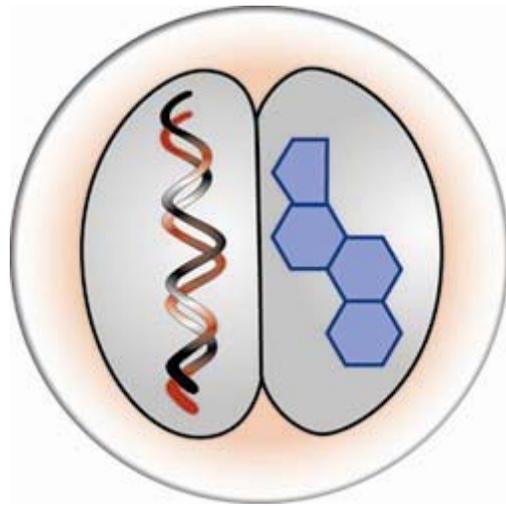


Endocrinology & Reproductive  
Physiology Program

# 2013 Annual Research Symposium



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### Event Acknowledgements

- Dr. Manish Patankar, PhD (Event Chair)
- Dr. Ian Bird, PhD (Program Director)
- Session Hosts: Roxanne Alvarez, Amanda Hankes, Fatou Jallow and Mayra Pastore
- Tiffany Bachmann, MA – Student Services Coordinator
- Staff at the Fluno Center

## Schedule of Events

9:00 AM – 9:15 AM	<b>Welcome Remarks</b>
9:15 AM - 9:35 AM	<b>Student Session 1:</b> Roxanne Alvarez “ The Role of Agonist-Induced Hyperpolarization in Initial and Sustained Phase Ca <sup>2+</sup> Entry in P-UAEC”
9:35 AM – 9:55 AM	<b>Student Session 1:</b> Fatou Jallow “Prolactin/TGF $\alpha$ Induce Tumorigenesis and ER $\alpha$ -Dependent Proliferation of Mammary Epithelial Cells in the Absence of Estrogenic Ligand
9: 55 AM – 10:15 AM	<b>Student Session 1:</b> Dr. Dan Mandel “ Safety of 17-hydroxyprogesterone Caproate in Rhesus Gestation”
10:15 AM - 10:30 AM	<b>Break</b> - Foyer
10:30 AM – 11:30 AM	<b>Keynote Presentation: Dr. Robert Lane, MD</b> Chair, Department of Pediatrics & Pediatrician in Chief Medical College of Wisconsin and Children’s Hospital of Wisconsin  Title: “Epigenetics – An Accessible Translational Mechanism”
11:30 AM - 12:15 PM	<b>Lunch</b> – Executive Dining Room (pending confirmation)
12:30PM – 2:00 PM	<b>Invited Speaker: Dr. Joel Eisner, PhD</b> Director of Product Development Viamet Pharmaceuticals  Title: “Selective P450C17 (CYP17) lyase inhibition: Its role in the treatment of castration-resistant prostate cancer (CRPC) and disorders of androgen excess. “  <i>Note: There will be time to discuss alternative careers in industry following the scientific talk</i>
2:00 PM – 3:00 PM	<b>Poster Session</b> and <b>Refreshment Break</b> – Foyer
3:00 PM - 3:20 PM	<b>Student Session 2:</b> Danielle Fontaine “ Tcf19 is a Novel Islet Factor Important in Cell Survival and Proliferation”
3:20 PM - 3:40 PM	<b>Student Session 2:</b> Lei Wang “Functional analysis of the endocytic adaptor FCHO in clathrin-coated vesicle formation”
3:40 PM – 4:00 PM	<b>Student Session 2:</b> Adriana Rodriguez “ The Role of FGF Signaling in the Establishment of the Fetal-Maternal Vasculature”
4:10 PM - 4:45 PM	<b>Closing Remarks and awards</b>
4:45 PM – 5:00 PM	Poster Take Down

## Keynote Speaker Dr. Robert Lane, MD

Chair, Department of Pediatrics & Pediatrician in Chief  
Medical College of Wisconsin and Children's Hospital of Wisconsin

### *"Epigenetics – An Accessible Translational Mechanism"*

**Bio:** Dr. Lane earned his MD from Northwestern University School of Medicine in 1989 and served an internship and residency in pediatrics there between 1989 and 1992. He was a fellow in the pediatrics scientist training program at the University of Chicago from 1992 until 1994 and earned a master of science degree in healthcare administration from the University of Texas – Dallas in 2011.

Certified by the American Board of Pediatrics with a subspecialty in neonatal-perinatal medicine, Dr. Lane is a prominent researcher in the fields of epigenetics (the study of heritable alterations in gene expression caused by mechanisms other than changes in DNA sequence) and uteroplacental insufficiency (insufficient blood flow to the placenta during pregnancy). He has received several national grants, including awards from the National Institutes of Health's National Institute of Child Health and Human Development, and the National Heart, Lung and Blood Institute, and from the American Diabetes Association.

A member and past president of the Perinatal Research Society, Dr. Lane belongs to numerous other organizations including the American Academy of Pediatrics, American Association for the Advancement of Science, American Diabetes Association, American Pediatric Society, and the Society for Pediatric Research. Dr. Lane possesses an extremely strong publication record including original peer-reviewed journal articles, book chapters and abstracts, and has participated in a significant number of invited lectures, workshops, site visits and grand round presentations, both nationally and internationally.

## Invited Guest Speaker Dr. Joel Eisner, PhD

Director of Product Development, Viamet Pharmaceuticals

*“Selective P450C17 (CYP17) lyase inhibition: Its role in the treatment of castration-resistant prostate cancer (CRPC) and disorders of androgen excess.”*

**Bio:** Dr. Eisner is a Director of Product Development at Viamet Pharmaceuticals where he leads the clinical and pharmacology activities for their CYP17 oncology clinical program. Previously he was Principal of Cato BioVentures, the life sciences venture capital parent of Cato Research. He focused on relationships with early-stage life science companies. He also served as an Integrated Drug Development Scientist of Cato Research, and as Vice President of Corporate Development of Hemodynamic Therapeutics and Echo Therapeutics (NASDAQ: INSM), both portfolio companies. Dr. Eisner is an Affiliate Scientist at the National Primate Research Center, University of Wisconsin-Madison. Prior to joining Cato BioVentures and Cato Research, Dr. Eisner was a Clinical Operations Manager at Northwestern University Medical School and Brigham and Women's Hospital. Prior to that he served as a Clinical Research Scientist for Insmmed, Inc. (NASDAQ: INSM). Dr. Eisner has 20 years' research experience in the field of endocrinology and reproductive physiology, and his areas of expertise include steroidogenesis, obesity, reproductive endocrinology, lipid abnormalities, diabetes, women's health, and infertility. He has 18 years' clinical research experience in both academic and pharmaceutical settings. Dr. Eisner is a member of the Endocrine Society and the Androgen Excess Society.

## Abstracts for Oral Presentations

### The Role of Agonist-Induced Hyperpolarization in Initial and Sustained Phase Ca<sup>2+</sup> Entry in P-UAEC

Roxanne Alvarez, Fu-Xian Yi, Bikash Pattnaik, Ronald R Magness, Ian M Bird

Uterine artery endothelial cells from pregnant ewes (P-UAEC) show periodic Ca<sup>2+</sup> bursting in response to ATP via enhanced activation of TRPC channels. We have reported that ATP stimulates a change in membrane potential (Vm) in UAEC (SGI 2012), and TRPC3 is known to be sensitive to Vm. We now investigate if ATP-stimulated changes in Vm play a role in regulating [Ca<sup>2+</sup>]<sub>i</sub> bursts. **Objective:** To determine if the dose dependent initial release of Ca<sup>2+</sup> (from ER) or sustained Ca<sup>2+</sup> bursting (TRPC Capacitative Ca<sup>2+</sup> Entry) is related to changes in Vm in P-UAEC. **Methods:** Ovine P-UAEC (passage 4) were grown in 35 mm glass bottom dishes (>90% density) and loaded with Fura-2 (Ca<sup>2+</sup> dye) followed by DIBAC4 (Vm dye). Simultaneous imaging of [Ca<sup>2+</sup>]<sub>i</sub> and Vm was acquired for 5 min basal and 30 min ATP (0-100uM) stimulation. Area under the curve was evaluated for both the initial Ca<sup>2+</sup> peak and the sustained Ca<sup>2+</sup> phase. Changes in Vm were reported as total change over 30 min. Dose response curves were constructed, and possible correlations between the initial Ca<sup>2+</sup> peak, sustained phase and Vm were identified. **Results:** The dose dependency of ATP for the initial peak and sustained phase Ca<sup>2+</sup> were parallel, but not coincident. The EC<sub>50</sub> for the initial Ca<sup>2+</sup> peak (26uM) was higher than the EC<sub>50</sub> for the sustained Ca<sup>2+</sup> phase (4.9uM). The Vm change was significant at 10uM (EC<sub>50</sub> = 3.2uM). At 3uM ATP a submaximal sustained Ca<sup>2+</sup> entry was observed, but there was barely an initial Ca<sup>2+</sup> peak and an undetectable change in Vm was noted. At 10uM ATP the Vm change was significant. **Conclusion:** These results suggest submaximal Ca<sup>2+</sup> bursting can occur without membrane potential change when there is minimal Ca<sup>2+</sup> release from the ER, but a maximal Ca<sup>2+</sup> CCE response coincides with both an initial peak and Vm hyperpolarization ≥ 11mV. The possibility that changes in Vm stimulate TRPC3 channel activation to enhance Ca<sup>2+</sup> bursting may explain why the dose dependency for sustained phase Ca<sup>2+</sup> entry is not always coincident with the initial Ca<sup>2+</sup> release from the ER. Future research of the regulatory mechanism behind endothelial CCE can be achieved by targeting ion channels that regulate Vm. In preeclampsia, and possibly other hypertensive disorders, future targeted therapies may be focused on manipulating endothelial Vm to support Ca<sup>2+</sup>-driven vasodilation. NIH R01 HL079020, HL49210, HD38843.

## Prolactin/TGF $\alpha$ Induce Tumorigenesis and ER $\alpha$ -Dependent Proliferation of Mammary Epithelial Cells in the Absence of Estrogenic Ligand

Fatou Jallow<sup>1</sup>, Kathleen A. O'Leary<sup>1</sup>, Debra E. Rugowski<sup>1</sup>, Ruth Sullivan<sup>1,2</sup>, Kerstin W. Sinkevicius<sup>3</sup>, Geoffrey L. Greene<sup>3</sup> and Linda A. Schuler<sup>1,2</sup>

<sup>1</sup>Department of Comparative Biosciences and <sup>2</sup>UW Comprehensive Cancer Center, University of Wisconsin-Madison, <sup>3</sup>The Ben May Department for Cancer Research, University of Chicago

**Background:** Initial or acquired resistance to anti-estrogen therapy is the most common cause of mortality in estrogen receptor positive (ER $\alpha$ +) breast cancer patients. The mechanisms that lead to resistance are not well understood, but one postulated mechanism is that ER $\alpha$  is activated in the absence of estrogenic ligand. Prolactin (PRL) and transforming growth factor  $\alpha$  (TGF $\alpha$ ) are two cytokines/ growth factors, which are elevated in the local breast cancer environment. They cooperatively activate signaling cascades, which can phosphorylate ER $\alpha$ .

**Hypothesis:** PRL plus TGF $\alpha$  increase ER $\alpha$ -dependent proliferation of normal mammary epithelium and tumorigenesis in the absence of estrogenic ligand.

**Methods:** To investigate the ability of PRL and TGF $\alpha$  to functionally activate ER $\alpha$  in the absence of ligand *in vivo*, we utilized genetically modified mouse models. The NRL promoter was used to drive PRL and TGF $\alpha$  synthesis in mammary epithelia. A "knocked-in" ER $\alpha$  with a mutation in the ligand-binding pocket (G525L) prevents the binding of endogenous 17 $\beta$ -estradiol, but retains the ability to be activated by phosphorylation and responds to the synthetic ER $\alpha$  agonist, propyl pyrazole triol (PPT). Beginning at d.10 of age, females were treated with PPT to promote normal ductal elongation. At week 10 after birth, PPT treatment was discontinued, and half of the NRL-PRL/TGF $\alpha$  (PRL/TGF $\alpha$ ) and NRL-PRL/TGF $\alpha$ /ER $\alpha$ (G525L) (PRL/TGF $\alpha$ /ER $\alpha$ (G525L) females were treated with ICI 182,780 (ICI) to block estrogenic signals until tumor end stage or 9 months of age.

**Results:** The combination of the PRL and TGF $\alpha$  transgenes induced proliferation of ductal epithelial cells 5-fold higher than wildtype, regardless of the ER $\alpha$  allele, evaluated by nuclear Ki-67 staining. Inhibition of ER-dependent signals with ICI reduced proliferation in both PRL/TGF $\alpha$  and PRL/TGF $\alpha$ /ER $\alpha$ (G525L) epithelium about 30%. These findings indicate that PRL/TGF $\alpha$  acts partially but not completely through ligand-independent activation of ER $\alpha$  in ductal epithelium. In contrast to the effect on ductal proliferation, ICI reduced tumor latency in PRL/TGF $\alpha$  females and tumor incidence in PRL/TGF $\alpha$ /ER $\alpha$ (G525L) females. Analysis of transcript levels by qRT-PCR in PRL/TGF $\alpha$  tumors showed that ICI increased ER $\alpha$  (p= 0.03) and decreased progesterone receptor (p= 0.02) transcripts. ER $\beta$  mRNA was undetectable in all tumors. Transcripts for integrins associated with differentiation were also altered by ICI treatment, which reduced CD29 and CD61 mRNA, and raised CD49f mRNA (p = 0.04, 0.5 and 0.03, respectively). Further, ICI increased Stat5b mRNA (p= 0.007), and tended to reduce Stat5a mRNA (p=0.06).

**Conclusions:** Our findings suggest that PRL and TGF $\alpha$  can functionally activate ER $\alpha$  in the absence of estrogenic ligand *in vivo*, and that this pathway constitutes a significant portion of their cooperative stimulation of proliferation of normal mammary epithelium. However, inhibition of ER signaling accelerated PRL/TGF $\alpha$ -induced tumorigenesis, and the resulting tumors displayed characteristics of reduced differentiation, including patterns of integrin and Stat5 isoform expression. Stat5a is expressed at higher levels than Stat5b in the normal mammary gland, and is considered a good prognostic factor in breast cancer. Although Stat5b cannot compensate for Stat5a in normal mammary physiology, it has been connected to growth factor signals in tumor aggression. Our data indicate that PRL and TGF $\alpha$  may contribute to resistance to aromatase inhibitors by activation of ER $\alpha$  in the absence of ligand. However, our findings also underscore the differentiative effects mediated by ER. Together, our studies suggest that additional study of the crosstalk between PRL, growth factors and ER in the development, progression and therapeutic responsiveness will lead to novel approaches to breast cancer.

Supported by CDMRP W81XWH-08-1-0513, NIH R25 GM083252 and UWCCC Core Grant P30 CA014520

## Safety of 17-hydroxyprogesterone Caproate in Rhesus Gestation

Mandel, D.C, Shah, D., Bird, I, Abbott, D.

Preterm birth affects one of eight live born pregnancies in the United States. It is one of the most common prognostic factors for development of chronic long-term disease, and carries significant morbidity and mortality to the developing fetus. 17-hydroxyprogesterone caproate (17-OHPc) is an FDA approved medication for the prevention of recurrent preterm birth. It is the only method of primary prevention available in clinical practice. 17-OHPc has been validated for safety in human gestation but its mechanism of action remains unclear. There are many potential difficulties surrounding the approval and performance of prospective drug trials in human pregnancy. In order to elucidate the action by which 17-OHPc may prevent preterm birth, animal models should be utilized that share homology in the signals and mechanisms that lead to onset of parturition. 17-OHPc must also be validated for safety in the chosen model. The rhesus macaque is a well-established model of human steroid hormone production and physiology. The rhesus has similar adult adrenal cortical function, fetal adrenal development, and placental steroidogenic function making it an ideal model to study hormonal regulation of parturition. We hypothesize that 17-OHPc affects maternal adrenal steroidogenesis and responsiveness within the hypothalamic-pituitary-adrenal axis. This would modulate placental-fetal hormone production, which is felt to play a key role in signaling the onset of parturition. Two previously published reports have raised concern with 17-OHPc use in rhesus pregnancy, suggesting risk for development of gestational diabetes and that it may be embryo/fetal toxic. This has not been demonstrated in human gestation, and there is no pathophysiologic basis for this to occur in the rhesus. As such, we sought to demonstrate safety of 17-OHPc in rhesus pregnancy. Local IACUC and Primate Center Executive Committee approval was obtained for our study. Two pregnant rhesus females received 17-OHPc at human dose and frequency equivalent and multiple measures of safety were obtained. Safety measures included fasting maternal glucose levels, maternal weight gain, serial fetal biometry, amniotic fluid and placental assessment as obtained by trans-abdominal ultrasonography, in addition to information on pregnancy outcome including birth-weight, neonatal gender, and if stillbirth/miscarriage occurred. These findings were compared to a group of eight pregnant rhesus receiving vehicle only. No statistically significant difference in these outcome measures was observed, suggesting that 17-OHPc does not adversely affect rhesus gestation. Further studies are needed with larger numbers of animals and different gestational age windows utilized.

## Tcf19 is a Novel Islet Factor Important in Cell Survival and Proliferation

Danielle A. Fontaine, Justin Bushkofsky, Amelia K. Linnemann<sup>1</sup>, Kimberly A. Krautkramer<sup>1</sup>, Amy L. Whillock, Dawn Belt Davis

Tcf19 is a putative transcription factor, initially described as a growth-regulated gene expressed at the G1/S transition. More recently, single nucleotide polymorphisms in TCF19 demonstrate genetic linkage to type 1 diabetes. Tcf19 is expressed in both mouse and human islet, and its expression is increased in non-diabetic obesity. In obesity, there is increased demand for insulin production and increased endoplasmic reticulum (ER) stress. The ER stress inducer, thapsigargin, led to a ~10-fold increase in Tcf19 expression. Knocking down Tcf19 using siRNA in INS-1 cells led to increased basal levels of apoptosis and increased susceptibility to thapsigargin-induced apoptosis. We also saw decreased expression of genes associated with ER homeostasis after Tcf19 knockdown including Bip, p58IPK, Edem1, and Calreticulin. There was also an increase in pro-apoptotic gene expression after Tcf19 knockdown, including Bim, Bid, Nix, Gadd34, and Pdia2. Given its initial description as a growth-regulated gene, we also examined the role of Tcf19 in cell proliferation. When Tcf19 is knocked down, there was a 45% decrease in proliferation as measured by <sup>3</sup>H-thymidine uptake. Chromatin immunoprecipitation on INS-1 cells overexpressing Tcf19 showed that Tcf19 is bound to promoter regions of Mki67, FoxM1, and Cyclin A2. We have shown that Tcf19 is important for adequate growth and survival and acts as a transcription factor that regulates key genes in cell cycle and ER homeostasis. We propose that Tcf19 may mediate the ability of beta cells to maintain adequate insulin production in the face of autoimmune attack.

## The Role of FGF Signaling in the Establishment of the Fetal-Maternal Vasculature

Rodriguez AM, Jin DX, Downs KM

Absence or improper alignment of communication between the three major circulatory systems during development can lead to a number of birth defects and early pregnancy loss. However, despite its importance, little is known about how the connection between the fetal, umbilical and yolk sac arteries is established and regulated. In the mouse, the pre-eminent model system of mammalian development, these arterial blood vessels are initially connected in a precise anatomical location within the fetal-umbilical interface through the Vessel of Confluence (VOC). The VOC is a blood vessel that appears to arise uniquely and independently from the arterial blood vessels at their fusion site, becoming visible at the precise embryonic stage of 4-somite pairs (~Embryonic day (E) 8.25); the VOC then enlarges, extending towards and connecting with the fetal dorsal aortae, umbilical artery and omphalomesenteric “yolk sac” artery by 6-somite pairs (~E8.5) to establish a communication link between the three major circulatory systems. In this study, we discovered a novel role for Fibroblast Growth Factor (FGF) signaling in establishing the fetal-umbilical-yolk sac arterial connection. Through a combination of whole embryo culture, pharmacological inhibition, immunohistochemistry, and 3-D analysis, we demonstrate that the attenuation of FGF signaling in living mouse embryos can disrupt development of the VOC. Embryos were continuously exposed to 40nM PD173074, a potent non-toxic Fibroblast Growth Factor (FGF) receptor inhibitor, in a whole embryo culture system from early neural plate/presomite stages through 6-somite pairs (~E7.25-E8.5) after which the inhibited embryos and untreated controls were assessed for the extent of arterial fusion at the VOC site. Although FGFs are a major signaling family involved in vascularization, application of the inhibitor beyond the neural plate/presomite stages (~E7.25-E8.0) did not affect VOC formation. Intriguingly, the FGF-sensitive developmental time window was 24 hours prior to the establishment of the fetal-umbilical-yolk sac arterial connection and 12 hours prior to the VOC’s overt presence. These results suggest that FGF signaling plays an early role in the establishment of the fetal-umbilical-yolk sac arterial connection, and thus provides new insight into the molecular mechanisms involved in the regulation of vasculogenesis as a whole and the fetal-umbilical-yolk sac arterial connection, in particular.

## Functional Analysis of the Endocytic Adaptor FCHO in Clathrin-coated Vesicle Formation

Lei Wang, Jon Mayers, Anjon Audhya

Clathrin-mediated endocytosis (CME) is used by all eukaryotic cells to internalize extracellular macromolecules, typically taking advantage of cell surface receptors. Many human diseases such as cancer, neuronal diseases, diabetes and cardiovascular diseases are caused by dysfunction of CME. Clathrin-coated vesicles (CCV) are used as transporters for a variety of cargos in this dynamic process, so understanding the mechanism of vesicle formation is crucial. Even though the process of CCV formation has been extensively studied, the mechanism of the initiation step remains unclear. Two major endocytic adaptor/complexes have been proposed to act as nucleators of CCV formation: the AP-2 adaptor complex and FCH only domain proteins (FCHO1/2). Preliminary data suggests that *C. elegans* protein FCHO-1 forms a stable complex with EHS-1 and ITSN-1 both in vitro and in vivo and serves as a key regulator of CME in cooperation with AP-2. *C. elegans* which lacks of all three endocytic adaptor proteins displayed 30% embryonic lethality compare to wild type animals, in those animals, cargo internalization is also impacted. This suggests that the FEI adaptor complex plays an important role in Clathrin-mediated endocytosis. We further hypothesize that the theoretical FEI adaptor complex functions at plasma membrane to induce membrane curvature necessary for CCV formation and select cargoes. The FEI complex may also function redundantly with the AP-2 adaptor complex.

## Poster Session Abstracts

### Interaction Between Gonadotropin-releasing Hormone Neurons and Aromatase Expressing Neurons in the Monkey Stalk-median Eminence.

Garcia JP, Keen KL, and Terasawa E

Gonadotropin-releasing hormone (GnRH) in the hypothalamus plays a key role in regulation of reproductive function (1). While the concept that GnRH release controlled by positive and negative feedback of gonadal steroids has been well established (2-4), a recent study in our laboratory indicates that neuroestradiol, synthesized and released in the stalk-median eminence (S-ME) region, is also involved in regulation of pulsatile GnRH release (5). Specifically, infusion of letrozole, an aromatase inhibitor, into the S-ME suppresses spontaneous as well as the estradiol benzoate-induced GnRH release (5), suggesting a physiological role for estradiol converted from testosterone by aromatase P450 in the S-ME. However, presently it is unclear whether the effect of neuroestradiol on GnRH release is direct or indirect through other neurons, such as kisspeptin neurons. Therefore, in this study we investigated the neuroanatomical interaction between GnRH neurons and aromatase expressing neurons using fluorescent immunohistochemistry. The basal hypothalamus of a female rhesus monkey was fixed with 4% paraformaldehyde and exposed to a cryoprotectant, coronally cut at 15  $\mu\text{m}$  thickness, and mounted on slides. Tissue sections were exposed to antibodies specific to GnRH (LRH13) and P450 (R-8-1 provided by Dr. Koh Shinoda) for 2-3 days. For visualization of GnRH and P450, tissue sections were further exposed to Alexa Fluor 594 and 488 (Invitrogen) conjugated secondary antibodies, respectively. Preliminary results indicate that 1) GnRH perikarya were found ventrolateral to the arcuate nucleus (ARC) while dense fibers were found ventrolateral in the S-ME; 2) P450 positive perikarya were found in neurons located in the ARC and ventromedial nucleus as well as S-ME, whereas P450 positive fibers were found in the lateral portion of the S-ME; 3) there was no colocalization of P450 in GnRH perikarya; and 4) close contacts of GnRH fibers and P450 positive neurons were observed with a confocal microscope at 0.2  $\mu\text{m}$  optical slices. These results are interpreted to mean that there is a direct interaction between GnRH neurons and P450 neurons, such that neuroestradiol released in the S-ME can directly influence GnRH release. (Supported by NIH grants R01 HD15433 and R01 HD11355 for ET, R25 GM83252 for JPG, and P50 OD011106 for WNPRC)

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5. Kenealy BP, Kapoor A, Guerriero KA, Keen KL, Zeigler TE, Terasawa E. Estradiol released in the hypothalamus of ovariectomized female monkeys (*Macaca Mulatta*): A possible role in control of gonadotropin releasing hormone release. Feature Poster Presentation at the 95th Annual Meeting of the Endocrine Society held at San Francisco, CA, 2013; Abstract # FP04-5.

## TNF alpha Induces Gap Junction Dysfunction in Sheep Uterine Artery Endothelial Cells; a Model for Preeclampsia

Hankes AC, Boeldt DS, Yi FX, Grummer MA, Magness RR, Bird IM

During pregnancy, there is an adaptation of the uterine artery endothelial function to increase sustained  $Ca^{2+}$  signaling in uterine artery endothelial cells (UAEC). Increase in  $Ca^{2+}$  signaling increases nitric oxide production causing vasodilation of the uterine arteries to increase blood flow to the uterus, in order to meet the needs of the growing fetus. Failure of proper adaptation can result in hypertensive diseases of pregnancy such as preeclampsia (PE). PE is a hypertensive disease affecting 5-8% of pregnancies nationwide. PE is characterized by a loss of the normal vasodilation in pregnancy and is associated with increases in growth factors and cytokines such as VEGF and TNF, which are known to inhibit Cx43 function. We have established in sheep UAEC pregnancy enhanced vasodilator production in response to multiple agonists. Pregnancy adaptation is due largely to a specific increase in gap junction mediated cell-cell communication, specifically Connexin43 (Cx43). Agonists such as VEGF and TNF inhibit Cx43 function through kinase activity. While TNF is known to inhibit vasodilation in PE subjects via reactive oxygen species (ROS) production, in UAEC at 10ng/mL dose or below ROS production is not seen (Yi, SGI 2005). VEGF is known to transiently phosphorylate Cx43 at inhibitory sites, while TNF phosphorylates Cx43 via the Src sensitive site  $\gamma$ 265, long-term. We have previously found that VEGF inhibits pregnancy adapted  $Ca^{2+}$  bursts in pregnant UAECs (P-UAECs), and hypothesize that TNF would similarly inhibit ATP stimulated  $Ca^{2+}$  bursts. In recent experiments, pre-treatment of P-UAECs with TNF (10ng/mL) reduces the sustained phase of a subsequent ATP induced  $[Ca^{2+}]_i$  response to levels observed in non-pregnant UAECs (NP-UAEC). Cells pre-exposed to TNF showed a dose dependent inhibition of subsequent ATP stimulated  $Ca^{2+}$  bursts (to 63% of control,  $P < 0.001$  10ng/ml). This inhibition was more potent than VEGF (to 79% of control, 10ng/ml). When cells are pre-exposed to PP2, a Src family inhibitor, the cells recovered back to control levels of  $Ca^{2+}$  bursts. TNF stimulated Cx43 phosphorylation at  $\gamma$ 265, and this response was fully reversed by PP2 ( $P < 0.05$ ). These results suggests TNF acts through Src to phosphorylate Cx43 at  $\gamma$ 265, inhibiting Cx43 function in a manner reversible by a Src inhibitor. While VEGF inhibits Cx43, TNF inhibition of bursting is greater and is rescued by PP2. This supports the hypothesis that inhibition of Cx43 in PE can be mediated via multiple factors, and the TNF effect is predominantly mediated via the Src pathway. Thus, physiologic TNF inhibition occurs more through the Src pathway rather than ROS. Therefore, treatments aimed at Src may be more effective in PE subjects than antioxidants treatments. Funded by NIH HL079020, HL49210, HD38843.

## Identification of Genetic Variants in the Steroid Metabolism Pathway that Regulate Circulating Sex Hormone Concentration

Kentaro Hayashi and Craig S. Atwood

The focus of our studies has centered on pathways that regulate steroidogenesis, since it is postulated that the endocrine dyscrasia associated with menopause, and andropause in men, is central to senescent changes leading to age-related diseases. Indeed, the incidence of a range of age-related diseases in both genders is elevated in those with lower circulating concentrations of sex steroids. Therefore, identifying the underlying genetic factors that regulate basal circulating sex steroid concentrations is of scientific, prognostic and diagnostic importance. To address which genetic factors regulate basal circulating sex steroid concentrations, we obtained 135 matched serum and DNA samples from age-matched women ( $n = 64$ ; age = 76.6) and men ( $n = 67$ ; age = 76.5). These samples were analyzed for 17 beta-estradiol (E2) and follicle-stimulating hormone (FSH) concentrations and 115 single nucleotide polymorphisms in genes that regulate sex steroid synthesis, catabolism, inactivation and elimination. Our data indicate a wide variation in the concentration of circulating sex steroids, including E2, in both post-menopausal women (range: 12-42 pg/mL) and age-matched men (range: 12-70 pg/mL). Moreover, age-matched males had significantly higher circulating concentrations of E2 than post-menopausal females (mean =  $37.9 \pm 12.3$  pg/mL vs.  $21.8 \pm 8.3$  pg/mL;  $p < 0.0001$ ). Recursive partitioning analyses of these results stratified by splitting the sample into either high or low circulating sex steroid concentration revealed that males ( $n = 35$  high, 34 low) containing 1 or 2 T alleles in an FSHR exonic polymorphism (rs6165) and who also were T allele homozygous in an HSD17B1 intronic polymorphism (rs12602084) had lower circulating E2 concentrations 100% ( $n = 11$ ) of the time. This data makes biological sense since any functional change in signaling via this FSHR missense mutation (Ala to Thr, position 281) might be anticipated to modulate E2 concentrations. Similarly, intronic-induced changes in 17 beta-HSD expression, which converts E1 and androstenedione/T into E2, also would modulate E2 concentrations. In females ( $n = 30$  high, 32 low), the best split was for an intronic SNP in LHR (rs4073366; LHR2); those who were heterozygous (G/C) were 82% (9 of 11) likely to have lower circulating E2 concentrations, while those homozygous for LHR2 (CC or GG) and homozygous for an intronic KISS1 polymorphism (T/T; rs2510) had high circulating E2 concentrations 81% (16 of 21) of the time. While none of these SNPs alone were associated with E2 concentration, cumulatively these SNPs appear to regulate E2 synthesis leading to a lower circulating E2 concentration in males and females. Interestingly, the LHR2 (homozygous) and FSHR SNPs identified in these analyses also were found to associate with AD in our earlier published studies. These results support the utility of identifying gene-gene interactions in identifying complex human traits such as circulating sex steroid concentration.

## Endothelin Signaling Promotes Osteogenesis via Changes in the Cellular miRNA Environment which Induces IGF-1 and PGE2 while Derepressing Wnt Signaling

M.G. Johnson, J. Kristianto, A. Gustaveson, K.Konicke, B.Yuan, and R.D. Blank

Endothelin (ET1) promotes the growth of osteoblastic breast and prostate cancer metastases, an effect previously shown to be due in part to derepression of canonical WNT signaling. Conversion of big ET1 to mature ET1, catalyzed primarily by endothelin converting enzyme 1 (ECE1), is necessary for ET1's biological activity. We previously identified *Ece1*, encoding ECE1, as a positional candidate gene for a pleiotropic quantitative trait locus affecting femoral size, shape, mineralization, and biomechanical performance. To test the hypothesis that ET1 signaling regulates osteogenesis in the normal state as well as in cancer, we exposed TMOB osteoblasts to 25 ng/ml big ET1 prior to and over the course of in vitro differentiation. Cells were grown for 6 days in growth medium and then switched to mineralization medium for an additional 15 days, by which time they form mineralized nodules. Cells were harvested every three days following the switch to mineralization medium. We measured mRNA levels of genes involved in the ET1 signaling axis, production of paracrine factors involved in osteogenesis, and miRNA expression. Data were analyzed by the rank sum test or nonparametric ANOVA. TMOB cells exposed to big ET showed greater mineralization than control cells ( $N = 6$ ,  $p = 0.008$ ). The mineralization difference was specific to ET1 signaling, as it was blocked by pharmacological inhibition of ECE1 or endothelin receptor A. Under normal mineralization conditions, *Ece1* mRNA expression showed no change over the course of mineralization, ET1 was repressed and endothelin receptor A was induced. Addition of big ET1 repressed expression of all three genes. IGF1 levels were significantly (1.3-1.8X) higher over time in the presence of big ET1 ( $p < 0.001$ ). PGE2 levels were significantly increased over time (1.2-1.4X) in the presence of big ET1 while DKK1 and SOST production were repressed over time by 30-40% by big ET1 ( $p < 0.001$ ). Big ET1 repressed anti-osteogenic miRNAs including miRNA 335-5p, which targets *Igf1* and *Runx2* transcripts, while miRNAs that target proteins involved in inhibition of bone catabolism were induced by big ET1 exposure. Modulation of canonical WNT signaling could not fully account for ET1's osteogenic effects, as big ET1 produced a greater mineralization than treatment with LiCl. Conclusion: Our data show that osteoblasts express all the elements needed for ET1 signaling over the course of differentiation and that ET1 signaling promotes mineralization. Moreover, they suggest that ET1's osteogenic effects are mediated in part via IGF1 and PGE2 induction, potentially through changes in miRNA expression previously unrecognized ET1 osteogenic mechanisms.

## Estradiol released in the Hypothalamus of Ovariectomized Female Monkeys (*Macaca Mulatta*): A possible Role in Control of Gonadotropin Releasing Hormone Release

Kenealy BP, Kapoor A, Guerriero KA, Keen KL, Garcia JP, Kurian JR, Zeigler TE, and Terasawa E

Estrogens play a pivotal role in regulating GnRH neuronal function in female mammals. In primates, systemic administration of estradiol benzoate (EB) induces negative feedback effects with a latency of ~2 h and positive feedback effects with a latency of ~24 h. Earlier *in vitro* studies from this lab, however, show that estradiol (E2) results in a rapid stimulatory action, similar to a neurotransmitter effect, in cultured GnRH neurons derived from fetal olfactory placodes. Therefore, in the present study, we examined whether 1) brief infusion of EB into the hypothalamus induces GnRH release *in vivo*, and if so, 2) whether there is any physiological significance in the hypothalamus using a microdialysis method. A microdialysis probe was inserted into the median eminence-stalk region of the medial basal hypothalamus (MBH) in ovariectomized (OVX) female rhesus monkeys through a cranial pedestal as described previously. EB at 10-100 nM was infused into the MBH through the microdialysis probe for 20 min, while dialysate samples were continuously collected at 10 or 20 min intervals for up to 12 h. GnRH and E2 in the dialysates were measured by RIA and liquid chromatography-mass spectrometry (LC/MS), respectively. Results indicate that infusion of EB into the MBH for 20 min stimulated GnRH release with a latency of 10 min, similar to those seen *in vitro*. To our surprise, EB also induced an increase in the release of E2 immediately following the EB-induced GnRH increase and following each subsequent GnRH pulse. While E2 levels prior to EB challenge were either not seen or were sporadically pulsing peaking at 100-250 pg/ml, EB-induced E2 release reached 700-2000 pg/ml (peak levels). Elevated levels of E2 are of hypothalamic origin, because 1) animals were OVX for several months, 2) LC/MS distinguishes E2 from EB, 3) E2 release was pulsatile, and 4) a series of experiments examining if EB metabolizes into E2 indicate that this was not the case. Accordingly, we focused on examining the concept that E2 release observed in our studies is hypothalamic neuroestrogen. First, we found that electrical stimulation of the MBH in OVX monkeys stimulated not only GnRH release, but also E2 release. Second, infusion of an aromatase inhibitor, letrozole (100 nM), through the microdialysis probe resulted in suppression of pulsatile GnRH release and letrozole blocked EB-induced GnRH as well as EB-induced E2 increases. It is therefore concluded, that neuroestrogen is synthesized and released in the MBH and appears to play a role in regulating pulsatile GnRH release. The relationship between hypothalamic neuroestrogen and circulating ovarian E2 remains to be investigated.

## Role of Ece1 in Mediating Maternal Cardiovascular Adaptation to Pregnancy

Jasmin Kristianto, Shannon Phillips, Jacqueline Fisher, Jing Wu, Suzanne Litscher, Robert Blank

Preeclampsia (~8% of all pregnancies), fetal loss (~15% of all pregnancies) and intrauterine growth restriction (IUGR) (~10% of newborns) are common pregnancy complications. Our laboratory has isolated a pleiotropic quantitative trait locus (QTL) in mouse chromosome 4 in recombinant congenic mice (HcB-8 and HcB-23) mice that harbors differentially expressed Ece1 (the gene encoding ECE1) alleles resulting in differences in litter size, placental weight, and frequency of fetal loss. Recent data in congenic mice in which the QTL has been isolated are consistent with those obtained in HcB-8 and HcB-23. Chromosome 4 congenic (C4C) females have smaller litter sizes than C3H females ( $5 \pm 1.5$  v  $7 \pm 1.5$ , respectively,  $p = 0.034$ ) as collected at 17.5 dpc. However, C4C 17.5 dpc females have heavier placental weight relative to C3H 17.5 dpc females ( $0.126 \pm 0.117$  v  $0.108 \pm 0.103$ , respectively,  $p = 0.009$ ) with no significant differences in 17.5 dpc embryos weight. These findings suggest the possible differences in placental efficiency between the two strains. The Doppler ultrasonography embryonic measurements of 17.5 dpc females show that C4C embryos have slower umbilical vein velocity than C3H embryos ( $145.6 \pm 29.7$  v  $219.9 \pm 40.3$ , respectively,  $p = 0.025$ ). Furthermore, the embryonic heart rate is also slower in the C4C embryos in comparison to the C3H embryos ( $204 \pm 41.8$  v  $315.8 \pm 64.5$ , respectively,  $p = 0.027$ ). Therefore, we hypothesized that chromosome 4 QTL mediates vascular adaptation in response to pregnancy that manifests in differences in reproductive phenotypes.

## Steroidogenic Factor 1 Drives Aggressive Prostate Cancer Cell Proliferation and Plays a Critical Role in Tumor Growth

Samantha R Lewis, William A Ricke, Joan S Jorgensen

The dependence of prostate cancer on androgens provides a targeted means of treating advanced disease. Unfortunately, androgen deprivation therapies that block gonadal steroid synthesis ultimately become ineffective, leading to the deadly form of prostate cancer. While there are likely many ways this transition to treatment refractive cancer can occur, one important factor is the ability of prostate adenocarcinoma cells to acquire machinery for de novo steroidogenesis and therefore, fuel their own growth. The mechanisms by which prostate cancer cells initiate and maintain steroidogenesis are unknown. We hypothesize that Steroidogenic Factor 1 (NR5A1, ADBP4, SF1), a key regulator of steroidogenesis in normal endocrine tissues, is expressed in castration resistant prostate cancer where it stimulates aberrant steroidogenesis and fuels malignant growth. Notably, SF1 is not expressed in normal prostate tissue. Our results indicated that SF1 was absent in benign prostate cell lines as expected, but present in aggressive prostate cancer cell lines. When ectopic SF1 expression was induced in benign prostate epithelial cells (BPH1), increased steroidogenic enzyme expression, steroid synthesis, and cell growth was observed. Converse experiments using shRNA-mediated knockdown of SF1 in an aggressive prostate cancer cell line (WR3) diminished steroidogenic activity and inhibited cell growth. SF1 depleted cells also exhibited signs of decreased cell cycle progression and defects in cell division. Xenograft studies were performed to evaluate the role of SF1 in tumor growth. Results comparing control versus SF1 deficient prostate cancer cells showed that knockdown of SF1 substantially impaired tumor growth under the kidney capsule in both castrated and intact nude mouse hosts. Based on these data, we conclude that aberrant SF1 expression in aggressive prostate cancers stimulates steroidogenesis and promotes aggressive tumor growth. These findings present a new potential mechanism and therapeutic target for deadly castration resistant prostate cancer. This work was supported by The University of Wisconsin-Madison Graduate School and NIH 5T32-HD041921-07.

## Vascular Growth and Endothelial Junction Protein Expression in Human Placentas from the First Trimester, Normal Term and Preeclamptic Pregnancies

Yan Li, Ying-jie Zhao, Qing-yun Zou, Kevin Zhang, Kai Wang, Jing Zheng

**Background:** Preeclampsia is a major cause of maternal and neonatal morbidity and mortality; however, the etiology of which remains poorly understood. Endothelial dysfunction in maternal vasculature, especially increased endothelial permeability caused by dissembled endothelial junction proteins, is intimately associated with preeclampsia. Nonetheless, it is still unclear if vascular growth and endothelial dysfunction in placentas significantly impact on preeclampsia. Hypothesis: Vascular growth and expression of endothelial junction proteins in placentas are involved in the pathophysiological event of preeclampsia. **Methods:** Expression of platelet endothelial cell adhesion molecule-1 (PECAM-1 or CD31) and vascular endothelial-cadherin (VE-Cad), two functionally critical proteins in endothelia, was evaluated using immunohistochemistry and Western blotting in placentas from the first trimester (FT), normal (N) and preeclamptic (PE) pregnancies. Using CD31 as an endothelial marker, capillary number density (CND) and capillary area density (CAD) in N and PE placentas were also analyzed. **Results:** Both CD31 and VE-Cad were immunolocalized primarily in endothelia of FT, N, and PE placentas. However, no differences in CND and CAD were found between N and PE placentas. Using the same antibodies as those used in immunohistochemistry, Western blotting revealed the levels of CD31 and VE-Cad in N placentas were 3.7 and 8.0 fold higher ( $P \leq 0.05$ ) than those in FT placentas, respectively. VE-Cad levels were similar between N and PE placentas. CD31 was undetectable in PE placentas, while CD31 was easily detected in N placentas. These data confirm the robust vascular growth in placentas during pregnancy and indicate similar vascular growth between N and PE placentas. However, the failure to detect CD31 using Western blotting suggests abnormal formation of CD31 in PE placentas, which may potentially adversely impact placental endothelial communication and function. **Conclusion:** Dysfunction of placental endothelial junction likely plays a significant role in preeclampsia, although we were unable to find major differences in vascular growth between N and PE placentas (Supported by NIH HD38843).

## Characterization of CYP1 Flavonoid Substrates

Maguire M, Liu M, Jefcoate CR

Flavonoids are natural dietary compounds found in most plant material. Normal dietary intake depends on age, sex, ethnicity and other factors that affect dietary choices. Flavonoids have been investigated with respect to anticarcinogenic and antioxidant capabilities. They participate as substrates and/or inhibitors of receptors and the metabolizing enzymes, cytochrome P450s (CYPs), in particular the CYP1 family. While activity has been repeatedly demonstrated on isolated liver microsomes, we use a semi-high throughput cell-based ethoxyresorufin-o-deethylation (EROD) assay. A distinct structure-function relationship indicated the poly-hydroxyl flavone structure exhibits both selectivity and specificity to human (h)CYP1B1 over hCYP1A1. Functionally, we are able to show that DNA damaging, CYP1B1-mediated DMBA metabolism is inhibited by coadministration of certain flavonoids by measuring phospho-p53 expression in cells expressing only a single P450 or murine cell lines. Additionally, we show that a selection of flavonoids act permissively, leading to upregulated NF- $\kappa$ B signalling in the presence of a stimulus in a reporter cell line. The varied in vitro capabilities of flavonoids exemplifies the complexity that translates in vivo and the necessary considerations when using flavonoids as treatment options for cancer or other oxidant-mediated diseases.

## Post-translational Regulation of eNOS via Estrogen Receptors- $\alpha$ / $\beta$ and GPER in Uterine Artery Endothelial Cell

Mayra B. Pastore, Saira Talwar, Rosalina Villalon-Landeros and Ronald R. Magness

Uterine endothelial nitric oxide production is partly responsible for maintenance of vasodilatation during physiologic states of high estrogen levels (e.g. Pregnancy). Estrogen receptors (ER- $\alpha$ / $\beta$ ) are nuclear transcription factors; however, they induce nongenomic vasodilatory responses via activation of endothelial Nitric Oxidize Synthase (eNOS). Recently, the receptor known as GPER has been implicated in estrogenic regulation of the vasculature. It is unknown however if eNOS regulation is dependent on ER- $\alpha$ / $\beta$  and/or GPER. We hypothesize that ER- $\alpha$ / $\beta$  and GPER localize to the plasma membrane of Uterine Artery Endothelial Cell (UAECs) and change eNOS phosphorylation patterns indicative of activation. Vehicle or estradiol-17 $\beta$  treated UAECs were fixed and labeled with immunogold for visualization of caveolae, ER- $\alpha$ , ER- $\beta$ , and GPER using Transmission Electron Microscopy. UAECs were treated with vehicle or increasing concentrations of estradiol-17 $\beta$ , ER- $\alpha$  (PPT) or GPER (G1) agonists. GPER expression and changes in eNOS stimulatory phosphorylation sites Ser1177, Ser635 vs. inhibitory site Thr495 were evaluated via Western-blot. Immunogold-labeling revealed that ER- $\alpha$ / $\beta$  localize to the plasma member, the cytosol and the nucleus. GPER was also detected. Increasing estradiol-17 $\beta$  concentrations augmented eNOS phosphorylation at Ser1177, Ser635 with decreased phosphorylation at Thr495. PPT (0.1nM and 100nM) only increased the eNOS phosphorylation at Ser635. G1 (100nM and 1 $\mu$ M) increased eNOS phosphorylation Ser1177 not Ser635 but decreased Thr495 phosphorylation at all doses. In conclusion: 1) ER- $\alpha$ / $\beta$  localize to the plasma membrane, cytosol and nucleus, 2) estradiol-17 $\beta$  mediates changes in eNOS multi-site phosphorylation, 3) ER- $\alpha$  mainly mediates an increase in stimulatory phosphorylation of eNOS and 4) eNOS multi-site phosphorylation also occurs via GPER and ER- $\alpha$ / $\beta$ -independent mechanism.

## Protein Kinase C-delta (PKC $\delta$ ) Regulates Pro-inflammatory Chemokine Expression Through the NF $\kappa$ B Pathway in Vascular Smooth Muscle Cells

Jun Ren, Qiwei Wang, Stephanie Morgan, Bo Liu

**Objective** - Inflammation is a key contributor to a plethora of vascular diseases including atherosclerosis and aneurysm. Pro-inflammatory chemokines released by vascular smooth muscle cells (SMCs) play a critical role in vascular inflammation. Previously, we showed protein kinase C-delta (PKC $\delta$ ) was upregulated in SMCs of inflamed arteries. PKC $\delta$ -/- mice are resistant to inflammation as well as apoptosis in models of abdominal aortic aneurysm. We hypothesize that PKC $\delta$  modulates the inflammatory response in part through regulation of pro-inflammatory chemokine expression in SMCs.

**Method and Results** - To identify PKC $\delta$ -regulated chemokines, we used microarray to compare transcription profiles of SMCs infected with AdPKC $\delta$  or AdNull. Results showed that 45 out of 203 differentially expressed genes have known inflammatory response roles. RT-PCR analyses confirmed the regulatory effect of PKC $\delta$  on selected inflammatory chemokines, including MCP1/CCL2, CCL7, CXCL16 and CX3CL1. Using a mouse aneurysm model, we showed all of these chemokines were elevated in aortae of wildtype, but not PKC $\delta$ -/- mice. We postulate that PKC $\delta$  regulates chemokine expression via the NF $\kappa$ B pathway. Using MCP-1 as a prototype, a detailed analysis showed PKC $\delta$  induced MCP1 mRNA expression ( $4.25 \pm 0.57$  fold,  $n=5$ ,  $p < 0.05$ ) and protein secretion ( $4.41 \pm 0.21$  fold,  $n=3$ ,  $p < 0.05$ ). Analysis of pre-mRNA and mRNA indicated that PKC $\delta$  stimulated MCP1 expression at the transcription level rather than by controlling its mRNA stability. Using a NF $\kappa$ B inhibitor (Andrographolide) or siRNA knockdown of NF $\kappa$ B subunit p65 eliminated PKC $\delta$ 's effect on MCP1 expression. Physical interaction between PKC $\delta$  and p65 was shown in cultured SMCs and aneurysmal aortae by the in situ proximity ligation assay. PKC $\delta$ /p65 complexes were detected largely outside the nucleus, indicating the two pathways intersect in the cytosol. This notion is supported by the lack of effect of PKC $\delta$  translocation peptides on MCP1. Furthermore, we demonstrated that PKC $\delta$  enhanced Ser536 phosphorylation and DNA-binding affinity of p65.

**Conclusion** - Our results show PKC $\delta$  plays a key role in inflammation through the NF $\kappa$ B-mediated chemokine expression pathway in vascular SMCs and may serve as a suitable target for novel anti-inflammatory therapy.

## Endogenous Estrogen Inhibition Using Letrozole in Ovine Pregnancy Reduces Placental Iron Transport and Fetal Weight and Alters Fetal Iron

Mary Y. Sun, Katie Meyer, Jason Austin, Sharon Blohowiak, Ronald Magness, Pamela Kling

**Background:** Estrogen levels from the placenta rise dramatically during increasing gestation, impacting fetal growth and development. Limited in vitro data show that estrogen modulates iron homeostasis through increased expression of endothelial nitric oxide synthase (eNOS), thereby enhancing iron transporter transferrin receptor (TfR), and ultimately facilitating cellular iron uptake. Letrozole is a potent clinical aromatase inhibitor that suppresses estrogen production. Therefore we hypothesized that letrozole-mediated decreases in estrogen production during pregnancy reduce both fetal weights and fetal iron levels. **Methods:** In late gestation (day 120±5 Term=147 days) sheep, we studied a loading dose of letrozole 20 mg IM (day 0), followed by prolonged administration of 125 ug/kg/day for 12 days vs. control (vehicle). Maternal and fetal plasma hormone levels were measured using ELISA and LC-MS. Placentae were harvested for western blot analyses of TfR and eNOS. Fetuses were weighed and crown rump, abdominal girth, and thoracic girth were measured. Maternal and fetal whole bloods were analyzed for red blood cell (RBC) count, hemoglobin, and zinc protoporphyrin (ZnPP), a measure of incomplete RBC iron incorporation. Moreover, fetal liver and kidney were obtained for tissue iron analyses. **Results:** Two hours after giving the letrozole load, we observed lower maternal and fetal circulating estrogen ( $P<0.05$ ); whereas progesterone levels were unaffected. Reductions in maternal plasma estrogen were confirmed using LC-MS, however, the C19 steroid levels of testosterone, DHEA and androstenedione were unaltered by letrozole. Placental TfR expression was reduced in the letrozole group compared to control ( $P<0.05$ ), however placental eNOS trended higher ( $P>0.05$ ). Compared to controls, ovine fetuses were  $11.4\pm 0.05\%$  lighter and the ponderal index was  $10.0\pm 0.43\%$  lower in the letrozole group ( $P<0.05$ ). Although letrozole did not alter the crown rump length, abdominal girth was 6% lower within fetuses of this group ( $P<0.05$ ). Letrozole fetuses also exhibited higher ZnPP levels, but similar RBC count and hemoglobin. Fetal liver iron concentration was similar and kidney iron concentration was higher in the letrozole group compared to control. **Conclusions:** Maternal estrogen reduction with aromatase inhibition resulted in reduced placental iron transporter expression, leaner fetuses, impeded RBC iron incorporation, and altered tissue iron, indicating a role for estrogen in controlling fetal growth, body composition, and iron metabolism during pregnancy. NIH HL87144-Supplement (PJK), HL49210, HD50578, and HD38843 (RRM).

## 4-Methoxyestradiol Induced Proliferation of Uterine Artery Endothelial Cells Occurs via Activation of p38, p42/44 and JNK MAPKs and Independent of Estrogen and Adrenergic Receptors

R Villalon Landeros, SO Jobe, J Zheng, RR Magness Dept. Ob/Gyn Perinatal Res Labs, Univ of WI-Madison

During pregnancy, uterine angiogenesis is partly regulated by estrogens and estrogen metabolites. 4-Methoxyestradiol (4-ME2), the direct metabolite of 4-hydroxyestradiol (4-OHE2) synthesized by catechol-O-methyltransferase (COMT), induces proliferation in pregnant uterine artery endothelial cells (P-UAECs). We reported (Jobe et al, 2011) that 4-OHE2 induces P-UAEC proliferation via  $\beta$ -adrenergic receptors ( $\beta$ -AR) and independent of estrogen receptors (ERs). We thus determined if 4-ME2 will follow its two immediate precursors by stimulating angiogenic activity via ERs or ARs. Methods: P-UAECs from late pregnant ewes were treated with or without blockers (10 $\mu$ mol/L) against  $\alpha$ -AR (phentolamine),  $\beta$ -ARs (propranolol) or ERs (ICI 182,780). To determine signaling pathways for 4-ME2 actions, we evaluated activation of mitogen-activated protein kinases (MAPK) or PI3K signaling cascades. Functionality of MAPK signaling pathways was also determined by treatment of P-UAECs with protein kinase blockers (2.5/5  $\mu$ mol/L) against p42/44 (PD98059), P38 (SB203580), JNK (SP600125) and the PI3K (LY294002) pathway followed by 4-ME2 (0.1  $\mu$ mol/L). Cell proliferation was evaluated via 5-Bromodeoxyuridine assays. Phosphorylated and total p42/44, p38, JNK, MAPKs, and PI3K were evaluated using western blots. Results: Blocking  $\alpha$ -AR,  $\beta$ -AR and ERs with phentolamine, propranolol or ICI 182,780 did not affect proliferation responses to 4-ME2. Pretreatment with PD98059, SB203580 and SP600125 all abrogated ( $P < 0.05$ ) 4-ME2-induced P-UAEC proliferation. However, LY294002 pretreatment ( $P > 0.05$ ) did not alter 4-ME2-stimulated proliferation. Western blots revealed proliferation-specific and time-dependent activation of phosphorylated p42/44, p38 and JNK MAPKs, not PI3K. As well total p42/44, p38, JNK and PI3K were unchanged by 4-ME2. Conclusions: Thus 4-ME2 stimulates P-UAEC proliferation independent of either ERs or ARs. The 4-ME2 mechanism for proliferation converges at the level of p42/44, p38 and JNK MAPKs and is independent of PI3K signaling. These findings provide insights into complexities of estrogen metabolite signaling during pregnancy via 4-ME2, whose activity is independent of classic ERs or ARs, but still activates distinct MAPK signaling cascades indicating that estrogen metabolites have the capacity to bypass these common receptors and yet still induce angiogenesis. HL49210, HD38843, HL87144, R25GM083252.

## IL6 and IL8 Differentially Regulate Human Endothelial Functions

Qing-yun Zou, Ying-jie Zhao, Yan Li, Dong-bao Chen, Jing Zheng

**Background:** Preeclampsia (PE) is a multi-organ syndrome that contributes greatly to neonatal mortality and maternal health without a definite cause. However, PE is associated with increased production of cytokines interleukin 6 (IL6) and 8 (IL8) and decreased production of vasodilator nitric oxide (NO) in placenta. It is known that IL6 and IL8 are potent regulators of vasculogenesis and angiogenesis. Nevertheless, it is unclear if IL6 and IL8 regulate expression and activation of fetoplacental endothelial NO synthase (eNOS), which is a major NOS isoform responsive for NO production in placenta. **Hypothesis:** IL6 and IL8 play important roles in regulating cell viability and migration as well as eNOS expression and phosphorylation in fetoplacental endothelial cells during pregnancy. **Methods:** Viability and death of human umbilical cord vein endothelial (HUVE) cells after serum starvation were determined using positive staining of acetomethoxy derivate of calcein (calcein AM) and ethidium homodimer-1 (EthD-1) as indexes, respectively. HUVE cell migration was evaluated using the BD FluoroBlok Trans-wells system. Expression and phosphorylation of eNOS were evaluated using Western blotting. **Results:** IL6 and IL8 did not affect HUVE cell viability and death. As compared to the control, IL6 increased HUVE cell migration by ~ 30%, while IL-8 slightly decreased it by ~ 10%. We also observed that while IL6 had no effect on the phospho-eNOS (Thr495) (indicative of eNOS inactivation) and phospho-eNOS (Ser1177) (indicative activation), IL-8 time-dependently increased the levels of phospho-eNOS (Thr495) (~2.2 folds), but not phospho-eNOS (Ser1177). **Conclusion:** IL6 and IL8 differentially regulate migration and eNOS activation in fetoplacental endothelium cells. IL8-induced increases in phospho-eNOS (Thr495) may inhibit eNOS activation, possibly partially contributing to the decreased fetoplacental perfusion in PE. (Supported by NIH PO1 HD38843).

## ATP and Forskolin Stimulate Phosphorylation of Cx43 and eNOS in Uterine Artery Endothelial Cells via Pregnancy-specific Cyclic Nucleotide Mechanisms

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Pregnancy is associated with endothelium-mediated vasodilation in response to a plethora of agonists. We have demonstrated that PGI<sub>2</sub> and NO regulate the pregnancy-specific production of cAMP and cGMP in uterine artery endothelium (Uendo), respectively. Furthermore, both UAendos and uterine artery endothelial cells (P-UAECs) produce substantially more NO and PGI<sub>2</sub> in response ATP via Ca<sup>++</sup> mediated mechanisms. Additionally, we have previously shown that both cAMP and cGMP increase the gap junction (GJ) assembly through the GJ protein Cx43 Ser365 as well as endothelial nitric oxide synthase (eNOS) Ser635 phosphorylation in P-UAECs. Therefore we hypothesized that ATP increases cAMP and cGMP mediated phosphorylation of Cx43 and eNOS. Validated P-UAECs were pretreated with the inhibitors 100nmol/L Sildenafil (PDE5 inhibitor) or 1umol/L Isogladine maleate (PDE4 inhibitor) followed by treatment with vehicle or with 100 umol/L of ATP or 100umol/L of Forskolin. Evaluation using Enzyme-linked immunosorbent assay (ELISA) showed that pretreatment with PDE4 and PDE5 inhibitors increased cAMP production in response to Forskolin. However, Forskolin pretreatment did not effect GMP production. These data also demonstrate that ATP clearly increases both cAMP and cGMP in the presence of either PDE4 or PDE5 inhibitor. Western blotting revealed activation of phosphorylated Cx43 and eNOS in the presence of Forskolin and ATP. Our findings indicate that increased pregnancy-enhanced gap junction communication between cells is mediated by cAMP and cGMP in response to endogenous ATP to enhance vasodilation. NIH GM083252, HL49210, HD38843, HL87144, R25GM083252

## Iron: A Possible Link between Obese Pregnancies and Asthma Predisposition at Birth

Natalie C. Dosch, Rachel Weigert, Elyssa Guslits, Shannon Murray, Theresa W. Guilbert, Christopher L. Coe, Pamela J Kling, UW Department of Pediatrics

**Background:** Maternal pre-pregnancy obesity is associated with a diagnosis of wheezing during infancy and ultimately asthma diagnosis in offspring, yet no clear mechanism has been described. We found that lower fetal iron status after obese pregnancy. A link between deficient cord blood iron and recurrent wheezing in infancy has been reported. Combining these three observations, we hypothesized that fetal iron deficiency in obese pregnancy alters developmental inflammatory processes, predisposing to asthma. Our aim was to examine fetal iron status and lymphocyte cytokine expression profiles birth in obese vs. control pregnancies. **Methods:** We examined iron status in cord blood collected from C/S from obese and control pregnancies. Lymphocytes were isolated and stimulated with PHA and we examined cytokine expression profiles after 24 hr. incubation in normal and low iron conditions. **Results:** Preliminary cord blood from 11 control and 12 obese pregnancies showed similar Hb and ZnPP/H, measures of erythrocyte iron, but a non-significant trend for lower ferritin in obesity. The obese group has similar cord white count, but higher cord eosinophil counts than control  $p < 0.05$ . Production of the Th1 cytokine, interferon-gamma was lower in obesity and completely inhibited by low iron conditions, in contrast to IL-8, similar between groups and different iron conditions. **Discussion:** The combination of higher eosinophil counts and lower Th1-stimulated cytokine expression are consistent with a relative dysregulation of the balance between Th1 and Th2 cytokines in offspring of obese pregnancy. A predominance of Th2 can create an atopic phenotype that predisposes to asthma and allergies.

## 2013 ERP Program Faculty Directory

<b>Name</b>	<b>Research Interests</b>
Abbott, David	Neuroendocrine function, Polycystic Ovary Syndrome
Alarid, Elaine	Estrogen response
Atwood, Craig	Hormone regulation of aging and Alzheimer's Disease
Audhya, Jon	Membrane development and organization
Barry, Terence	Aquaculture, fish reproduction
Bird, Ian	Uterine blood flow, eNOS
Bosu, William	Folliculogenesis, Corpus luteum function
Davis, Dawn	Basic and translational research on diabetes and obesity
Downs, Karen	Developmental and genetic control of fetal and extraembryonic lineage formation during mouse gastrulation, use of mammalian stem cells in gene therapy
Drezner, Marc	Phosphatonins, Hormones
Duello, Theresa	Health Disparities in Underrepresented Populations
Ginther, Oliver	Equine reproduction
Golos, Thaddeus	Placenta biology, stem cells
Hernandez, Laura	Lactation biology
Jefcoate, Colin	stAR protein
Jorgensen, Joan	Gonad formation
Kessel, Julie	Neonatology
Khatib, Hasan	Genomic imprinting, genetic development of embryos in cattle, genetic traits that impact health and milk quality in cattle.
Kimple, Michelle	Signal transduction, Diabetes pathophysiology
Kling, Pamela	Neonatal development, Growth factors
Kreeger, Pamela	The use of mathematical, and computational techniques to address cellular signaling questions relevant to women's health
Levine, Jon	Polycystic Ovary Syndrome
Liu, Bo	Molecular mechanism underlying vascular inflammation, molecular mechanism underlying occlusive vascular diseases, and development of new materials for biomedical applications (gene delivery and vascular grafts)
Magness, Ronald	Shear stress
Martin, Thomas	Cell Signaling, neuropeptides
Ntambi, James	Genetic regulation of metabolism
Odorico, Jon	Stem cells, Pancreatic islet development
Parrish, John	Sperm regulation and function, Equine Reproduction
Patankar, Manish	Epithelial Ovarian Cancer (EOS)
Payseur, Bret	Genetics of hybrid sterility

Pelegri, Francisco	Cellular and molecular level processes involved in early vertebrate development
Peterson, Richard	Prostate disease
Salih, Sana	Molecular Determinants of Oocyte Development, Fertilization, and Early Embryogenesis in Humans
Schuler, Linda	Prolactin, Growth hormones
Shah, Dinesh	Maternal-Fetal Medicine, mechanisms of preeclamptic hypertension
Terasawa, Ei	Neuroendocrinology
Thomson, James	Stem Cells
Vezina, Chad	Prostate Disease
Watters, Jyoti	Molecular mechanisms employed by microglia, Central Nervous System
Wiltbank, Milo	Hormonal interaction; intracellular regulation of cell death and steroidogenesis in the corpus luteum; regulation of ovarian function in dairy cattle.
Xu, Wei	Dissecting the epigenetic mechanisms controlling estrogen responsiveness
Zheng, Jing	Endothelial cell function

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