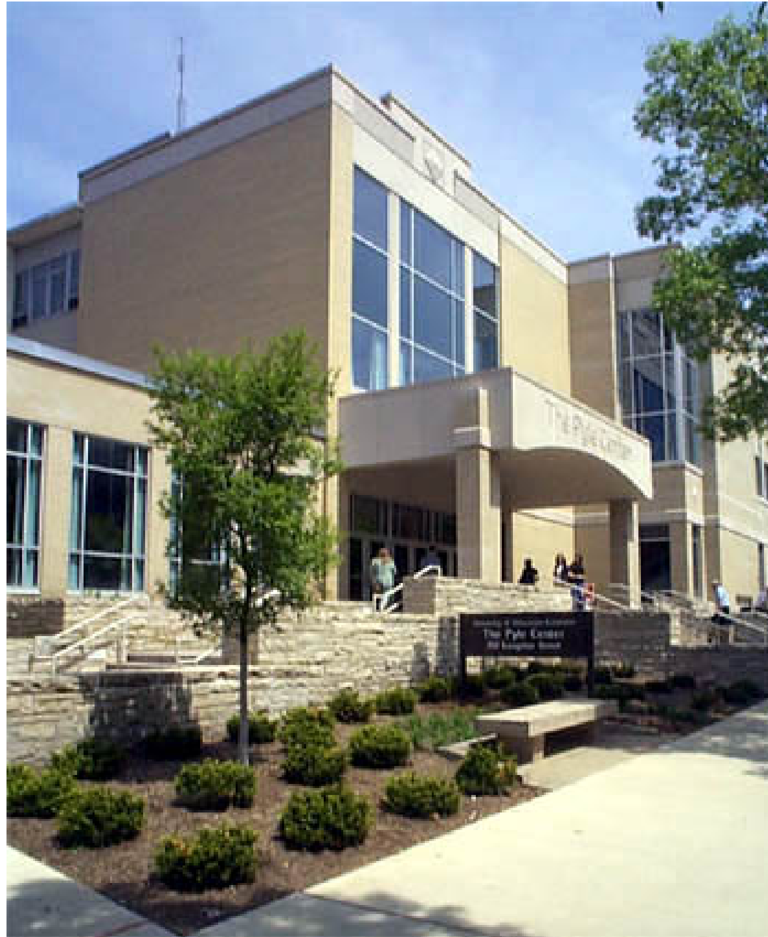
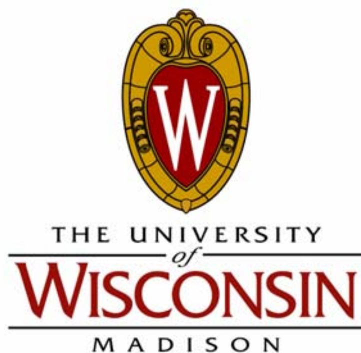
Endocrinology – Reproductive Physiology Annual Research Symposium





November 21, 2005
University of Wisconsin - Madison
The Pyle Center

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Schedule

8:00-9:00 **Registration, Poster Set-up**
Registration – Pre-function Area Outside Room 325 & 326
Poster Set-up – Alumni Lounge

9:00-10:00 **Distinguished Faculty Lecture:**
Room 325 & 326
"Regulation of Macrophage Activation States: Association with Asthma and the Role of Extracellular Nucleotides and Rhinovirus"
Dr. Paul Bertics, Professor, Department of Biomolecular Chemistry, University of Wisconsin - Madison

10:00-10:15 **Coffee Break**

10:15-11:15 **Oral Session I**
Room 325 & 326
Chairs: Dr. Manish Patankar, Department of Obstetrics and Gynecology
Dr. Gabriela Cezar, Department of Animal Science

10:15 "*Induction of Meiotic Maturation in Mouse Oocytes by Adenosine Analogs"*
Jing Chen, Department of Biological Sciences, Marquette University

10:35 "*The Effects of Scrotal Insulation on Bovine Sperm Nuclear Shape as Determined by Fourier Harmonic Analysis and its Relation to In Vitro Fertilization"*
Lefric Enwall, Parrish Lab, ERP Program

10:55 "*Use of Laser Microdissection and Pressure Catapulting for Derivation of Bovine Embryonic Stem Cell Lines"*
Jessica Quam, Cezar Lab, Department of Animal Science

11:15-11:30 **Coffee Break**

11:30-12:30 **Invited Speaker's Lecture:**
Room 325 & 326
"RNA interference: A Powerful Technique for Protein Knockdown"
Dr. Thomas Yeager, Senior Research Scientist, Promega Corporation, Madison, WI

12:30-1:30 **Lunch**
Alumni Lounge

Schedule Continued

1:30-2:30 **Oral Session II**
Room 325 & 326

Chairs: Dr. David Abbott, Department of Obstetrics and Gynecology
Dr. Craig Atwood, Department of Medicine

1:30 "*Possible Role of G Protein-Coupled Receptor GPR30 in Estrogen Action in Primate LHRH Neurons*"
Sekoni Noel, Terasawa Lab, ERP Program

1:50 "*Concentrating Reticulocytes to Assess Iron Deficiency in Neonates*"
Melinda Chen, Kling Lab, Department of Pediatrics

2:10 *"CYP17 Activity In cDNAs Isolated From Marmoset and Rhesus Adrenals"*
J Christina Pattison, Bird Lab, ERP Program

2:30-2:45 **Coffee Break**

2:45-3:45 **Keynote Lecture**
Room 325 & 326
"Molecular Profiling of Human Endometrium across the Menstrual Cycle"
Dr. Linda Giudice, Chair, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California – San Francisco

3:45-4:00 **Break**

4:00-5:00 **Poster Session and Reception**
Alumni Lounge
4:00-4:30 Odd numbered posters
4:30-5:00 Even numbered posters

5:00 **Closing Remarks**
Alumni Lounge

Distinguished Faculty Lecture:
Paul J. Bertics, Ph.D.

Accomplishments of Paul Bertics: Dr. Bertics has had a long record in the study of growth factor and cytokine action, as evidenced by his publication record (over ninety peer-reviewed publications that have focused on the analysis of cell signaling and protein kinases). His research program has made fundamental contributions to our understanding of the mechanisms associated with epidermal growth factor receptor (as well as nucelotide, LPS and IL-5 receptor) structure, regulation and interaction with other signaling pathways. His national recognition is also reflected by his service on numerous editorial boards (such as Endocrinology) as well as by his appointment to three major grant review panels (Regular Member of the NIH CBY-2 and CDF-3 Study Sections, the American Cancer Society Cell Cycle and Growth Control Advisory Committee, and the VA Merit Review Board for Oncology). Dr. Bertics is the current chair of Medical School Admissions, he is the current chair of the Medical School Research Committee, he is a member of the Executive Committee of the UWCCC, and he serves as the Group Leader for Cell Signaling within the UWCCC. Bertics is in an excellent position to direct the proposed research related to EGF receptor assessment in various tissue/tumor specimens before and after chemotherapy, and his UWCCC link allows for integration of information into the cancer research community in the institution and nationally.

Abstract 1

Regulation of Macrophage Activation States: Association with Asthma and the Role of Extracellular Nucleotides and Rhinovirus

Paul J. Bertics
Biomolecular Chemistry, University of Wisconsin, Madison, WI

Macrophage activation is central to normal immune function, but it can also contribute to the development and progression of many inflammatory and fibrotic disorders. Recent studies have revealed that human and murine macrophage activation is a heterogeneous process wherein, depending on the stimuli, different classes of activated macrophages can be generated that exhibit diverse physiologies and immunological functions. One class of stimuli that can modulate macrophage function is termed "classical" activators, and includes interferon-γ (IFN-γ) and Gram-negative bacterial lipopolysaccharide (LPS). Together, these stimuli promote a "classically" or type 1 activated macrophage that releases high levels of cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and IL-6, as well as the chemokines interferon-induced protein-10 (IP-10) and monocyte chemoattractant protein-1 (MCP-1). Conversely, other classes of activated macrophages, often termed "alternatively-activated" macrophages, can be generated by exposure to factors such as IL-4, IL-13, glucocorticoids or antibody-antigen complexes. These different patterns of macrophage activation result in the liberation of distinct profiles of chemokines and cytokines, e.g., IL-10 release is readily observed with certain alternatively-activated macrophages but is minimally detected with classically-activated macrophages. Our studies have revealed that, in addition to LPS, classically-activated macrophages are generated following cell exposure to the human rhinovirus-16 (RV-16) as well as to ligands for the nucleotide receptor P2X7. For example, our studies reveal that, similar to LPS, RV-16 or extracellular nucleotide challenge of macrophages from non-asthmatic individuals leads to the activation of transcription factors (NF- B, CREB) and MAP kinases (Jun kinases and p38), and promotes the release of pro-inflammatory factors, including TNF-α, IP-10, and MCP-1. Furthermore, although the activation of the nucleotide receptor P2X7 amplifies many LPS responses, naturally-occurring P2X7 mutations that decrease receptor function lead to a reduction in the capacity of LPS to induce a classically-activated macrophage phenotype and directs these cells towards an alternatively-activated phenotype wherein TNF-α production is reduced but IL-10 production is elevated. Interestingly, because IL-4 and IL-13 are often elevated in asthma and can promote alternatively-activated macrophage phenotypes, we postulate that airway macrophages from asthmatic patients are also directed towards an alternatively-activated phenotype that affects their resolution of infection and prolongs airflow obstruction. In this regard, we have observed that RV-16 challenge of airway macrophages from asthmatic patients results in the elaboration of mediators that is characteristic of alternatively-activated cells. Altogether these studies reveal that macrophage status and activation profiles are modulated by factors such as RV and extracellular nucleotides (which are released at sites of inflammation, platelet degranulation and cell damage/lysis). Accordingly, an understanding of macrophage behavior and contribution to the inflammatory and fibrogenic events associated with disorders such as asthma and viral/bacterial infections is likely to provide novel insight into therapeutic approaches for these disease states.

Oral Session I

Room 325 & 326
10:15 – 11:15

Chairs:
Dr. Manish Patankar
Dr. Gabriela Cezar

Abstracts 2-4

Abstract 2

Induction of Meiotic Maturation in Mouse Oocytes by Adenosine Analogs

Jing Chen, Stephen M. Downs

Department of Biological Science, Marquette University, Milwaukee, WI

In this study, we show that the adenosine analogs, 8-bromo-adenosine (8-Br-Ado) and methylmercaptopurine riboside (MMPR), but not adenosine, are very potent inducers of mouse oocyte maturation. Both 8-Br-Ado and MMPR stimulated oocyte maturation in oocytes maintained in prophase I arrest by a variety of meiotic inhibitors in dose-dependent fashion. Compound C, an inhibitor of AMP-activated protein kinase (AMPK), eliminated the effects of MMPR and 8-Br-Ado on oocyte maturation, which suggests that active AMPK may play a role in this process. Acetyl-CoA carboxylase (ACC) is an important substrate of AMPK, and western analysis of its phosphorylation state is commonly used as an indirect assay for AMPK activity. Western analysis using anti-phospho-ACC showed that GV-stage oocytes after 2h treatment with MMPR and 8-Br-Ado have increased levels of phospho-ACC compared to the untreated control. The results indicate that AMPK is activated in 8-Br-Ado- or MMPR-treated oocytes before meiotic resumption. Oocytes treated with adenosine for 2h also contained high levels of phospho-ACC, but these levels decreased with longer culture (4-6h). This suggests that maintaining a high level of active AMPK is required for GVB. 8-Br-Ado and AICAR, an AMPK activator, significantly increased the percentage of oocytes maturing spontaneously to the metaphase II stage, but MMPR blocked this process. The inhibition of polar body formation by MMPR may be due to suppression of purine de novo synthesis. However, other purine de novo synthesis inhibitors, azaserine and aminopterin, had no effect on polar body formation. Taken together, these data support the idea that adenosine analogs promote meiotic maturation in mouse oocytes through activation of AMPK.

Supported by funds from the NIH (HD040392).

Abstract 3

The Effects of Scrotal Insulation on Bovine Sperm Nuclear Shape as Determined by Fourier Harmonic Analysis and its Relation to In Vitro Fertilization

Lefric E Enwall [1], Abdulah Kaya [1, 2], Lindsey N Geiger [1], Josh R Schindler [1], Jennifer L Orchard [1], David L Northey [1] and John J Parrish [1]

[1] University of Wisconsin Madison, WI ; [2] Selçuk University Konya , Turkey

The objective was to examine the relationship of sperm shape as determined from Fourier Harmonic shape analysis (FHA) with in vitro fertilization (IVF) and embryonic development. Semen was collected and frozen from two scrotal-insulated bulls beginning 21 days prior to scrotal insulation as control days and for 80 days after scrotal insulation (ASI) to encompass the entire period of spermatogenesis in the bull. Insulation of the bull testis causes heat-induced anomalies in sperm morphology. Semen samples were used in an IVF system to fertilize oocytes (n = 12,590) collected from an abattoir and subsequent cleavage and embryo development (n = 8,429) was also recorded. There were significant time-dependent decreases in fertility and embryo viability associated with scrotal insulation. Reduced ($p<0.05$) fertilization, cleavage, and embryo development occurred over days 16-35 corresponding to early spermiogenesis through late meiosis. Individual days representing late spermatocytogenesis were also reduced ($p<0.05$) for cleavage and embryonic development. FHA also detected shifts in sperm nuclear morphology that correspond to the decreases of fertility and embryo viability seen in the IVF data. When scrotal insulation produced an effect of reduced fertility in sperm undergoing spermiogenesis, there was a decrease in harmonic amplitude (HA) 0 and an increase in HA 3 and 5. This indicates that sperm nuclei were smaller and more tapered in the anterior and posterior regions. In contrast, effects of scrotal insulation during meiosis caused decreases in HA 2, 3, and 5 indicating that sperm nuclei got shorter overall and wider in the posterior region. Objective measurements of sperm shape were related to in vitro sperm fertility.

Abstract 4

Use of Laser Microdissection and Pressure Catapulting for Derivation of Bovine Embryonic Stem Cell Lines

Jessica Quam and Gabriela Cezar
Department of Animal Sciences, University of Wisconsin-Madison

Embryonic stem (ES) cells are defined by there abilities to self-renew indefinitely in culture and to differentiate into many cell types (Gjorret and Maddox-Hyttel, 2005). Because of their pluripotent capabilities, ES cells have given new hope to cell therapy for diseases such as diabetes, heart failure, and neurodegenerative diseases (Vats *et al*, 2005). Extensive successful research has been done to develop mouse ES cells lines, however the mouse model may not serve as the best comparison to humans for in vivo studies.. This has brought about and increased interest in developing stem cell lines in domestic livestock species. Domestic animals can be more physiologically similar to humans, and allow for more extensive long-term research (Gjorret and Maddox-Hyttel, 2005). There has been widespread research in the development of bovine ES cells; however high variability in the techniques used and results obtained remain. Inner cell mass (ICM) cells in the bovine embryo are tightly surrounded by trophoblast cells, which will later derive the placenta (Maddox-Hyttel *et al,* 2003). This close association may be one of the reasons that few bovine ES cell lines have been established. Pickering *et al* (2003) indicated that there is a risk that not all of the trophoblast cells may be removed during mechanical dissociation and that they could overgrow and inhibit the growth of the inner cell mass cells in culture. My project proposes a new method for the removal of inner cell mass cells from bovine blastocysts. This new method employs the use of laser microdissection and pressure catapulting (LMPC). LMPC platform is a pulsed UV-A laser that allows isolation of specific cells in a non-contact and contamination free manner (Burgemeister, 2005). Viability of the cells extracted and surrounding cells is not compromised and the isolated cells are removed via "cold ablation." The second aim of my project will be to determine if the presence of trophoblast cells does indeed affect the ability of bovine stem cells to propagate in vitro. This will be accomplished by co-culture of bovine ICM cells with trophoblast cells to recreate the embryonic microenvironment ex vivo.

References

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Pickering, S.J., Braude, P.R., Patel, M., Burns, C.J., Trussler, J., Bolton, V., Minger, S. 2003. Preimplantation genetic diagnosis as a novel source of embryos for stem cell research. *Reproductive BioMedicine Online.* 7: 353-364

Vats, A., Bielby, R.C., Tolley, N.S., Nerem, R., and Polak, J.M. 2005. Stem Cells. *The Lancet*. 366: 592-602.

Invited Speaker:
Thomas Yeager, Ph.D.

Dr. Yeager received his B.S. in Biology from the University of Alaska-Fairbanks. Afterwards he spent two years studying microbiology and analytical chemistry on the EXXON Valdez oil spill. He then went on to obtain his Ph.D. in Cellular and Molecular Biology from the University of Wisconsin-Madison where he studied the effects of tumor suppressor genes in bladder cancer progression. His post-doctoral research focused on the role of telomeres and telomerase in immortalization at the Children's Medical Research Institute in Sydney, Australia. He has been working at Promega for the last four years where he is a Senior Research scientist studying RNAi.

Abstract 5

RNA Interference: A Powerful Technique for Protein Knockdown

Thomas Yeager, Ph.D.
Promega Corporation, Wisconsin, Madison

RNA interference (RNAi) is a process that occurs naturally within cells by which small, double-stranded RNA can specifically suppress gene expression. Its use in research has grown rapidly in recent years, creating a powerful tool for studying gene function in the complex environment of a cell. RNAi can be initiated by the transient introduction of small synthetic double-stranded RNAs (siRNA) or by transcription based approaches in which short hairpin RNAs (shRNA) are generated from a DNA template. These tools and methods have been applied to evaluate TRAIL induced apoptosis in HeLa cells. Vectors expressing shRNAs against genes critically involved in this apoptosis pathway have been used to experimentally alter TRAIL induced apoptosis. Our ddRNAi experiments involve two basic steps: (1) Finding an effective shRNA target sequence; and (2) expressing that target sequence in cells to direct gene silencing by RNAi. To address target site screening, we developed a reporter-based system (siCHECK™ System) that allows for quantitative and rapid identification of effective siRNA and short hairpin RNA (shRNA) target sequences. Once a target site was chosen, the cognate shRNA was cloned into psiSTRIKE™ or psiLentGene™-2 Vectors to express hairpin sequences ***in vivo***. The vectors encode antibiotic-resistance genes, allowing transient or stable expression of the shRNA.

Oral Session II

Room 325 & 326
1:30-2:30

Chairs:
Dr. David Abbott
Dr. Craig Atwood

Abstracts 6-8

Abstract 6

Possible Role of G Protein-Coupled Receptor GPR30 in Estrogen Action in Primate LHRH Neurons

S.D. Noel[1], K.L. Keen[1], S.I. Frost[1], E.J. Filado[3], E. Terasawa[1,2]
[1]Wisconsin National Primate Res. Ctr. and [2]Dept. Pediatrics, Univ. Wisconsin-Madison, Madison, WI, [3]Dept. Medicine, Brown Univ., Providence, RI 02903

Activity of luteinizing hormone releasing hormone (LHRH) neurons is modulated by estrogen (E_2). Previously we have shown that E_2 causes rapid actions in LHRH neurons derived from the olfactory placode of rhesus monkey embryos: E_2 increased the frequency and synchronization of intracellular calcium ($[Ca2+]_i$) oscillations in cultured LHRH neurons with a short latency and this rapid action was partially insensitive to ICI 182,780. Because GPR30, an orphan G protein-coupled receptor, has been shown to mediate E_2 action in several breast cancer cell lines, in the present study we examined the possible role of GPR30 in the rapid action of E_2 in primate LHRH neurons. Immunocytochemistry and RT-PCR analysis showed that GPR30 protein was expressed in LHRH neurons in female adult hypothalamus, and GPR30 mRNA was expressed in olfactory placode cultures and the medial basal hypothalamus of adult female monkeys. The resulting PCR product was cloned and sequenced. The rhesus monkey form of GPR30 cDNA from a 600 bp fragment had 96% identity to human GPR30 cDNA and the deduced amino acid sequence corresponding to the peptide encoding the second intracellular loop through the third extracellular loop had 99% identity to human GPR30 amino acid sequence. To assess the possible involvement of GPR30 in E_2 action, we investigated the effects of pertussis toxin (PTX), which inhibits the G_i subunit of adenylate cyclase, on the E_2-induced $[Ca2+]_i$ changes in LHRH neurons. Preliminary data suggest that the effects of E_2 on $[Ca2+]_i$ oscillations were blocked by PTX. The results suggest a potential role of GPR30 in E_2 action in LHRH neurons.

Supported by NIH grants HD15433, HD11355, and RR00167.

Abstract 7

Concentrating Reticulocytes to Assess Iron Deficiency in Neonates

Melinda E. Chen, Sharon E. Blohowiak, Pamela J. Kling
Dept of Pediatrics and Center for Perinatal Care, University of Wisconsin, Madison

The protoporphyrin molecule, when bound to iron, becomes heme. With insufficient iron delivery, zinc substitutes and forms zinc protoporphyrin (ZnPP). ZnPP when expressed as a ratio to erythrocyte heme, shows promise as a clinical measure of iron deficient erythrocyte production in fetuses and newborns. Whole blood contains a mixed population of erythrocytes, with varying times in circulation. Immature red blood cells, called reticulocytes, have just entered the circulation. We hypothesized that if we were able to concentrate the percentage of reticulocytes in whole blood, the sensitivity of ZnPP to identify iron deficient erythropoiesis would improve. We concentrated reticulocytes in random human cord blood, and newborn and adult Sprague Dawley rat blood by centrifuging whole blood at 4K rpm for a total of one hour and collecting the top 6.25%. This top fraction contains the lighter reticulocytes. ZnPP was measured with the Aviv hematofluorometer in paired pre- and post-centrifugation samples from human cord and postnatal rats. Additionally, ratios from dam fed rats were compared to rats fed an iron deficient formula. Both inter- and intra-assay variation was assessed. Paired *t*-tests, unpaired *t* tests and repeated measures ANOVA were used. A p-value of <0.05 was considered significant. The concentrating step increased ZnPP in human cord blood by 10.1 µM/M, newborn rat blood by 40 µM/M, 8-day rat by 64.1 µM/M, 12-day rat by 85.0 µM/M, 18-day rat by 36.1 µM/M, and rat adults by 10.9 µM/M ($p < 0.01$ for all from before concentration). Pre-concentration ZnPP from 12-day old rats fed an iron deficient formula was similar to dam fed controls, but after concentrating, were higher (232.9 ± 24.0 vs. 164.1 ± 9.1, $p<0.005$). We observed similar ZnPP ratios assayed in different sample aliquots on the same day (intra-assay variation) or up to 10 subsequent days (inter-assay variation). The sensitivity of ZnPP to assess acute changes in iron availability may be improved by concentrating reticulocytes. If evidence continues to show reliability, we plan to determine the utility of fetal ZnPP after concentrating reticulocytes in additional populations, including fetuses.

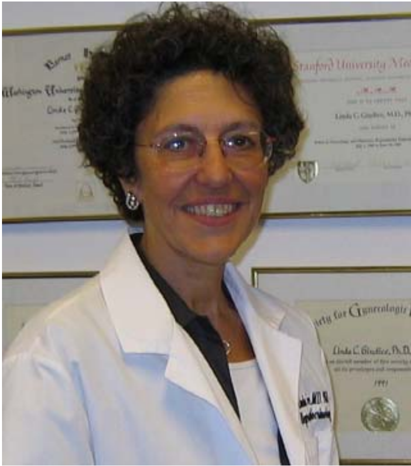
Abstract 8

CYP17 Activity In cDNAs Isolated From Marmoset and Rhesus Adrenals

J Christina Pattison[1]*, Ian M Bird[1]
[1]*Perinatal Research Labs, UW-Madison, Madison, WI, 53715, USA*

Marmoset monkeys differ from humans in having low circulating levels of DHEA. Males do not express a ZR and females exhibit a rudimentary ZR. Our interest lies in the mechanism by which this phenomenon occurs. To date we have isolated and sequenced the marmoset and rhesus CYP17 protein coding cDNAs. While numerous differences exist between the marmoset and human sequences, the specific mutations reported in familial 17,20-lyase deficiency are not present in the marmoset sequence. However, in the vicinity of the reported mutations, and between two predicted regulatory residues, lies an isoleucine-to-valine mutation. In this study we expressed our CYP17 cDNAs in HEK-293 cells. The marmoset and rhesus constructs translated immunodetectable protein, as determined by immunocytochemistry. 17alpha-hydroxylase activity appeared to be similar in both marmoset and rhesus constructs, with a preference for pregnenolone over progesterone. 17,20-lyase activity was measured by metabolism of pregnenolone substrate. Both constructs were equally capable of converting pregnenolone to 17alpha-hydroxypregnenolone and DHEA. Additionally, both constructs converted progesterone to 17alpha-hydroxyprogesterone, but not androstenedione. In conclusion, marmosets are not 17,20-lyase deficient in the basal state. It remains to be seen whether regulation of the protein (i.e. phosphorylation) induces similar 17,20-lyase activity in the marmoset vs. the rhesus constructs.

Keynote Lecture: Linda C. Giudice, M.D., Ph.D.



Dr. Giudice is Professor and Chair of the Department of Obstetrics, Gynecology and Reproductive Sciences at University of California, San Francisoc. She is a biochemist and gynecologist/reproductive endocrinologist whose research has focused for over a decade on endometrial biology and placental-uterine interactions and has clinical interests in endometriosis, implantation and ovulatory disorders, infertility, and assisted reproduction. Dr. Giudice received her BS from Columbia University and her PhD in Biochemistry from the University of California at Los Angeles. She was a postdoctoral fellow at Rockefeller University and also at the NIH. She subsequently received her MD from Stanford University and did her residency in OB/GYN at Stanford and Washington University in St. Louis and her fellowship in Reproductive Endocrinology and Infertility (REI) at Stanford. She is currently the Director of the REI Division, the Center for Research on Women's Health and Reproduction, and the *Women's Health @ Stanford* Program at Stanford University. She has trained more than 65 undergraduate and graduate students, and post-doctoral fellows. Since 1990, her laboratory has been continuously funded by the NIH for research on human endometrium, and she is a co-editor of the textbook, *The Endometrium.* At the national level, Dr. Giudice has been a member of two NIH study sections and the March of Dimes study section C, and chaired the NIH Population Research Committee. She is currently Steering Committee Chair of the NIH Specialized Cooperative Centers Program for Reproductive Research and of the NIH Reproductive Medicine Network. In 2002 she was elected to the Institute of Medicine of the National Academy of Sciences. She is Chair of the Reproductive Health Drugs Advisory Committee to the FDA, immediate past President of the Society for Reproductive Endocrinology and Infertility and sits on the Executive Board of the Reproductive Scientist Development Program and on the Frontiers in Reproduction Board of Scientific Counselors. She was Chair of the Gordon Research Conference (GRC) on Reproductive Tract Biology and was on the Council of the GRC. She was recently elected President of the Society for Gynecologic Investigation and sits on its Executive Council. She is on the Board of Directors of the Society for Women's Health Research and was the Board of Directors of the American Society for Reproductive Medicine. She also sits on the editorial boards and is associate editor of numerous professional journals. Dr. Giudice is a sought-after lecturer and consultant for issues related to research, mentoring, education, and policy in women's health and reproduction.

Abstract 9

Molecular Phenotyping of Human Endometrium Distinguishes Menstrual Cycle Phases and Underlying Biological Processes

Linda C. Giudice, MD, PhD
Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, CA

Histologic evaluation of human endometrium has long been the gold standard for clinical diagnosis and management of women with endometrial disorders. However, several recent studies have questioned the accuracy and utility of such evaluation, primarily because of significant intra- and inter-observer variability. We have proposed that biochemical or molecular signatures of endometrium is a powerful approach to endometrial evaluation. To this end, we investigated whole genome molecular phenotyping (54,600 genes/ESTs) of this tissue sampled in 28 normo-ovulatory women, using high-density oligonucleotide microarrays. Unbiased principal component analysis (PCA) of all samples revealed that samples self-cluster into four groups consistent with histologic phenotypes of proliferative (PE), early secretory (ESE), mid-secretory (MSE), and late secretory (LSE) endometrium. Independent hierarchical clustering analysis revealed equivalent results, with 2 major dendrogram branches corresponding to PE/ESE and MSE/LSE and sub-branching into the 4 respective phases with heterogeneity among samples within each sub-branch. Kinetic clustering analysis of genes revealed 4 major patterns of gene expression, and gene ontology analysis of these clusters demonstrated cycle-phase specific biological processes and molecular functions. Six samples with ambiguous histology were identically assignable to a cycle phase by both PCA and hierarchical clustering. Additionally, pair-wise comparisons of relative gene expression across the cycle revealed genes and gene families that clearly distinguish the transitions of PE→ESE, ESE→MSE, and MSE→LSE, including receptomes and signaling pathways. Select genes were validated by quantitative RT-PCR. Overall, the results demonstrate that endometrial samples obtained by two different sampling techniques (biopsy and curetting hysterectomy specimens) from subjects who are as normal as possible in a human study and including those with unknown histology, can be classified by their molecular signatures. Furthermore, these signatures cluster and correspond to known phases of the menstrual cycle with identical results using two independent analytical methods and without requiring a priori histologic assessment. Also, the results enable global identification of biological processes and molecular mechanisms that occur dynamically in the endometrium in the changing steroid hormone milieu. The results underscore the potential of gene expression profiling for developing molecular diagnostics of endometrial normalcy and abnormalities and identifying molecular targets for therapeutic purposes in endometrial disorders.

Poster Session

Alumni Lounge
4:00-5:00

Abstracts 10-32

Abstract 10

Female Germline Development and Stem Cell Differentiation

Maria Giakoumopoulos and Thaddeus Golos
Wisconsin National Primate Research Center and Dept. Ob/Gyn, University of Wisconsin-Madison, Madison WI 53715

Primordial germ cells (PGCs) are the precursors to the gametes and are thought to arise independently of the ectodermal, endodermal, and mesodermal embryonic germ layers. Gene expression in PGCs at the time of gonadal development has been characterized, and a subset of genes including *DAZL, VASA, SCP3,* and *GDF9* are useful markers of the germ cell lineage. The aim of this work is to create a culture system for the differentiation of follicles and oocytes from human and rhesus embryonic stem cells (hESC, rESC respectively). RT-PCR was used with hESC and rESC at the undifferentiated (0%) state, spontaneously differentiated state (90%), and embryoid bodies (EBs) to assay for a panel of follicle/oocyte-specific and enhanced genes. Also, hESC differentiation marked by *GDF9* and *SF1* promoters driving the expression of fluorescent proteins to demonstrate spontaneous germ and follicular cell differentiation is currently in use. Our results show that undifferentiated H1 hESC express *STELLAR, NANOS, PUM1; STELLAR* and *NANOS* following spontaneous differentiation; and *NANOS* in EBs. In rESC, *NANOS* and *TEKT1* are expressed in the undifferentiated cell as well as in differentiated derivatives. Future directions may include oocyte co-culture with ovarian stromal and granulosa cells to develop an *in vitro* model of the ovarian follicular developmental environment as a model for morphogenesis.

Abstract 11

A Three-Dimentional *In Vitro* Model on Primate Embryo Implantation and Early Pregnancy Development

T Chang, B Gerami-Naini, JG Drenzek, GI Bondarenko, OV Dovzhenko, M Durning, MA Garthwaite, TG Golos

National Primate Research Center, and Department of Obstetrics and Gynecology, University of Wisconsin-Madison

The initiation of embryo invasion into the endometrium and the formation of placenta from proliferation and differentiation of trophoblasts and mesenchyme are essential and critical toward establishment of a successful pregnancy. The detailed mechanisms relevant to cellular localization and actions during the time of embryo invasion and placentation are yet to be well studied due to lack of tools to visualize and examine real-time primate implantation and maternal-embryo interactions. Nonhuman primate models are more feasible than rodent models for understanding human reproduction physiology and pathology, as well as bridging the gap between animal models and human medicine. Therefore, an *in vitro* nonhuman primate model is in need to enhance the study of pregnancy initiation.

We have reported a novel *in vitro* three-dimensional (3-D) model to study implantation phenomena by utilizing IVF generated rhesus monkey (*macaca mulatta*) embryos in a Matrigel culture system. Expanded pre-hatching blastocysts were embedded in 3-D microenvironment constructed by Matrigel and feeder layer cells in conditioned medium. Hatching of blastocysts initiated short after embedding, following by enlargement of embryo mass, invasion and proliferation of trophectoderm cell layers, cystic formation, and cellular outgrowths. Trophoblast-like structures with protrusion and branches growing from the surface of embryo implants were observed beginning at the end of first week post embedding. Rapid proliferation of the trophoblast-like cellular structures during the second and third week post embedding has shown evidence of interactions between the embryo and the microenvironment, which is similar to placental development. Immunohistochemical staining with chorionic gonadotropin (CG) and other markers, combining immunoassays with CG and progesterone, indicated positive characteristics of trophoblastic cell lineages from growing embedded embryos. Immunoassay has also revealed a curve of elevation and decline in CG and progesterone secretion, similar to early pregnancy *in vivo*, from those embryos showing trophoblast-like features. In addition, our study found indicators for establishing normal and prolonged embryo growth in such an *in vitro* system.

This 3-D in vitro system has shown capacity to study implantation by using IVF generated rhesus embryos and Matrigel platform. Proceeding projects and future directions on optimizing microenvironment conditions and investigating growth factors critical for peri-implantation stage development will be beneficial to facilitate our understandings of nonhuman primate biology and human reproduction, with potential to accelerate therapeutic treatments for women suffering from early pregnancy loss and related pathological issues.

Abstract 12

Detecting Expression of Proliferation, Differentiation and Apoptosis Markers in Trophoblast Cells Derived from Embryoid Bodies Grown in Three-Dimensional Matrix Structure and in Suspension Culture

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Trophoblast turnover includes proliferating cytotrophoblasts, differentiation of cytotrophoblasts into syncytiotrophoblasts and apoptosis. Our preliminary studies indicated that embryoid bodies (EBs) derived from human embryonic stem cells (hESC) embedded into a three-dimensional Matrigel (MG) environment promoted pronounced levels of placental hormone secretion compared to EBs grown in suspension culture. In the current study, our aim is to further examine the capacity of EBs as a model for studying the early stages of feto-maternal interaction and placental development. By using a panel of markers, we identified levels of proliferation, differentiation, and apoptosis among EBs grown in suspension and EBs embedded into Matrigel for different periods of time.

We used of Ki67 immunohistochemistry (IHC) staining to evaluate the level of cell proliferation among EBs in suspension and EBs embedded into Matrigel. To determine the level of cell differentiation, we determined the expression of Octamer-binding transcription factor 4 (Oct-4), a marker of pluripotency, via IHC. We determined cytokeratin 18 neopeptide (CTK 18) expression as an indirect approach to detect the level of apoptosis.

Ki67 staining showed proliferation found in outer layer as well as inner parts of the EBs in suspension during the early stages: EB one day old (EBd1) and EB eight days old (EBd8). Ki67 staining for the EBs embedded in Matrigel is mainly visible in the outer layer of cells. Similarly, Oct4 expression is maintained around the outer edges of the EBs, suggesting that anchorage of EBs to the ECM may play an important role in keeping the cells in a stable pluripotent state. Cytokeratin 18 neopeptide detection by antibody M30 in EBs grown in suspension is rare in early stages, mainly focused in a few larger areas at later stages (60 days) of culture. M30 staining among EBs embedded in Matrigel was not observed consistently and only a few cells were stained by M30.

In conclusion, Ki67 and Oct4 immunostaining revealed EBs embedded into Matrigel for long period showed higher level of proliferation and undifferentiated cells than EBs cultured in suspension. M30 immunostaining displayed low level of apoptosis in both EBs cultured in suspension and EBs embedded into Matrigel. Yet, the early stages of EBs cultured in suspension displayed lower level of apoptosis than the EBs cultured for longer period of time.

This work was supported by NIH grants HD34215 and RR14040 to T.G.G., and HD041921 to B.G.-N.

Abstract 13

Overexpression of a Major Histocompatibility Complex Alloantigen in the Rhesus Monkey Placenta

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Major histocompatibility complex (MHC) class I molecules may play a role in allowing the fetus to evade an attack by the maternal immune system. Primate implantation is relatively invasive when compared to other species. The placenta attaches to the maternal decidua, is bathed in maternal blood, and is in contact with maternal immune cells. Extravillous cytotrophoblasts invade maternal spiral arteries and remodel them to allow for continuous blood flow to the fetus. Nonclassical MHC class I molecules include human leukocyte antigen (HLA)-E, HLA-F, and HLA-G. These molecules exhibit low polymorphism, a low level of expression, and are found in the placenta. HLA-G is known to interact with decidual NK cell receptors to deliver an inhibitory signal to spare the placenta from an immune attack by the mother. Classical MHC class I molecules include HLA-A, HLA-B, and HLA-C. HLA-A and HLA-B exhibit high polymorphism, a high level of expression, and are not found in the placenta. Conversely, HLA-C exhibits low polymorphism and is found in the placenta. The purpose of this experiment is to overexpress a classical MHC class I alloantigen, Mamu-A*01, in the rhesus monkey placenta. By overexpressing Mamu-A*01 in the rhesus monkey placenta we will be able to test the hypothesis that maternal fetal immune tolerance is dependent on the lack of highly polymorphic MHC class I molecules in the placenta. It would be expected that the expression of this alloantigen in the rhesus monkey placenta would cause a T cell response to nonself, an NK cell response to the lack of an inhibitory signal, or both types of responses. Two vectors have been constructed that both have elongation factor-1α as the promoter and Mamu-A*01 as the transgene. One plasmid is within a lentiviral backbone and the other plasmid expresses green fluorescent protein. Two cell lines were used for transfection experiments. They are K562 and 721.221 cells, which both do not express MHC class I molecules. After undergoing antibiotic selection, expression of Mamu-A*01 has been confirmed by RT-PCR, Western blot, and flow cytometry. The next step will be to incorporate Mamu-A*01 into an SIV-based lentiviral vector. The lentivirus will be microinjected into rhesus monkey embryos and transferred to a recipient monkey. It is important to note that neither the mother or father nor the recipient monkey will be Mamu-A*01 positive. Understanding the mechanisms of maternal fetal immune tolerance while using the rhesus monkey model may lead to therapies that would improve fetal growth or development and combat complications of pregnancy that are a result of aberrant implantation such as miscarriage, pre-eclampsia, and intrauterine growth retardation.

Abstract 14

Human Monocytes and Alveolar Macrophages Release Interferon γ-Inducible Protein 10 (IP-10) following Rhinovirus (RV) Challenge

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Rationale: Respiratory infections with human RV are a major cause of asthma exacerbations and likely contribute to the overall pathogenesis of asthma. Secretion of IP-10 (CXCL10), which is an enhancer of activated T-lymphocyte and natural killer cell chemotaxis, is significantly increased in nasal-lavage fluid of humans challenged with human major group rhinovirus – 16 (RV-16) and the levels correlate with symptom severity, viral titer, and T-lymphocyte numbers. However, the cellular source and regulation of IP-10 production in response to RV exposure are not known. Activation of alveolar macrophages, the predominant immune cell in the airway lumen, likely alters the hormone milieu within the inflammatory microenvironment in response to RV respiratory infections. Accordingly, this study tested the hypothesis that human monocytes/macrophages release IP-10 following exposure to RV-16 and that this process is associated with STAT1 and Jun kinase activation and the release of type 1 interferon, which are important for the induction of IP-10 expression.

Methods: Primary human blood monocytes and bronchoalveolar lavage macrophages were pre-treated with or without SP600125 (selective inhibitor of Jun kinases) or anti-CD118 (type 1 interferon receptor blocking antibody) and incubated with purified-human RV-16 (MOI=0-100) for up to 72 h. Tissue culture medium was examined for the presence of IP-10 via ELISA, and cell lysates were immunoblotted for activated STAT1.

Results: Exposure of human monocytic cells to RV-16 results in the release of IP-10 and phosphorylation/activation of STAT1 in a time- and dose-dependent manner ($n \geq 3$). Pretreatment of human monocytic cells with SP600125 or anti-CD118 attenuates RV-induced release of IP-10 ($n \geq 3$).

Conclusions: These studies demonstrate that RV interaction with monocytic cells stimulates STAT1 phosphorylation and release of IP-10. These events are mediated, at least in part, by the activation of Jun kinase pathways and are likely secondary to the autocrine/paracrine release of type 1 interferons.

Abstract 15

Fetal Androgen Excess Programs for Insulin Resistance, Type 2 Diabetes and Increased Adiposity in Female Rhesus Monkeys

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Prenatal androgenization (PA) during early (E) or late (L) gestation induces metabolic and reproductive defects in adult female rhesus monkeys that resemble those of polycystic ovary syndrome (PCOS) women. Fetal male testosterone levels were induced in fetal females by administering to their mothers daily subcutaneous injections of 10mg testosterone propionate for either 15-35 or 15-25 consecutive days, starting on gestation days 40-44 (EPA), or 100-115 (LPA), respectively. Newborn weights of EPA and LPA females are similar to those of controls, as are heart rate and blood pressure in later life. By mid/late reproductive years, EPA females exhibit insulin resistance, diminished glucose-stimulated insulin release and elevated fasting glucose, as well as total free fatty acid levels, versus age- and body mass index-matched controls, along with an increased incidence of type 2 diabetes. LPA females show hyperglycemia, alone, with preferential accumulation of total body and abdominal fat. Fetal androgen excess appears to program *in utero* susceptibility to several PCOS-like defects through dysregulation of crucial cellular or molecular metabolic processes.

Supported, in part, by NIH R01-RR013635, P50-HD044405, U01-HD044465 and T32-AG000265.

Abstract 16

LH Receptor Expression in Luteinized Granulosa Cells Obtained Following Ovarian Hyperstimulation for In Vitro Fertilization in a Nonhuman Primate Model for Polycystic Ovary Syndrome (PCOS)

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PCOS is a complex human disorder of unknown etiology. It is the leading cause of anovulation and type 2 diabetes in young women. Prenatal androgen excess may reprogram multiple organ systems, including the ovary, resulting in PCOS. Our lab is investigating the basis for poor oocyte quality in PCOS women. We are using the prenatally androgenized female rhesus monkey, a well-established animal model for PCOS. These monkeys exhibit LH hypersecretion and they respond to recombinant hCG-induced luteinization in an exaggerated fashion. Such altered ovarian response to gonadotropin stimulation may contribute to diminished oocyte developmental competence. This study was initiated to determine whether LH receptors are over-expressed in luteinized granulosa cells and thus provide a potential mechanism for abnormal luteinization.

Abstract 17

Uterine Artery (UA) Shear Stress in the Proliferative and Secretory Phases of the Menstrual Cycle

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Shear stress is the most potent physiologic stimulus for elevating endothelial NO production for inducing flow-mediated vasodilatation. Shear stress = 4*Blood Flow * viscosity/πr^3. At a given vascular cross sectional area, shear stress is directly proportional to rises in blood flow and viscosity and inversely proportional to internal radius cubed. Elevations in uterine blood flow (UBF) are seen in the menstrual cycle secretory vs proliferative phase (Obstet Gynecol 94:695, 1999). In this study we measured changes of *in vivo* shear stress during the menstrual cycle and tested the *hypothesis* that compared to the proliferative phase, secretory phase UBF rises account for elevation in shear stress.
Methods: During the proliferative and again in the secretory phase (n=31 subjects; 55 total cycles). UA blood flow velocity and internal radius were measured bilaterally (5 replicates/side) using high-resolution color Doppler ultrasound; angle of sonoincidence < 60. Heparinized blood was collected and viscosity measured using a torque viscometer
Results: The estrogen/progesterone ratio was elevated in proliferative vs. secretory phase women because progesterone fell; estradiol was unaltered.

	Menstrual Cycle Phase (***<0.001)	
	Proliferative; D=11 (n=55)	**Secretory; D=22 (n=55)**
Estradiol (pg/ml)	78.8 ± 7.0	95.7 ± 5.5 NSD
Progesterone (ng/ml)	0.539 ± 0.034	10.9 ± 0.7 ***
Estrogen/Progesterone	195 ± 25	10.2 ±0.7 ***
Viscosity (P)	0.0562 ±0.001	0.0585 ± 0.007 NSD
UA Internal Radius (cm)	0.098 ± 0.002	0.099 ± 0.002 NSD
Mean UBF (ml/sec)	0.33 ± 0.021	0.45 ± 0.03 ***
Peak Systolic UBF (ml/sec)	1.02 ± 0.06	1.27 ± 0.08 ***
Mean Shear Stress (dynes/cm^2)	28.8 ± 1.95	37.6 ± 3.11***
Peak Systolic Shear Stress (dynes/cm^2)	91.2 ± 5.22	106.8 ± 5.79***
Mean UBF (ml/min)	19.9 + 1.25	26.9 + 1.89
Peak Systolic UBF (ml/min)	31.3 + 3.46	1.27 + 0.08

Neither viscosity of blood samples nor internal radius of the UA was altered by the ovarian cycle. By contrast, proliferative phase women showed significant and proportional rises in Mean UBF and Shear Stress (36% & 31%) and Peak Systolic UBF and Shear Stress (25%and 17%). **Conclusions:** Compared to the estrogen/progesterone ratio-dominated proliferative phase, the secretory phase (state of high progesterone with significant luteal estrogen production) elevations in UBF and shear stress are observed. We provide the first *in vivo* estimates of UA shear stress and suggest that changes in ovarian steroid may mediate UBF thereby contributing to elevations in shear stresses and thus endothelial nitric oxide production.

Supported by funds from the NIH (HL492710, HD33255, HD3884, HL63101).

Abstract 18

Blood Rheologic Properties and Uterine Blood Flow Changes During the Ovarian Cycle in Women and Sheep

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Body: Blood rheologic properties are an integration of visco-elastic forces between RBC's and plasma proteins. Viscosity is used to calculate fluid shear stress; i.e. the frictional forces on the artery wall that is a potent physiologic stimulus for elevating endothelial NO. Uterine blood flow (UBF) is altered cyclically during the ovarian cycle and in sheep this is estrogen-mediated. Purpose: To compare UBF changes and rheologic properties of blood in women and sheep in the ovarian cycle. Methods: Heparinized blood was collected from Follicular/proliferative phase women (D-11; n=9) and again in their luteal/secretory phase (D-22; n=9) and from follicular phase (periovulatory D-0, n=11) and luteal phase sheep (D-10-11 post ovulation, n=12). UBF was measured in women by color Doppler ultrasound and in sheep by transonic flow probes in the main uterine artery (UA). Because blood is a non-Newtonian fluid and hematocrit plays a major role, viscosity was analyzed in plasma (0%) and whole blood at endogenous (30-40%), low (15-29%) and high (41-55%) hematocrits using a torque viscometer.

	Human		Sheep	
	Follicular/Proliferative	Luteal/Secretory	Follicular	Luteal
Endogenous Hct (%)	36 ±0.5[a]	37 ±0.4[a]	35 ±1.6%[b]	36 ±0.9%[a]
Viscosity (cP)	5.61 ±0.1[a]	5.85 ±0.7[a]	3.70 ±0.11[b]	4.38 ±0.04[b]
UA Diameter (mm)	1.96 ±0.04[a]	1.98 ±0.04[a]	3.39 ±0.16[b]	3.05 ±0.19[b]
UBF (ml/min)	19.9 ±1.25	26.9 ±1.89 (P<0.01)	31.9 ±4.7 (P<0.01)	7.0 ±0.425

Results: In both species endogenous hematocrit, viscosities and internal UA diameters were not altered by the ovarian cycle. Viscosities of blood samples were increased as in vitro hematocrit was elevated (P<0.001). Although the endogenous hematocrits were unaltered by cycle in humans and sheep, women had a higher viscosity and smaller UA diameter (P<0.01). UBF patterns were opposite in the two species; Luteal > Follicular in women and Follicular > Luteal in sheep. Conclusion: The physiologic sequence contributing to these UBF differences is uncertain. The striking differences in UBF patterns may manifest because luteal phase women have both high estradiol and progesterone whereas sheep do not have elevations in corpus luteum-derived estradiol. Another possibility is a substantially greater follicular phase angiogenic response of the human endometrium followed by luteal phase thickening, and consequent vascular remodeling of the stratum functionalis, not seen in the ovine uterus, which does not undergo menstruation.

NIH HL49210, HD33255, HD38843, HL63101.

Abstract 19

Pregnancy and Ovarian Steroid Regulation of Angiotensin II Type 1 and Type 2 Receptor Expression In Ovine Uterine Artery Endothelium and Vascular Smooth Muscle

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While pregnancy is clearly associated with refractoriness to infused AII in the uteroplacental unit, there is still dispute over the mechanism by which AT_1R and AT_2R may mediate this response in the uterine artery. This is in large part due to incomplete knowledge of levels of AT_1R and AT_2R expression and function in UA Endo in the nonpregnant and pregnant states, combined with the disagreement on whether AII may act through release of adrenomedullary catecholamines. We have previously described an increase in AT_1R in UA Endo but not UA VSM during pregnancy as compared to the nonpregnant intact ewe. We report that the pregnancy associated increase in AT_1R expression in UA Endo is regulated by ovarian steroids. Using a recently developed antibody to AT_2R we now show there is no change in AT_2R in UA Endo or VSM associated with ovarian function, and while AT_2R is not changed in UA Endo by pregnancy, there is a significant decrease observed in UA VSM at that time. We also examined changes in receptors in UA Endo and VSM in E2β primed ewes in view of the common use of this model as a control for physiologic studies. In contrast to our findings in nonprimed nonpregnant or pregnant animals, we observed a significant increase in both AT_1R and AT_2R in UA Endo in response to the supra-physiologic priming with E2β. In order to address the possible functionality of AT_1R or AT_2R in UA Endo we used the UAEC model of UA Endothelial cells maintained in culture to passage 4. Differences in expression of AT_1R or AT_2R were normalized at passage 4 in P-UAEC and NP-UAEC. Treatment with AII activated PLC in both NP and P-UAEC but signaling through the ERK pathway was dramatically enhanced in P-UAEC compared to NP-UAEC. To our surprise, both phosphoinositol turnover and ERK2 phosphorylation responses failed to display the expected dose-responses. Inhibition of AII-stimulated ERK 2 phosphorylation with antagonists DUP 753 (AT_1R, 10uM) and PD 123319 (AT_2R, 10uM) failed to selectively inhibit ERK2 phosphorylation. We observe that (i) an effect of pregnancy is an increase in the AT_1R/ AT_2R ratio in both UA Endo and VSM but through apparently distinct mechanisms. (ii) the ovex animal model is similar to the luteal state for AT_1R and AT_2R expression, while the E2β primed model does not resemble the nonpregnant or pregnant state. (iii) there is a real possibility that AII may mediate its effects either through a complex AT_1R - AT_2R interaction or via an as yet unidentified non AT_1, non AT_2 receptor. *Supported by USDA 0002159, NIH-HL56702, HL49210.*

Abstract 20

Use of Ciclohexamide (CHX) to Inhibit Estradiol 17 Beta (E2)-Induced Prostaglandin-F2 Alpha (PGF) Production in Bovine Endometrium

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The PGF induces luteolysis in cattle. However, regulation of PGF production is not well known. Several studies show evidences for a role of E2 in this context (1; 2), but mechanisms by which E2 acts are still unclear. Production of PGF in response to E2 injection in cows increases after 3 h (1; 3; 4). Based on the lag of time from injection to stimulus, the hypothesis of the present study was that E2–stimulated PGF production depends on new protein synthesis. Objective of *Experiment 1* was to identify concentrations of E2 capable of stimulating endometrial PGF production *in vitro*. Objective of *Experiment 2* was to verify whether E2-induced PGF production was dependent on protein synthesis. Cross-bred beef cows were slaughtered on day 17 of a synchronized estrous cycle. Endometrial explants (±100 mg) were cultured in six well plates containing 3 mL of Ham-F10 medium at 37,5^{o}C. After 1 h of incubation, explants received treatments in triplicate. Medium samples were collected at experimental hours 1 and 12 and concentrations of PGF were measured by radioimmunoassay.

*Experiment 1:*Endometrial explants from each of 4 cows were randomly distributed to receive 0, 10^{-15}, 10^{-14}, 10^{-13}, 10^{-12} or 10^{-11} M E2. Production of PGF was calculated subtracting concentrations at hour 12 from that at hour 1, and analyzed by ANOVA. Independent variables were cow and concentration of E2. There was interaction between cow and concentration of E2 ($P<0.01$) indicating that responses to E2 treatments varied among animals. The E2 concentrations to most effectively stimulate PGF production were 10^{-12} and 10^{-11} M.

*Experiment 2:*It was determined in preliminary studies that 200µg/ml of CHX (a protein synthesis inhibitor) effectively inhibited PGF synthesis in endometrial tissue, while not affecting its viability. Endometrial explants from each of 3 cows were randomly distributed in a 3x2 factorial arrangement to receive combinations of E2 (0, 10-12 or 10-11 M) and CHX (0 or 200µg/ml). Production of PGF was analyzed by ANOVA. Independent variables were cow, concentration of E2, concentration of CHX, time, well and interactions. The absence of an interaction between concentration of E2 and time ($P>0.05$) indicated that although there was production of PGF in culture overtime, it was similar in presence or absence of E2. The CHX blocked PGF production in culture (interaction of CHX and time; $P<0.01$), but this effect was independent of E2 concentration (there was no interaction of CHX by E2; $P>0.05$). In conclusion, PGF production by endometrial explants is dependent on protein synthesis. Whether E2-estimulated PGF synthesis is dependent on protein synthesis remains to be determined.

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Abstract 21

Effects of Leptin on Gene Expression in Mammary Epithelial Cells

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It is known that leptin is produced by the mammary gland, and that mammary epithelial cells express leptin receptors. The role of leptin is still not fully understood but it is thought to play an autocrine/paracrine and/or endocrine role in mammary develop-ment. Our research focuses on what genes might be regulated by leptin. Normal murine mammary gland (NMuMG) cells were either treated with 100 ng/mL leptin for 0, 2, 4, 6, 8, or 12 hours or left untreated. Subsequent reverse transcription-polymerase chain reaction (RT-PCR) and 5% polyacrylamide gel electrophoresis (PAGE) was used to determine the presence of nucleoporin 153 (NUP153), SP1 cofactor, Parkin (ubiquitin ligase), and AP4β1 with GAPDH used as an internal control. Preliminary results from quantitative PCR (qPCR) show a 100-1000 fold increase in NUP153, while there appears to be only a slight increase in SP1 cofactor and a slight decrease in Parkin.

Abstract 22

The Pathophysiological Basis of Vitamin D Resistant Diseases in Man

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Renal phosphate wasting disorders constitute a substantial portion of the Vitamin D resistant diseases in man. Central to the pathogenesis of these diseases is the elaboration of a hormone(s) from the family of hormones recently named the 'phosphatonins'. The identification of these hormones and the mechanism underlying their enhanced production remains unknown. Several relatively recent observations, however, have provided the building blocks about which to hypothesize the mechanism underlying the prototypic vitamin D resistant disease, X-linked hypophosphatemia (XLH). These observations include: 1) identification of the gene underlying XLH, the Phosphate Regulating gene Homologous to Endopeptidases coded on the X-chromosome-(*PHEX*) gene, which encodes a membrane bound endopeptidase located primarily in the osteoblasts and osteocytes; 2) recognition that Fibroblast Growth Factor (FGF-23) produced primarily in osteocytes, and Frizzled Related Protein-4 (FRP-4) produced in bone and kidney, may serve as the phosphatonins in XLH; and 3) documentation that knockout of Dental Matrix Protein-1 (DMP-1), located in osteocytes and other tissues, results in a renal phosphate wasting disorder. Based on these data, the pathophysiological cascade in XLH may include: defective PHEX function in osteocytes → a decrease in osteocyte DMP-1 production → increased osteocyte FGF-23 and /or FRP-4 production → increased circulating levels of these phosphatonins, causing abnormal renal phosphate reabsorption (phosphate wasting) and 25(OH)D1α-hydoxylase regulation, classic phenotypic characteristics of XLH. Of course serial investigation of this hypothesis may result in alteration in the schema outlined. Nevertheless, the studies undertaken will unequivocally provide essential information to unravel the pathophysiological basis of XLH.

Initially, characterization of DMP-1 knockout mice will be accomplished by: 1) analyzing renal phosphate transport and 25(OH)D1α-hydoxylase mRNA, protein and enzyme activity, as well as assessing production and circulating levels of FGF-23 and FRP-4. As hypothesized, the results of these studies may be linked to the pathophysiology of XLH. To confirm such links, we will cross transgenic DMP-1 mice with *hyp*-mice, the murine homologue of XLH, to determine if DMP-1 over expression rescues the *HYP* phenotype. Further, using the CRE-lox approach and DMP-1 cre mouse, we will and selectively knockout *PHEX* in osteocytes and thereby determine if a *PHEX* abnormality exclusively in osteocytes underlies the disease phenotype, further linking XLH to a DMP-1 defect in osteocytes.

Abstract 23

Fetal Androgen Excess Accelerates Pulsatile Luteinizing Hormone (LH) Release in a Nonhuman Primate Model for Polycystic Ovary Syndrome (PCOS)

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Since exposure of the developing female hypothalamus to androgens produces permanent alterations in the ability of GnRH release, LH excess found in hyperandrogenic PCOS women may be a consequence of programming of the developing fetal hypothalamus by androgens, leading to hypersecretion of gonadotropin-releasing hormone (GnRH) in adulthood. In order to test this hypothesis, we studied 7 adult, prenatally androgenized (PA) female rhesus monkeys, and 3 age- and weight-matched controls. PA monkeys exhibit many of the pathophysiological features of PCOS. Four of the PA monkeys were exposed to androgen excess during early (E) gestation (25-35 days, starting on gestation days 40-43) and 3 were exposed during late (L) gestation (15-25 days, starting on gestation days 100-115). Plasma LH levels were determined from a series of blood samples obtained through an indwelling femoral catheter at 10-min intervals from 0600h-1600h, followed by an additional GnRH challenge test in which GnRH was infused at 1600h. LH levels in plasma were assessed by RIA and LH pulses were determined by PULSAR. Mean (±SEM) LH levels were similar in EPA (0.9±0.2 ng/ml), LPA (0.6±0.2 ng/ml) and control females (1.1±0.2 ng/ml). Both EPA and LPA females also did not differ from control females with regard to baseline LH levels, LH pulse amplitude, and LH response to GnRH. Comparison of LH parameters between control and PA female groups, however, suggested ~2-fold increase in frequency of pulsatile LH release in EPA, but not LPA females. Early gestation exposure to androgen excess thus appears to increase the frequency of pulsatile LH release without apparent concomitant changes in pituitary gonadotrope responsiveness to GnRH. The acceleration of LH pulse frequency in EPA, but not LPA females, implies that early prenatal androgen exposure programs accelerated GnRH pulsatility in adult female monkeys. Our non-human primate study has direct implications for PCOS women, as these results suggest that not all women with PCOS will exhibit LH hypersecretion, a prediction that is borne out in the heterogenous presentation of elevated LH levels in women with PCOS.

Supported by NIH SCOR Grant P50 HD044405.

Abstract 24

The Effect of Oxytocin on mGnRHI and cGnRHII in Salmonoids

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Oxytocin has been shown to impact the release of Gonadotropin Releasing Hormone (GnRH I) in several model systems. My focus is the regulation of GnRH release in fish, specifically salmonoids. Fish express 3 distinct variants of the GnRH molecule: seabream GnRH (sbGnRH), chicken GnRH II (cGnRH II), and salmon GnRH (sGnRH). Each variant has been reported to be released from a differing region of the brain. Brook trout brain tissue was divided into three regions (nucleus olfactoretinalis, preoptic area, and midbrain) and placed into an in vitro perifusion system to collect samples linearly for 10 hours. The brain sections were exposed to one day of media and samples were collected for 5 hours. On the second day the tissue was exposed to 1 hour of media followed by 10 minutes of oxytocin and 3 hours of media. Samples were collected every 10 minutes and mGnRH I and cGnRH II levels were determined by radioimmunoassay. Oxytocin showed no effect on the release of either GnRH isoform tested. Differential release from the various brain regions has not yet been observed. This study represents the first observation of GnRH release over time in Salmonoids.

Abstract 25

The Effects of Cyclic AMP as an Intracellular Signal Molecule Involved in GnRH Release

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Cyclic AMP is an intracellular signal molecule involved in GnRH release. GnRH is a decapeptide that is released from neuroterminals in the hypothalamus in discrete pulses in the median eminence. After GnRH is released it is degraded by neuropeptidases: EP 24.15 is a peptidase that breaks GnRH between the fifth and sixth amino acid, leaving GnRH 1-5 and GnRH 6-10 metabolites. GnRH 1-5 metabolite has been shown to be a positive feedback mechanism to the hypothalamus. Treatment with GnRH 1-5 increases GnRH and cAMP production. We are investigating the communication between neurons that may be conducted by cyclic adenosine monophosphate (cAMP). cAMP is a second messenger hormone that may be used to signal the release of GnRH to other neurons. When GnRH and it receptor bind the receptor also binds to a complex of proteins (the G proteins). Through many steps ATP may bind to a Calcium channel which may cause conversion of ATP to cAMP. Increased cAMP can lead to an increase in GnRH release. We plan on testing the affects of GnRH 1-5 on the production of cAMP in the cell and the release of GnRH decapeptide by running an Enzyme-linked immunosorbent assay (ELISA) to measure cAMP levels and Radioimmunoassay to measure GnRH levels concurrently. Tissue will then be sent to a collaborating lab to run a superarray analysis. They will extract mRNA and make a cDNA library. This will allow us to look at every gene that is being up-regulated and down-regulated in our samples to further explain what is happening in the GnRH neurons in response to GnRH 1-5 stimulation.

Abstract 26

Human Gonadotropin-Releasing Hormone Modulates Reproduction in *Caenorhabditis elegans*

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A number of similarities exist between higher organisms and nematodes with regard to the neuroendocrinology of reproduction. Though reproduction in *Caenorhabditis elegans* is well studied, the endocrinology of reproduction in *C. elegans* is largely unknown. Estrogen receptor and a G-protein coupled receptor (LGR) homologous to human follicle stimulating hormone (FSH) have previously been reported for *C. elegans*, but identification of a complete hypothalamic-pituitary-gonadal (HPG) axis has not been contemplated since gonadotropin releasing hormone receptor (GnRHR) and its ligands (GnRH) central to this axis have not been reported. To test whether GnRHR is conserved in nematodes, we cultured *C. elegans* (N2 strain) in *E. coli* (OP50) with and without agonists and antagonists of GnRHR. Compared to untreated worms, 1 μM GnRH increased egg production 42 %, while the GnRHR inhibitor acyline (1 mM) blocked egg production (-13 %). These results suggest the presence of a homologous GnRHR in this nematode. Homology analysis indicated a 401 amino acid rhodopsin-like G-protein coupled receptor (F54D7.3) in *C. elegans* with 46.9 % nucleotide homology to that of human GnRH1R. Like its human homolog, a structural analysis predicted that this homologous protein has a 7 transmembrane domain, 5 potential N-linked glycosylation sites and phosphorylation sites for protein kinases. Immunoblot analysis of homogenate prepared from *C. elegans* using antibodies made against this GnRH1R *C. elegans* homologue as well as against human GnRH1R indicated the presence of a 46 kDa protein equivalent to the calculated molecular weight of the GnRH1R *C. elegans* homologue. Thus reproduction in *C. elegans* is modulated via signaling through an evolutionarily conserved GnRHR homologue and implies that an endogenous nematode GnRH-like peptide exists. We have identified a putative GnRH oligopeptide (~4 kDa) in *C. elegans* using an antibody with high specificity to human, but not other analogs, of GnRH. The GnRH and its receptor homologues were immunohistochemically located in the head and the reproductive structures, respectively. The single most parsimonious tree resulting from our phylogenetic analysis supports a relatively early origin of the GnRHR in evolutionary history and shows a close relationship between *C. elegans* GnRHR and the GnRHRs and adipokinetic hormone receptors of arthropods. Our results thus indicate that *C. elegans* has the major functional components of the HPG axis that controls reproduction in higher organisms. These observations pave the way for the use of *C. elegans* as a model system for studying reproductive hormonal signaling.

Abstract 27

Human Extra-pituitary Neurons Possess GnRH I Receptors that Respond to GnRH by Expressing Luteinizing Hormone

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Extra-pituitary luteinizing hormone (LH) has recently been found localized to pyramidal neurons of the aged human and rat brain. Extra-pituitary LH is a marker of neoplasia and levels of neuron LH are further elevated in pyramidal neurons of the Alzheimer's disease (AD) brain compared with age-matched control brain (Bowen, 2002). Pyramidal neurons of the AD brain display characteristics of neuron re-entry into the cell cycle. The source of this extra-pituitary LH and its role in promoting cell division is unclear. The de novo synthesis of LH by pyramidal neurons of the brain is possible given the presence of GnRH I receptors on extra-pituitary neurons and that the secretion of hypothalamic GnRH I following menopause/andropause is markedly increased. To test this hypothesis, M17 neuroblastoma cells cultured in serum free conditions were treated with GnRH I (0-10uM) for 6 h and cell lysates examined by immunoblot. M17 neuroblastoma cells express 3 LH variants (~30, 46 & 60 kDa). LH expression was modulated such that at low GnRH I concentrations LH expression increased, but at high GnRH I concentrations LH expression decreased. GnRH I receptor (GnRHR) expression was not altered at any GnRH I concentration over this short time period, indicating that LH expression was independent of GnRH I receptor expression. We next characterized the expression of GnRH I receptor on human extrapituitary neurons by immunocytochemistry using the monoclonal antibody F1G4 in age-matched control and AD brains. GnRH I receptor immunoreactivity was detected in the cell body as well as along the apical dendrites of pyramidal neurons in the CA2, CA1 and End plate but was clearly reduced in the subiculum of the hippocampus. Staining also was evident in cortical neurons, including those located in the entorhinal cortex and lateral geniculate body, but was not observed within the granular layer of the dentate gyrus or in other neuronal cell types. No differences in immunocytochemical staining were observed between control and AD tissues, although immunoblot analysis of brain homogenates indicated decreases in the expression of the ~30 and 120 kDa GnRH I receptor variants, but not the mature, functional 64 kDa variant. Our results indicate that human extra-pituitary neurons possess GnRH I receptors that respond to GnRH I by increasing LH expression. Although neuroblastoma cells did not secrete LH, the above observations indicate that extra-pituitary neurons develop characteristics of neoplastic cells with age, consistent with findings that neurons of the AD brain display numerous markers of cell cycle re-entry.

Abstract 28

Optimization of Differentiation Conditions for Endothelial Differentiation from Human Embryonic Stem (ES) Cells

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Human ES cells are able to differentiate in vitro to a variety of cell types including endothelial cells. However, the endothelial differentiation rate reported for human ES cells is extremely low (~2% cells expressing $CD31^+$). To improve the endothelial differentiation, in this study, we tested three conditions for endothelial differentiation from human ES cells. These studies may generate important information on the mechanisms underlying endothelial differentiation, and also will potentially provide highly purified endothelial cell supply for basic biomedical research and clinic applications.

Methods: Human ES cells (H9) were plated on Matrigel. After culture in the ES media overnight, ES cells were induced to differentiate in the EBM2 media in the absence (control, designated as T1) or presence of the EGM2-MV bullet kit without (T2) or with bone morphogenetic protein-4 (BMP4; T3). After 7-15 days of treatment the cells were analyzed for their uptake of LDL, and expression of CD31 and vascular endothelial cadherin (VEcad) by flowcytometry.

Results: All three treatments induced cell differentiation. No LDL uptake was observed in undifferentiated human ES cells. When analyzing using flowcytometry, we observed that all three treatments increased the cell number exhibiting positive LDL uptake (59%) after 7 days of treatment, but without significant difference between treatments across days. Only a few of $CD31^+$ (0.18%) and $Vecad^+$ (1.9%) cells were found in T1 at Day 15. No significant difference in the numbers of $CD31^+$ and $Vecad^+$ cells was found between T2 and T3 at each individual day of treatment. Both T2 and T3 time-dependently increased $CD31^+$ and $Vecad^+$ cell numbers, rising from Day 7 ($CD31^+$: 16.0%; $Vecad^+$: 23.3%) to Day 10 ($CD31^+$: 42.2%; $Vecad^+$: 43.1%), and then declining at Day 15 ($CD31^+$: 5.4%; $Vecad^+$: 33.5%). The dual $LDL^+/CD31^+$ and $LDL^+/Vecad^+$ cells in T1 were 0.6% and 1.9% respectively. The numbers of dual $LDL^+/CD31^+$ (2.4%) and $LDL^+/Vecad^+$ (14.1%) cells were similar in T2 and T3 across days of treatment, whereas these cell numbers were much fewer than their corresponding single staining over the experiment period.

Conclusions: 1) The EGM2-MV bullet kit either without or with BMP4 greatly promotes human ES cells differentiation towards endothelial cell fate; 2) Day 10 appears to be an optimal time point selected to isolate endothelial precursors; and 3) LDL is not a suitable marker used to identify endothelial cell precursors derived from human ES cells.

Abstract 29

Heat Stress Induces AMPK Activation and Meiotic Maturation in Mouse Oocytes

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AMP-activated protein kinase (AMPK), a stress response enzyme, has recently been implicated in the regulation of meiotic resumption in mouse oocytes. AICAR, a common pharmacological activator of AMPK, triggers meiotic resumption in arrested mouse oocytes in vitro. We have previously shown that a variety of cellular stresses are able to induce germinal vesicle breakdown (GVB), and the present study focuses on oocytes exposed to a brief heat shock. Our experiments reveal that 1) a 60 minute pulse of oocytes at 42C optimally triggers GVB; 2) the heat-treated increase in maturation is dose-dependently blocked by compound C, an inhibitor of AMPK; 3) heat-treated denuded oocytes (DO) and oocyte-cumulus complexes (OCC) exhibit increased AMPK activity prior to GVB that is blocked by compound C; and 4) heat-induced meiotic resumption occurs in the presence of three different meiotic inhibitors (dbcAMP, hypoxanthine, and isobutylmethylxanthine). Also, we have been unable to detect changes in other stress-activated protein kinases, such as p38 and JNK MAPK, as well as ERK1/2, in association with heat-induced or AICAR-induced maturation. Furthermore, DO and CEO subjected to heat stress progress meiotically to metaphase II and extrude a polar body, indicating the stress is not damaging the meiotic spindle or the oocyte's ability to complete maturation. Taken together, these data suggest a short exposure to heat stress is sufficient to trigger AMPK activation and meiotic induction in mouse oocytes. Heat-induced maturation occurs under a variety of inhibitory conditions through the activation of AMPK and not kinases from the MAPK family.

Supported by funds from the NIH (HD040392).

Abstract 30

The Role of Calcium in Maturation and Activation of Horse Oocytes

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Previous reports indicate that the Ca concentration in follicular fluid of the mare is several times higher than that of the cow (White et al, 2004). In addition, the first equine foals produced by nuclear transfer were obtained culturing and activating the oocytes in media with $CaCl_2$ concentrations 3 to 6 times the standard of 1.4 mM (Woods et al, 2003). The objective of this experiment was to determine the effect of calcium concentration in maturation media on the free intracellular calcium response to Ionomycin of mature equine oocytes. Compacted cumulus oocyte complex were collected from slaughterhouse ovaries and matured for 30 h in TCM-199 based media at 38.5 ºC in 5 % CO_2 in air. There were four maturation treatments with $CaCl_2$ concentrations of 1.4 mM (1X); 4.2 mM (3X); 8.4 mM (6X); and 14 mM (10X). Ten oocytes were used per treatment per replicate. Four replicates were used for parthenogenetic activation and seven replicates for calcium imaging. For parthenogenetic activation oocytes were separated from cumulus cells and visually evaluated for maturation state. Oocytes exhibiting polar bodies (M-II) were placed in Ionomycin (10µM) for 5 minutes, followed by 6-(Dimethylamino)purine (2 mM) for 4.5 hours, and placed in culture media (DMEM/F12 based media). Cleavage and 8-cell embryo development rates were recorded after 48h of culture. Metaphase II, cleavage and 8-cell embryo rates were compared by ANOVA after arcsin transformation. For calcium imaging oocytes were separated from cumulus cells, M-II oocytes were selected and loaded with Fura 2-AM (2µM) for 45 min, followed by Fura-free medium for 30 min. Oocytes were placed in medium with 1.4 mM $CaCl_2$ and 10-fluorescence image pairs (340 and 380nm) were obtained to establish resting calcium level (baseline). Ionomycin (10µM) was added and 70 image pairs were recorded at 5-second intervals to determine free intracellular calcium concentration ($[Ca_i]$). The peak $[Ca_i]$ was determined for each oocyte, as well as the time in seconds from Ionomycin addition to the maximum $[Ca_i]$. Data were analyzed by ANOVA and differences among means compared by Tukey's test. In the parthenogenetic activation experiment, there were no differences among treatments ($p>0.05$) for maturation rates before Ionomycin addition (M-II mean average 49%); or cleavage (17 %), and 8-cell embryo (12%) rates after 48h of post-activation culture. The baseline $[Ca_i]$ means were 166.57 ^{a}, 172.23 ^{a}, 186.18 ^{a}, 233.43 ^{b}, and peak $[Ca_i]$ means were 785.13 ^{cd}, 917.94 ^{de}, 965.93 ^{de}, 1064.25 ^{e} nM for 1X, 3X, 6X and 10X respectively (different letters differ, $p<0.05$). The time from Ionomycin to peak $[Ca_i]$ response did not differ among treatments with an average of 40 seconds (ANOVA $p>0.05$). Maturation and early development rates of parthenogenetic embryos are not affected by 1.4 – 14 mM $CaCl_2$ in maturation media when oocytes are activated at 1.4 mM $CaCl_2$. Resting $[Ca_i]$ only increased when oocytes were matured in 14 mM $CaCl_2$. The peak value of the $[Ca_i]$ induced by Ionomycin was only affected when the $CaCl_2$ concentration in maturation media was 14 mM. The difference in developmental competence of oocytes cultured at 4.2 vs. 1.4 mM $CaCl_2$ observed by Woods et al (2003) seems not to be related to a higher resting $[Ca_i]$.

Abstract 31

A Proposed Model to Study the Effects of Endometriosis on Follicle and Oocyte Development

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The effects of endometriosis on oocyte quality and early embryo development are poorly understood. Interestingly, recent data from oocyte donation programs suggest that endometriosis is somehow interfering with oocyte maturation and/or early embryo development but not implantation or later development. Investigation into the mechanism of this effect, however, is limited, primarily due to the lack of an appropriate study model. In an attempt to understand this phenomenon, we are proposing a murine follicle culture system to test the effects of endometrium on oocyte and embryo development *in vitro*. Lenie et al (BOR in press) have described an *in vitro* culture system for growth and maturation of isolated murine preantral follicles. Using a variation of this model, we plan to explore this effect by exposing developing murine follicles to peritoneal fluid from women with and without endometriosis. Outcomes measures assessed will include oocyte survival, fertilization rates, embryo cleavage rates, and blastocyst formation rates. We hypothesize that exposure to peritoneal fluid will alter one or more of these outcomes.

Abstract 32

The Role of MUC16 in the Progression of Ovarian Cancer

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The large molecular weight mucin, MUC16, is found on the surface of ovarian cancer cells, as well as on the surface of lymphocytes in blood and ascites of ovarian cancer patients. CA125 is an epitope present on MUC16 that is monitored in the serum of patients for the progression of ovarian cancer. The genetic sequence of this mucin was only recently discovered, which has opened the doors to new research on this glycoprotein. The biological function and significance of MUC16 in ovarian cancer is the main focus of our lab. Three specific aims presented here include the role of MUC16 in the metastasis of ovarian cancer in the peritoneal cavity, the effect of MUC16 on the inhibition of NK cells, and the development of a model to study MUC16/NK cell interaction using NKL (natural killer leukemia) cells.

Mesothelin is a GPI-anchored glycoprotein expressed on the surface of mesothelial cells that line the peritoneal cavity. Using a recombinant form of this protein, we have shown in Western blotting overlay experiments that mesothelin binds to MUC16 that has been partially purified from patient ascites and from the OVCAR-3 human ovarian cancer cell line. Treating MUC16 with enzymes and using lectins (plant proteins with specific carbohydrate chains), we have determined the carbohydrate structure present on MUC16 that mesothelin is specific for. This observation leads to possibly one mechanism by which the cancer is spreading throughout the peritoneal cavity.

It has been shown that MUC16 inhibits the cytolytic function of NK cells (Patankar, *et. al.*2005 Gyn.Onc. paper in press.). To determine what receptors are involved in this inhibitory effect, we isolated lymphocytes from patient peritoneal fluid (ascites) and peripheral blood. We have shown that peripheral fluid lymphocytes from ovarian cancer patients express decreased levels of CD16 and NKp46, two NK cell activating receptors.

NK cells are expensive to isolate and make up only 5-10% of the blood population. Because the presence of MUC16 affects NK cell function, we need a model to study the interaction between MUC16 and NK cells. Therefore, we have used NKL (natural killer leukemia) cells as a substitute for NK cells in some *in vitr*o experiments. NKL cells when stimulated by IL-2 are highly proliferative and cytotoxic. To test the cytotoxic effect of these cells on ovarian cancer cells, OVCAR-3 cells which produce MUC16 on their surface, and MUC16 negative clones were plated on a 12 well plate. When confluent, NKL cells were added to these cultures. At 24, 48, and 72 hours, cells and colonies were counted. The results indicated that cells that expressed MUC16 on their surface were more resistant to NKL attack compared to $MUC16^{neg}$ cells.

These experiments have given us an initial idea of how MUC16 is functioning biologically in ovarian cancer. We hope to extend these experiments to further characterize the effects of MUC16 in hopes of possible treatment options.

Abstract 33

All the Right Moves: Phylogenetically Widespread Natricine Promiscuity

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Promiscuity and the occurrence of multiple paternity within litters of offspring are characteristic of many species within the animal kingdom, and are analogous to the mating strategies of cross-pollinating plants. Specific knowledge of reptile mating systems, however, is lacking. Previous independent investigations determined multiple paternity to be characteristic of four disjunct natricine snake species, including *Thamnophis sirtalis*, *Nerodia sipedon*, *Thamnophis butleri*, and *Thamnophis elegans*. We investigated the various untested clades and subclades of North American natricine snakes to determine the phylogenetic extent of multiple paternity within this subfamily. Representative species tested included members of the semifossorial clade (*Storeria dekayi*, *Storeria occipitomaculata*), watersnake clade (*Nerodia rhombifer*, *Regina septemvittata*) and gartersnake clade (*Thamnophis radix* – widespread subclade; *Thamnophis sauritus* – ribbon and common gartersnake subclade; *Thamnophis melanogaster* – Mexican subclade). Using primers originally developed for *Thamnophis* and *Nerodia*, DNA from litters with known mothers was amplified at highly variable microsatellite loci. After identifying maternal alleles in offspring genotypes, the number of paternal alleles per litter was counted. The presence of more than two paternal alleles (the maximum when a sire is heterozygous) within any single litter indicated that multiple paternity is widespread throughout Natricinae, with the exception of the Mexican subclade, represented by *T. melanogaster*. This study is the first to examine multiple paternity across a widespread, related group of vertebrates.

Keywords: multiple paternity, snake, natricine, microsatellite DNA

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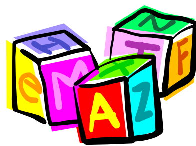

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