expression. To explore further the role of ID4 in regulating SSC functions, we have generated a novel transgenic mouse line possessing an *Id4* conditional overexpression transgene. Using this resource, we discovered that ID4 overexpression at the prospermatogonial precursor stage of development leads to formation of a foundational SSC pool but the emergence of progenitor spermatogonia is suppressed. Further exploration revealed that ID4+ population is mostly quiescent and ID4 levels influence cell cycle progression. Outcomes of RNA-seq analysis have revealed that ID4 levels are associated with major alterations in the transcriptome and functional classification indicates roles in repressing cell metabolism and enhancing migration and motility. Altogether, our findings indicate that the stem cell state in spermatogonia is dictate by ID4 which influence key cellular processes including cell cycle progression, bioenergetics, and migration. Moreover, the findings suggest that the transition from SSC to progenitor states is controlled by levels of ID4. This research has been supported by grant HD061665 awarded to JMO from the National Institutes of Health.

Leydig cell gene expression changes with the cycle of the seminiferous epithelium in the mouse.

Estela Jauregui, Traci Topping, Debra Mitchell, Cathryn Hogarth, and Michael Griswold.
School of Molecular Bioscience, Washington State University, Pullman, WA, USA.

Androgens, produced by Leydig cells, and retinoic acid (RA), synthesized by multiple cell types, are both needed for normal spermatogenesis in mammals. Our laboratory has demonstrated that RA levels cycle across the seminiferous epithelium (Hogarth, 2014), but there is no information regarding whether Leydig cell function also cycles. Therefore, our objective was to determine whether Leydig cells display cyclic changes in gene expression. To accomplish this, we utilized our novel WIN 18,446/RA treatment regime to synchronize male germ cell development and RiboTag/*Cyp17*iCre-positive male mice to investigate changes in Leydig cell gene expression across the cycle of the seminiferous epithelium. Immunohistochemistry confirmed that polyribosomes in Leydig cells contained HA tags in the experimental RiboTag/*Cyp17*iCre-positive adult male mice. Additionally, qRT-PCR was performed using cell specific gene primers to demonstrate that a 74-fold enrichment for Leydig cell transcripts with minimal contamination from other cell types could be isolated from RiboTag/*Cyp17*iCre-positive testes. Using microarray analysis, we identified 2,687 enriched Leydig cell genes with a raw score of greater than 200 ($P<0.05$ for $N=2$) and >1.5-fold change for immunoprecipitated versus total RNA samples. From these enriched Leydig cell genes, 36 genes cycle with at least 2-fold change across the seminiferous epithelium. Interesting, the 36 genes included the steroidogenesis gene *cyp21a1,* the cholesterol metabolism gene *cyp7a1,* and the transcription regulator gene *Egr1.* All of these genes are needed for Leydig cell function. Additionally, 57 genes associated differently with ribosomes across the twelve stages of spermatogenesis. This list included several aldehyde dehydrogenase genes (*aldh1a7*, *aldh3a1*, and *aldh3b1*), retinoic acid receptor responder 1 (*rarres1*), androgen receptor (*ar*), and a gene involve in steroidogenesis (*cyp21a1*). Functional annotation clustering was performed on the 36 cycling genes and revealed that most were associated with regulation of transcription (9 genes), or ion binding (14 genes). As a result, this study has, for the first time, mapped the Leydig cell "translatome" across the cycle of the seminiferous epithelium and inferred that Leydig cell function may fluctuate with the cycle of the seminiferous epithelium. Further studies will involve confirmation of whether Leydig cells express specific genes when in the vicinity of a particular stage of the cycle and investigation of whether cyclic Leydig cell gene expression contributes to the regulation of spermatogenesis. Supported by Supplement Supplement NIH Grant 11H-2428-0287 to MDG and EA.

Development of a high throughput siRNA screen to identify novel transcription factors involved in spermatogonial stem cell renewal

Tessa Lord and Jon M. Oatley
Centre for Reproductive Biology, School of Molecular Biosciences, Washington State University, Pullman, WA, USA.

Spermatogonial stem cells (SSCs) act as a reservoir in the testes from which undifferentiated progenitor spermatogonia arise that will transition through a process of differentiation to produce spermatozoa. As such, impairments to the self-renewing capacity of SSCs is catastrophic for male fertility, culminating in an azoospermic phenotype. Despite the critical role of SSCs in the continuation of spermatogenesis, relatively few regulating factors have been identified that are involved in the maintenance of this population, primarily due to a lack of tools available to distinguish the rare SSCs from their undifferentiated progenitor counterparts. Recently however, our laboratory has characterized 'Inhibitor of DNA binding 4' (ID4) as a SSC-specific transcription factor that is indispensable for maintenance of the SSC pool. Further, we have developed an ID4-GFP reporter mouse model from which primary spermatogonial cultures can be established, allowing for SSC cells to be easily distinguished from progenitors. Thus, while the field has previously been reliant on single gene analysis using time-consuming spermatogonial transplantation techniques to assess changes in SSC content, the ID4-GFP cell line provides a rapid means to identify alterations to the SSC pool, through a direct readout of GFP fluorescence. The dynamics of the spermatogonial pool can also be more intricately assessed using this model, by monitoring the distribution of cells within the GFP+ population itself; specifically analyzing the subpopulations of GFP dim, mid, and bright fluorescence.

We are in the process of conducting a large-scale siRNA library screen that targets over 1400 transcription factors in our ID4-GFP cultures, performing high-throughput analyses to assess the effects of transcription factor knockdown on SSC population dynamics. ID4-GFP cultures are transfected overnight in a 96 well culture dish and maintained in this format on feeder cells for 6 days post-transfection. At day 6, fluorescence intensity and distribution of the GFP population is assessed using a flow cytometer with plate-reader capacity, and compared to that of a control culture that has been transfected with non-targeted siRNA. Using this methodology, we have demonstrated significant disruption to the ID4-GFP population in response to knockdown of transcription factors such as Retinoblastoma protein that have been previously identified for their role in SSC renewal; particularly within the 'GFP-bright' contingent in which the majority of cells with regenerative capacity are thought to reside. Further, we have begun the identification of novel transcription factors that appear to be involved in maintaining the SSC-to-progenitor ratio by virtue of their knockdown causing a shift in the distribution of ID4-GFP fluorescence in our spermatogonial populations. This high-throughput approach to identifying novel factors involved in SSC maintenance is not only important to bridge the gap in knowledge in the reproductive biology field, with potential ramifications for elucidating causes for idiopathic azoospermic infertility; but also has the capacity for adaptation to study stem cell dynamics in other tissue types.

Investigating the Potential Causes and Consequences of Chromosomal Instability During Primate Pre-Implantation Development

Shawn L. Chavez[1,2]

[1]Division of Reproductive & Developmental Sciences; Oregon National Primate Research Center; Beaverton, Oregon

[2]Departments of Obstetrics & Gynecology and Physiology & Pharmacology, Oregon Health & Science University School of Medicine; Portland, Oregon

Since the introduction of human *in vitro* fertilization (IVF) over 35 years ago, a major challenge has been to identify the embryo(s) most suitable for transfer and likely to result in a normal term pregnancy. Despite significant efforts to improve embryo selection techniques, the average live birth rate is still only ~30% per IVF cycle. One of the primary contributors to IVF failure is thought to be whole chromosomal abnormalities (aneuploidy) that arise during pre-implantation development. Indeed, DNA microarray and more recently, next generation sequencing (NGS) studies, have shown that one or more cells in ~50-80% of cleavage-stage human embryos are aneuploid. While mouse embryos exhibit considerably less aneuploidy at ~1-10%, the aneuploidy frequency in embryos from other mammalian species more closely related to humans such as non-human primates remained unknown and directly addressing this question is essential for potential translation to early human embryogenesis. Chromosomal mis-segregation in human oocytes during meiosis has long been considered the primary cause for aneuploidy and embryonic loss, especially in cases of advanced maternal age. However, even if a meiotic error occurs, there is evidence that mis-segregation can be corrected later in oocyte maturation by removal of the extra set(s) of chromosomes following polar body extrusion. Moreover, several studies of cleavage-stage human embryos from women of average maternal age have established that mitotic errors occur at an equal or greater propensity than meiotic errors. Thus, human embryonic aneuploidy is a complex process that can originate from multiple sources, but exactly how mis-segregation events further impact embryo chromosomal stability and subsequent pre-implantation development is not well defined. Using a combination of live cell imaging, novel gene reporter/targeting technologies and single-cell whole genome/transcriptome analyses, the Chavez laboratory aims to determine how chromosomal abnormalities may arise or be resolved during primate pre-implantation development. Chavez and colleagues are also evaluating whether chromosomal aberrations can be detected earlier in primate oocytes, sperm, and/or fertilized oocytes via biomedical optics or other non-invasive methods to avoid the unnecessary creation, culture and transfer of embryos that are unlikely to advance in development. Additional aims of this research are to assess the long-term implications, including transgenerational inheritance of epigenetic changes or defects, of ovarian stimulation, *in vitro* oocyte maturation, IVF treatment and extended embryo culture in offspring conceived from Assisted Reproductive Technologies (ART) and subsequent generations. Ultimately, the goals of these studies are to enhance our understanding of normal embryogenesis across different species and improve IVF outcomes for the approximately 1 in 10 reproductive age couples in our population who are infertile.

Featured Speakers:

Session III: Gonadotropins-
Synthesis, Secretion, and Signaling

Cecily V Bishop[1], Xin Li[2]

1. Division of Reproductive & Developmental Sciences, Oregon National Primate Research Center, Beaverton, OR; 2. Advanced Imaging Research Center, Oregon Health & Science University, Portland, OR

Effects of CG and VEGF on Vascular Function of the Primate Ovary.

The goal of these studies was to determine if VEGF neutralization before or after hCG administration restores ovarian vascular parameters in a nonhuman primate model of ovarian hyperstimulation syndrome (OHSS), thus preventing onset of OHSS symptoms. In addition, to ascertain if VEGF neutralization alters production of angiogenic factors by macaque granulosa cells (GCs) *in vivo* and *in vitro*. Rhesus females (n=8) underwent baseline evaluation of ovarian/luteal vascular flow and blood volume (BV) and permeability to serum albumin at mid luteal phase during normal menstrual cycles analyzed by contrast enhanced ultrasound and Dynamic Contrast Enhanced-MRI. In 2 subsequent cycles females were treated with a Controlled Ovarian Stimulation (COS) protocol and randomly assigned to Control (n=5), VEGF neutralizing agent Avastin 19±5hrs before hCG (single IV bolus; 10mg/kg; n=6), and Avastin 3-4 days post-hCG (single IV bolus; n=4). Contents of one follicle/ ovary were aspirated 36 h post-hCG and collected for follicular fluid analyses. Remaining follicles were aspirated, and GCs were isolated and cultured with FSH (2.5 ng/ml) and LH (100 ng/ml) for 24 h. Production of free VEGF, angiopoietin 1 (ANGPT1), ANGPT2, and progesterone (P4) were analyzed in the follicular fluid and media samples. Vascular flow, BV, and permeability of ovaries were analyzed 6-8 days post-hCG in the first COS cycle (n=2-3/group; similar to mid-luteal phase). Abdominal fluid (OHSS symptom) was present in 4/5 Control, 2/6 Avastin pre-hCG, and 3/4 Avastin post-hCG females. Ovarian BV in females exposed to Avastin post-hCG was not significantly different from any other group ($P<0.3$). Evaluation of COS ovaries showed increased leakage of serum albumin in 2 COS control females who developed OHSS compared to Control female without OHSS and normal menstrual cycles. Neutralization of VEGF at any time point reduced ovarian permeability to serum albumin to near those of normal menstrual cycles, even in 1 Avastin post-hCG female with OHSS. There were no differences in the total number of oocytes and MII-stage oocytes recovered between treatment groups ($P>0.7$). VEGF levels in follicular fluid were reduced 78-fold ($P<0.009$) by Avastin pre-treatment before hCG trigger. Levels of ANGPT2 were 36 to 48-fold higher than levels of ANGPT1. After 24 h of culture media levels of VEGF were also reduced 4-fold ($P<0.007$) in granulosa cells (GCs) that were exposed to Avastin *in vivo*. Media levels of P4 ($P<0.02$) and ANGPT1 ($P<0.03$) were reduced following Avastin exposure *in vivo*. ANGPT2 concentration was not altered ($P>0.4$), but ratio of ANGPT2/1 increased ($P<0.012$) in Avastin-exposed GCs compared to control. Ovarian vascular parameters in nonhuman primates associated with development of OHSS symptoms in COS cycles are prevented by neutralization of VEGF. Blockage of VEGF action prior to hCG bolus prevents development of OHSS symptoms. The elevated ANGPT 2/1 ratio observed *in vitro* in absence of elevated VEGF both *in vitro* and *in vivo* suggests the angiogenic signaling environment is altered to promote vessel degeneration following VEGF neutralization. Further studies utilizing this nonhuman primate model could provide insights into prevention of ovarian hyperstimulation syndrome in women. Support: R21 HD078819 (CVB), P51OD011092 (ONPRC).

Neuroendocrine control of ovulation in primates: a role for two distinct populations of GnRH neurons?

Henryk F. Urbanski[1], and Donald I. Brown[2]

[1]Oregon National Primate Research Center, OHSU, Beaverton, USA
[2]Universidad de Valparaíso, Valparaíso, Chile

Gonadotropin-releasing hormone (GnRH) neurons represent the primary neuroendocrine link between the brain and the rest of the reproductive system, and traditionally it has been assumed that a single population of GnRH neurons controls both pusatile LH release as well as the preovulatory LH surge. This view has profoundly influenced our strategies for contraception and for the treatment of infertility in women. Recent data from our laboratory, however, questions the validity of this fundamental assumption. Using the female rhesus monkey as a translational animal model, we found that: 1) Primates express two distinct molecular forms of GnRH, both of which are highly effective at stimulating LH release; 2) GnRH-I and GnRH-II, are encoded on different chromosomes, and the neurons that secrete them have completely distinct locations in the hypothalamus; 3) GnRH-I neurons respond to estrogen exclusively in a negative manner, while GnRH-II neurons respond to estrogen exclusively in a positive manner. Taken together, these data suggest that different aspects of reproductive function in primates are orchestrated by two distinct populations of GnRH neurons, with GnRH-II neurons playing the primary role in mediating the estrogen-induced preovulatory LH surge. On the one hand, these findings suggest that it may be possible to develop a novel reversible contraceptive, which uses pharmacological agents to selectively silence GnRH-II neurons - thereby blocking ovulation while leaving the rest of the reproductive axis relatively unperturbed. On the other hand, blockade of ovulation without blocking the underlying pulsatile pattern of GnRH-I release may result in a pathological condition in which the ovaries develop multiple cysts, thereby providing a novel nonhuman primate model in which to study the etiology of polycystic ovary syndrome (PCOS).

Investigating the role of luteinizing hormone in nonreproductive tissues in dogs

Michelle A. Kutzler, DVM, PhD, DACT
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Elective sterilization of pet dogs is a common surgical procedure performed in veterinary practice. The main benefit of sterilization is population control and the reduction in euthanasia of unwanted dogs. The most common methods for sterilizing female and male dogs are ovariohysterectomy (spay; which removes both the ovaries and the uterus) and castration (neutering; which involves removing the testicles), respectively. There is mounting evidence supporting the long-term health complications associated with surgical sterilization with gonad removal. Gonadal removal results in the inability for gonadal steroid hormones to feed back upon the hypothalamus and anterior pituitary, and this results in a rapid increase in luteinizing hormone (LH) concentrations. In the dog, LH concentrations stabilize approximately one year post-surgery at 7-fold increases. LH concentrations remain permanently elevated for the life of the dog. The negative health effects of gonadectomy varies between individuals. Canine ovary removal is associated with urinary incontinence. These effects are likely due to persistently supraphysiologic elevations in gonadotropin concentrations and extragonadal receptor-ligand binding as suppressing LH concentrations via GnRH immunization following gonadectomy will restore continence in spayed incontinent dogs. In women, anterior cruciate ligament rupture is associated with gonadal hormones. In dogs, anterior cruciate ligament rupture is more common after gonadal hormone removal with spaying or neutering. Gonadal hormones mediate growth plate closure in long bones and studies have demonstrated that growth plate closure is delayed as a result of prepubertal spaying. Research has shown that other factors such as obesity, breed predilection, and abnormal angulation of the stifle can increase the risk of ACL injury; but they point out that recent studies indicate that joint laxity may differ under varying hormonal stimuli suggesting a possible cause and effect mechanism. There is also growing concern among veterinarians that the lack of sex hormones in a sterilized dog can foster increased rates in osteosarcoma. A study by Ru and coworkers (1998) noted that neutered dogs were at 2.2 times greater risk of developing osteosarcoma than sexually intact dogs. Osteosarcoma is an uncommon tumor in dogs with an overall incidence rate at 0.2%. Incidences of osteosarcoma have been found to increase by 1.3-2 fold following a gonadectomy, suggesting a hormonal link between the hormone producing ovaries and cancer prevention in specific breeds, especially the Doberman Pinscher, Great Dane, Rottweiler, Irish Setter, and the Irish Wolfhound. Lastly, Waters and colleagues (2009) have shown that shortened longevity is another possible complication associated with ovary removal in dogs. Female Rottweilers who kept their ovaries for at least six years were 4.6 times more likely to reach exceptional longevity—that is, live 30% longer on average—than females with shortened ovary exposure. This study also found that the increased longevity was independent of cause of death, suggesting that a network of processes regulating the intrinsic rate of aging is under ovarian feedback. In conclusion, any surgery that sterilizes an animal changes the animal in both positive and negative ways. Gonads are not just gamete producing organs, but are important endocrine organs to the whole body.

Canine Hemangiosarcoma Expresses Luteinizing Hormone (LH) Receptors

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Throughout most of the developed world, surgical sterilization via gonadectomy has become a common tool for combating the overpopulation of unwanted dogs as well as to eliminate the risk of reproductive diseases in pet dogs. However, canine gonadectomy increases the risk of several non-reproductive long-term disorders possibly due to a loss in negative feedback to the anterior pituitary, which results in supraphysiologic circulating concentrations of LH. In addition to its reproductive hormone action, LH is a powerful mitogen in extragonadal tissues with LH receptors. Studies have confirmed the presence of LH receptors in vascular endothelial and smooth muscle cells in humans. Hemangiosarcoma is a rapidly growing, highly invasive cancer arising from the lining of blood vessels (mostly commonly the spleen in dogs). Gonadectomized (spayed) female dogs have two times the risk for developing hemangiosarcoma compared to unaltered females. We hypothesized that LH receptors would be present in vascular cells of canine hemangiosarcoma. The aim of study was to investigate if LH receptors were expressed in primary and metastatic lesions of canine splenic hemangiosarcoma using immunohistochemistry. Formalin-fixed surgical biopsies submitted to the Oregon State University Veterinary Diagnostic Laboratory were paraffin-embedded and sectioned (6 μm) onto charged slides. Testicular tissue from a separate dog obtained following castration was used as a positive control. All slides were deparaffinized, rehydrated, subjected to heat-induced epitope retrieval (#S1700, Dako). Endogenous peroxidase activity was inactivated with 3% H_2O_2 and nonspecific binding was blocked with 1% horse serum. Goat polyclonal anti-human LHR antibody (SC-26341, Santa Cruz Biotechnology) was applied at a 1:50 dilution. Negative controls from each tissue were treated in the same way except in absence of primary antibody. Slides were then reacted with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA) and incubated with preformed avidin-biotin-peroxidase complex (#PK6105, ABC kit, Vector Laboratories) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories). Slides were counter-stained with hematoxylin, dehydrated, and mounted. Images were digitally captured at 400X magnification. LH receptor expression (cytoplasmic and granular) was found in splenic stromal cells of a primary tumor from one dog (identified by arrows on figure) but not in a primary splenic tumor or a mesenteric metastatic tumor from two other dogs. There was no positive staining in the negative sections. This is the first report shows that LH receptors are present in canine hemangiosarcoma and provides evidence for how gonadectomy may increase the incidence of cancer in dogs.

Keywords: Dog, Gonadectomy, Immunohistochemistry

Featured Speakers:

Session IV: Alterations in Steroid Hormone Signaling by Exogenous Factors

Title: Impact of RU486 on phosphatidylinositol hydrolysis in the *ovine* corpus luteum

Mary Meaker-Smallman, Theresa Filtz, Fred Stormshak. Oregon State University, Corvallis, OR.

It is well known that the corpus luteum (CL) functions to produce progesterone (P_4) needed to maintain pregnancy. However, whether or not P_4 receptors in the CL allow P_4 to act in an autocrine or paracrine fashion is presently unknown. To determine the impact of blockage of P_4 receptors in the CL, we antagonized these receptors using a high-affinity synthetic P_4 receptor antagonist, RU486. RU486 was administered via the ovarian artery on day 8 of the estrous cycle. The corpora lutea of RU486-treated ewes (n=5) and untreated control ewes (n=5) were incubated with and without $PGF_2\alpha$ (prostaglandin) *in vitro* in the presence of radiolabeled [^{3}H]-myo-inositol. We know that $PGF_2\alpha$ stimulates the PIP_2 pathway and we hypothesized that possible P_4 receptors if blocked by RU486 in the CL might result in a change in the incorporation of [^{3}H]-inositol into the phosphoinositides via activation of the PIP_2 pathway. Our data indicated that the incorporation of [^{3}H]-inositol into the phosphoinositides of the PIP_2 pathway was similar in both treatment groups, suggesting that RU486 indirectly had the same effect as $PGF_2\alpha$. Analysis of variance revealed a significant $PGF_2\alpha$ x RU486 interaction ($p<0.05$). If receptors for P_4 are indeed present in the CL, then RU486 binding to these receptors may be relieving progesterone's ability to suppress the PIP_2 pathway. Serum P_4 concentration (ng/ml) in both control and RU486 treated ewes was significantly greater prior to as compared to after injection into the ovarian artery.

The kids are (not) alright: multigenerational estrogen exposure disrupts meiosis and reproductive tract morphology in male mice.

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There is growing concern about the long-term effects of environmental contaminants on reproductive health. Existing data from clinical and experimental studies indicate a link between exposure to endocrine disrupting chemicals (EDCs) and declining male fertility. Recently, our laboratory found that brief estrogenic exposures coinciding with the establishment of the male germ stem cell population permanently decreases meiotic recombination in all descendant spermatocytes. Because genetic and epigenetic changes to the germ cell can be passed from parent to offspring, we hypothesize that estrogen-induced dysregulation of meiotic recombination is heritable. Moreover, we suspect that subsequent developmental exposure in successive generations may exacerbate the effect. Using outbred mice, we have devised multi- and transgenerational exposure paradigms in which three successive generations (F0-F2) of males are treated with either ethinyl estradiol or placebo. Our results indicate transgenerational inheritance of an ancestral exposure effect in unexposed grandsons in all estrogen-exposed lineages. Additionally, one family shows an additive effect of multigenerational exposure in F1 and F2 estrogen-exposed sons. In most families, meiotic recombination rate negatively correlates with the accumulation of estrogenic insults across generations. Moreover, multiple exposures across subsequent generations increased the incidence of meiotic errors that would result in spermatocyte elimination. These data provide insight into the link between reduced sperm counts and developmental estrogenic exposures. Unexpectedly, we observed defects in reproductive tract morphogenesis, particularly the vas deferens, associated with multiple exposures. Overall, this study demonstrates that continual exposure to EDCs in mammals poses serious threats to male reproduction and fertility.

Kiss1 expression is modulated by estrogen and endocrine disruptors in immortalized female AVPV- and arcuate-specific neuronal kisspeptin cell lines.

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Environmental and Molecular Toxicology, College of Agricultural Sciences[1], Comparative Health Sciences Program[2], Department of Biomedical Sciences, College of Veterinary Medicine[3], Oregon State University, Corvallis OR 97331

Ovulation requires preovulatory surges of gonadotropin-releasing hormone (GnRH) from preoptic hypothalamic neurons, initiated by elevated ovarian estradiol (E_2). Rising E_2 activates a subset of sexually dimorphic kisspeptin (Kiss-1) neurons in the female, located in the anteroventral periventricular nuclei (AVPV). Conversely, estradiol negative feedback on GnRH secretion is mediated by a neuroanatomically separate population of Kiss-1 neurons in the arcuate nuclei. Kisspeptin stimulates GnRH expression and secretion *in vivo*, and the development of this system is critical for the initiation of puberty. To elucidate how phenotypically similar Kiss-1 neuronal populations react differentially to E_2 exposure, we have generated two immortalized Kiss-1 cell lines from *kiss1*-GFP post-pubertal female mice. These cell models recapitulate *in vivo* differential responsiveness to E_2, with KTaV-3 (AVPV-derived) demonstrating ~6-fold increases in *kiss1* expression under higher E_2 doses (5pM – 50pM E_2), while *kiss1* expression in KTaR-1 cells is suppressed up to 80% under lower E_2 concentrations (2pM – 10pM). Further, we have found that baseline expression of estrogen receptor α (ERα/*esr1*) is significantly different between these lines, with KTaR-1 cells exhibiting a 5-fold higher expression of *esr1* relative to KTaV-1, whereas estrogen receptor β (ERβ/*esr2*) is not differentially expressed. Additionally, we are exploring the impact of endocrine disrupting class of perfluorinated alkyl substances (PFASs) on these neurons, with preliminary results illustrating *kiss1*, *esr1*, and *esr2* transcriptional activation and/or repression at relevant doses of perfluorooctanoic acid, perfluorooctanesulfonic acid, and perfluorohexanoic acid in the two lines. Finally, we are probing temporal patterns of *kiss1* and core clock gene expression in these lines in response to estradiol, and find distinct antiphasic patterns of *bmal1* and *per2* in KTaV-3 cells irrespective of E_2 exposure. Treatment of KTaV-3 cells with 25pM E_2, however, elicited distinct patterns of *kiss1* expression over time in contrast to vehicle, suggesting differential coupling of intracellular oscillators to *kiss1* transcriptional activity in the presence of E_2. Ongoing delineation of responsiveness to E_2 in these lines could reveal novel molecular mechanisms underlying differential expression patterns demonstrated *in vivo* between these neuronal populations. Furthermore, investigating the impact of select PFASs on transcriptional activity of *kiss1*, *esr1*, and *esr2* between these two cell lines could elucidate the consequence of estrogen mimicry during sex-steroid sensitive developmental phases.

Circadian dysregulation is associated with a loss of estrogen receptor expression in mammary tissue: implications for an etiology of breast cancer.

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Ambient urban nighttime lighting and increasing use of digital technology has been associated with adverse human health outcomes, including sleep disruption, metabolic disease, and cancer. Using canine and murine models, we are exploring alterations in circadian clock gene expression patterns in mammary cells resulting from exposure to light at night (LAN) as a potential etiology of hormone-dependent mammary cancer. LAN has been correlated with an increased risk of breast cancer in epidemiological studies, likely by inducing deregulation of cellular clocks, comprised of feedback loops of transcription factors controlling the timing of gene expression. We and others found that normal breast and prostate tissues exhibit robust clock cycling, while established tumor lines demonstrate a loss of oscillatory gene expression. We derived a canine mammary tumor line (cMAM) removed from a patient at the OSU Veterinary Teaching Hospital, observing a rapid and progressive loss of clock rhythmicity, evaluated via expression rhythms of the core clock genes *bmal1* and *per2*. Loss of circadian clock cycling occurred concomitant with severely dampened expression levels and loss of rhythmic expression of the estrogen receptors (ER) alpha (ERα) and beta (ERβ). Since abundance of ERβ is often associated with decreased metastasis, lower proliferation rates, and decreased mortality in breast cancer patients, we explored whether over-expression of clock components could rescue expression of this receptor. Transient transfection of CLOCK and BMAL1 increased both basal *esr2* expression levels and rhythms, suggesting that loss of clock cycling may underlie the loss of ERβ abundance in these cancers. Additionally, we used transgenic m*Per2*::luciferase knock-in mice to investigate effects of LAN on clock cycling in mammary chain and other tissues in females. Strikingly, 21 days of LAN exposure (18:6 LD) significantly altered patterns of *Per2*-driven bioluminescence specifically in the mammary chain, lengthening the period to ~42h, while exerting no effect on other peripheral tissues in comparison to mice exposed to a normal 12:12 light-dark (LD) cycle. Additionally, mice exposed to 3 weeks of extended LAN also exhibited significant decreases in both ERα and ERβ expression specifically in mammary chain at time of terminal sacrifice. Taken together, these results suggest that LAN exerts robust disruptive effects on the mammary clock, which then contributes to a loss of ER expression. We are continuing to explore this potential model of cancer etiology by investigating epigenetic changes resulting from LAN, to determine if deregulation of clock cycling combined with altered ER transcriptional modulation may lead to abnormal gene expression patterning and further epigenetic modification, phenomena associated with disrupted DNA damage repair, increased mutation accumulation, and tumor development.

Featured Speakers:

Session V: Effects of Nutritional Intervention on Reproduction

Nutrigenomic intervention to prevent mastitis in dairy animals

Massimo Bionaz, Department of Animal and Rangeland Sciences, College of Agricultural Sciences, Oregon State University.

Dietary compounds affect gene expression *via* interactions with transcription factors. Among the most relevant for nutrigenomics are the peroxisome proliferator-activated receptors (PPAR). PPAR subtypes are activated by long-chain fatty acids (LCFA) and play pivotal roles in regulating lipid and glucose metabolism and inflammatory response. Among LCFA, palmitate and stearate are the strongest PPAR agonists. The long-term goal of our lab is to study the possibility of activating PPAR by LCFA to increase performance and disease resistance in dairy animals. There is indirect evidence suggesting that, among the three PPAR isotypes, PPARγ regulates milk fat synthesis and may help to prevent mastitis. In order to test the possibility to improve the response to mastitis in dairy animals *via* activation of PPARγ, we run two experiments in lactating Saanen dairy goats. In both experiments we injected goats daily with the PPARγ synthetic agonist 2,4-thiazolidinedione (TZD) or saline and induced subclinical mastitis in half of the goats receiving TZD and in half of the goats receiving saline, leaving the other goats as control. We measured milk yield, milk components (including milk somatic cells or SCC), rectal temperature, leukocytes phagocytosis, metabolic and inflammatory profiling in blood, expression of specific genes in mammary epithelial cells (MEC) and adipose tissue and adipocytes size by histology. In the first experiment we did not observe any effect on milk fat synthesis but a lower amount of SCC and a better liver response to inflammation and higher neutrophils activity in TZD-treated animals. Gene expression data suggested a lack of activation of PPARγ in MEC and adipose tissue. Subsequent *in vitro* experiments indicated that the activation of PPARγ by TZD is determined by the availability of the vitamin A metabolite 9-*cis*-retinoic acid. In order to test if vitamin A can improve the response to TZD we performed the second *in vivo* experiment. Preliminary results from the second study indicated that goats fed adequate amount of vitamin A have a better overall response to mastitis and TZD injection provided some relatively minor benefits. Data indicated a more robust liver response to inflammation supported by a larger haptoglobin surge after IMI and greater immunoglobulin in blood in TZD-treated goats. Contrary to the first study, in the second study we observed a large decrease in NEFA due to TZD injection. More analyses are underway; however, the data so far produced in the two studies indicated that activation of PPARγ improves the overall response to mastitis. The results obtained in goats can be even more relevant in dairy cows, considering that mammary tissue of dairy cows has a higher expression of PPARγ compared to goats, suggesting a possible greater sensitivity to its agonists, as partly confirmed by *in vitro* studies. LCFA activate PPAR but it remains to be identified the most potent LCFA agonist for each of the three PPAR isotypes. This information is critical for practical nutrigenomic interventions at the farm level to harness the benefit of PPAR activation.

Maternal Creatine Supplementation for Protection against Perinatal Brain Injury in the Non-Human Primate.

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Perinatal asphyxia, leading to hypoxic-ischemic encephalopathy, can result in significant long-term motor, sensory, cognitive and behavioural deficits. The creatine-phosphocreatine shuttle is essential for the maintenance of cellular ATP, particularly under hypoxic conditions. Creatine supplementation during pregnancy has therefore been proposed as a potential neuroprotective therapy for perinatal hypoxic-ischemic insults. We have previously demonstrated that maternal dietary creatine supplementation significantly reduces mortality and multi-organ morbidity in a rodent model of birth asphyxia. In this pilot study, we aimed to gather preliminary data on: [1] the safety and efficacy of prolonged maternal dietary creatine supplementation for the mother and fetus using a non-human primate model; and [2] if exposure to creatine *in utero* protects the fetal brain from the effects of acute hypoxia at birth. At 104-106 days gestation (d GA) (term ~165 days) rhesus macaques (n=4) were surgically instrumented with fetal ECG electrodes, and catheters inserted into the amniotic sac, maternal femoral vein and artery. This allowed for continuous monitoring of maternal blood pressure (BP) and fetal heart rate (HR), uterine contractility and routine sampling of maternal blood and amniotic fluid. From 115d GA NHPs received creatine orally (0.3g/kg/day for 8 days, followed by 0.075g/kg/day until delivery; n=2), or vehicle (n=2) as controls. Maternal blood, urine and amniotic fluid was sampled throughout supplementation, to assess creatine content, basal metabolic status, and renal and hepatic functions. At 147-148d GA one creatine-treated and one control fetus underwent a 12-minute umbilical cord occlusion (UCO), to induce a hypoxic insult before delivery. One creatine treated non-UCO infant was survived to 10-days post-term age equivalent for neonatal monitoring, and the other non-UCO control was sent straight to necropsy at delivery. Dietary creatine supplementation increased maternal plasma and amniotic fluid creatine levels by ~60%. Preliminary analysis suggests that creatine treatment had no affect on maternal health parameters, including BP and uterine activity. After UCO the creatine supplemented neonate had a 30 min Apgar score of 8, compared to 2 for the control-UCO infant. The control-UCO infant displayed wrist flexion, reduced forelimb motor coordination and a reduced suckling reflex from birth until 5-days term equivalent, behaviours not observed in the creatine supplemented infant after UCO. This pilot study suggests that maternal dietary creatine is safe for the NHP mother and fetus, and can reduce neonatal morbidity following a hypoxic insult at birth. Future studies will aim to establish the mechanisms for the maintenance of mitochondrial function, neuroprotective capacity of creatine in the non-human primate and long-term developmental outcomes of neonates exposed to hypoxic-ischemic insults following maternal creatine supplementation.

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Effects of OmniGen-AF® on superovulation response and embryo quality in donor beef cows.

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Superovulation is a required yet costly and biologically stressful procedure in cattle embryo transfer. Inconsistent number of ova recovered and fertilization rates prevent industry adoption, therefore limiting the potential use for genetic improvement. Inflammation and immune system dysregulation have been suggested to be one cause of this variability. Therefore, the objective of this study was to evaluate OmniGen-AF® (OG) supplementation on superovulatory response, embryo quality and serum cortisol in beef cattle embryo donors using two doses of follicle stimulating hormone (FSH). Twenty-four cross-bred beef cows were randomly assigned to one of four treatment groups utilizing a 2x2 factorial design with FSH dose (200 or 400 mg) and OG supplementation (0 g/h/d or 56 g/h/d) for a feeding period of 49 days. The superovulation protocol was started on Day 28 of feeding and ova were nonsurgically recovered 7 days after estrus and artificial insemination. Good to excellent quality morulae and blastocysts were either fixed for staining or cultured to evaluate *in vitro* embryo development and plasminogen activator (PA) production. In cows superovulated with 400 mg FSH, feeding OG had a tendency to decrease the percent of degenerate embryos recovered (P = 0.08). Embryos recovered from cows superovulated with 400 mg FSH and fed OG produced more total PA, with peak PA production tending to be higher at 72 h of culture (P= 0.08), compared to all other groups. In addition, serum cortisol concentration was lower (P = 0.049) in donor cows fed OG at the last breeding of the superovulation protocol when compared to controls. In summary, feeding OmniGen-AF may ameliorate negative effects of the higher FSH dose used in superovulation protocols resulting in more transferable and fewer degenerate embryos. Also based on PA production, there is a potential for healthier embryos (if supplemented with OG and dosed with 400mg FSH), with a greater likelihood of developing beyond hatching in an embryo transfer procedure.

Key words: OmniGen-AF®, Embryo, Superovulation, FSH

Percentage of milk fat, lactose and protein is affected by diurnal variation in dairy goats

Fernanda T. Rosa, Johan S. Osorio, Jayant Lohakare, Misagh Moridi, Erminio Trevisi, Massimo Bionaz

Diurnal variation in milk synthesis in dairy goats is not known but can have important implication for nutrigenomic interventions to improve milk synthesis. The diurnal variation in milk synthesis was evaluated in 12 *Saanen* multiparous goats in early to mid-lactation. Six goats were treated with intrajugular injection of 2,4-thiazolidinedione (TZD) at 10AM and 6 goats received saline as control. Goats received a NRC-compliant diet at 8AM. All goats received intramammary infusion with Streptococcus uberis 10 days before to induce sub-clinical mastitis in the right half with the left half used as control. Goats were milked every 2 hours from 7AM to 7PM. Besides milk yield, milk samples were collected for components analysis and jugular blood samples were collected for analysis of NEFA, triacylglycerol (TAG), urea (BUN), and glucose. Data was analyzed using a GLIMMIX procedure of SAS with time, treatment, and time x treatment as fixed effect for blood parameters and addition of udder halves and relative interaction as fixed effect for the milk parameters. Goat was used as random effect. Significance was declared with Tukey's adjusted P-value<0.05. TZD injection did not affect any parameter. SCC was not affected by time but was higher in the right half of both groups (2.71 vs. 3.58±0.15). However, the % of milk fat peaked at 9AM (4.9%) and decreased afterwards. The % of milk protein and milk urea (MUN) peaked at 11AM (2.6% and 28.2 mg/dL, respectively) and % lactose (4.4%) and solid nonfat (SNF; 7.8%) between 1 and 3PM. None of the blood metabolites were affected by treatment or Treatment x Time interaction but all were highly affected by time. Glucose consistently increased until 1PM, urea reached a peak at 11AM, NEFA decreased through 1PM and increased afterwards while TAG consistently decreased through the day. The sum of TAG+NEFA, as index of available fatty acids, was affected by treatment with higher value in TZD compared to control. Significant (P<0.01) positive correlations were observed for blood glucose with milk lactose %, NEFA with SNF, TAG with SCC, BUN with milk fat and MUN. Negative correlations were observed for glucose with milk fat% and TAG and SNF. Our data highlight the diurnal variation in milk synthesis in goat. Surprising suggestion by the data are that milk fat synthesis does not appear to be driven exclusively by availability of fatty acids and protein synthesis does not seem to be affected by availability of glucose.

Diurnal, milk synthesis, goat

Pubertal exposure to western style diet and androgens does not alter reproductive neuroendocrine sensitivities.

C. True, D. Takahashi, C. Cameron, D. Stouffer

Patients with polycystic ovary syndrome (PCOS) show reproductive dysfunction at each level of the hypothalamic-pituitary-gonadal axis. Increased LH pulse frequency and amplitude are thought to reflect alterations at the hypothalamus and pituitary, respectively. Early exposure to androgens is hypothesized to contribute to adult PCOS phenotypes; however, the critical period during which androgens program these defects is unclear. In addition, obesity is a contributing component of PCOS and weight loss improves reproductive neuroendocrine function. The current study examined whether exposure to both androgens and a western-style diet in peripubertal rhesus macaques increased sensitivity of the hypothalamus or pituitary to neuroendocrine stimuli. Female rhesus macaques began treatment at 2.5 years of age, close to the time of menarche. The study consisted of four treatment groups (n=10): animals receiving a control diet with 16% of calories from fat and cholesterol implants (C), animals receiving a control diet and testosterone implants to achieve a serum level between 1-1.5 ng/mL (T), animals receiving a western-style diet with 36% of calories from fat and cholesterol implants (WSD), and animals receiving both a western-style diet and testosterone implant (WSD+T). WSD+T treatment increased weight gain, fat mass and insulin resistance compared to WSD or T alone, consistent with observations in PCOS patients. Kisspeptin and GnRH stimulation tests were performed at the mid-luteal and early follicular phase, respectively, following two years on the treatment paradigms. Neither hypothalamic responsiveness to exogenous kisspeptin or pituitary responsiveness to exogenous GnRH were altered across treatment groups. These findings indicate that although diet and androgen exposure during puberty were sufficient to alter metabolic function, this treatment protocol did not alter sensitivities of the reproductive neuroendocrine axis. This is in contrast to previously described work demonstrating increased hypothalamic and pituitary neuroendocrine drive in monkeys treated with androgens during the prenatal period. We hypothesize that the critical developmental window for androgen programming of reproductive neuroendocrine dysfunction occurs prior to puberty.

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POSTER PRESENTATIONS

(in alphabetical order)

The Timing of the Synchronized Onset of Spermatogenesis using WIN 18,446/RA Mimics the Timing of the First Round of Spermatogenesis in the Mouse Testis

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Retinoic acid (RA), the active metabolite of vitamin A, is known to be required for the transition of the undifferentiated A spermatogonia to differentiating A1 spermatogonia. The first round of spermatogenesis initiates in response to RA and occurs in patches along the length of the seminiferous tubule. However, very little is known about the individual differentiating spermatogonial populations and their progression through the cell cycle due to the heterogeneous nature of the onset of spermatogenesis. In this study, we utilized WIN 18,446 and RA as tools to generate testes enriched with different populations of spermatogonia to further investigate: 1) the undifferentiated to differentiating spermatogonial transition, 2) the progression of the differentiating spermatogonia through the cell cycle, and 3) Sertoli cell number in response to altered RA levels. Detection of Ki67 (cell cycle marker) and LIN28 (undifferentiated spermatogonia marker) within cross sections of testis tubules isolated from WIN 18,446 only treated male mice (N = 3) indicated that the majority of undifferentiated spermatogonia were arrested in G0. WIN 18,446/RA-treated neonatal mice allowed to recover for 2 hour intervals between 2 and 72 hours following RA injection (n=1) were then used to determine when and how synchronously mitoses occurred in the differentiating spermatogonial population following treatment. Based on the timing of the S phase windows (EdU incorporation) identified during the first 72 hours, larger recovery intervals were chosen through 148 hours post RA injection (n=1) to map all S phases that occur between the A to A1 transition and the formation of the preleptotene spermatocytes. Five differentiating spermatogonial S phase windows were identified between 2 and 148 hours post treatment. During the 2 to 72 time period, EdU co-localization with markers of differentiating spermatogonia was detected during three different recovery windows: 16 to 18 hours, 46 to 48 hours, and 70 and 72 hours. The fourth and fifth S phase windows were detected between 94 and 96 hours and 120 and 122 post RA injection, respectively. Preleptotene spermatocyte formation occurred between 144 and 146 hours post treatment (n=3). These data suggest that the onset of spermatogenesis following synchronization mimics the timing of the first round of spermatogenesis in the wild type animal. In addition, a slight increase in Sertoli cell number was observed following RA treatment, possibly implicating a role for RA in Sertoli cell cycle progression. This study has thoroughly examined the onset of synchronized spermatogenesis by investigating both the germ and Sertoli cell response to RA. In addition, this study has, for the first time, mapped the cell cycle kinetics of both the undifferentiated and differentiating spermatogonial populations in the neonatal testis and identified the precise timing of when specific individual differentiating spermatogonial populations are enriched within the testis following synchrony. Taken together, the data from this study can be utilized to further investigate both the asynchronous initiation and synchronized onset of spermatogenesis. This work was supported by NIH Grant R01 HD10808 to MDG.

Examination of estrogen receptor isoforms involved in differential regulation of hypothalamic kisspeptin expression.

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The hypothalamic neuropeptide kisspeptin (Kiss-1) acts as a central component of the reproductive axis. A critical factor in pubertal progression and normal reproduction, this peptide is an afferent stimulator of gonadotropin-releasing hormone (GnRH), and is responsive to gonadal steroids, integrating steroid hormone signals and modulating GnRH secretion accordingly. Kiss-1 exhibits a sexually dimorphic expression pattern, found , in neurons of the arcuate (Arc) nuclei in both male and female rodents, and in the anteroventricular periventricular (AVPV) nuclei in females. In females, estradiol (E_2) differentially regulates *kiss1* expression in arcuate and AVPV Kiss-1 neurons, inhibiting *kiss1* expression in the arcuate, while inducing it in the AVPV. Both characterized nuclear receptor subfamily isoforms, ER-α (*esr1*) and ER-β (*esr2*), have been localized to Kiss-1 neurons, and additional evidence suggests that rapid, non-genomic estrogen actions may also exert effects on these cells. Previous *in vivo* studies implicate ERα as crucial for the stimulatory effect of E_2 in the AVPV, while multiple mechanisms may act to inhibit *kiss1* expression in the arcuate. To explore in more detail the respective contribution of ER-α and ER-β on expression of *kiss1*, our laboratory has generated two immortalized cell lines, KTaR-1 and KTaV-3, representative models of the arcuate and AVPV Kiss-1 populations, respectively. Preliminary results indicate that that basal expression of *esr1* is higher in KTaR-1 cells relative to KTaV-3 whereas *esr2* basal expression is not different between the two lines. Quantitative PCR reveals that both the ER –β selective agonist (2,3-*bis*(4-Hydroxyphenyl)propionitrile (DPN)) and the ER-α selective agonist (propylpyrazole triol (PPT)) in KTaR-1 cells suppress *kiss1* expression after 4 hours, with a return to baseline after 24 hours, with a far more potent repression by the ER-α ligand. These results implicate ER-α as the predominant nuclear receptor isoform responsible for the repressive effects of E_2 in Kiss-1 arcuate neurons *in vitro*. Evaluation of respective ER subtypes in KTaV-3 cells and investigation into underlying mechanisms of differential regulation are ongoing.

Germ Cell Development Proceeds for Several Spermatogenic Cycles in a Lowered RA Environment

My-Thanh Beedle, Debra Mitchell, Traci Topping, Cathryn Hogarth, and Michael Griswold.

Bis-(dichloroacetyl)-diamines are compounds that inhibit the conversion of aldehydes to acids. Prolonged treatment with one of these compounds, WIN 18,446 (WIN), can reversibly inhibit spermatogenesis, making WIN an intriguing target as a novel male contraceptive. Our laboratory recently demonstrated that exposure of adult male mice to 125ug of WIN for 8 consecutive days resulted in an 80% reduction in testicular RA levels. However, it is not completely understood if spermatogenesis can proceed in a lowered RA environment. To investigate the effects of lowered RA levels on germ cell development, 2 day old male mice were given 100ug WIN daily for 7 days and then placed in one of three different treatment groups: 1) vehicle control (DMSO) injection on day 8 and then maintained on WIN during injection recovery, 2) RA injection on day 8 only (WIN/RA), or 3) RA injection on day 8 and maintained on WIN during injection recovery (WIN/RA/WIN). For all three strategies, the animals were left to recover from their injection across two spermatogenic cycles (8 and 16 days). Histological analysis of testes from the WIN/DMSO/WIN group demonstrated that this regime was sufficient to block spermatogonial differentiation, as there was an absence of both CKIT and STRA8 signal in all animals analyzed. Surprisingly, testes from the WIN/RA/WIN group displayed STRA8-positive spermatogonia and preleptotene spermatocytes at 8 and 16 days during the lowered RA recovery window, albeit in smaller numbers compared to the WIN/RA group. Meiotic spreads were performed at 8 days post injection to determine the effect of continued WIN exposure on meiotic progression. In the WIN/DMSO/WIN group no meiotic cells were detected. Surprisingly, in both the WIN/RA and WIN/RA/WIN groups, germ cell development was found to advance to the zygotene stage of meiosis. These data suggest that in a presumably lowered RA environment, STRA8 expression can be triggered and meiotic progression can still ensue. To determine if the severity of these effects was dose-dependent, we analyzed testicular morphology following WIN maintenance of increasing concentrations (100ug, 150ug, 200ug and 300ug) for 8 days post the RA injection. All treatment doses resulted in the appearance of STRA8-positive spermatogonia and preleptotene spermatocytes. However, a significant decrease in the intensity of STRA8 staining and the appearance of vacuoles were seen in the testes of animals treated with the 300ug dose and their health was severely compromised. Collectively, these findings suggest that once the testis receives an RA signal, continued WIN exposure is unable to immediately block subsequent spermatogonial differentiation completely. Based on these observations we hypothesize that the initial RA signal allows the formation of preleptotene spermatocytes independent of the RA environment and that these cells then provide a source of RA in a manner that is partially immune to the effects of WIN. Future studies will be directed towards understanding which cell types are sources of RA within the postnatal testis and the whether the effects of WIN are cell-specific.

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Determining the Onset of Spermatogenesis and Folliculogenesis in Free-Roaming, Unowned Cats

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Free-roaming unowned (FRU) cat populations around the world continue to grow. Our laboratory is interested in determining if an underlying biological cause exists for the exuberant reproductive success observed in this animal. Faya and colleagues have shown that female and male laboratory-raised cats produce significant amounts of gonadal steroid hormones in fecal samples by 3.5 months of age. We have hypothesized that FRU cats are reaching reproductive capacity at this age. The objective of this study was to examine FRU kittens presented for surgical sterilization for reproductive markers (spermatogenesis in males and folliculogenesis in females). For males (under 4 months n=10; 4-6 months n=8), a routine castration was performed. For females (under 4 months (FRU n=10) and 4-6 months (FRU n=7)), a routine ovariohysterectomy was performed. In both genders, gonads from each cat were hemi-sectioned, formalin-fixed, paraffin-embedded, cut into sections (6 μm), and stained with hematoxylin and eosin. The slides were blindly evaluated at 200X by the same observer (KP for males; EB for females) using bright field microscopy. For males, the Yoshida spermatogenesis scoring system was used. For females, the largest follicle diameter was measured. Mean ± SD was determined for each parameter. A Student's t-test was used to compare the two age groups for each sex. Significance was defined as $p<0.05$. Spermatogenesis score in under 4 month old and 4-6 months old FRU tomcats was 4.5±2.0 and 8.1±2.7, respectively. The largest follicle size in under 4 month old and 4-6 months old FRU queens was 581.6±53.6 μm and 469.4±113.9 μm ($p<0.05$). In the FRU cats studied, folliculogenesis appears to occur before 4 months of age in females, but spermatogenesis does not occur until after 4-6 months of age in males. Our results support the findings of Faya for females. However, in FRU males the age at the onset of spermatogenesis does not appear to coincide with the age at which significant levels of fecal testosterone are observed in laboratory cats. The implication of our results indicates that sterilization programs need to include female kittens in their efforts.

Growth Hormone Immunoactivity in Canine Adenomas and Adenocarcinomas

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Growth hormone (GH) of pituitary origin is an important global regulator of mammary gland development, as it induces insulin-like growth factor-1 in the mammary stroma and epithelium to stimulate ductal branching. In dogs, GH is also secreted by normal mammary tissue and may induce proliferation of mammary tumor cells. It is now widely accepted in human medicine that GH functions in the mammary gland in an autocrine and paracrine manner as local growth factors in tumor formation. Therefore, the objective of this study was to compare GH immunoactivity in archived canine mammary adenoma and adenocarcinoma submissions to histopathologic grading. We hypothesized that canine mammary adenocarcinomas would be more likely to be GH positive than canine mammary adenomas. Formalin-fixed specimens from spontaneously occurring mammary adenomas and adenocarcinomas from 24 female client-owned dogs were submitted to the Oregon State University Veterinary Diagnostic Laboratory for histopathologic diagnosis. Mammary tumors were histologically classified and morphologically described according to the World Health Organization system. Information pertaining to the reproductive status of the patient at the time of mammary tumor diagnosis was obtained from each of the submitting veterinarians. A canine anterior pituitary was used as a positive control for GH. Tissues were paraffin-embedded, and sectioned (6 μm) onto charged slides. All slides were deparaffinized and rehydrated. Endogenous peroxidase activity was inactivated with 3% H_2O_2 and nonspecific binding was blocked with DAKO serum-free protein block (X0909). Polyclonal rabbit anti-human GH antibody (DAKO A0570) was applied at a 1:200 dilution. A universal rabbit negative control (DAKO) (N1699) was used on pituitary and mammary tissues. Slides were then reacted with DAKO ENV ™ with anti-rabbit horseradish peroxidase (K4003) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories). Slides were counter-stained with hematoxylin, dehydrated, and mounted. Tumor type and reproductive status at time of tumor diagnosis were compared individually between tumors that were negative or positive for GH using a two-tailed Fisher's exact test. Significance was defined as $p < 0.05$. Eight of the thirteen submissions with mammary adenocarcinoma were GH positive compared to six of the eleven submissions with mammary adenoma. There was also no significant relationship between tumor type and GH presence. Reproductive status (e.g. ovariohysterectomized versus intact) at the time of tumor removal was compared with GH expression in mammary neoplasia and found to be not significant. The mammary tumor GH immunoactivity in the current study (55%) is lower than that reported by van Garderen and colleagues (1999) (87%). However, in the van Garderen study, two of the GH negative samples were from progesterone-depleted castrated bitches, which led these authors to speculate that progestogens induced biosynthesis of mammary GH. In the current study, nine of the fourteen bitches with GH positive mammary tumors were ovariohysterectomized at the time of diagnosis, meaning that factors other than progestogens contribute to biosynthesis of mammary GH. More research in this area is needed.

Conditional ablation of *Pgrmc1* and *Pgrmc2* results in faulty decidualization

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Progesterone receptor membrane component (PGRMC) 1 and PGRMC2 are non-classical progesterone (P4) receptors that promote cell survival and play roles in regulating mitosis and opposing apoptosis. PGRMC1 and PGRMC2 are abundantly expressed at the maternal:embryo interface during murine pregnancy. Both genes are differentially regulated by steroid hormones in estrous and menstrual cycling species, including women. Furthermore, PGRMC1 was recently shown to mediate the actions of P4 in promoting endometrial, ovarian, and breast cancer cell survival and chemoresistance while enhancing growth of xenograft tumors developed from these cell lines. We have established that conditional ablation of *Pgrmc1* and/or *Pgrmc2* from female reproductive tissues using *Pgr-cre* mice results in subfertility with fewer pups per litter and fewer litters overall in single and double conditional knockout (*Pgrmc1/2*$^{d/d}$) mice compared to control (*Pgrmc1/2*$^{f/f}$) mice. The purpose of this study was to investigate the cause for this subfertility phenotype. This phenotype appears to be uterine in origin in single and double conditional knockout mice based on three lines of evidence: 1) *Pgrmc1/2*$^{d/d}$ mice have a normal time between litters, suggesting intact hypothalamic-pituitary functions and estrous cyclicity; 2) *Pgrmc2*$^{d/d}$ mice have the same number of implantation sites on day 10 of pregnancy (DOP 10) and similar serum P4 levels as controls, suggesting normal ovulation, luteinization, fertilization, and implantation; and 3) *Pgrmc2*$^{d/d}$ mice have resorption of approximately 40% of implantation sites following implantation, a time when the uterine deciduum is expanding. Based on these findings, it was hypothesized that PGRMC1/2 are important functional elements for normal uterine decidualization. In order to test this hypothesis, artificial decidualization was performed using pseudopregnant *Pgrmc1/2*$^{f/f}$ and *Pgrmc1/2*$^{d/d}$ female mice. *Pgrmc1/2*$^{d/d}$ mice displayed faulty decidualization, with an average decidualized horn wet weight of 0.26 ± 0.08 g compared to 0.61 ± 0.08 g in *Pgrmc1/2*$^{f/f}$ mice ($p = 0.015$, n=3-5) on day 9 of pseudopregnancy. This occurred despite no difference in serum P4 between *Pgrmc1/2*$^{f/f}$ (9.39 ± 1.18 ng/ml) and *Pgrmc1/2*$^{d/d}$ (11.11 ± 4.65 ng/ml) mice ($p = 0.775$, n=3-4). Preliminary RNA-seq analysis of decidual tissue on DOP7 revealed that 543 genes were differentially regulated (334 downregulated; 209 upregulated) between *Pgrmc2*$^{f/f}$ and *Pgrmc2*$^{d/d}$ mice ($p < 0.05$, fold change ≥ 1.5, n=3); some of these changes were validated by qPCR. Gene ontology analysis established that changes in gene expression included major categories such as serine hydrolases, lipid metabolism, and growth factor signaling. Insufficient growth factor support, disrupted lipid metabolism, and dysregulated serine hydrolase activity could all contribute to the observed faulty decidual response and subsequent embryonic death in *Pgrmc1/2*$^{d/d}$ mice. In summary, our *in vivo* studies demonstrate a critical role for PGRMC1 and PGRMC2 in uterine decidualization and pregnancy. This study was supported in part by NIH OD016564 and RR030264, as well as the NSF Graduate Research Fellowship Program (GRFP1347973).

Aneuploidy Tolerance in Rhesus Macaque Pre-Implantation Embryos Via Micronuclei Formation, Cellular Fragmentation, and Blastomere Exclusion.

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Although the use of *in vitro* fertilization (IVF) to treat human infertility has more than doubled in the past decade, IVF success rates have remained relatively unchanged for several years. A primary contributor to IVF failure is the presence of unbalanced whole chromosomes, or aneuploidy. Previous microarray-based studies determined that 50-80% of human embryos are aneuploid at the cleavage-stage. During early mitotic divisions, many human embryos also sequester mis-segregated chromosomes into micronuclei and concurrently undergo cellular fragmentation. We hypothesize that cellular fragmentation represents a response to mis-segregated chromosomes that are encapsulated into micronuclei. Here, we utilized the rhesus macaque pre-implantation embryo as a model to study human embryonic aneuploidy using Eeva™ time-lapse microscopy for evaluating cell divisions, next generation single-cell/-cellular fragment DNA-Sequencing (DNA-Seq), and confocal imaging of nuclear structures. Results from our time-lapse image analysis demonstrated that while the timing intervals for the first two mitotic divisions were significantly different between rhesus and human embryos that reach the blastocyst stage ($p<0.0001$ and $p=0.001$, respectively), the third mitotic division is similar between the two species. We also found considerable differences in the timing of the first three mitotic divisions between rhesus blastocysts and those that arrested prior to this stage in development ($p<0.0001$, $p=0.0252$, and $p<0.0001$, respectively). By examining the chromosome content of each blastomere from cleavage-stage embryos via DNA-Seq, we determined that rhesus embryos have an aneuploidy frequency up to ~80% (n=15) with most embryos having mosaicism for aneuploid and euploid blastomeres. Several embryos also exhibited reciprocal whole chromosome gains/loses among blastomeres, indicating that these embryos had undergone mitotic errors at the early cleavage stage. In addition, findings of a reciprocal sub-chromosomal deletion/duplication between blastomeres suggest that chromosomal breakage occurs in certain embryos as well. Furthermore, we observed that chaotic aneuploidy often arises from embryos undergoing multi-polar divisions and that one of the blastomeres may receive only a few chromosomes following a tri-polar division. Using confocal microscopy and immunostaining for the nuclear envelope protein, LAMIN-B1, we determined that fragmented cleavage-stage rhesus embryos contain micronuclei and that cellular fragments can enclose DNA as shown by DAPI staining. Our DNA-Seq analysis of cellular fragments confirmed that fragments can encapsulate whole and/or partial chromosomes lost from blastomeres. When we immunostained embryos with gamma-H2AX, a marker of chromatin fragility, we observed distinct foci solely in micronuclei and DNA-containing cellular fragments. This suggests that micronuclei containing mis-segregated chromosomes can be ejected from blastomeres through the process of cellular fragmentation and once sequestered, these chromosomes may become highly unstable and undergo degradation. Finally, we also observed that some embryos prevented cellular fragments or large blastomeres from incorporating into the inner cell mass or trophectoderm at the blastocyst stage (9.8%, n=5). Upon imaging one of these blastocysts, multiple nuclei and intense gamma-H2AX foci were found in a large unincorporated blastomere. Altogether, our findings demonstrate that the rhesus embryo may respond to segregation errors by eliminating chromosome-containing micronuclei via cellular fragmentation and/or selecting against aneuploid blastomeres that fail to divide during pre-implantation development. Funding: P50HD071836 (NCTRI); P51OD011092 (ONPRC).

Exploring Coral and Sea Anemone Reproduction through Spatial Expression Patterns of a Gonadotropin-Like Hormone Receptor

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Coral reefs, which provide support and habitats for many ocean ecosystems, have declined sharply in recent years due to temperature stress from climate change and other environmental factors limiting the rate of successful reproduction. While the ecological conditions required for successful coral reproduction have been heavily studied, there remains much to be learned about the physiological mechanisms behind their reproductive processes. In order to elucidate reproductive physiology in corals, this project attempts to identify spatial expression patterns of the leucine-rich repeat-containing G protein-coupled receptor, LGRA2, in cnidarians (corals and sea anemones) by conducting *in situ* hybridization tests on the sea anemone *Aiptasia pallida* as well as stably transfected human embryonic kidney cells (HEK 293) as controls. The mammalian orthologs of the LGRA2 receptor, luteinizing hormone receptor (LHR) and follicle stimulating hormone receptor (FSHR), activate signaling pathways important in reproductive cycles and development of secondary sexual characteristics when exposed to the gonadotropins luteinizing hormone (LH) and follicle stimulating hormone (FSH). Expression of mammalian LHR and FSHR occurs in the gonads, while LH and FSH are released from the anterior pituitary. However, cnidarians lack a comparable central nervous system and use vastly different reproductive strategies from mammals, including synchronized spawning and hermaphroditism. We have shown that activation of the LGRA2 receptor can trigger similar signaling pathways to LHR and FSHR in transfected HEK 293 cells when exposed to mammalian LH and FSH, suggesting that it may also participate in reproduction in corals and anemones. Preliminary *in situ* hybridization tests using antisense probe show positive staining for the expressed receptor near gonadal regions of a sexually developed individual. Negative controls using sense probe show no staining. Similar results were found in stably transfected HEK 293 cells, with antisense probe staining along cell membranes and sense probe producing no staining. It is our hope that further animal replicates will strengthen our understanding localization of the LGRA2 gene's expression in an ancestral sea anemone, providing insight into the evolution of reproductive hormone receptors, aiding in the control of cnidarian reproduction in laboratory settings, and assisting with reef conservation and repopulation efforts.

Elucidating the Hormonal Regulation of the Claudin Genes in the Ovary

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The ovary is a dynamic organ that responds to many hormonal signals. When these hormonal signals are disrupted, it is possible to develop ovarian disease states such as Polycystic Ovarian Syndrome (PCOS). PCOS patients suffer from a multitude of symptoms including high levels of testosterone. Excess testosterone may misregulate genes in the ovary and disrupt ovarian function. Claudin 3 (*Cldn3*) and *Cldn11* have been shown to be regulated by androgens in the testis, while studies in ovarian cancer cells suggests a coregulatory mechanism could exist between *Cldn3* and *Cldn4* in the ovary. The objective of this study was to characterize the hormonal regulation of *Claudin* gene expression in the ovary. The ovaries of estrogen receptor alpha knockout (αERKO) mice have high serum testosterone concentrations, therefore *Claudin* expression was measured in these ovaries. Experiments were conducted using qRT-PCR to monitor the expression of *Cldn3*, *4*, and *11* in wild-type and αERKO mouse ovaries. Comparisons were made using a students t-test. These experiments indicated that *Cldn3*, *4*, and *11* were expressed significantly more in αERKO mice than their wild-type counterparts ($p<0.05$, n=5 for WT and αERKO groups). Further experiments characterized *Cldn* expression in the ovaries of mice treated with Dihydrotestosterone (DHT) for 90 days, a common rodent model for PCOS. DHT treated mice were found to express *Cldn3* and *Cldn11* significantly higher than wild-type mice. *Cldn4* expression decreased in DHT treated mice when compared to the wild-types($p<0.05$, n=4 for WT and DHT groups). These findings suggest that *Cldn3* and *Cldn11* are upregulated by testosterone in the ovary. The data also indicates *Cldn4* is regulated via different mechanisms than the other *Cldn* genes in the mouse ovary. DHT reduces expression of *Cldn4*, while increases are observed in the absence of ERα. These findings provide a first look into the regulation of the *Claudin* genes in the ovary, while providing a basis for future research to explore how they may contribute to the PCOS.

Matrix metalloproteinase type-2 (MMP2) and type-9 (MMP9) immunoexpression in bovine caruncular tissue at the time of calving, 2 hours post-calving, and 4 hours post-calving.

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As bovine parturition nears, the placentome undergoes changes that allow placental delivery within 12 hours after calving. These changes include the expression of enzymatic proteins (e.g. MMPs) within the placentome that are capable of breaking down collagen, allowing for the separation of the cotyledon from the caruncle. Dysregulation of either MMP can reduce collagen breakdown and result in placental retention. The objective of the study was to use immunohistochemistry to semiquantify MMP2 and MMP9 expression in bovine caruncles following a normal delivery. We hypothesized that MMP2 and MMP9 immunoexpression peak at calving and decrease within 4 hours after calving. Caruncles were collected from five cows as previously described by McNeel and coworkers (2013). Tissues were formalin-fixed, paraffin-embedded, and sectioned (6 μm) onto charged slides. All slides were deparaffinized, rehydrated, and subjected to either Proteinase-K (#S3020, Dako) or heat-induced epitope retrieval (#S1700, Dako) for MMP2 and MMP9, respectively. Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide and nonspecific binding was blocked with Protein Block serum (X0909, Dako). MMP2 (LS-B2799) was applied at a 1:1000 dilution and MMP9 (LS-A9461) at a 1:100 dilution. Negative controls from each tissue were treated with a universal negative antibody (#N1699, Dako). Slides were then reacted with One Step Horse Radish Peroxidase-Conjugated Polymer Anti-Rabbit IgG (ImmunoBioScience) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories). Slides were counter-stained with hematoxylin, dehydrated, and mounted. Utilizing bright-field microscopy at 50X magnification, immunoexpression of MMP2 and MMP9 was scored (0-3) across four quadrants of the caruncle. The average score for each time point in each individual was used to calculate the average ± SEM. These preliminary results show that there was a trend for MMP2 expression to be lower at two hours post calving ($p=0.07$) but this difference did not persist at 4 hours ($p=0.11$). For MMP9, there was a trend for decreased expression by 4 hours post calving ($p=0.06$). These results differ from that of Takagi and coworkers (2007) who found that MMP2 and MMP9 mRNA expression was lower at parturition compared to six hours postpartum, although protein expression was not determined in their study. Additional studies are underway to determine if acupuncture administered at the time of calving increases MMP expression.

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Effect of an Antigen Stabilization Agent and Peroxidase Substrates on Canine GnRH ELISA Results

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Enzyme-linked immunosorbent assays (ELISAs) are important research tools for determining amounts of antibodies and proteins (antigens) in biological samples. ELISAs utilize principles of antigen-antibody complexing as well as enzyme-substrate activation to detect the presence and/or quantify the amount of the substance being tested. The conformation and activity of the substances being tested can be effectively preserved (antigen stabilization agent) while simultaneously reducing non-specific binding by blocking the surface of the plate to other substances. The criteria for choosing a peroxidase substrate depends on the sensitivity of the substrate (i.e., lower limit of detection). The peroxides substrate 3,3',5,5'-tetramethylbenzidine (TMB) oxidizes more quickly than 2,2'-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS), which could increase non-specific binding. Therefore, we hypothesized that an ELISA using an antigen stabilization agent with TMB would provide more consistent results than an ELISA without an antigen stabilization agent or using ABTS. A 96-microwell plate was coated with 5 µg/mL of human LH-RH (Sigma, 71447-49-9) diluted in sodium bicarbonate buffer (pH 8.0) and incubated overnight at 4°C. For the first experiment, the plate was washed with phosphate-buffered saline (NaCl, NaH2PO4, and Na2HPO4) containing 0.05% Tween-20 (TPBS) (pH 8.0) and then an antigen stabilization agent (StabilCoat™ #SG01-0025, SurModics) was added to half of the 96-microwell plate. Per manufacturer's instructions, the plate was covered and incubated for 15 minutes at room temperature. The antigen stabilization agent was then removed by aspiration and the plate was processed according to the canine GnRH ELISA protocol developed in our laboratory. Briefly, negative and positive control samples and unknown samples were incubated at room temperature for one hour and then washed with TBPS. To compare the effects of peroxidase substrate, either ABTS (#50-66-18, KPL) or TMB (#34028, Thermo Science) was reacted with the horseradish peroxidase (HRP). Plates were read at 405 nm and 450 nm (respectively) by spectrophotometer (FLUOstar Omega, BMG Labtech Inc) immediately and every 60 seconds for 10 minutes following peroxidase substrate treatment. This experiment was repeated three times. Treatment with antigen stabilization agent produced lower optical density (O.D.) values compared to wells not treated with an antigen stabilization agent. Because the plates were assayed immediately after coating with the antigen stabilization agent, its beneficial effects (i.e., preserving dried proteins) could not be realized and instead it functioned to block specific binding. Treatment with TMB also produced lower O.D. values compared to those treated with ABTS. These results contradict an internal report by KPL (https://www.kpl.com/docs/techdocs/ML108.pdf) but in the current study, the manufacturer for both substrates was different. In addition, TMB resulted in O.D. values that were below the cut-off, which is the sum of the mean of the negative control samples added to their standard deviation (SD) that has been multiplied to a constant (*f*) that varies by the number of replicates and the selected confidence interval (mean + SD * *f*). The high background results from TMB may be due to its reported higher sensitivity. For the canine GnRH assay, samples treated with ABTS in the absence of the antigen stabilization agent resulted in a predictable physiologic response. Research supported by the Ministry of Higher Education and Scientific Research in Iraq for graduate school financial assistance.

Glycolysis optimized conditions improve the regenerative quality of spermatogonial stem cells during long-term in vitro maintenance

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Spermatogonial stem cells (SSCs) are a rare subset of the undifferentiated spermatogonial population and their activities provide the foundation for continual sperm production. A remarkable feature of SSCs is their capacity to regenerate the spermatogenic lineage following transplantation into the testes of a sterile recipient. A potential clinical application of SSC transplant is to treat infertility in cancer patients with germline elimination caused by cytotoxic effects of chemotherapy. The number of SSCs that can be collected from testicular tissue is low, thus, application is dependent on the ability to expand a primary population in vitro. At present, culture methodologies are available for rodents and are being used as the basis for development of similar conditions for human SSCs. However, the percentage of rodent primary culture populations that possess regenerative capacity is low (1-10%) and the ability to regenerate complete colonies of spermatogenesis declines during long-term culture (4-6 months). Thus, there is need to refine culture conditions to optimize SSC maintenance and a potential avenue to address this is to match conditions to SSC bioenergetics. Based on the outcomes of RNA-Seq analysis, we discovered that expression of key genes involved in the regulation of glycolysis are up-regulated in SSCs. In the current study, we explored the impacts of altering culture conditions to favor utilization of glycolysis as the primary bioenergetics process on maintenance of SSCs in primary cultures of mouse undifferentiated spermatogonia. Conventional culture conditions include maintenance in 21% O2 and media containing free fatty acids, which promote utilization of beta-oxidation as the primary bioenergetics process. To produce glycolysis optimized (GO) conditions, we cultured cells in 10% O2 and removed free fatty acids from the media. Transplantation analyses at 2-month intervals revealed that the capacity to regenerate complete colonies of spermatogenesis was improved over the 6-month culture period in the GO condition compared to the conventional condition. These findings indicate that matching culture conditions to the bioenergetics effects stem cell fidelity in primary cultures of mouse undifferentiated spermatogonia. Importantly, this information may be useful in devising culture conditions for SSCs of higher order mammals including humans. This research was supported by grant HD061665 awarded to JMO from the NICHD.

The kids are (not) alright: multigenerational estrogen exposure disrupts meiosis and reproductive tract morphology in male mice.

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There is growing concern about the long-term effects of environmental contaminants on reproductive health. Existing data from clinical and experimental studies indicate a link between exposure to endocrine disrupting chemicals (EDCs) and declining male fertility. Recently, our laboratory found that brief estrogenic exposures coinciding with the establishment of the male germ stem cell population permanently decreases meiotic recombination in all descendant spermatocytes. Because genetic and epigenetic changes to the germ cell can be passed from parent to offspring, we hypothesize that estrogen-induced dysregulation of meiotic recombination is heritable. Moreover, we suspect that subsequent developmental exposure in successive generations may exacerbate the effect. Using outbred mice, we have devised multi- and transgenerational exposure paradigms in which three successive generations (F0-F2) of males are treated with either ethinyl estradiol or placebo. Our results indicate transgenerational inheritance of an ancestral exposure effect in unexposed grandsons in all estrogen-exposed lineages. Additionally, one family shows an additive effect of multigenerational exposure in F1 and F2 estrogen-exposed sons. In most families, meiotic recombination rate negatively correlates with the accumulation of estrogenic insults across generations. Moreover, multiple exposures across subsequent generations increased the incidence of meiotic errors that would result in spermatocyte elimination. These data provide insight into the link between reduced sperm counts and developmental estrogenic exposures. Unexpectedly, we observed defects in reproductive tract morphogenesis, particularly the vas deferens, associated with multiple exposures. Overall, this study demonstrates that continual exposure to EDCs in mammals poses serious threats to male reproduction and fertility.

Luteinizing hormone receptor expression in the canine femoral head ligament, hyaline cartilage, and subchondral bone

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In the United States, surgical sterilization has become a common tool for managing canine overpopulation as well as preventing reproductive diseases in canine companions (e.g. mammary gland cancer, and prostate hyperplasia/prostatitis). However, surgical sterilization with gonadectomy is associated with long-term health problems in dogs. Gonadectomy significantly increases the incidence of canine hip dysplasia by 1.5-2 fold.[1] In the intact dog, luteinizing hormone (LH) secreted from the anterior pituitary gland stimulates the synthesis of gonadal hormones (e.g. testosterone, estrogen), which negatively feedback to regulate LH secretion. Conversely, in the gonadectomized dog, there remains no negative feedback due to the absence of gonads to complete the feedback loop. Thus, LH concentrations can be over 30 times higher than in normal adult dogs. Although the main role of LH remains within the reproductive system, LH receptors (LHR) are present in many non-reproductive tissues (e.g. bladder, skin, thyroid) and have been associated with several problematic conditions such as canine urinary incontinence. We hypothesized that there are LHR in the coxofemoral joint, specifically the femoral head ligament, hyaline cartilage, and subchondral bone. The objective of the study was to use immunohistochemistry to determine if LHR were present in these tissues. The femoral head was removed from six dogs postmortem using an osteotome. The femoral head ligament was dissected free using a scalpel blade and hyaline cartilage adjacent to the ligament insertion site was shaved off using the same scalpel blade. These tissues were fixed in 10% buffer formalin. The femoral head was decalcified in Cal-Ex (Fisher Scientific, Waltham, MA) for 7 days and then moved to formalin. Similar tissues from all six dogs were combined on the same section with a formalin-fixed canine testicle collected from an unrelated dog during a routine castration (positive control). These tissues were then paraffin-embedded and sectioned (6 μm) onto charged slides. All slides were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval (#S1700, Dako, Carpinteria, CA). Endogenous peroxidase activity was inactivated with 3% hydrogen peroxide and nonspecific binding was blocked with 1% horse serum. Goat polyclonal anti-human LHR antibody (SC-26341, Santa Cruz Biotechnology, Dallas, TX) was applied at a 1:50 dilution. Negative controls from each tissue were treated in the same way except in the absence of primary antibody. Slides were then reacted with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA) and incubated with preformed avidin-biotin-peroxidase complex (#PK6105, ABC kit, Vector Laboratories) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories). Slides were counter-stained with hematoxylin, dehydrated, and mounted. Immunoexpression of LHR was detected utilizing bright-field microscopy at 400X magnification. Although the number of positive cells varied between dogs, LHR expression was present in all three tissue types examined. The etiopathogenesis for the increased incidence of hip dysplasia in gonadectomized dogs remains unknown. However, the results from this preliminary study suggest that persistent LHR activation in such structural support tissues within the hip joint may play a role. LHR activation is known to stimulate nitric oxide release,[2] which in the coxofemoral joint of predisposed breeds could result in excessive joint laxity and thus the gradual degeneration and symptoms associated with hip dysplasia.

[1] Torres de la Riva G, Hart BL, Farver TB, et al. Neutering dogs: effects on joint disorders and cancers in golden retrievers. PLOS One 2013;8(2):e55937.
[2] Greene JM, Ginther OJ. Circulating nitric oxide metabolites during luteolysis and the effect of luteinizing hormone on circulating nitric oxide metabolites in heifers. Theriogenology 2015;83(2):213-221.

Ancestral Atrazine Exposure Leads to Multi-generational and Transgenerational Increases in Disease and Phenotypic Change

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Some environmental compounds are known to promote the epigenetic transgenerational inheritance of adult onset disease in subsequent generations (F1-F3) following ancestral exposure during fetal gonadal sex determination. In the current study F0 generation gestating rats were exposed to 25 mg/kg IP of the herbicide atrazine, or to control vehicle DMSO, at the time of fetal sex determination (E8-E14). Adult onset disease and changes in phenotype were evaluated in F1, F2 and F3 generation progeny. Testis, prostate, ovary and kidney were examined histologically for abnormalities. Preliminary data suggest that atrazine-exposed lineage animals, compared to those of the control lineage, have histological changes in organs, a higher rate of tumor formation in the F2 generation, and an increase in the length of gestation leading to the birth of the F3 generation. This supports the idea that exposure of gestating rats to atrazine results in multigenerational and transgenerational changes in disease incidence and phenotypes. These studies are supported by grants from the Gerber Foundation and the National Institute of Health.

Ancestral Exposure to the Toxicants DDT, Vinclozolin, and Atrazine has Transgenerational Effects on Age of Puberty

Authors: **Marlee Lawley**, Deepika Kubsad, Margaux McBirney, Ryan Thompson, Maggie Unkefer, Eric Nilsson, and Michael Skinner

Ancestral exposure to environmental factors such as toxic compounds has been shown to have epigenetic transgenerational effects on such things as disease inheritance and developmental milestones. The current study was designed to determine the effects of ancestral exposure to the compounds DDT, Vinclozolin, and Atrazine on the milestone of age of puberty, in a rat model. Pregnant F0 generation animals were exposed to toxicants during gestational days 8-14, and subsequent generations bred out to F3. When compared to control lines, the F2 generation males showed an earlier age of puberty in DDT and Atrazine lineages, and later date of puberty in Vinclozolin lineages, while the F2 females showed a later date of puberty in DDT and Vinclozolin lineages. The F3 males showed a later date of puberty in DDT and Atrazine, and observations indicate that in subsequent generations, the compounds DDT, Vinclozolin, and Atrazine have the capacity to affect, through epigenetic transgenerational inheritance, timing of developmental milestones. Financial support provided by grants from the Templeton Foundation and the National Institute of Health.

Kallikreins in Mouse Uterus: The Final Essential Step for Semen Liquefaction?

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Back ground: Semen hyperviscosity (non-liquefied semen) contributes to 11.8 to 32.3% of male infertility. The liquefaction of seminal coagulation liberates sperm and increases their mobility. Liquefaction is thought to be modulated by the proteolytic activity of the kallikreins (KLKs), a serine protease family secreted from seminal vesicles and prostates.
Objective: In this study we hypothesized that KLKs derived from the uterus are functionally required for semen liquefaction and female reproduction. We reported previously that conditional ablation of *Esr1* (encoding estrogen receptor α) from mouse uterine epithelium (*Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$) causes infertility due in part to a dramatic reduction in the number of sperm entering the oviduct.
Methods: WT and *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ female mice reproductive tracks were studied after mating with healthy fertile male. Histological analysis and microarray analysis were performed to test physiological abnormalities and gene expression pattern of the tissues.
Results: At 0.5 days post coitus (dpc), a completely solidified semen was observed in the absence of epithelial ESR1 in the uterus compared to a watery liquefied semen in control uteri (*n*=4-5 mice/genotype). Moreover, *Klk1b5* and *Klk1* transcripts were significantly less in *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ compared to control littermates at 0.5 dpc (*n*=4-7 mice/genotype). In addition, the collagen (one of the KLK substrates in the semen) content was detected at a higher level in the lumen of *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ compared to controls at 0.5 dpc (*n*=4-5 mice/genotype). Although matrix metalloproteinases (MMPs) in the uterus could potentially contribute to semen liquefaction in female reproductive tract, we found that this is unlikely as *Mmp2* was expressed at significantly higher level and *Mmp9* was not differentially expressed in *Wnt7a*$^{Cre/+}$;*Esr1*$^{f/f}$ compared to *Esr1*$^{f/f}$ uteri (*n*=4-7 mice/genotype).
Conclusion: our findings suggest that 1) deletion of *Esr1* in the uterine epithelial cells causes a lack of uterine KLKs expression; 2) KLKs derived from the semen alone are not sufficient to liquefy the semen in female reproductive tract; and 3) uterine KLKs are potentially required for complete liquefaction in the female reproductive tract and successful pregnancy. Potential impact of this proposed work would be toward development of novel therapeutic approaches for contraception or for treating semen hyperviscosity to improve fertility.

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Rapid recovery from metabolically driven reproductive inhibition is correlated with changes in multiple metabolic hormones.

Cloe Moctezuma, Cadence True, Kevin L. Grove

It has been well established that reproductive function is tightly regulated by metabolic status, such that ovarian cycling is disrupted when energy output exceeds energy input. Despite years of research, the metabolic cues responsible for this inhibition of reproduction remain elusive. Recovery of reproductive function occurs rapidly with restoration of a balanced energy state, indicating that acute changes in hormones can relieve this metabolically driven inhibition. The current study investigated potential metabolic hormone levels that correlate with 1) reproductive inhibition during calorie restriction or 2) restoration of reproduction following a return to an *ad libitum* fed state. Sexually mature female rats were divided into three groups: control animals with *ad lib* food intake (CT; $n = 8$), calorically restricted (CR; $n = 7$) females restricted to achieve a 15 – 20% loss of body weight, and a refed (RF; $n = 8$) group that underwent CR followed by a 14 day return to *ad lib* feeding. Blood was assayed for luteinizing hormone (LH), follicle-stimulating hormone (FSH) and a panel of metabolic hormones. Group differences were analyzed by one-way ANOVA. CR caused reproductive dysfunction as evidenced by decreased uterine and ovarian weights, a trend toward lower LH levels, elevated FSH levels, and a decrease in the number of estrous cycles compared to the CT group. Lowered LH levels rapidly recovered within 24 hrs of refeeding. Compared to controls, an increase of insulin and c-peptide levels was observed in RF animals within 24 hours of refeeding and remained significantly elevated after two weeks of *ad lib* food access. Leptin levels were significantly decreased with CR as expected; however, leptin levels recovered to CT levels within just 24 hours of refeeding. Gastric inhibitory peptide (GIP) levels were found to be suppressed with CR, but significantly elevated compared to CT animals by 24 hours of refeeding. Serum interleukin-6 (IL-6) was significantly elevated in CR animals compared to CT and this increase dropped rapidly within 24 hrs of refeeding, and returned to CT levels after two weeks of *ad lib* food access. While alterations in leptin and insulin have previously been implicated in regulating reproductive function, depression of GIP with CR followed by significant elevation following a 24 hour refeed could provide a novel stimulatory signal between metabolism and reproduction. In addition, elevation of IL-6 could indicate that increased stress-induced inflammatory signaling contributes to reproductive dysfunction during negative energy balance. Furthermore, alterations in multiple systems known to regulate reproductive function highlights that infertility during negative energy balance is likely driven by a multitude of signals.

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Ancestral atrazine exposure promotes the epigenetic transgenerational inheritance of altered stress/anxiety behavior

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Atrazine is a widely used herbicide that was shown to promote transgenerational epigenetic effects in later generations (F3) following fetal exposure during gonadal sex determination in the F1 generation. The purpose of this study was to determine if atrazine exposure in the F1 generation induced behavioral changes in the F3 generation. Pregnant F0 rats were exposed to 25 mg/kg IP of atrazine or the control substance DMSO, from embryonic days 8-14. Rats were bred out to the F3 generation to identify if atrazine exposure had an epigenetic transgenerational effect. One year old F3 generation atrazine and control groups were tested behaviorally using an Elevated Plus Maze (EPM) and open field test. Both the duration in which a rat resided in an open or closed arm and the number of attempts a rat had in an open or closed arm were analyzed. Preliminary data suggests that the F3 generation atrazine rats exhibited a different behavioral response to the EPM than the F3 generation control lineage. Males from the F3 generation atrazine group revealed lower levels of stress/anxiety when compared to the control group; however, females in the F3 generation atrazine group revealed higher levels of stress/anxiety. These results support the hypothesis that when an F0 gestating female is exposed to atrazine, the following generations can experience behavioral changes. These studies are supported by grants from the Gerber Foundation and the National Institute of Health.

Dissecting Inherent Differences in the Expression and Function of DNA Methyltransferase 3A (DNMT3A) Isoforms During Primate Gametogenesis

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DNA methylation is mediated by a family of DNA methyltransferases (DNMTs), which catalyze the transfer of a methyl group (-CH3) to the 5-position of cytosine residues within CpG dinucleotides. Besides establishing and/or maintaining genomic imprinting patterns, previous studies in rodents suggest certain DNMTs may also contribute to the regulation of germ cell differentiation. More specifically, targeted disruption of both active isoforms of the *de novo* methyltransferase, DNMT3A, by conditional knockout technology in mouse germ cells has shown to result in aberrant gametogenesis and poor embryo development. Whether DNMT3A plays a similar role in both humans and non-human primates is unknown and the focus of this study. By performing Western Blot analysis of the four different human DNMT3A isoforms, we determined that DNMT3A2, which is transcribed from an alternative intronic promoter, is predominantly expressed in human fetal gonads. Using fluorescent *in situ* Hybridization Chain Reaction (HCR) imaging, we demonstrated that DNMT3A2 primarily localizes to the seminiferous tubules of adult human and vervet monkey testes at the mRNA level. Upon further analysis, we observed positive DNMT3A2 HCR signals in germ cells throughout spermatogenesis as early as the spermatogonial stage and late in development during residual body formation. In order to test the function of DNMT3A2, we nucleofected male 8.5 embryonic germ (EG) cells from C57BL/6 mice with DNMT3A2 specific morpholino antisense oligonucleotides. Morpholino-induced knockdown of DNMT3A2 significantly reduced the expression of the germ cell-specific protein, mouse vasa homologue (Mvh/Ddx4), and the meiosis-associated synaptonemal complex protein 1 (SCP1). Taken together, this suggests that DNMT3A2 expression and function in gametogenesis is conserved across mammalian species and confirms a role for particular DNMTs in germ cell development outside of genomic imprinting. Given the differences in the genetic requirements for germ cell development between humans and mice and the ethical limitations of studying human germ cell development *in vivo*, it also suggests that non-human primates can serve as suitable models for devising novel therapeutic strategies in the diagnosis and treatment of human infertility.

Title: Longitudinal Serum Chemistry Panels in Young Adult Female Rhesus Monkeys Treated with and without Western-Style Diet and Androgen.

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In order to better understand the causes, symptoms, and treatment of infertility in women with polycystic ovary syndrome, we are examining effects of chronic elevated testosterone (T) and a western-style diet (WSD) beginning at puberty in a primate model. As part of this study, we also examined serum lipid and chemistry panels to consider effects of treatment and aging. Female rhesus monkeys were treated from menarche with a normal diet (n=20) or a WSD (n=20), as well as subcutaneous implants of cholesterol (n=20) or T (n=20) to elevate serum T 3-4 fold. This resulted in four treatment groups: control (C), T, WSD, and WSD+T (n=10/group). Lipid and chemistry panels were evaluated prior to treatment (baseline), after 1 month, and after 2 years. Lipid panels were also evaluated at 1 year. All data were analyzed using the Mixed Models function of SAS (v9.4). All parameters were significantly different ($p<0.05$) over time, reflecting treatment and aging effects. WSD alone affected more parameters (potassium, sodium, chloride, magnesium, blood urea nitrogen, creatinine, alanine aminotransferase, high-density lipoprotein [HDL], low-density lipoprotein [LDL], total cholesterol, triglycerides) than T alone (albumin, HDL). There was significant WSD-by-T interactions for three parameters (chloride, creatinine, LDL). To date, lipid panel parameters do not display evidence of metabolic syndrome. HDL and total cholesterol were chronically elevated in the WSD groups, whereas LDL levels were highest at 1 month and fell at 1 and 2 years. This could reflect systemic acclimation to WSD. For T alone, a significant increase in HDL occurred at 1 month above C. These observations stand in contrast to human studies where both WSD and T are independently associated with low HDL. Chemistry panel glucose was considered unreliable because of different processing time; however independently measured glucose indicated no treatment effect. Osmolality (O=2*[Na+K]+[BUN/2.8]+[Glu/18]) displayed a WSD-by-time interaction, and indicated that WSD groups were likely more hydrated. Lower potassium levels are expected with adequate hydration, however osmolality is lower in WSD groups while potassium levels are mildly elevated. Given that WSD contains animal fat, this could be consistent with mild metabolic acidosis. Regardless, because low potassium levels are associated with diabetes, we do not observe evidence of diabetes when consulting potassium or glucose. However, metabolic testing indicated that there were significant WSD-by-time and T-by-time increases in fasting insulin levels. Alkaline phosphatase showed age-related changes: values were significantly elevated only at baseline and 1 month in all groups, potentially reflecting changes in the isoenzyme responsible for bone deposition. This dataset provides valuable reference ranges for young female rhesus monkeys, and provides insight into pathophysiological changes that occur when WSD or T are added at menarche. Continued surveillance of serum parameters will indicate how systems change in developing phenotypes.

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AMPK signaling plays a functional role in postnatal regenerative development of the female reproductive tract following parturition

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Signaling pathways that coordinate prenatal and postnatal development of the female reproductive tract are incompletely defined. AMP-activated protein kinase (AMPK) is a highly conserved heterotrimeric complex that acts as a cellular energy gauge in mammals. Based on recent observations of AMPK expression in reproductive tissues, we hypothesized that AMPK is functionally required for development of the female reproductive tract. This hypothesis was tested by conditionally ablating genes that encode the two catalytic subunits of AMPK, *Prkaa1* and *Prkaa2,* using *Amhr2-cre* and *Pgr-cre* mice*.* The requirement of AMPK for prenatal development of the reproductive tract was evaluated using *Amhr2-cre* mice, in which *Prkaa1/2* were ablated around e14. Ablation of *Prkaa1/2* using *Amhr2-cre* mice resulted in normal prenatal development. Similarly, ablation of *Prkaa1/2* using *Pgr-cre* mice resulted in normal postnatal development in nulliparous female mice. To further assess the requirement of *Prkaa1/2* for postnatal development following parturition, *Prkaa1/2*$^{fl/f}$ and *Prkaa1/2*$^{d/d}$ female mice were placed into a six-month breeding trial. While the first parity was comparable between *Prkaa1/2*$^{fl/fl}$ and *Prkaa1/2*$^{d/d}$ female mice in terms of the number of pups generated (p=0.8619), all subsequent litters were dramatically reduced in *Prkaa1/2*$^{d/d}$ female mice (p=0.0015). Furthermore, *Prkaa1/2*$^{d/d}$ female mice experienced a decrease in the number of litters during the breeding trial (p=0.0054) and an increase in time between parturitions (p=0.0286). Reproductive senescence occurred after the fourth parity in all *Prkaa1/2*$^{d/d}$ female mice. Histological evaluation of the reproductive tracts of nulliparous and multiparous *Prkaa1/2*$^{d/d}$ mice indicated that disrupted ovarian function is likely not responsible for the observed premature reproductive senescence given that folliculogenesis and ovulation were maintained beyond the fourth parity. Furthermore, estrous cyclicity between the *Prkaa1/2*$^{fl/f}$ and *Prkaa1/2*$^{d/d}$ mice did not differ and age was not a factor in the faulty fertility. Serum progesterone levels were consistent between *Prkaa1/2*$^{fl/f}$ and *Prkaa1/2*$^{d/d}$ female mice during pregnancy, suggesting normal luteal function. Interestingly, uteri from multiparous *Prkaa1/2*$^{d/d}$ mice became structurally disorganized with loss of mesenchymal tissue, aberrant placement of glandular epithelium and development of stromal fibrotic lesions as assessed by trichrome stain. The presence of hemosiderin suggests the infiltration of macrophages possibly due to vascular hemorrhaging. We suspect that faulty repair of the endometrium following parturition caused by excessive fibrosis in the stromal compartment and potentially vascular abnormalities are causally responsible for premature reproductive senescence in *Prkaa1/2*$^{d/d}$ female mice. It is concluded that AMPK plays a pivotal role in the postnatal developmental programming that coordinates endometrial regeneration following parturition.

Improving daily sperm production in bulls via transient hypothyroidism in pre-pubertal development

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Advanced reproductive technologies such as artificial insemination and semen sexing could be more widely applicable to cattle production if daily sperm production (DSP) in bulls could be increased. A strong correlative with DSP is the number of testicular Sertoli cells (SC). Hence, a possible way to increase DSP is by increasing SC number. Induction of temporary hypothyroidism during neonatal life in both rats and mice leads to an increased SC number and in association testicular size and sperm production, after puberty. In bulls, there is a negative correlation between the level of the thyroid hormone thyroxine in neonatal life and testicular circumference after puberty. Therefore, it is logical to postulate that temporary hypothyroidism induced during pre-pubertal development when Sertoli cell proliferation usually ceases in the bull could increase SC number, testicular circumference and DSP. To test this, we devised a treatment strategy to induce hypothyroidism during the developmental window of time when Sertoli cell proliferation ceases. First, we conducted immunostaining for an SC marker (Sox9) and marker of proliferation (Ki67) on cross-sections of testes from calves at 1-10 months of age to define the developmental point when Sertoli cell proliferation ceases as 4.5-5 months of age. Next, we treated Angus calves (n=4) with the drug Methimazole (2mg/kg body weight) from 4-6 months of age to temporarily induce hypothyroidism. Controls were age matched calves (n=3) that did not receive treatment. After six weeks of treatment, serum levels of the thyroid hormones thyroxine and triidothyronine in the treatment group vs the control group were 59.3±11 ng/ml vs 151±28 ng/ml and 0.0997±0.0487 vs 1.317±0.398, respectively, demonstrating effectiveness at inducing hypothyroidism. At 20 months of age, total sperms/ejaculate (51.66% more) and semen volume (48.07% more) were significantly greater for treated bulls compared to the control. However, sperm viability, individual sperm motility, progressive sperm motility and mass motility were not significantly different between the two groups. Moreover, ultrasonography of reproductive tract at this age showed more maturation of the tract as compared to the observation at 12 months of age. In addition to ongoing semen evaluation, future analyses will include in depth examination of sperm viability via comparing mitochondrial integrity, acrosomal function, sperm capacitation, and sperm survival during cryopreservation. Serum collected throughout the experiment will be analyzed for levels of thyroxine, triidothyronine testosterone, anti mullerian hormone, inhibin and cortisol. Post orchiectomy analysis, at 27 months of age of the bulls, will include: epididymal sperm counting, weight of epididymides, weight of testes, weight of accessory sex glands, number of SC and spermatids per gram of testes, number of SC and germ cell in cross section of seminiferous tubules, and diameter of seminiferous tubules. Collectively, findings to date suggest that induction of a transient hypothyroidic state during pre-pubertal development in bulls leads to an increase in sperm production after puberty. Thus, this treatment paradigm could materialize into a commercially viable tool for improving the reproductive efficiency of bulls with desirable genetics thereby having a positive impact on cattle production systems worldwide.

Elevated Testosterone Levels in Mice Deficient for the Chromatin Modifying Enzyme Kdm5c

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The X-linked gene *Kdm5c* encodes a histone demethylase that is involved in epigenetic regulation of gene expression. By removing the methylation modifications from lysine 4 on histone H3, the Kdm5c enzyme suppresses the transcription of its targeted genes. When *Kdm5c* is mutated, its protein products are either absent or function poorly, which results in up-regulation of genes normally repressed by this demethylase. Mutations of *Kdm5c* cause many symptoms, including intellectual disability, aggression and disruptive behavior, as well as male infertility. The goal of this ongoing project is to uncover the molecular basis for the disease phenotypes.

These experiments utilized mice with a germline transmitted constitutive deletion of *Kdm5c*, i.e. *Kdm5c* deficient mice ($Kdm5c^{Def}$). They underwent behavioral testing, including a test of aggression. In comparison to wild type (WT) mice, $Kdm5c^{Def}$ mice displayed higher levels of aggression, reduced sociability, and memory deficits. Consistent with the finding of high aggression, the serum and testicular concentrations of testosterone were elevated in the $Kdm5c^{Def}$ mice, which in turn led to the up-regulated expression of androgen responsive genes in the brain, as shown by RNA-seq analysis. To investigate the cause for the increase of testosterone, expressions of steroidogenic genes were quantified in testes with RT-qPCR, and found to be either decreased in $Kdm5c^{Def}$ mice or similar between the two genotypes, making these genes less likely to be responsible for the increased levels of testosterone. Only one steroidogenic gene, *Srd5a1*, showed higher levels of mRNA expression in $Kdm5c^{Def}$ mice, which was concordant with the higher levels of H3K4 methylation at its promotor in the mutant mice. Histological examination revealed fewer testosterone producing Leydig cells in the $Kdm5c^{Def}$ mice as well as irregularities in spermatogenesis.

The findings of this study help us to better understand the role of *Kdm5c* and epigenetic chromatin remodeling in steroid production with direct relevance to spermatogenesis, male infertility, and behavior. Ultimately, this study might suggest chromatin targeted new treatments for male infertility as well as neuropsychiatric symptoms, such as aggression, that are related to abnormal testosterone levels. (Supported by an NIH R01 MH096066 to JX & YS)

Canine Thyroid Gland Expresses Luteinizing Hormone Receptors

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Hypothyroidism is a complex disease with a net effect of inadequate thyroid hormone receptor activation. The incidence of hypothyroidism is 10-15% in postmenopausal women and 30% in gonadectomized dogs. Circulating luteinizing hormone (LH) concentrations are significantly and persistently elevated in both of these populations. LH receptors are expressed in normal human thyroid glands. We hypothesized that LH receptors were also present in normal canine thyroid glands. The aim of this study was to determine if LH receptors were expressed and to quantify the level of cellular expression. Thyroid and bladder tissue were removed from two dogs (2 months old Labrador Retriever female and 6 months old Labrador Retriever mix spayed female) postmortem, formalin-fixed, paraffin-embedded, and sectioned (6 μm) onto charged slides. Testicular tissue from a separate dog obtained following castration was treated in the same manner. All slides were deparaffinized, rehydrated, subjected to heat-induced epitope retrieval (#S1700, Dako, Carpinteria, CA). Endogenous peroxidase activity was inactivated with 3% H_2O_2 and nonspecific binding was blocked with 1% horse serum. Goat polyclonal anti-human LHR antibody (SC-26341, Santa Cruz Biotechnology, Dallas, TX,) was applied at a 1:50 dilution. Negative controls from each tissue were treated in the same way except in absence of primary antibody. Slides were then reacted with biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA) and incubated with preformed avidin-biotin-peroxidase complex (#PK6105, ABC kit, Vector Laboratories) followed by Nova Red Peroxidase substrate (#SK4800, Vector Laboratories). Slides were counter-stained with hematoxylin, dehydrated, and mounted. The percentage of cells positive for LHR was determined at 400X magnification. Canine thyrocytes expressed LH receptors in 5% and 19% of the cells counted. LH receptor expression was also evident in bladder tissue from the same animals as well as in positive control testis. There was no positive staining evident in any of the negative control tissue sections. This is the first report of LH receptor expression in the canine thyroid. Additional studies are needed to determine if the unregulated hypersecretion of LH after gonadectomy is responsible for the incidence of hypothyroidism observed in these dogs. Research supported by the Ministry of Scientific Research and Higher Education in Libya for graduate school financial assistance and Dr. Howard Meyer of Chippewa Kennels for research supplies.