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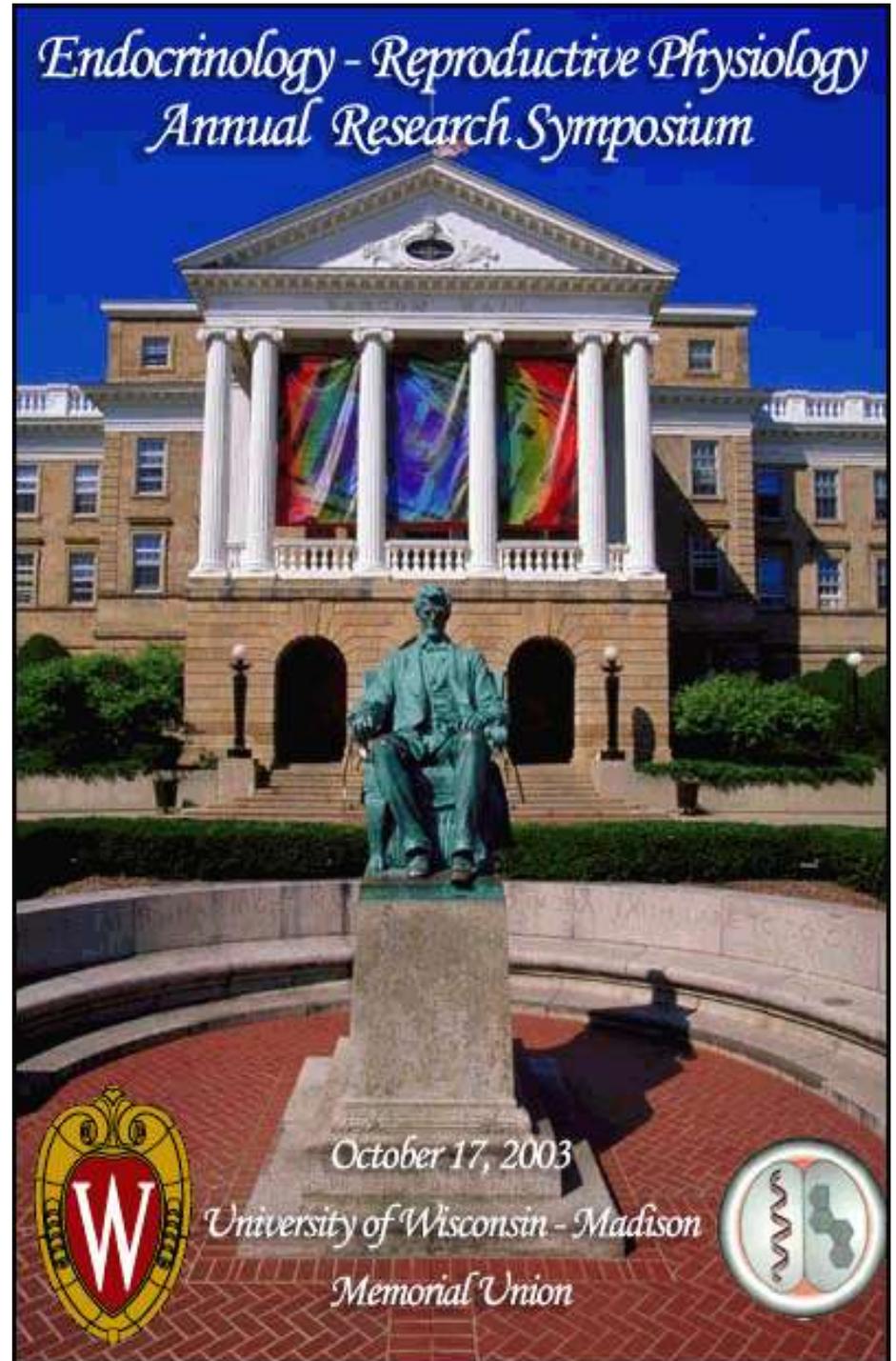
*Department of Obstetrics and  
Gynecology*

Department of Animal Health  
and Biomedical Sciences



Animal  
Sciences  
Department

**ZYMANGO**



October 17, 2003

University of Wisconsin - Madison

Memorial Union



## Notes

## Schedule

|            |   |
|------------|---|
| 8:00 am –  | <b>Registration, Poster Set-up, and Breakfast</b>   |
| 9:00 am    | Great Hall  |
| 9:00 am    | <b>Opening Remarks:</b><br>Class of '24 Reception Room<br><b>Dr. Ian Bird</b> , Endocrinology – Reproductive Physiology Program Director  |
| 9:00 am –  | <b>Opening Lecture:</b>   |
| 10:00 am   | Class of '24 Reception Room<br><i>“Hormonal Control of Gene Expression in the Ovarian Granulosa Cell”</i><br><b>Dr. Kelly Mayo</b> , Professor, Biochemistry, Molecular Biology, and Cell Biology, Northwestern University  |
| 10:00 am – | <b>Coffee Break</b>   |
| 10:15 am   | Great Hall<br>Sponsored by Animal Health and Biomedical Sciences Department   |
| 10:15 am - | <b>Concurrent Oral Sessions I and II:</b>   |
| 11:15 am.  |   |
|            | <b>Stem Cell Research:</b><br>Class of '24 Reception Room:<br>Chairs: Dr. Jing Zheng, Assistant Professor, Ob/Gyn<br>Dr. Jon Odorico, Assistant Professor, Surgery<br>10:15: <i>“Endothelial Cells Derived from Human Embryonic Stem Cells”</i><br><b>Yang Song</b> , Zheng Lab, ERP Program<br>10:45: <i>“Trophoblast Differentiation in Embryoid Bodies Derived from Human Embryonic Stem Cells”</i><br><b>Behhad Gerami-Naini</b> , Golos Lab, ERP Program   |
|            | <b>Hypothalamus/Pituitary Axis:</b><br>Capital View Room<br>Chairs: Dr. Ei Terasawa, Professor, Primate Center and Pediatrics<br>Dr. Theresa Duello, Associate Professor, Ob/Gyn<br>10:15: <i>“Estrogen Induces a Rapid Increase in the Frequency of Intracellular Calcium Oscillations in GT1-7 Cells”</i><br><b>Sekoni Noel</b> , Terasawa Lab, ERP Program<br>10:45: <i>“Acetaminophen Treatment of Cultured Rat Hypothalamic Explants Inhibits Pulsatile GnRH Release through Competitive Inhibition of COX1 and COX2 Enzymes”</i><br><b>Lauren Schmidt</b> , Woller Lab, UW-Whitewater |
| 11:15 am – | <b>Break</b>  |
| 11:30 am   |   |
| 11:30 am - | <b>Invited Speaker’s Lecture:</b>   |
| 12:30 pm   | Class of '24 Reception Room<br><i>“Real-time Determination of the Blood Flow and Local Secretion of Steroids, Prostaglandins and Vasoactive Peptides in the Bovine Preovulatory Follicle”</i><br><b>Dr. Tomas Acosta</b> , Research Scientist, AHABS, University Of Wisconsin - Madison   |

*Opening Speaker:*  
*Dr. Kelly E. Mayo*

Dr. Mayo is Professor of Biochemistry, Molecular Biology and Cell Biology at Northwestern University in Evanston, Illinois, where he has been a member of the faculty since 1985. He is jointly appointed in the Department of Neurobiology and Physiology. His laboratory studies gene regulation in the mammalian neuroendocrine system, and is interested in the synthesis and actions of hormones



that control key physiological processes such as growth and reproduction. His work on the actions of the brain peptide growth hormone-releasing hormone (GHRH) led his research team to identify a receptor for this peptide that is expressed in the growth hormone-expressing cells of the pituitary gland, and to demonstrate that a mutation in this receptor is responsible for a form of dwarfism in the mutant mouse strain *little*. His current work in this area focuses on understanding the regulation of GHRH receptor gene expression in the pituitary gland and investigating the signaling pathways activated by the GHRH receptor in pituitary cells. A second research area in the Mayo laboratory investigates hormones produced by the ovary that regulate reproductive function in mammals, focusing on the peptide hormones inhibin and activin.

His research team cloned genes encoding the multiple subunits ( $\alpha$ ,  $\beta_A$  and  $\beta_B$ ) of these two related hormones and established how their synthesis is controlled during the reproductive cycle of the rat. His current work in this area focuses on the molecular and cellular events underlying regulated gene expression in the ovary, and on animal models of pathophysiology related to inhibin and activin action. At Northwestern University, Dr Mayo is Director of the Center for Reproductive Science and Director of the Cellular and Molecular Basis of Disease Training Program. Nationally, he has significant involvement with the Endocrine Society, the Society for the Study of Reproduction, and a summer laboratory course at the Marine Biological Laboratories, *Frontiers in Reproduction: Molecular and Cellular Concepts*.

## ERP Faculty Contact Information

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## Concurrent Oral Sessions I and II

### *Stem Cell Research*

Class of '24 Reception Room

Chairs: Dr. Jing Zheng

Dr. Jon Odorico

### *Hypothalamus / Pituitary Axis*

Capitol View Room

Chairs: Dr. Ei Terasawa

Dr. Theresa Duello

## Abstracts 2-5

### Insulin Secretion and Action in Prenatally Androgenized Male Rhesus Monkeys.

\*CM Bruns<sup>1</sup>, ST Baum<sup>2</sup>, RJ Colman<sup>2,5</sup>, JR Eisner<sup>6</sup>, JW Kemnitz<sup>2,3,5</sup>, R Weindruch<sup>1,5,7</sup>, DH Abbott<sup>2,4</sup>.

<sup>1</sup>Medicine; <sup>2</sup>Wisconsin Primate Research Center; <sup>3</sup>Physiology; <sup>4</sup>Ob/Gyn; <sup>5</sup>Institute on Aging, University of Wisconsin, Madison, WI; <sup>6</sup>Cato Research, Durham, NC; <sup>7</sup>Geriatric Research, Education and Clinical Center, Veterans Administration Hospital, Madison, WI.

Women with polycystic ovary syndrome (PCOS) and their male siblings demonstrate abnormalities in insulin secretion and action. The prenatally androgenized (PA) female rhesus monkey, an animal model for PCOS, manifests similar glucoregulatory deficits. To determine whether prenatal androgen excess induces glucoregulatory deficits in male rhesus monkeys similar to those found in male siblings of women with PCOS, 7 PA and 7 control (C) male rhesus monkeys underwent a tolbutamide-modified, frequently sampled, intravenous glucose tolerance test (FSIGT). Both groups were similar in age (PA:12.46[7.25,17.66]; C:9.77[7.76,11.78] yrs; mean[95%CI]), body weight (PA:12.75[10.73,14.77]; C:12.79[10.75,14.84] kg), and BMI (PA:44.99[35.72,54.26]; C:43.15[38.54,47.75] kg/m<sup>2</sup>). Insulin and glucose values from the FSIGT were analyzed using the Modified Minimal Model method of Bergman. One PA male was identified as an outlier with elevations in both insulin sensitivity index (S<sub>I</sub>) (p<0.05) and disposition index (DI) (p<0.0001) and thus was excluded from analyses of Minimal Model parameters. There were no significant differences in basal circulating levels of glucose (PA:63.75[57.68,69.82]; C:58.84[55.56,62.12] mg/dl) or insulin (PA:53.20[32.53,73.87]; C:45.62[28.43,62.82] μU/ml). The acute insulin response to glucose (AIR<sub>G</sub>) was similar in the two groups (PA:94.41[32.40,275.11]; C:153.82[124.98,189.30] μU/ml). However, both S<sub>I</sub> (PA:0.80[0.18,3.64]; C:3.06[1.79,5.24] x10<sup>-4</sup>·min<sup>-1</sup>·μU<sup>-1</sup>·ml<sup>-1</sup>; p<0.05) and DI (PA:226.38[106.81,345.95]; C:509.21[354.69,663.72] min<sup>-1</sup>; p<0.02) were diminished in PA males. When the PA outlier was included in analyses of parameters derived from the Minimal Model, neither S<sub>I</sub> nor DI were significantly different between the male groups. Plasma total insulin levels after tolbutamide infusion (22-180 min), as determined by area under the curve (AUC) analysis, were increased in PA compared to C males (PA:14655.48[9374.84,22910.58]; C:8491.80[6163.72,11699.23] μU/ml·158min; p<0.03). Prenatal androgen excess may produce impairments in insulin secretion and action in male rhesus monkeys, as demonstrated in females. PA male rhesus monkeys.

### Trophoblast Differentiation in Embryoid Bodies Derived from Human Embryonic Stem Cells

B Gerami-Naini<sup>1</sup>, OV Dovzhenko<sup>1\*</sup>, M Durning<sup>1</sup>, RH Xu<sup>2</sup>, JA Thomson<sup>1,2,3</sup>, and TG Golos<sup>1,4†</sup>

Wisconsin National Primate Research Center<sup>1</sup>, WiCell Research Center<sup>2</sup>, University of Wisconsin-Madison, and the Departments of Anatomy<sup>3</sup> and Obstetrics & Gynecology<sup>4</sup>, University of Wisconsin Medical School, Madison WI 53715

Trophoblast differentiation and early placental development are essential for the establishment of pregnancy, yet these critical events are not readily investigated in human pregnancy. We used embryoid bodies (EBs) prepared from human embryonic stem (hES) cells as an *in vitro* model of early human development. The levels of hCG, progesterone and estradiol-17β in medium from hES cell-derived EBs grown in suspension culture for 1 week were higher than unconditioned culture medium or medium from undifferentiated hES cells or spontaneously differentiated hES cell colonies. EBs were explanted into Matrigel “rafts” and cultured for up to 53 days. During the first 7-10 days of growth in Matrigel, small protrusions appeared on the outer surface of EBs, some of which subsequently extended into multicellular outgrowths. The secretion of hCG, progesterone, and estradiol-17β began to increase on approximately day 20 of Matrigel culture, and secretion remained dramatically elevated over the next 30 days. EBs maintained in suspension culture failed to demonstrate this elevation in hormone secretion. Suspension cultured and Matrigel-embedded EBs exhibited widespread expression of cytokeratins 7/8, demonstrating extensive epithelial differentiation as well as consistent hCG expression. We propose that hES cell-derived EBs may be a useful model for investigation of human trophoblast differentiation and placental morphogenesis.

**Aspirin treatment of cultured rat hypothalamic explants inhibits pulsatile GnRH release through irreversible inhibition of COX1 and COX2 enzymes.**

**N Sabel, L Schmidt, D Waechter-Brulla, M Woller**

*Department of Biological Sciences, University of Wisconsin-Whitewater*

The release of pulsatile GnRH is influenced by a variety of signal molecules such as norepinephrine, Neuropeptide Y, GABA, and NMDA. Several studies have indicated that one important class of signal molecule with a role in the control of GnRH release is prostaglandins. We have treated cultured rat hemihypothalami in a perfusion system from hours 1-3 of culture with media containing aspirin at a dose approximating that suggested for humans (100µg/ml aspirin). Other wells of tissue were treated with vehicle (media without acetaminophen) for the same time period (hours 1-3). From hours 0-15, samples were collected at 10 min intervals, chilled immediately, frozen at 80C, and later assayed to measure GnRH concentration. The results of each experiment were blocked for the following times of the study: Pre-infusion hours 0-1 of culture; 1 hr infusion hours 1-3 of culture; 0-2 Post-infusion hours 3-5 of culture; 2-4 Post-infusion hours 5-7 of culture; 4-6 Post infusion hours 7-9 of culture; 6-8 Post infusion hours 9-11 of culture; 8-10 Post infusion hours 11-13 of culture; 10-12 Post infusion hours 13-15 of culture. Treatment with aspirin suppressed GnRH release significantly from the first block after treatment (0-2 Postinfusion;  $p < 0.001$  vs. preinfusion and vehicle) through the last block after treatment (10-12 Post infusion;  $p < 0.001$  vs. preinfusion and vehicle). These results are consistent with aspirin acting as an irreversible inhibitor of the COX1 and COX2 enzymes, which are critical for the synthesis of endogenous prostaglandins. In the future, we plan to extend our sample time to 24 hr to determine if the hypothalamic tissue will recover from the suppression of GnRH release induced by the 2 hr aspirin treatment. This work represents strong evidence for a role of prostaglandins in the coordinated release of pulsatile GnRH. This work was funded in part by an UW-Whitewater Undergraduate Research Grant to NS and LS.

**Acetaminophen treatment of cultured rat hypothalamic explants inhibits pulsatile GnRH release through competitive inhibition of COX1 and COX2 enzymes.**

**L Schmidt, N Sabel, D Waechter-Brulla, M Woller**

*Department of Biological Sciences, University of Wisconsin-Whitewater*

It has been reported in several model systems that prostaglandins can influence the release of GnRH. We have treated cultured rat hemihypothalami in a perfusion system from hours 1-3 of culture with media containing acetaminophen at a dose approximating that suggested for humans (20µg/ml acetaminophen). Other wells of tissue were treated with vehicle (media without acetaminophen) for the same time period (hours 1-3). From hours 0-15, samples were collected at 10 min intervals, chilled immediately, frozen at -80C, and later assayed to measure GnRH concentration. The results of each experiment were blocked for the following times of the study: Pre-infusion hours 0-1 of culture; 1 hr infusion hours 1-3 of culture; 0-2 Post-infusion hours 3-5 of culture; 2-4 Post-infusion hours 5-7 of culture; 4-6 Post infusion hours 7-9 of culture; 6-8 Post infusion hours 9-11 of culture; 8-10 Post infusion hours 11-13 of culture; 10-12 Post infusion hours 13-15 of culture. Treatment with acetaminophen suppressed nRH release significantly from the first block after treatment (0-2 Post infusion;  $p < 0.001$  vs. preinfusion) through the 4th block after treatment (6-8 Post infusion;  $p < 0.001$  vs. preinfusion), and recovered most of the GnRH release (8-10 Post infusion) to all of the GnRH release (10-12 Post infusion). These results are consistent with acetaminophen acting as a competitive inhibitor of the COX1 and COX2 enzymes, which are critical for the synthesis of endogenous prostaglandins. This study represents one step in critically evaluating the role of prostaglandins in the coordinated release of pulsatile GnRH from disparate neurons in the hypothalamus of the rat. This work was funded in part by an UW-Whitewater Undergraduate Research Grant to LS and NS.

### Effects of exogenous VS. endogenous Estradiol-17 $\beta$ and Progesterone on Blood Viscosity in Sheep

TJ Stow<sup>1</sup>, TM Phernetton<sup>1</sup>, D Meister<sup>1</sup>, L Gathers<sup>1</sup>, and RR Magness<sup>1,2,3</sup>

Departments of Obstetrics and Gynecology<sup>1</sup>, Animal Sciences<sup>2</sup>, and Pediatrics<sup>3</sup> School of Medicine and College of Agriculture & Life Sciences, University of Wisconsin-Madison, Madison, WI 53715

Blood viscosity, a parameter controlling shear stress, affects vascular endothelial cell shape and production of vasodilators (e.g. Nitric Oxide and PGI<sub>2</sub>). Experiments were conducted to evaluate effects of estradiol-17 $\beta$  (E<sub>2</sub> $\beta$ ) and progesterone (P<sub>4</sub>) on blood viscosity in pregnant (Preg; Gestational age = 120-130, n = 20; high E<sub>2</sub> $\beta$  + P<sub>4</sub>), follicular (Fol, n = 11; high E<sub>2</sub> $\beta$  low P<sub>4</sub>), luteal (Lut, n = 12; low E<sub>2</sub> $\beta$  high P<sub>4</sub>), and ovariectomized (n = 24) sheep. Ovariectomized ewes received no hormone replacement (Control, n = 6), E<sub>2</sub> $\beta$  (n = 4), or P<sub>4</sub> (n = 4) therapy. Blood samples were collected by jugular catheter during the estrous cycle and pregnancy, and analyzed for viscosity at various shear rates (6–134/sec) and hematocrits (20–40%). Ovariectomized ewes were administered P<sub>4</sub> via a CIDR pessary and E<sub>2</sub> $\beta$  was infused through jugular catheter for 10 days during which time 4 blood samples were collected and analyzed for viscosity. Magnitude of blood viscosity at similar physiological hematocrits and shear rates were: Ovariectomized Controls = P<sub>4</sub> = Luteal which were less than (P<0.05) E<sub>2</sub> $\beta$  = Fol = Preg. Individual and overall group comparisons demonstrate: 1) Ovariectomy and P<sub>4</sub> treatment did not substantially alter blood viscosity; 2) physiologic states of high estrogen (E<sub>2</sub> $\beta$  treatment, Fol, and Preg) significantly elevated blood viscosity. Because Preg is a state of both elevated E<sub>2</sub> $\beta$  and P<sub>4</sub>, whereas Fol phase sheep only have elevated E<sub>2</sub> $\beta$ , these data suggest E<sub>2</sub> $\beta$  rather than P<sub>4</sub> is primarily responsible for the observed increase in blood viscosity. It is known that elevated blood viscosity will increase shear stress especially when blood flow has risen. Moreover, shear stress will increase vascular endothelial cell production of vasoactive hormones in order to increase arterial diameter and lower vascular resistance. *Support: NIH HL49710, HD33255, HL57653, HD38843*

### Real-time determination of the blood flow and local secretion of steroids, prostaglandins and vasoactive peptides in the bovine preovulatory follicle

TJ Acosta

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Changes in ovarian blood flow are involved in the cyclic remodeling of the ovary that occurs during follicular growth, ovulation, and development of the new corpus luteum (CL). Therefore, two *in vivo* experiments were conducted to assess changes in ovarian blood flow and factors that may induce these changes in cattle. The objective of the first study was to characterize the real-time changes in blood flow within the follicle wall associated with ovulation and during early CL development. The objective of the second study was to examine local changes in the secretion of steroids, prostaglandin (PGs) and vasoactive peptides within the follicular wall and in the ovarian venous plasma (OVP) during the periovulatory period.

In the first study, cycling cows were examined by transrectal color and pulsed Doppler ultrasonography to determine the blood flow within the wall of the preovulatory follicle and in the early CL. Ultrasonographic examinations began 48 h after a luteolytic injection of PGF<sub>2</sub> $\alpha$  during the mid luteal phase of the estrous cycle. Cows showed a clear rise in the plasma levels of LH (LH-surge) followed by ovulation 26 to 34 h later. In the color Doppler image of the preovulatory follicle, blood flow before the LH-surge was detectable only in a small area at the base of the follicle. However, an acute increase follicular blood flow was detected synchronously with the initiation of the LH-surge.

In the second study, the local release of vasoactive peptides, such as angiotensin (Ang) II, endothelin (ET)1 and atrial natriuretic peptide (ANP) were determined by surgical implantation of a microdialysis system (MDS) within bovine follicles *in vivo*. Prostaglandin F<sub>2</sub> $\alpha$  concentrations in OVP were high during the pre-LH surge period and dropped towards the onset of the LH surge. Acute increases in PGF<sub>2</sub> $\alpha$  concentrations in the OVP and in the MDS perfusates were observed around the time of ovulation. Follicular Ang-II release increased at the beginning of the LH surge. Concentrations of Ang-II peaked in the OVP at 24 to 48 h after the peak of LH surge and remained at a higher level than that in the JVP. These results demonstrate for the first time the local release of Ang-II, ET-1 and ANP from the bovine mature follicle *in vivo*. In addition, these data show that Ang-II and PG concentration in the OVP acutely increases around the time of ovulation.

Collectively, the complex structural, secretory and functional changes that take place in the ovary around ovulation are closely associated with a local change in the blood flow within the wall of the preovulatory follicle. Overall, these results suggest that the actions of vasoactive peptides, PGs, and steroids play key roles in the LH-triggered ovulatory cascade in the bovine preovulatory follicle.

## Maternal-Fetal Immune Tolerance of Pregnancy in Primates

**J Drenzek and TG Golos**

*Wisconsin National Primate Research Center and the Department of Obstetrics and Gynecology  
University of Wisconsin Medical School, Madison, WI*

The challenge of establishing maternal-fetal immune tolerance begins at implantation. Trophoblasts make intimate contact with the uterine epithelium by invading maternal endometrium vessels to initiate blood flow to the intervillous space. During pregnancy, the fetus expresses both maternal and paternal major histocompatibility complex (MHC) class I classical molecules such as human leukocyte antigen (HLA)-A, HLA-B, and HLA-C. Trophoblasts express nonclassical MHC class I molecules such as HLA-G in humans and *Macaca mulatta* (Mamu)-AG in rhesus monkeys. Suggested actions of nonclassical placental molecules include protecting the fetus and placenta from a maternal immune response, promoting normal implantation and placental development, and regulating the cytokines in the pregnant uterus. My hypothesis is that placental MHC class I molecules down-regulate a uterine immune response to promote healthy pregnancy. This is important in the generation of maternal-fetal immune tolerance. My projects are designed to modify placental function in the rhesus monkey. I plan to knockdown endogenous gene expression of Mamu-AG by using RNA interference (RNAi). Mamu-AG is considered to be a homolog of HLA-G. Mamu-AG is expressed in the placenta and has several features in common with HLA-G including a shortened cytoplasmic domain and expression in multiple isoforms. I also plan to manipulate gene expression by overexpressing the alloantigen Mamu-A\*01 through the use of lentiviral vectors. Mamu-A\*01 is a nonplacental expressed transgene. My current work has centered on performing transient transfections on suspension cells using the plasmid that I have constructed. This plasmid contains human elongation factor 1 alpha (EF1 $\alpha$ ) as the promoter and Mamu-A\*01 as the reporter gene. I have been using the FuGENE 6 transfection method to transfect 721.221 cells, which is a mutant cell line in which the cells do not express surface MHC class I molecules. I have also used K562 cells, which is an erythroleukemia cell line derived from a chronic myeloid leukemia patient. I have yet to obtain satisfactory gene expression efficiency using the FuGENE 6 transfection method. Upon efficient transient transfection of this plasmid, I will be able to prepare lentivirus and microinject it into rhesus embryos.

## Stem Cells and Gametes: the Cycle of Life

**AA Kiessling**

*Department of Surgery, Harvard Medical School*

For many decades, cleaving eggs have been recognized as the source of totipotent embryonic stem (ES) cells, including daughter eggs. If the cleaving egg was fertilized with a Y-chromosome bearing sperm, the totipotent ES cells are also the source of daughter spermatozoa. This cycle requires elegantly orchestrated gene expression. The egg is not the penultimate totipotent cell because it is, itself, a highly differentiated cell with its own peculiar cell cycle. Therefore, the first step in the derivation of totipotent ES cells is de-differentiation of the egg. If it is fertilized, the process of de-differentiation must also include the sperm nucleus. In mammals, the totipotent state lasts only a few cell cycles, on the order of four or five doublings. The cycle of life, therefore, includes a fleeting interlude of totipotent cells amidst a lifetime of committed cells, most fully differentiated. Understanding the totipotent interlude may be the key to fully understanding the potential pitfalls faced by an activated egg.

**Acute changes in plasma progesterone following prostaglandin F<sub>2α</sub> or oxytocin treatment during the mid or late luteal phase in cycling pony mares**

**MD Utt<sup>1,3</sup>, OJ Ginther<sup>1</sup>, TJ Acosta<sup>1</sup>, MC Wiltbank<sup>2,3</sup>**

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Prostaglandin F<sub>2α</sub> (PG) and oxytocin (OT) have been implicated as having a pivotal role in the luteolytic mechanism of the mare. The capacity of these hormones to alter circulating levels of progesterone immediately following injection may be differentially affected when administered at differing times of luteal development. The objective of this study was to determine the effect of prostaglandin F<sub>2α</sub> (PG) or oxytocin treatment, during the mid (8 d post ovulation) and late (13 d post ovulation) luteal phase, on plasma progesterone concentrations during the 60 min following treatment. Pony mares (300 kg) were randomly assigned to receive PG (2.5 mg, i.v.), OT (10 i.u., i.v.), or control (SAL; 0.5 ml sterile saline, i.v.) treatments 8 d (n = 7, n = 6, n = 7; respectively) or 13 d (n = 7, n = 7, n = 7; respectively) post ovulation. Prior to administration of experimental treatments, mares were fitted with an indwelling jugular vein catheter. Two blood samples were taken prior to administration of experimental treatments to determine baseline progesterone concentration prior to treatment. Following treatment, samples were collected at 1 min intervals from 1 to 5 min post treatment and at 5 min intervals from 5 to 60 min post treatment. Data are expressed as the change in plasma progesterone (ng/ml) relative to the average of the two baseline samples. Because there was no effect of day of experimental treatment (d 8 or d 13) or a day by time interaction within PG, OT, or SAL treatments (P > 0.05), data for treatment on experimental d 8 and d 13 within PG, OT, or SAL treatment were combined. Treatment with PG resulted in increased plasma progesterone relative to baseline within one minute following treatment (1.24 ± 0.34 ng/ml; P < 0.05) and peaked within 10 min (1.95 ± 0.36 ng/ml; P < 0.05). Plasma progesterone concentrations of mares treated with PG were significantly lower than baseline values in PG treated mares by 40 min following treatment. Oxytocin treatment did not significantly alter plasma progesterone concentrations. In conclusion, data from this experiment demonstrate an acute increase in plasma progesterone concentrations in pony mares following PG treatment prior to the decrease in progesterone normally associated with PG administration.

**Erythropoietin Regulates Growth and Function of Lung Epithelial Cells**

**M Litchman, AK Kapur, DP Carlton and PJ Kling.**

*UW Perinatal Center and Department of Pediatrics*

Lung development and maturation are regulated by a number of different molecular signals, including growth factors. Erythropoietin is a circulating growth factor traditionally considered important in the regulation of hematopoietic cell growth, but now recognized as mitogenic for variety of different cell types. Because erythropoietin and the receptor for erythropoietin are expressed in the human lung during development, we considered the possibility that erythropoietin might play a role in the regulation of lung growth. To test this notion, we studied the effect of erythropoietin on A549 cells, an immortal adenocarcinoma cell line that possesses morphological and biochemical characteristics similar to the respiratory epithelium in the distal airspace. We found that after 4 days of treatment, erythropoietin (50 U/mL) increased A549 cell protein by 25% (control: 47.8 ± 7.7 vs erythropoietin: 58.5 ± 5.8 mg/well; mean ± SD, n = 10, p < 0.05). The increase in protein was accompanied by a similar proportional increase in cell number, indicating that erythropoietin treatment resulted in cell hyperplasia and not simply hypertrophy (control: 1.54 ± 0.36 vs erythropoietin: 2.05 ± 0.38 million cells/well, n = 10, p < 0.05). To see if this increase in growth was accompanied by changes in cell function, we studied whether erythropoietin influences transmembrane Na movement. Na transport, and particularly Na transport through channels that are inhibited by the diuretic amiloride, is an important characteristic of the respiratory epithelium because of the relationship between Na movement and the clearance of water from the distal airspaces. We found that erythropoietin (50 U/mL, 4 d exposure) increased the rate of total Na uptake by 30% (control: 214 ± 128 vs erythropoietin: 278 ± 169 nmol Na/mg protein/5 min, n = 10, p < 0.05) and that most of the increase in Na transport could be accounted for by movement through amiloride-sensitive pathways (control: 180 ± 122 vs erythropoietin: 238 ± 159 nmol Na/mg protein/5 min, n = 10, p < 0.05). Thus, erythropoietin accelerates growth and increases Na transport in respiratory epithelial cells *in vitro*. We speculate that erythropoietin plays a physiological role in normal lung development.

### Relationship Between AT1-R and P450c17 Expression In The Marmoset Adrenal.

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Previously we have described the characterization of P450c17 and related protein expression in the marmoset adrenal (Endo 2002). The marmoset model is of particular interest due to its relative lack of a functional zona reticularis (ZR) compared to both humans and rhesus. One particularly important modulator of P450c17 expression in the mammalian adrenal is angiotensin II (AII), acting through the angiotensin II type 1 receptor (AT1-R). Therefore, in order to understand the distribution of P450c17 in the marmoset adrenal, we performed immunohistochemistry on adrenals from six adult male marmosets to investigate the expression of the AT1-R. The AT1-R stained most strongly in the zona glomerulosa (ZG), as was expected since AII stimulates aldosterone production in this region. The ZG is negative for P450c17, where AT1-R is highest, consistent with an enzyme expression pattern that favors aldosterone production. The AT1-R was expressed at a reduced level throughout the remainder of the cortex, sometimes up to the cortico-medullary interface. We have previously shown that P450c17 stains consistently throughout the zona fasciculata/reticularis (ZF/R), thus the expression of AT1-R in the ZF/R, although at much lower levels, is not surprising and is consistent with the possible role in modulating P450c17 expression. The AT1-R was undetectable in the medulla. Consequently, the marmoset can be grouped with other species whose medulla does not express AT1-R. Adrenocortical cells freshly isolated from whole adrenal glands stained for P450c17 and AT1-R show P450c17 expression at a consistent level in all cells while AT1-R expression is lower and more diffuse. Long-term stimulation of these cells with Forskolin, AII, TPA and the combination of Forskolin with AII or TPA showed dramatic increases in P450c17 expression in response to Forskolin, but not in response to AII or TPA. The Forskolin-induced expression of P450c17 was blocked by addition of either AII or TPA with Forskolin. These changes in P450c17 expression are consistent with other work done on ovine, bovine and human adrenocortical cells, indicating normal endocrine function of the marmoset adrenal. Further studies will be necessary to determine the extent to which AII, and other agonists, regulate P450c17 expression in the marmoset. Funded by NIH grants MH60728, HL 64601, and HL 56702.

### Increased Proceptive Sexual Behavior in the Female Common Marmoset after Intracerebroventricular (icv) Administration of GnRH II.

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GnRH II (pGlu-His-Trp-Ser-Trp-Gly-Leu-Arg-Pro-Gly•NH<sub>2</sub>), an evolutionarily conserved gonadotropin releasing hormone first isolated from chicken brain, plays a role in the reproductive behavior of a number of non-primate species. To investigate whether GnRH II similarly plays a role in primate sexual behavior, six female marmosets (2-4 years of age) were surgically implanted with an indwelling cannula in the third ventricle of the brain for intracerebroventricular (icv) administration of GnRH II. Subject animals were also ovariectomized, implanted subcutaneously with silastic capsules (3 females with empty implants and 3 with estradiol filled implants) and pair-housed with their mate. Behavioral sex tests were conducted to (1) determine behavioral responses to icv infusions of 0 (vehicle), 1, and 10 µg of GnRH II and (2) determine if GnRH II effects are estrogen dependent. Females were out of visual contact from their male pairmate for 90 minutes after icv infusion before the start of the behavioral test. At the start of each test, male pair-mates were introduced into the test cage through a remote door and behaviors were scored for 30 minutes by observers behind a one-way mirror. For analysis, mean values from each animal at each dose were used in a repeated measures analysis of variance with post hoc tests to determine significant differences between means. GnRH II significantly increased (p=0.043) the total number of proceptive [sexual solicitation] behaviors (tongue flicking, proceptive stare and frozen posture) a female exhibited towards her male pairmate during behavioral tests. Proceptive behavior was virtually absent when females receive vehicle infusion in both estradiol + and - conditions. GnRH II stimulated proceptive behavior at 1µg (significantly higher than control treatment; p=0.009, but not different from 10µg). Infusion of 10µg GnRH II did not significantly increase female proceptive behavior over that observed following vehicle infusion (p=0.148). Proceptive behavior from GnRH II tended (p=0.067) to be more pronounced in estradiol - females, although the current statistical interaction of hormone and GnRH II dose is not significant (p=0.074). The reduced effect of GnRH II in estradiol + females may reflect the trend for fewer mounts preceding intromission and ejaculation by the male. The females may be more attractive, receptive or both in the estradiol + condition. When females receive no estradiol or GnRH II, male pair-mates fail to mount the female in 33% of the behavioral tests. In contrast, the mount failure rate was 11-12.5 % when females received either estradiol or GnRH II. GnRH II reduced the mount failure rate in estradiol -, but not in estradiol + females. Financial Support from Ardana Biosciences LTD.

### **Microfluidic Devices in Embryology**

**AL Reeder**

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Modern embryology encompasses several emerging areas of interest in human health including cloning, stem cells, chimeras, and *in vitro* fertilization. Nanotechnology is currently being applied to this area in order to optimize the assisted reproductive technologies listed above. And biomimetics, especially microfluidic technology, is at the forefront of technologic advancement in embryology. Microfluidic devices work on a micro or nano scale to simulate physiologic conditions. The goal of this nanotechnology is to provide individual cells with a unique, manipulatable environment.

Microfluidic technology is currently being applied in our laboratory to *in vitro* embryo production. We are optimizing culture and micromanipulation devices in the bovine *in vitro* fertilization process. My research utilizes microfluidic devices collaboratively designed and fabricated in Bio-Medical Engineering in order to treat embryos in a microenvironment that is more representative of *in vivo* development. Microfluidic culture devices have been optimized for murine embryo culture, and porcine work is in progress. Our laboratory is optimizing embryo culture devices for the bovine model and testing two micromanipulation devices. Utilizing cumulus cell removal devices, we have shown improved embryonic development when cumulus cells were removed by microfluidic manipulation as opposed to traditional vortex methods.

## **Poster Session**

**Great Hall**

**Abstracts 12-34**

### Ovine Endothelial Nitric Oxide Synthase Transiently Expressed in COS-7 Cells is Negatively Regulated by ERK-1/2 and Protein Kinase C

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While endothelial nitric oxide synthase (eNOS) is capable of activation by Ca<sup>2+</sup>/calmodulin (CaM), recent progress has suggested that eNOS phosphorylation increases sensitivity to [Ca<sup>2+</sup>]<sub>i</sub>. eNOS in uterine artery endothelial cells undergoes pregnancy-specific enhancement of activity associated with both increased Ca<sup>2+</sup> and ERK-1/2 signaling. The objective of this study was to determine the extent to which Ca<sup>2+</sup> and kinase signaling pathways influence eNOS activation alone or together. Therefore, we describe activation and phosphorylation of ovine eNOS cDNA transiently expressed in COS-7 cells. A23187-stimulated activation of eNOS was accompanied by phosphorylation of S1179 but not T497, suggesting increases in [Ca<sup>2+</sup>]<sub>i</sub> alone may not be the only mechanism of activation. In contrast, phorbol 12-myristate 13-acetate (PMA) did not elevate [Ca<sup>2+</sup>]<sub>i</sub> or increase eNOS activity but increased phosphorylation of both S1179 and T497. Both agonists also stimulated activation of Akt and ERK1/2.

Inhibitors of phosphatidylinositol 3-kinase (PI3-K), mitogen activated protein kinase-kinase (MEK), and protein kinase C (PKC) were used to determine the participation of these pathways in eNOS activation. Inhibition of PI3-kinase pathway did not change eNOS activity despite decreased p-S1179. Inhibition of MEK enhanced A23187- but not PMA-mediated eNOS activity but did not change T497 or S1179 phosphorylation. Finally, inhibition of PKC decreased basal and PMA-induced p-T497 and p-S1179 and increased A23187/PMA-stimulated eNOS activity. We conclude that when [Ca<sup>2+</sup>]<sub>i</sub> is not elevated, Akt signaling is not sufficient for eNOS activation. When [Ca<sup>2+</sup>]<sub>i</sub> is elevated, Akt activity may fail to contribute to eNOS activation, and ERK-1/2 activity may attenuate eNOS activation independent of phosphorylation of S1179 or T497.

### Transgenic Strategies for Non-human Primate Embryo and Placental Development

**T Chang\* and TG Golos**

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Transgenic technology in the non-human primate provides an animal model that can enhance the study of human biology including embryology, physiology, endocrinology and pathology. Numerous attempts and results from previous studies had not successfully established a consistent model for primate transgenesis. Therefore, efficient methods to produce sufficient expression of transgenes are important.

The first objective of current projects is producing overexpression of specific genes functioning in non-human primate placenta and embryo development by using lentiviral transgenic methods. Specific genes including IGF2, CG, and MHC-I, are required for normal and programmed primate placental and fetal development through pregnancy. Theories derived from rodent experiments suggested that regulation of insulin-like growth factor II (IGF-II) expression is controlled by epigenetic mechanisms including genomic imprinting. Chorionic gonadotropin (CG), a glycoprotein hormone, is the major embryonic signal in primates. During implantation and pregnancy in primates, CG is primarily secreted by the syncytiotrophoblast. Previous studies showed CG is essential for establishing implantation at the early pregnancy. The third target, MHC class I (MHC-I), is essential in maternal-fetal immune response and tolerance. Detailed roles and regulation of those genes have not been well understood in human and non-human primates. Through studies and prospective data obtained from the non-human primate model approach, it can provide information on specific functions of those genes.

The second project progressing is focusing on the down regulation of gene expression in primate cell lines and during embryo development by methods as RNA interference (RNAi) and/or antisense transgenic approaches. In the past decades the application of knock-out rodents has provided studies accelerating our knowledge on genetics and physiology. But again, the difference between rodent placenta and primate placenta physiology indicates that rodent model is not sufficient enough for understanding human biology. Though non-human primates is the best model, the well established knock-out method can not be applied on primates due to the long time frame of pregnancy, juvenile years to puberty and life span. There also lays the possibility of lethal situation during early fetal and placental development when homozygous genes by knock-out are generated. Therefore, down regulation by using antisense transgenic technology and newly developed RNAi technology on mammalian cells can be used as novel and promising methods to study gene expression and regulation in embryogenesis and pregnancy.

The current progress including successful transfection and overexpression of genes has been demonstrated in *in vitro* experiments, while *in vivo* experiments including microinjection into rhesus monkey and marmoset embryos, along with down regulation approaches, are being continuously proceed. Combining the progress of *in vitro* transgene regulation methods and developing efficient *in vivo* approaches focusing on primate embryo and placental development, transgenic technology in non-human primate can enhance our understanding on gene expression and regulation and will accelerate the knowledge of medical primatology and human reproductive biology.

### Reconstitution of frozen-thawed rooster sperm in seminal plasma

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The objective of this experiment was to determine if the addition of seminal plasma to a frozen-thawed avian species sperm could recover viability, as has been previously demonstrated in the ram. Semen was collected from roosters by massage technique and extended in Wisconsin Avian Extender (WISA) with 14% glycerol. Semen was then frozen in LN<sub>2</sub> and later thawed for 1 min. at 35°C. Seminal plasma was collected by centrifuging separate semen collections at 12000 rpm for 5 min, collecting the upper 0.5 ml of supernatant. This was then done a second time and the final supernatant frozen at -22°C until use. Thawed semen was diluted to 10 million/ml in WISA and added to three experimental treatments of 1:1, 1:2, and 1:3 volumes of semen to seminal plasma. The control was a 1:1 volume of semen to WISA without glycerol. 4-ml tubes were injected with CO<sub>2</sub> for one second and incubated at 39°C and 5% CO<sub>2</sub>. There were 3 replicates of four treatments (control, 1:1, 1:2, 1:3) with each treatment done in triplicate. Aliquots were assessed for motility and viability at 0, 3, and 6 hours of incubation. Motility was both video-recorded for replay and assessed at 10X while viability was assessed by the live:dead fluorescent staining assay. ANOVA analysis showed that there were significantly more sperm with intact membranes ( $p < 0.01$ ) for each treatment relative to the control at both 3 hours and 6 hours. At 3 hours, motility was significantly higher in 1:1 and 1:2 dilutions ( $p < 0.05$ ) than the control but 1:3 was not different ( $p < 0.05$ ). At 6 hours there were no significant differences between the motility of the seminal plasma treatments and the control ( $p < 0.05$ ). In conclusion, this experiment demonstrates that seminal plasma helps to restore the membrane integrity of rooster semen but that motility is not restored.

### Estrogen induced Chk-1 activation in MCF-10F cells line

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Estrogens have been shown to play a central role in breast cancer development, the carcinogenicity of estrogen on human breast cancer is has not been fully characterized. In this study, we utilized the estrogen-receptor negative human breast epithelial cell line, MCF-10F as our model to study the role of estrogen in breast cancer. Because the tumor suppressor protein p53 and cell cycle checkpoints protein play important roles in carcinogenesis. We investigate the effects of 17 $\beta$ -Estradiol (E2) on the level of p53 and Chk-1 protein in MCF-10F cells. Flow cytometry experiment revealed that E2 (10<sup>-6</sup>M) treatment caused an S phase arrest in both primary breast epithelia cells and MCF-10F cells. E2 (10<sup>-9</sup>M~10<sup>-6</sup>M) induced a 2-4 fold increase of Chk-1 protein, 2-4 fold increase of P53 in a dose dependent manner. The results indicate that the S-phase arrest of E2 treatment is estrogen receptor independent and through activation of Chk-1 pathway.

### Functional Characterization of HUVEC-C: Calcium Signaling, Mitogenesis, ERK 1/2 Activation, and Vasodilator Production

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The commercially available, immortalized human umbilical vein endothelial cell line (HUVEC-C) is potentially a useful tool to study endothelial cell function, yet little research has been done to characterize these cells. The purpose of this study is to determine if HUVEC-C maintain established endothelial cell characteristics and to functionally characterize mitogenesis, kinase activation, and vasodilator production. HUVEC-C is a valid endothelial cell model because they exhibit positive AcLDL uptake and express eNOS. Immunolocalization of PCNA and KI67 showed that the cells are healthy and proliferating under our culture conditions.

Ca<sup>2+</sup> signaling may be important for mitogenesis, kinase activation, and vasodilator production. Therefore, Ca<sup>2+</sup> imaging was performed to determine which agonists could cause a rise in the intracellular free calcium concentration ([Ca<sup>2+</sup>]<sub>i</sub>). ATP induced a rise in [Ca<sup>2+</sup>]<sub>i</sub> in a dose dependent manner. It appears that ATP binds to P2Y receptors rather than P2X receptors since after thapsigargin pretreatment emptied internal stores, ATP was unable to initiate a response. AII, bradykinin, EGF, and VEGF (but not bFGF) can cause a rise [Ca<sup>2+</sup>]<sub>i</sub> in a subset of the cells. Nifedipine pretreatment did not decrease the ATP-induced Ca<sup>2+</sup> response nor did BAYK8644 cause an increase in [Ca<sup>2+</sup>]<sub>i</sub>. This suggests L-channels are nonfunctional in HUVEC-C.

Thymidine incorporation assays were used to ascertain which agonists could induce mitogenesis. ATP, bFGF, EGF, and VEGF induced mitogenesis, while AII and insulin did not. This implies that a rise in [Ca<sup>2+</sup>]<sub>i</sub> is not essential for induction of mitogenesis since bFGF did not cause a rise in [Ca<sup>2+</sup>]<sub>i</sub> but did stimulate mitogenesis. Similarly, kinase activation must not require calcium mobilization because ATP, bFGF, EGF and VEGF all caused a rise in ERK 1/2 activation.

Western analysis revealed that the HUVEC-C express some of the key proteins necessary for vasodilator production. As in primary HUVEC, these cells express eNOS, HSP 90, cav 1 and 2. This data would suggest that HUVEC-C can produce nitric oxide therefore eNOS activation assays were performed to verify the model. The experiments revealed that EGF, VEGF, and ATP induced arginine to citrulline conversion. Furthermore, since ATP caused a robust increase (~8 fold), which is higher than is commonly seen in the literature for a physiological agonist, this is an excellent model with which to study eNOS activation. As in primary HUVEC, HUVEC-C also express cPLA2, COX-1, and COX-2 however, PGI2 was not detectable by western analysis. Research on primary HUVEC has demonstrated that PGI2 production is minimal thus this cell line is likely not a competent model with which to study PGI2 production.

### Insulin Receptor Isoform Expression in Mural Granulosa and Cumulus Cells From Human Ovarian Follicles

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The role of insulin receptors (IR) in human folliculogenesis is not well understood. Two IR isoforms exist, resulting from alternative splicing of exon 11 and differing by absence (IRA) or presence (IRB) of 12 amino acids in the alpha subunit. IRA is mitogenic and predominates in some fetal and tumor cells; IRB has metabolic effects and predominates in adipose, liver and muscle. To determine whether IRA and IRB exist in human luteinized mural granulosa (MG) and cumulus cells (CC) obtained during in vitro fertilization (IVF) and whether one IR isoform predominates, 11 nonhirsute ovulatory women without diabetes were studied [age, 31 years (range, 27-34 years); body mass index (BMI), 23.9 kg/m<sup>2</sup> (range, 18.5-39.8 kg/m<sup>2</sup>)]. All women received recombinant human follicle stimulating hormone (rhFSH) therapy with pituitary downregulation. At oocyte retrieval, MG and CC were obtained by aspirating the first follicle, which was chosen by size (i.e., ≥15 mm in diameter), with all such follicles containing an oocyte (10 metaphase II, 1 atretic). Cells were counted by hemocytometer before storage in TRIzol for later ribonucleic acid (RNA) extraction. Real-time polymerase chain reaction, using primers and probes specific for IRA, IRB and 28S ribosomal RNA, quantified gene expression in MG and CC of the same follicle, compared to standard curve dilutions. Multiple regression analysis compared log IRA and IRB messenger RNA (mRNA) transcript number by cell type (i.e., MG versus CC). There was no cell type effect on mean mRNA transcript number for IRA (MG, 15827; CC, 15717 per 10<sup>8</sup> 28S mRNA) or IRB (MG, 4365; CC, 2366 per 10<sup>8</sup> 28S mRNA)(p=0.5). In both cell types combined, mRNA expression of IRA was significantly greater than that of IRB (p=0.0004), with IRA representing 81% of all IR mRNA. In the same follicle, there was a positive correlation between MG and CC for mRNA expression of IRA (r<sup>2</sup>=0.80) and of IRB (r<sup>2</sup>=0.72). BMI and glucose tolerance were not correlated with IR mRNA expression. In luteinized MG and CC of normal women receiving rhFSH therapy for IVF, IRA and IRB transcription occurs, is similar between the two cell types, and is differentially regulated such that IRA is the predominant isoform. IR heterogeneity in luteinized granulosa cells introduces a novel regulatory mechanism by which insulin may direct mitogenesis, maturation and steroidogenesis of the developing human follicle.

### Evidence for the Involvement of Raf -1 in ERK 1/2 activation but not Prostacyclin production in an Ovine Uterine Artery Endothelial Cell Culture Model (UAEC).

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Many of the physiological changes during pregnancy coincide with changes at the level of intracellular signaling. Whether the physiological change leads to the signaling change or vice versa is highly speculative and a plausible argument can be made for either being primary. Our lab employs a unique Ovine Uterine Artery Endothelial cell culture model that retains some of the observed physiological changes (increased vasodilator production, refractoriness to infused vasoconstrictors) between pregnant (PUAEC) and nonpregnant (NPUAEC) ewes. An observation we have reported previously is that PUAEC have a relatively higher agonist induced activation of ERK 1/2 than NPUAEC. ERK 1/2 activation has been implicated in many different cellular functions, including vasodilator (Nitric Oxide, Prostacyclin) production and mitogenesis. Our lab addresses not only events “downstream” of ERK 1/2 activation, such as altered expression of pregnancy related genes and vasodilator production, but also events “upstream” of ERK 1/2 that lead to ERK 1/2 activation: which may differ depending on the treatment administered and the physiological condition of the animal. One protein often implicated in the initiation of the ERK 1/2 cascade by growth factors as well as more classic hormones is the protein kinase Raf. The actions of Raf phosphorylating/activating MEK1, which subsequently activates ERK 1/2 are well documented. In light of the difference in activation of ERK 1/2 between our P and NPUAEC, we decided to investigate the contribution of one of the two Raf isoforms we have identified in our model, Raf-1 (c-Raf). To examine the activity of Raf in our model, we employed a double step radioactive labeling protocol consisting of immunoprecipitating Raf from treated and control cultured cells followed by incubation with a competent MEK1 and the subsequent incubation with a GST-ERK1/2 protein. In our preliminary investigations, we observe no change in substrate P32 incorporation upon agonist stimulation (ATP, AII, bFGF, EGF, VEGF) and control treatments in both P and NPUAEC. In lieu of dominant negatives we are using a reasonably selective inhibitor of Raf-1, GW 5074, to investigate the contribution of Raf in prostacyclin (PGI<sub>2</sub>) production. Using an EIA to the stable metabolite of prostacyclin, 6-keto PGF<sub>1</sub> alpha, we observe no inhibition of ATP induced PGI<sub>2</sub> production with GW 5074 (.02-2U<sub>M</sub>). Although preliminary, we do see a trend toward dose dependent (.02-2uM) inhibition of bFGF and ATP stimulated ERK 1/2 activation using phospho-specific Western analysis. We observe no change in Raf S259 phosphorylation upon agonist stimulation: one of many possible regulatory phosphorylation sites on Raf. The extent of the contribution of Raf-1 in normal endothelial functioning in the pregnant and nonpregnant ewe is obviously difficult to assess. Our observations do not point to a difference in Raf-1 activity between the P and NPUAEC. Raf-1 activity also does not appear to be obligatory for PGI<sub>2</sub> production, however, it does seem to be involved in ATP and bFGF induced ERK 1/2 activation. What role Raf-1 may exert in P and NPUAEC nitric oxide production and other associated endothelial functions is also a matter of investigation in our lab at this time.

### Luteal Phase Defects in Prenatally Androgenized Female Rhesus Monkeys

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Ovulatory menstrual cycles are infrequent or absent in women with polycystic ovary syndrome PCOS). When ovulatory cycles occur, they can be accompanied by a deficient luteal phase (1). A similar dichotomy in ovulatory function is exhibited by a nonhuman primate model for PCOS, the prenatally androgenized (PA) female rhesus monkey. Luteal phase defects, however, are only reported for adolescent PA females (2). To investigate whether ovulatory function in mature adult PA monkeys is accompanied by luteal phase defects, 6 female rhesus monkeys, exposed to 15-35 days of testosterone propionate (TP) therapy, starting on gestational days 40-44, and 6 control (C) females were studied. Age (C:18.98±1.97 years; PA:20.931.49 years; mean ±SEM), body weight(C:8.54±1.00; PA:8.68±1.02kg), and BMI (C:37.61±5.20; PA:37.60±2.59 kg/m<sup>2</sup>) were similar in both female groups. Serum progesterone concentrations were determined by EIA in thrice weekly samples collected over 5-6 consecutive months, avoiding the summer period of potential oligomenorrhea. ovulatory menstrual cycles were confirmed by two or more serum progesterone levels ≥1 ng/ml within 15 days before menses. PA female monkeys exhibited fewer ovulatory cycles compared to controls (C:6.33±1.03; PA:3.83±1.94 cycles; p<=0.02) due to an extended (p<=0.03) duration of the overall cycle from 26.47±5.53 days in C females to 40.27 ±24.64 days in PA females. An increased duration of the follicular phase (C:11.68±5.00 days vs. PA:28.10±26.43 days) contributed significantly (p<=0.02) to the extended cycles of PA females. In addition, luteal phase abnormalities were detected. Peak progesterone levels (C:5.84±2.49; PA:3.82±2.14 ng/ml; p<=0.03) and total serum progesterone levels, as measured by area under the curve (AUC) analysis (C:42.38±16.79; PA:26.69±16.52 (ng/ml)·day; p<=0.03), were both diminished in PA compared to C females. Luteal phase duration also tended (p<=0.06) to be shorter in PA (13.46±4.02 days) compared to C (15.40±1.83 days) females. Our findings suggest that early prenatal exposure of female rhesus monkeys to TP not only impairs ovulatory function, but also compromises luteal function in adulthood. It remains to be determined whether deficient LH support of the corpus luteum or abnormal preovulatory follicular development provide a causal mechanism. Financial Support: NIH R01 RR13635.

### Effect of Fibroblast Growth Factors (FGFs) on Pancreatic Precursors from Embryonic Stem Cells

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Due to their potential to differentiate into all three embryonic germ layers including the endoderm from which the pancreas develops, embryonic stem (ES) cells may provide an unlimited supply of pancreatic islet cells for transplantation to treat diabetes. Though ES cells are capable of differentiating into insulin producing cells, culture conditions leading to their efficient generation have not been identified. Previous experiments have determined that human and mouse ES cells are capable of differentiating into focal clusters of PDX1+ cells, from which hormone positive cells emerge. Strategies that yield enriched populations of PDX1+ cells could be used to generate large numbers of islet cells for transplantation. We tested the hypothesis that exposure of differentiating ES cells to various FGFs could promote their differentiation into PDX1+ pancreatic precursor cells.

**Methods:** ES cells (D3 murine cell line or H9 human cell line) were differentiated into embryoid bodies (EBs) and then plated to allow further differentiation on gelatinized coverslips. In the case of H9 cells, EBs were cultured in the presence of FGFs 1, 2, 7 or 10 at concentrations of 25 and 50ng/mL for 14 or 28 days. In addition, EBs were formed in serum-replacement (SR) and FCS, or SR only, with no other growth factors added. D3 cells were grown in 1% FCS with 50ng/mL FGF10, or 10% FCS (control) starting at day 4 post plating.

**Results:** In general, the addition of different fibroblast growth factors led to different percentages of original EBs remaining at the end of the culture periods. In the set of experiments involving serum, timing and amount of PDX1 expression was influenced by the presence of FCS and SR. In murine ES cell cultures, FGF10 treatment led to a 2-3 fold increase in the mean number of PDX1+ cells. The first PDX1 positive cells appeared between 2 and 4 days post-plating, with both the absolute number of positive cells and number of clusters increasing over the observed period.

**Conclusions:** Fibroblast growth factors including FGF10 may promote differentiation of ES cells toward a pancreatic precursor cell fate.

### The effects of shear stress on eNOS regulation and the possible involvement of heat shock proteins

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Endothelial cells are normally exposed constantly to shear stress that is a mechanical force associated with flowing blood. Shear stress can modulate endothelial cell function by sequentially activating the mechanosensors, intracellular signaling pathways, specific transcription factors, and the expression of genes and proteins. One of the shear stress-induced factors is the potent vasorelaxant nitric oxide (NO). Shear stress also greatly upregulates the expression of endothelial nitric oxide synthase (eNOS). Heat shock proteins (HSPs) are families of proteins elevated in response to hyperthermia and other environmental stresses, although some are constitutively expressed. HSPs serve to stabilize proteins in abnormal configurations, and play a major role in the folding and unfolding of proteins. HSPs have also been suggested to act on intracellular signaling pathways. Therefore, it will be of interest to reveal the relationship among eNOS, shear stress, and HSP. We hypothesize that, in the presence of shear stress, the levels/activity of eNOS and HSPs will be augmented suggesting that the elevated level of HSPs induce endothelial cells to increase eNOS expression and/or its activity. In order to elucidate the effect of shear stress on endothelial cell HSP levels, we planned to use human uterine microvascular endothelial cells (HUtMVEC) and human umbilical artery endothelial cells (HUmAEC). With CELLMAX artificial capillary modules, HUtMVEC and HUmAEC cells were grown at 0.8 dynes/cm<sup>2</sup> and 1.5 dynes/cm<sup>2</sup>, respectively. When the cells reach confluence, they were exposed to shear stress of 15 dynes/cm<sup>2</sup> for 24 hours. The capillaries were then perfused with lysis buffer, and Western analysis was performed on the cell lysates for eNOS protein. Regardless of the level of shear stress, HUmAEC protein levels of eNOS were substantially greater than that observed in HUtMVECs. In fact, the level of eNOS expression in HUtMVEC was nearly non-detectable by Western analysis; this observation was confirmed at the level of mRNA using RT/PCR. Further results revealed that eNOS protein was elevated by shear stress in HUmAEC, but HUtMVEC still showed little to no eNOS expression. To date we have observed the presence of HSP-47 and HSP-70 in HUmAEC exposed to higher temperatures (42 degrees C for 30 min). The presence of HSP-47 in endothelial cells was surprising, as it is usually associated with folding of the collagen family of proteins. Further evaluations of how HSPs are involved in the endothelial responses to shear stress and whether heat shock itself can induce the increase of eNOS protein are in progress. *Support: NIH HL49710, HD33255, HLS7653, HD38843.*

## Effect of Macromolecules on Bovine Oocyte Vitrification

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Vitrification could become the procedure of choice to preserve oocytes of valuable cows. Most successful protocols include Fetal Calf Serum (FCS) as a component, which varies from batch to batch and may contain viruses. The aim of this study was to determine the effectiveness of different macromolecules to replace FCS in vitrification solutions for oocyte cryopreservation. Oocytes were matured as described below for 21 h, at 38.5°C, in 5% CO<sub>2</sub> in air, and then partially denuded in 100 IU/ml hyaluronidase in holding medium (Hepes-TCM 199 with 20% FCS to prevent zona hardening: HM). Oocytes in groups of 24 were held in HM before vitrification. All vitrification and warming procedures were carried out at 37±2 °C. Sub-groups of 4 oocytes were pre-equilibrated for 30s in 100 µl vitrification solution 1 (Hepes-TCM 199 with 10% DMSO, 10% Ethylene Glycol (EG) and the correspondent macromolecule). Then, oocytes were rinsed in two 100 µl drops of vitrification solution 2 (Hepes-TCM 199 with 20% DMSO, 20% EG, 0.5 M galactose and the correspondent macromolecule), loaded onto cryoloops (Lane et al., *Nat Biotechnol*, 1999;17:1234), and immersed in liquid nitrogen after 25s. Oocytes were warmed by plunging the cryoloop into 0.5 M galactose in HM and placed in 0.5, 0.25, 0.125 and 0 M galactose in HM, for 3 min each. Oocytes were placed back in maturation medium for 0.5 to 2 h to complete a total of 23 h incubation. Frozen-thawed semen from one of three bulls was used for fertilizations in chemically defined medium (Olson and Seidel, *J Anim Sci* 2000; 78:152; F-CDM) at 1 million sperm/ml. Zygotes were vortexed after fertilization, cultured in CDM-1 for 48 h, and then in CDM-2 for 144 h, at 38.5 °C, in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N. Experiment 1: Two maturation media were used: TCM 199 vs. CDM, both with the addition of 10% FCS, 1 µg/ml E<sub>2</sub>, 1 µg/ml LH, 15 ng/ml FSH. Macromolecule treatments were: A: no-macromolecule; B: 20% FCS; C: 6% BSA; D: 2% BSA; E: 18% Ficoll 70,000; F: 6% Ficoll 70,000; G: non-vitrified control; H: non-vitrified but cumulus partly removed control. Experiment 2: Maturation medium was CDM with the addition of 0.5% FAF-BSA, 1 µg/ml E<sub>2</sub>, 1 µg/ml LH, 15 ng/ml FSH and 50 µg/ml EGF. Macromolecule treatments were: A; B; G; H (see above); I: 20% PVP; J: 6% PVP; K: 1% PVA; L: 0.3% PVA. Cleavage was evaluated at 72 h post fertilization, and blastocyst rate was evaluated on days 7, 8 and 9. Each experiment was replicated six times. Data were arcsin transformed and analyzed by ANOVA in experiment 1, there were no differences between maturation media, so data were pooled. Cleavage rates were not different among treatments (P>0.05) ranging from 63 to 80%. LS means for blastocyst formation rate (per oocyte) was different in H vs. A and C, and G vs. A (P<0.05) A: 9; B: 18; C: 12; D: 21; E: 15; F: 15; G: 28 and H: 34%.

In Experiment 2, there were not significant differences in cleavage rates (70-87%; P>0.05), but blastocyst rates were different between H vs. L and A, and in G vs. L (P<0.05) A: 17; B: 23; I: 21; J: 22; K: 20; L: 10; G: 43; H: 34%. In conclusion, vitrification solutions for bovine oocytes containing no macromolecule 0.3% PVA and 6% BSA were inferior to non-vitrified controls. A wide range of other macromolecules and concentrations were, however, suitable for vitrification of bovine oocytes. *Theriogenology* 59(1):207

## Estrogen differentially modulates the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in murine BV-2 microglial cells

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Excessive microglial cell activation has been implicated in the progression and neuronal injury sustained as a result of multiple neurodegenerative diseases. Upon their activation, microglia synthesize and secrete toxic mediators and other substances involved in neuroinflammation. These include nitric oxide, primarily synthesized by the inducible form of nitric oxide synthase (iNOS); and prostaglandins, synthesized by cyclooxygenase-2 (COX-2). Because men and women have different predispositions to neurodegenerative disorders like Multiple Sclerosis and Alzheimers Disease, it has been hypothesized that estrogen may play a role in the etiology of these diseases. Estrogens have been shown to exert protective effects in the brain by multiple mechanisms, including modulating the activity of ER alpha in neurons. However, little work has been done to investigate the function of ER beta in neuroprotection. Furthermore, the role of microglia in mediating these estrogen responses is also poorly understood. Therefore, we tested the hypothesis that estrogen, acting through ER beta, exerts protective effects in the brain by modulating microglial cell expression of iNOS and COX-2. The murine microglial cell line BV-2 was used to study the role of ER beta in lipopolysaccharide (LPS)-induced iNOS and COX-2 expression. Due to the undetectable levels of ER alpha in these cells, their responses to estrogen are likely mediated through ER beta. Following estrogen and LPS treatment, mRNA and proteins were analyzed for iNOS and COX-2 by RT-PCR and immunoblot analyses. Our results indicate that estrogen differentially modulates the expression of iNOS and COX-2. These data suggest that estrogen can act through ER beta to exert protective effects in the brain by controlling inflammatory mediator production from microglial cells.

### Regulation of Epidermal Growth Factor Receptor Signaling in Response to Cholesterol Levels and the Disruption of Lipid Rafts

**BG Woods**

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Many transformed cells exhibit a decrease in the expression and/or integrity of detergent-resistant membrane microdomains called lipid rafts. These structures have high concentrations of cholesterol and other lipids, as well as various signaling molecules. The protein caveolin-1 has been shown to be necessary in cholesterol metabolism at the plasma membrane, as well as in sequestering signaling molecules in their inactive state in certain lipid rafts called caveolae. Our lab has demonstrated that disruption of these microdomains by cholesterol depletion leads to caveolin-1 phosphorylation in an EGF receptor and Src-related manner, potentially releasing signaling molecules held inactive by caveolin-1. Conversely, introduction of additional membrane cholesterol has been shown to attenuate the activation of ERK1/2 in certain cell types. These data support the hypothesis that increasing membrane cholesterol can enhance the rigidity of lipid rafts and thereby attenuate EGF receptor signaling. To test this concept we treated human epidermoid carcinoma A431 cells with varying doses of the cholesterol-chelating compound cyclodextrin in order to disrupt lipid/cholesterol rafts, or we treated the cells with a cholesterol-cyclodextrin complex in order to increase membrane cholesterol content and stabilize raft structure and/or formation. We observed that cholesterol depletion by cyclodextrin caused a dose-dependent increase in caveolin-1 phosphorylation with and without EGF treatment. Conversely, cell incubation with cholesterol-cyclodextrin complexes, which should elevate membrane cholesterol, promoted a decrease in caveolin-1 phosphorylation in response to EGF treatment fifteen fold. These data support the hypothesis that increasing plasma membrane cholesterol regulates EGF receptor and caveolin function, possibly via the modulation of the ability of lipid rafts to hold signaling molecules in their inactive states.

### Effects of Soluble EGFR in Mammary Epithelial Cells

**JJ Gavinski and LG Sheffield**

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Through alternative splicing and polyadenylation signals the epidermal growth factor receptor (EGFR) gene gives rise to multiple RNA transcripts lacking the transmembrane and intracellular domains. These alternative EGFR transcripts are therefore produced as secreted forms of the receptor. Soluble EGFR (sEGFR) binds epidermal growth factor (EGF), dimerizes in EGF's presence and suppresses ligand-dependent EGFR activation, cell signaling, and transformation. Soluble EGFR is present in normal human serum and highly expressed in some cancers, specifically ovarian cancer. However, its biological role remains unclear. The objective of our study was to investigate the ability of sEGFR to act as a signaling protein and induce biological effects in mammary epithelial cells. Purified, sEGFR bound radioiodinated EGF with 3.1 nM affinity. Binding assays conducted with intact NMuMG cells, which express transmembrane forms of EGF, also result in soluble EGFR binding with 20 nM affinity. Several cytoskeletal proteins exhibited increased tyrosine phosphorylation in response to sEGFR in NMuMG cells. Matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF-MS) identified cytokeratin 8, cytokeratin 18, gamma-actin and cytokeratin 19. In addition, 2-dimensional polyacrylamide gel electrophoresis followed by MALDI-TOF-MS determined that cytokeratin 8, phospholipase C alpha and tubulin alpha 6 content was increased by sEGFR. Further studies focusing on cytokeratins indicated that cytokeratin 8 expression is increased after 1 hour and maximum after 3 hours sEGFR treatment at both the mRNA and protein levels. We further investigated the physiological or functional significance these sEGFR-mediated effects on keratin proteins may have in mammary epithelial cells. Treatment of NMuMG cells with sEGFR reduced cell loss following 24 hour serum deprivation in comparison to their control counterparts, suggesting that sEGFR may play a protective role in prevention of cell loss. In conclusion, our studies have identified a possible role for sEGFR in the mammary gland, specifically a role in controlling cytokeratin expression, protein modification and possibly protection against cell loss.

# Concurrent Oral Sessions III and IV

## *Cell Signaling*

**Class of '24 Reception Room**

**Chairs: Dr. Paul Bertics**

**Dr. Lewis Sheffield**

## *Whole Animal Physiology*

**Capitol View Room**

**Chairs: Dr. Paul Fricke**

**Dr. David Abbott**

**Abstracts 8-11**

### **Local Uterine Blood Flow Responses to ICI 182,780 in Ovariectomized-E2b Treated, Intact Follicular Phase and Pregnant Sheep**

**RR Magness<sup>1,2,3</sup>, TC Gibson<sup>1</sup>, and TM Phernetton<sup>1</sup>**

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Estrogen dramatically increases uterine blood flow (UBF) in ovariectomized (ovx) ewes. Both the follicular phase and pregnancy are physiological states with high circulating estrogen levels. ICI 182,780 is reported to be a pure estrogen receptor (ER) antagonist. We hypothesized that an ER-mediated mechanism is responsible for in vivo rises in UBF in physiological states of high estrogen. We therefore examined the effect ER antagonist on exogenous and endogenous E2B-mediated rises in UBF. Sheep were surgically instrumented with bilateral uterine artery blood flow transducers, uterine and femoral artery catheters. Ovx animals (n=8) were infused with vehicle (95% EtOH) or ICI 182,780 (0.1-3.0ug/min) into one uterine artery for 10 min before and 50 min after an I.V. E2B bolus (1 ug/kg) and UBF was recorded for an additional hour. Intact, cycling sheep were synchronized using a vaginal progesterone controlled internal drug release (CIDR; 0.9g; 7 days), PGF2a (7.5mg I.M., 2x- 4 hours apart; Day 1) and 1000 IU PMSG (I.M.; Day 0=CIDR removal). At approximately 50hrs after CIDR removal and PMSG injection, or when UBF reached peak levels ICI 182,780 (1 or 2ug/min) was infused unilaterally (n=7). Late pregnant ewes were also given ICI 182,780 (0.23- 2.0ug/min; 60min) into one uterine artery (n=10). In ovx sheep, local infusion of ICI 182,780 did not alter systemic cardiovascular parameters [Mean arterial blood pressure (MAP) or heart rate (HR)], however, it decreased ipsilateral, but not contralateral, UBF responses to exogenous E2b by approximately 55% (P<0.01). In the follicular phase and late pregnant ewes models of high endogenous E2b, ICI 182,780 locally inhibited (60% and 36%, respectively) the elevated UBF in a time dependent manner; ipsilateral > contralateral (P<0.01). In late pregnant sheep ICI 182,780 also mildly and acutely (5-30 min) elevated MAP and HR (P<0.05). In conclusion, exogenous E2B-induced increases in UBF in the ovx animal and endogenous E2B-mediated elevations of UBF during the follicular phase and late pregnancy are partially mediated by ER activation. Support: HL49210, HD33255, HD38843.

*Keynote Speaker*  
*Dr. Ann Kiessling*

Ann A. Kiessling is Associate Professor of Surgery, Harvard Medical School. She studied chemistry and nursing as an undergraduate and received a Ph.D. in Biochemistry/Biophysics from Oregon State University in 1971. Her postdoctoral work explored relationships between viruses and cancer in the laboratories of Paul Neiman, Fred Hutchinson Cancer Center; Lloyd Old, Memorial Sloan-Kettering Cancer Center; and Mehran Goulian, University of California, San Diego. The cancer cell studies revealed the need to understand undifferentiated cells, which were not cancerous, which in turn led to studies of early cleaving mouse embryos in collaboration with Harry Weitlauf at the Oregon Health Sciences University. The dual interests in virology and embryology led to research in semen transmission of Human Immunodeficiency Virus as well as the creation of the first laboratory for Human In Vitro Fertilization in Oregon. Research in both areas has continued since her recruitment to Harvard Medical School in 1985. She has published over one hundred scientific papers in both areas of research and is the proud mother of four children.



**Estrogen Induced Increases in VEGF Modulate Uterine Artery Endothelial Cell Angiogenesis**

**AG King<sup>1</sup>, K Matsubara<sup>1,4</sup>, IM Bird<sup>1,3</sup>, J Zheng<sup>1</sup>, and RR Magness<sup>1,2,3</sup>**

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The endometrium of the luteal phase ovine uterus is considerably thicker than that of the follicular phase. This thickening is partly accomplished through proliferative angiogenic growth of the follicular phase uterus, when the ratio of estrogen (E2b) to progesterone is high. Therefore these angiogenic processes are critical to prepare the endometrium with a vascular network sufficient to maintain the subsequent development of the luteal phase endometrium. We hypothesized that vascular endothelial growth factor (VEGF), acting in an autocrine fashion through its receptors Flt-1 and KDR contributes to this angiogenesis in order to modulate this estrogen receptor (ER) mediated response. We first demonstrated the presence of cell-associated and secreted VEGF (Western and ELISA) as well as Flt-1 and KDR (immunocytochemistry) in uterine artery endothelial cells (UAEC). Capillary tube formation was evaluated in UAEC seeded onto Matrigel in phenol red-free/serum-free DMEM and incubated with or without E2B. ICI-182,780 (ICI) was used to block ER signaling and anti-Flt-1, anti-KDR antibodies and soluble Flt-1 (sFlt-1) were used to block VEGF binding to its receptors. We also quantified mitogenesis using a 3H-Thymidine incorporation assay. For the mitogenesis assays, UAEC were seeded onto Matrigel, pretreated with the Kd50 and Kd100 (from the tube formation assays) of these VEGF receptor blocking antibodies or sFLT-1 protein followed by treatment with E2B. E2B stimulated UAEC capillary tube formation and VEGF secretion at doses ranging from 0.1-100nM, with maximal rises of 1.75-2.0 fold at 10nM E2B. The E2B-induced increase of tube formation was blocked by 2.0uM ICI, anti-Flt-1, anti-KDR blocking antibodies, as well as sFlt-1 protein. E2B increased 3H-Thymidine incorporation, which was inhibited by anti-Flt-1 antibody and sFlt-1 protein, but not the anti-KDR antibody. Thus, an autocrine VEGF, Flt-1, KDR mechanism exists in UAEC whereby it can be activated by an ER mediated mechanism to modulate uterine artery angiogenesis. Support: NIH HL49710, HD33255, HL57653, HD38843.

## Invited Speaker Dr. Tomas Acosta

Dr. Tomas J. Acosta is a research associate in the Department of Animal Health and Biomedical Sciences at the University of Wisconsin-Madison. In addition to his appointment with the University of Wisconsin, Dr. Acosta also holds the position of Assistant Professor of Animal Physiology at Asuncion University in Paraguay. A native of Paraguay, Dr. Acosta grew up on his family farm. Following high school, Dr. Acosta began studying at Asuncion University and was awarded a D.V.M. in



1991. Dr. Acosta and his family moved to Japan in 1996 where he began work towards a Ph.D. at Obihiro University after receiving the Japan Fellowship for Foreign Researchers. It was during his Ph.D. program that Dr. Acosta developed a microdialysis system to assess autocrine/paracrine secretions from bovine, ovarian follicles *in vitro* and then *in vivo*. Hence the title of his doctoral thesis: autocrine /paracrine regulation of secretory mechanisms in mature bovine follicles during the periovulatory period. Following completion of his Ph.D., Dr. Acosta remained at Obihiro University for 2 years as a postdoctoral fellow of the Japanese Society for the Promotion of Science. During his stay

at Obihiro University, Dr. Acosta also participated in joint research projects between Japan and Germany, thus spending a few months in Munich, Germany. While conducting his research in Japan, Dr. Acosta utilized color flow Doppler ultrasonography to assess local changes in blood flow local of the follicle in addition to examining the effects of exogenous prostaglandin  $F_{2\alpha}$  on blood flow within the corpus luteum. Although most of Dr. Acosta's research in the past utilized the bovine as a model for study, his recent research endeavors have focused on the equine as a research model. Recently, he has been investigating changes in follicular blood flow during stages of follicle development in the mare.

## Positive Association Between Reduced Ovarian Responsiveness to Recombinant Human Follicle Stimulating Hormone (rhFSH) Therapy for In Vitro Fertilization (IVF) And Diminished Intrafollicular Testosterone (T) and Estradiol (E<sub>2</sub>) Levels

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**Objective:** To assess whether reduced ovarian responsiveness to rhFSH treatment for IVF is associated with abnormal intrafollicular steroidogenesis

**Design:** Prospective cohort

**Materials and Methods:** Eighteen normal women (mean age, 31 [range 27-37 years]) received gonadotropin releasing hormone (GnRH) agonist/rhFSH therapy followed by human chorionic gonadotropin (hCG) administration for IVF. Women were defined as having a high normal response (HN, n=11) or a low normal response (LN, n=7) to rhFSH therapy, based upon whether the total amount of rhFSH administered was less or greater than the mean amount of rhFSH given to all patients combined (2138, range 1088-3525 IU). Blood sampling for basal bioactive luteinizing hormone (bioLH) and immunoreactive FSH (iFSH) levels were performed during pituitary desensitization immediately preceding rhFSH therapy and repeated on the day of oocyte retrieval. At oocyte retrieval, follicle fluid (FF) uncontaminated by blood was aspirated from the first follicle of each ovary and was assayed for bioLH, iFSH, E<sub>2</sub>, T, dihydrotestosterone (DHT), androstenedione (A<sub>4</sub>), progesterone (P<sub>4</sub>) and 17-hydroxyprogesterone (17OHP<sub>4</sub>). Hormone values were adjusted for protein content and were logarithmically transformed as necessary to meet assumptions of regression analyses. Models were fit using generalized estimating equations accounting for more than one follicle per subject.

**Results:** Age, body mass index (BMI), basal serum bioLH and iFSH, maximum serum E<sub>2</sub> level and total number of oocytes retrieved were similar between female groups. As expected, duration and total amount of rhFSH administered were higher in women with a LN response (11±0.8 days; 2742±433 IU [mean±SD]) than in women with a HN response (9±1.5 days,  $P<0.01$ ; 1800±493 IU,  $P<0.001$ , with FF iFSH levels higher in women with a LN response (6.1±2.6 ng/mg) than in women with a HN response (3.2±1.4 ng/mg,  $P<0.001$ ). Despite comparable FF bioLH levels in both female groups ( $P=0.82$ ), women with a LN response had lower FF E<sub>2</sub> and FF T levels (E<sub>2</sub>: 2.1±1.1 ng/mg; T: 20.7±4.1 pg/mg), with higher FF P<sub>4</sub> levels (306.7±117.5 ng/mg), compared to women with a HN response (E<sub>2</sub>: 3.1±1.4 ng/mg,  $P<0.04$ ; T: 29.3±11.5 pg/mg,  $P<0.003$ ; P<sub>4</sub>: 210.3±96.7 ng/mg,  $P<0.0003$ ). The FF 17OHP<sub>4</sub>/P<sub>4</sub> ratio, as a marker of 17 $\alpha$ -hydroxylase activity, also was diminished in women with a LN response (0.04±0.02) compared to women with a HN response (0.07±0.03,  $P<0.001$ ). There were no female type differences in FF 17OHP<sub>4</sub>, A<sub>4</sub> and DHT levels, or FF E<sub>2</sub>/T and DHT/T ratios, as markers of aromatase and 5 $\alpha$ -reductase, respectively. However, when corrected for FF FSH, FF androgens were lower in women with a LN response as compared to women with a HN response.

**Conclusions:** Despite increased FF FSH levels, reduced ovarian responsiveness in normal women receiving GnRH analog/rhFSH therapy for IVF is associated with diminished intrafollicular T and E<sub>2</sub> levels, perhaps from impaired paracrine signaling of theca 17 $\alpha$ -hydroxylase.

### Estrogen Induces a Rapid Increase in the Frequency of Intracellular Calcium Oscillations in GT1-7 Cells

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Estrogen plays a pivotal role in control of the preovulatory LHRH surge. As a step toward understanding the positive feedback effects of estrogen on LHRH neurosecretion, the present study examines the effect of estrogen on intracellular  $Ca^{2+}$ ,  $[Ca^{2+}]_i$ , oscillations in a GT1 LHRH-secreting neuronal cell line. Cultured GT1-7 cells were primed with either estradiol benzoate (EB, 20 pM) or vehicle 24 hours prior to the experiments, labeled with fura-2, and examined for  $[Ca^{2+}]_i$  oscillations. After a control period, cells were exposed to EB at 0.1 or 1 nM for 10 min and recording was continued for an additional 30 min. In estrogen primed cells, EB at both doses consistently stimulated the frequency of  $[Ca^{2+}]_i$  oscillations during the EB exposure and this continued for at least 10 min after EB. The pulse amplitude was not altered with either dose. In contrast, in non-estrogen primed cells, EB at 1 nM weakly stimulated the frequency of  $[Ca^{2+}]_i$  oscillations, but 0.1 nM EB was not effective. Despite this rapid action of EB, exposure to 17 $\beta$ -estradiol-BSA conjugate failed to stimulate  $[Ca^{2+}]_i$  oscillations in both estrogen primed and non-primed cells. Moreover, the estrogen receptor antagonist, ICI 182,780, blocked the EB-induced frequency increase in  $[Ca^{2+}]_i$  oscillations. These results suggest that 1) estrogen induces a rapid increase in the frequency of  $[Ca^{2+}]_i$  oscillations, 2) estrogen-priming enhances sensitivity of GT1-7 cells to this rapid action of estrogen, 3) this effect appears to require the entrance of estrogen into the cells, rather than estrogen interaction with cell membrane receptors, and is mediated by a mechanism involving receptors sensitive to ICI 182,780. Supported by NIH grants HD15433 and HD11355

### Leptin and total free fatty acids are elevated in the circulation of prenatally androgenized female rhesus monkeys

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Adiposity in primates plays a crucial role in the regulation of reproductive function. Leptin and free fatty acids appear to serve as putative modulators of ovarian steroidogenesis. Both have been reported to be elevated in the circulation of women with polycystic ovarian syndrome (PCOS). To determine whether leptin and free fatty acids serve a similar role in nonhuman primates, two separate experiments were performed using prenatally androgenized (PA) female rhesus monkeys as a model for PCOS. Six to seven PA females previously exposed to testosterone excess (15-35 days) beginning on gestational days 40-44 and a similar number of normal (C) females underwent dual x-ray absorptiometry (DXA) for total body fat mass determination. All females also underwent serial blood sampling for leptin measurement during the early follicular phase of three consecutive menstrual cycles or a comparable time of anovulation (Experiment 1). The same two female groups also received an intravenous (iv) glucose tolerance test (gtt) with iv infusions of glucose (300mg, time=0min) and tolbutamide (5mg/kg, time=20min). PA females were similar in age and weight to C females and were matched to the latter female group by BMI. In Experiment 1, total body fat mass did not differ between the two female groups (PA:2350[2005-2695]g vs C:2209[1572-2846]g;mean[95%CI]). Nevertheless, serum leptin levels were elevated ( $P<0.04$ ) in PA (7.31[5.85-9.44]ng/ml) compared to C (4.35[3.15-5.99]ng/ml) females. In Experiment 2, basal serum total FFA levels tended ( $P<0.12$ ) to be greater in PA (693[481-999]mM/L) than C (426[305-596]mM/L) females. Furthermore, serum total FFA levels during ivgtt, as determined by area under the curve (AUC) analysis, were significantly ( $P<0.03$ ) increased in PA ( $8.28*10^4$ [ $6.40-10.71*10^4$ ]mM/L\*180min) compared to C ( $5.85*10^4$ [ $4.64-7.36*10^4$ ]mM/L\*180min) females. These differences between female groups in circulating leptin and FFA levels remained significant after adjusting for the confounds of total body fat mass or BMI, respectively. Therefore, hyperleptinemia and elevated serum FFA levels are present in PA female rhesus monkeys independent of adiposity and, if modulators of ovarian steroidogenesis, may play a crucial role in the regulation of reproductive function. Supported, in part, by NIH grants RR13635, RR14093 and RR00167.

***ERP Student Contact Information*****Endothelial Cells Derived from Human Embryonic Stem (ES) Cells in vivo****Y Song<sup>1\*</sup>, Y Wen<sup>1</sup>, B Gerami-Naini<sup>1,2</sup>, M Durning<sup>1,2</sup>, TG Golos<sup>1,2</sup>, and J Zheng<sup>1</sup>***Departments Ob/Gyn<sup>1</sup>, Wisconsin National Primate Research Center<sup>2</sup>, Univ. of Wisconsin, Madison, WI*

Reports on development of other kinds of cells differentiated from human ES cells are increasing, however, those about endothelial cells are still in narrow. Although in previous experiments we have proved that using a mixture of growth factors including VEGF and bFGF promoted differentiation of endothelial cells from human ES cells in vitro, yet in this study we will determine if human ES cells can differentiate into endothelial cells and form blood vessel structures in vivo after transplantation into sever combined immunodeficient (SCID) –beige mice and the percentage of endothelial cells occupied in total differentiation cells. Human ES cell (NIH Human ES Cell Registry Code: WA09) were cultured in suspension for 4 days to induce formation of embryoid bodies (EBs). EBs were disaggregated by trypsin, followed by treating cells with the EB differentiation media plus VEGF. After 30 days of treatment cells were used to examine the endothelial cell marker expression and isolate CD31 and Vecad positive cells by flow cytometry. For in vivo experiments, human ES cells are injected into the rear legs of SCID mice to form teratoma and immunohistochemistry and histochemistry are served to examine if there are endothelial cells and blood vessels induced in teratoma. The flow cytometry analysis revealed that approximately 2.2% of the total dispersed cells are positive for CD31 and almost 1.3% of total cells were positive for Vecad. These percentages of differentiation were very similar to those reported in other cells differentiated from human ES cells. Similar results were also obtained for Flk-1 positive cells. We use mouse monoclonal anti-human-specific nuclear antigen (H-SNA) and mouse monoclonal anti-VEcad antibodies as the primary antibodies to identify the endothelial cells and blood vessels. Teratoma formed by the human ES cells in SCID beige mice is stained by H-E. We observed that there were blood vessels with obvious erythrocytes inside the vessel lumen. These data indicate that there are functional blood vessels in teratoma. At adjacent sections we also found endothelial cells of blood vessels positive for both H-SNA and Vecad. This suggests to us that this blood vessel is originated from human cells. Based on this series evidence we can find human ES cells can differentiate in vitro into cells that express CD31, Flk-1 and VEcad. Some of blood vessels and VEcad positive cells were observed in teratoma tissues formed by human ES cells. In conclusion human ES cell can differentiate into endothelial cells in vitro. And human ES cells have the possible to differentiate into endothelial cells and form blood vessels in vivo.

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**Hormonal Control of Gene Expression in the Ovarian Granulosa Cell****KE Mayo***Department of Biochemistry, Molecular Biology & Cell Biology,  
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Ovarian folliculogenesis requires the concerted action of numerous intrinsic and extrinsic hormonal cues. The pituitary gonadotropins FSH and LH are key hormonal factors necessary for maturation, ovulation and luteinization of the follicle. The gonadotropins exert their actions by activating cell-signaling pathways that lead to changes in gene expression associated with alterations in ovarian cell proliferation and differentiation. In many cases, genes induced by FSH during growth of the follicle are subsequently repressed by LH during the periovulatory period. We are exploring the molecular mechanism by which FSH and LH exert these opposing actions on ovarian gene expression in the granulosa cell, and use as a model system the genes encoding the  $\alpha$  and  $\beta$  subunits of the reproductive hormones inhibin and activin. Inhibin is an endocrine hormone that acts to suppress FSH release from the pituitary gland, while activin stimulates pituitary FSH synthesis and secretion, largely through paracrine mechanisms. These functional antagonists are dimeric hormones belonging to the TGF $\beta$  superfamily of proteins, and they share common  $\beta$  subunits (inhibin= $\alpha\gamma_A$  or  $\alpha\beta_B$ , activin= $\beta_A\beta_A$ ,  $\beta_A\beta_B$  or  $\beta_B\beta_B$ ). During the rodent estrous cycle, FSH positively regulates inhibin synthesis in growing follicles, while the preovulatory LH surge strongly down-regulates inhibin synthesis. This removal of inhibin negative feedback provides an environment permissive to the secondary FSH surge on estrous morning, and plays a key physiologic role in recruitment of a new cohort of follicles into the growing pool. We previously demonstrated that FSH acts through a cAMP/CREB-dependent pathway to activate transcription of the inhibin  $\alpha$  subunit gene. In more recent studies, we found that this gene is also regulated by two orphan nuclear receptors, SF-1 and LRH-1, and that CREB and SF-1/LRH-1 act in a synergistic fashion to stimulate inhibin gene transcription. Using chromatin immunoprecipitation (ChIP) approaches, we find that after hormonal stimulation there is an ordered recruitment of these transcription factors, as well as several well-characterized transcriptional coactivators, to the inhibin gene promoter. Following the preovulatory LH surge, the transcriptional repressor ICER is induced in the ovary. We found that ICER can suppress FSH or cAMP-stimulated expression of the inhibin  $\alpha$  subunit promoter in granulosa cell transfection systems, and that it likely does so by competing with CREB for binding to the inhibin promoter CRE site, thus acting as a passive repressor. ChIP experiments demonstrate that the SF-1/LRH-1/coactivator complex dissociates from the promoter once ICER is induced and presumably binds. ICER is auto-regulated and only transiently expressed in the ovary, so it is likely that additional factors play a role in long-term repression of the inhibin  $\alpha$  subunit gene. One such factor appears to be C/EBP $\beta$ , in that the inhibin gene fails to fully down-regulate following an LH stimulus in C/EBP $\beta$  null mice. C/EBP $\beta$  acts to repress inhibin promoter activity in granulosa cell transfection systems, and binds to a unique upstream site in the inhibin promoter. Our studies suggest that a transcriptional switching mechanism underlies the divergent actions of FSH and LH on inhibin  $\alpha$  subunit gene expression. We are continuing to explore the assembly and composition of these transcriptional regulatory complexes, and to investigate the generality of these findings to other ovarian genes coordinately regulated with inhibin during the reproductive cycle.

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## *Schedule Continued*

## Notes

12:30 pm **Lunch**  
- 1:30 pm Great Hall

1:30 pm – **Keynote Lecture:**  
2 :30 pm Class of '24 Reception Room  
*“Stem Cells and Gametes: The Cycle of Life”*  
**Dr. Ann Kiessling**, Associate Professor, Surgery, Harvard Medical School

2:30 pm – **Break**  
2:45 pm  
2:45 pm – **Concurrent Oral Sessions III and IV:**  
3:45 pm

**Cell Signaling:**  
Class of '24 Reception Room  
Chairs: Dr. Lewis Sheffield, Professor, Dairy Science  
Dr. Paul Bertics, Professor, Biomolecular Chemistry  
2:45: *“Erythropoietin Regulates Growth and Function of Lung Epithelial Cells”*  
**Megan Lichtman**, Kling Lab, Pediatrics, UW-Madison  
3:15: *“Regulation of Epidermal Growth Factor Receptor Signaling in Response to Cholesterol Levels and the Disruption of Lipid Rafts”*  
**Benjamin Woods**, Bertics Lab, ERP Program

**Whole Animal Physiology:**  
Capital View Room  
Chair: Dr. David Abbott, Professor, Primate Center and Ob/Gyn  
Dr. Paul Fricke, Assistant Professor, Dairy Science  
2:45: *“Increased Proceptive Sexual Behavior in the Female Common Marmoset after Intracerebroventricular (icv) Administration of GnRH II”*  
**Deborah Barnett**, Abbott Lab, Primate Center, UW-Madison  
3:15: *“Effects of Macromolecules on Bovine Oocyte Vitrification”*  
**Celina Checura**, Wiltbank Lab, ERP Program

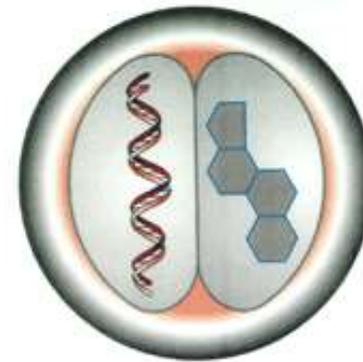
3:45 pm – **Break**  
4:00 pm  
4:00 pm – **Poster Session**  
5:00 pm Great Hall.  
5:00 pm **Closing Remarks**  
Great Hall  
Adjournment to the Union Terrace (weather permitting)

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*Friday, October 28, 2004*

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