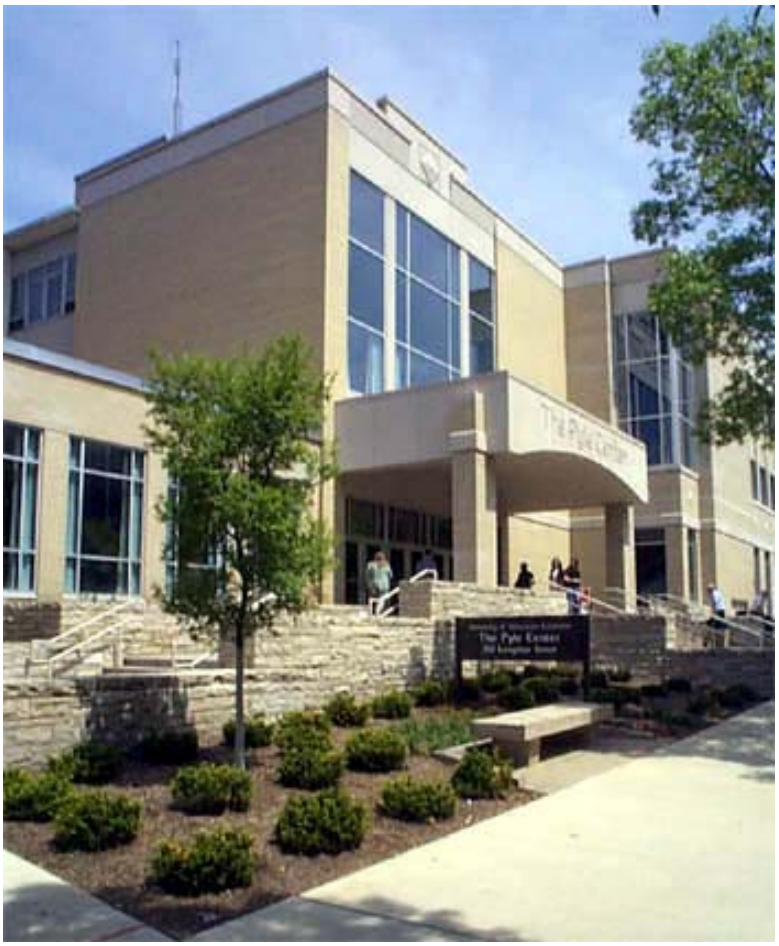
Endocrinology – Reproductive Physiology Annual Research Symposium



THE UNIVERSITY of WISCONSIN MADISON

November 17, 2006
University of Wisconsin - Madison
The Pyle Center

Table of Contents

Schedule

8:00-9:00 **Registration, Poster Set-up**
Registration – Pyle Center Lobby
Poster Set-up – Alumni Lounge

9:00-11:15 **Session 1: Current Topics in Endocrinology-Reproductive Physiology**
Gale VandeBerg Auditorium Room 121

9:00-9:40
"Regulated Expression of Estrogen Receptor in Breast Cancer"
Dr. Elaine Alarid, Associate Professor, Department of Physiology, University of Wisconsin - Madison

9:40-10:20
"Organizing Activity within the Murine Allantois: Building a Vascular Organ"
Dr. Karen Downs, Associate Professor, Department of Anatomy, University of Wisconsin – Madison

10:20-10:35 **Coffee Break**

10:35-11:15
"Culture of Human Embryonic Stem Cells in Defined Conditions"
Dr. Tenneille Ludwig, Assistant Scientist, Laboratory of Dr. James Thomson, University of Wisconsin-Madison

11:15-12:10 **Session II: Feature Abstracts**

11:15-11:25 Introduction by the ERP Symposium Abstract Selection Committee**: Dr. Ziming Yu, Dr. Svetlana Dambaeva, and Carla de Azevedo Piccinato**

11:25 "*Differentiation of Human Embryonic Stem Cells Induces the Processing of the Amyloid-β Precursor Protein towards the Amyloidogenic Pathway"*
Prashob Porayette, Atwood Lab, ERP Program

11:40 "*Establishment of a Functional Ovine Fetoplacental Artery Endothelial (OFPAE) Cell Line with a Prolonged Life Span"*
Yang Song, Zheng Lab, ERP Program

11:55 "*Genomic Elements of the Hypothalamic-Pituitary Gonadal Axis in Caenorhabditis elegans"*
Miguel J. Gallego, Atwood Lab, ERP Program

Schedule Continued

12:10-12:40 **Lunch Pick-up and Poster Session I,** Alumni Lounge

12:40-1:10 **Poster Session 1I**, Alumni Lounge

1:15-2:15 **Keynote Speaker:**
Gale VandeBerg Auditorium Room 121
"The Implications of Living and Dying for Sex"
Dr. Richard Bowen, O.T.B Research, Raleigh, North Carolina

2:15-2:30 **Coffee Break**

2:30-4:45 **Session III: Techniques Workshop**
Gale VandeBerg Auditorium Room 121

Moderators: **Dr. John Parrish**, Department of Animal Science
Jessica Quam, Cezar Lab, ERP Program

2:30-3:15 "*Quality Control in Quantitative Real Time PCR: Design, Control, and Analysis Considerations"*
Dr. Lewis Sheffield, Professor, Department of Dairy Science

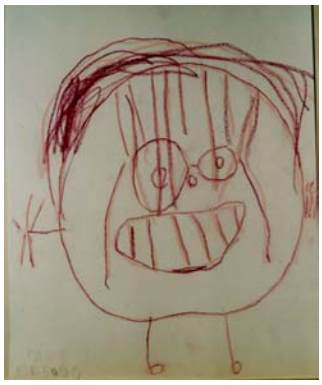
3:15-4:00 "*Proteomic Analyses Using Mass Spectrometry"*
Dr. Mark Scalf, Assistant Scientist, Department of Chemistry

4:00-4:45 *"The Production of Transgenic and Knock-out Mice"*
Dr. Anne Griep, Professor, Department of Anatomy, Biotechnology Center

4:45-5:30 **Social and Awards Presentation**, Alumni Lounge

Session I: Current Topics in Endocrinology-Reproductive Physiology: Speaker Biosketches

Elaine Alarid earned her B.A. in genetics and Ph.D in physiology from the University of California at Berkeley. She then took two consecutive postdoctoral positions at the University of California at San Francisco. Dr. Alarid is now an Associate Professor at the University of Wisconsin-Madison in the Physiology department. The focus of her research is on understanding the molecular mechanisms governing the activity of estrogen receptor (ER), a member of the nuclear receptor transcription factor family that is critical in normal reproduction and is implicated in the pathogenesis of breast cancer.



(Portrait provided courtesy of Dr. Downs' daughter at age 3).
Karen Downs obtained her B.S. in Biology, M.S., and Ph.D in Yeast Genetics at the University of Illinois at Chicago. Her postdoctoral work was done at the University of California, San Francisco, and the University of Oxford, England, in mouse embryology. She is now an Associate Professor at the University of Wisconsin-Madison in the Anatomy department. Dr. Downs' research focuses on the cellular, molecular and genetic components of differentiation during placental morphogenesis.

Tenneille E. Ludwig obtained a B.S. (1992) in Animal Sciences and M.S. (1994) in Reproductive Endocrinology from Washington State University. She earned a Ph.D. (2001) in Embryology and Developmental Biology with a minor in Bioethics from the University of Wisconsin-Madison. Dr. Ludwig is a senior scientist in Dr. James Thomson's laboratory, and has worked closely with him for more than 5 years. She is the lead author on the seminal publication describing the development of a defined, feeder-independent, animal-product free culture system for human ES cells published in Nature Biotechnology earlier this year. She has authored or co-authored more than a dozen media development papers in multiple journals including Nature Biotechnology, Nature Methods, Nature Medicine and Stem Cells. Dr. Ludwig is an internationally recognized expert in human ES cell culture and media optimization, has been an invited lecturer on the topic both nationally and internationally, and has served as an invited participant in the International Stem Cell Initiative since its inception.

Session II: Feature Abstracts

Suppression of Phosphatase 2A (PP2A) Expression Does Not Affect FGF2- and VEGF-Stimulated Cell Proliferation in Ovine Feto-Placental Artery Endothelial (OFPAE) Cells

Yang Song[1], Dong-bao Chen[2], Jing Zheng[1]

Department of Obstetrics and Gynecology, Perinatal Research Laboratories[1], University of Wisconsin, Madison, WI 53715; Department of Reproductive Medicine[1], University of California San Diego, CA 92093

The angiogenic factors, FGF2 & VEGF, are key regulators of placental angiogenesis. Cellular responses to FGF2 and VEGF are mediated by a complex signaling network involving protein kinases (i.e., ERK1/2 & AKT) and phosphatases (i.e., PP2A & PP2B), both of which are equally vital for modulating biological actions induced by FGF2 and VEGF. We recently observed that activation of the MEK/ERK1/2 and PI3K/Akt pathways attributed partly to FGF2- & VEGF-stimulated OFPAE cell proliferation. PP2A can dephosphorylate ERK1/2 & AKT1, resulting in inactivation of these protein kinases. In this study, we tested a hypothesis that PP2A modulates FGF2- and VEGF-stimulated OFPAE cell proliferation. Small interfering RNA (siRNA) specifically targeting PP2A catalytic subunit α was used to suppress PP2A expression in OFPAE cells. Transient transfection (2 days) of PP2A siRNA, but not scrambled siRNA specially decreased ($p < 0.05$) PP2A protein levels by ~ 70% as compared with the scrambled siRNA, but did not alter PP2B, NOS3, and GAPDH protein levels. This suppressive effect on PP2A expression maintained at least for 6 days after the transfection. The PP2A activity, measured using a PP2A immunoprecipitation phosphatase assay kit, was decreased ($p < 0.05$) by ~ 6 fold by PP2A siRNA, but not scrambled siRNA. Specificity of this PP2A activity was confirmed using okadaic acid (a widely used PP2A inhibitor; Ki 0.2 nM) at 1 nM. We also confirmed the stimulatory effects of FGF2 and VEGF (2 days of treatment) on OFPAE cell proliferation. However, suppression of PP2A did not significantly affect FGF2- and VEGF-stimulated OFPAE cell proliferation. Hence, our data indicate that PP2A does not play a major role in regulating FGF2- and VEGF-stimulated OFPAE cell proliferation. Together with our previous data emphasizing an important role of MEK/ERK1/2 and PI3K/AKT1 in FGF2- and VEGF-stimulated OFPAE cell proliferation, our current data also suggest that PP2A alone is not sufficient to inactivate the MEK/ERK1/2 and PI3K/AKT1 signaling pathways in OFPAE cells. *(Supported by NIH grants HL64703 to JZ, & HL74947 & HL70562 to DBC).*

Differentiation of Human Embryonic Stem Cells Induces the Processing of the Amyloid-β Precursor Protein towards the Amyloidogenic Pathway

Prashob Porayette[1], Miguel J Gallego[1], Sivan Vadakkadath Meethal[1], Richard L. Bowen[2] and Craig S. Atwood[1]

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The function of the amyloid-β precursor protein (AβPP) and its cleavage product amyloid-β that deposits in the brain during Alzheimer's disease (AD) remains unclear. Since the expression and processing of AβPP to soluble and insoluble fragments has been reported to increase in the brain during embryonic development, we examined how developmental hormones and differentiation may alter the processing of AβPP in human embryonic stem (hES) cells. Undifferentiated H9 hES cells expressed mRNA for AβPP as determined by RT-PCR while immunoblot analyses indicated the presence of both the immature and mature full length forms of AβPP. The developmental hormone human chorionic gonadotropin (hCG) increased AβPP expression and its processing to 47-kDa and 25-kDa fragments in undifferentiated hES (H9) cultures. These results suggested that AβPP may play a vital function during the development of the germ layers and subsequent differentiation into different cell lineages. To test this hypothesis, we differentiated H9 hES cells for 10 days on 1) mouse embryonic fibroblast feeder layers, 2) Matrigel supplemented with mTeSR1 media, and 3) with rocking in the presence of serum to induce embryoid body formation (homologous to the blastocyst stage). We also differentiated hES cells over 19 days into rosettes (which consist of ~70 % columnar neuroectodermal cells). While the expression of the immature and mature full length AβPP decreased with differentiation (except in the rosettes), differentiation increased the processing of full length AβPP to 47-kDa, 25-kDa and the C-99 fragments. These results indicate that differentiation induces the activity of β-secretase, and implies that amyloid-β has a functional role during embryogenesis. The rosettes, but not other differentiated lineages, also expressed hyperphosphorylated tau, a biochemical feature that characterizes the formation of neurofibrillary tangles in AD. Together, these results demonstrate that hES cells express AβPP and that developmental and differentiation signals regulate the processing of AβPP and the phosphorylation of tau. The role of these processes in the development of germ layers and subsequent differentiation into different cell lineages is currently under investigation.

Genomic Elements of the Hypothalamic-Pituitary Gonadal Axis in *Caenorhabditis elegans*

Miguel J. Gallego[1], Sivan Vadakkadath Meethal[1], Ryan J. Haasl[1], Jean Y. Sgro[2] and Craig S. Atwood[1]

[1]Section of Geriatrics and Gerontology, Department of Medicine, University of Wisconsin-Madison and Geriatric Research, Education and Clinical Center, Veterans Administration Hospital, Madison, WI, 53705. [2]Biotechnology Center, University of Wisconsin, Madison, WI 53705.

Although a previous report has suggested the presence of a putative sex steroid receptor in *C. elegans*, a hypothalamic-pituitary-gonadal (HPG) axis such as found in higher organisms has not been contemplated in the nematode. We undertook this study to determine if hypothalamic, pituitary and gonadal genomic elements were present in *C. elegans.* Our sequence analyses indicated two hypothalamic gene candidates, one of 1206 bp (AF039712; 401 aa, AAK21401) and another of 1140 bp (NM_074165; 379 aa, NP_506566) with 46.9 % and 44.7 % nucleotide similarity to that of human gonadotropin-releasing hormone receptor 1 (GnRHR1) and GnRHR2, respectively. Like their human homologues, the *C. elegans* GnRHR homologues belong to the rhodopsin family of G-protein coupled receptors (GPCR) and contain several structural motifs similar to that of human GnRHR's: 7 transmembrane domains, 3 intracellular and extracellular loops, and amino acid residues representing PKC phosphorylation sites and G protein coupling sites. Overall, our analysis showed that 60 % of the functionally important amino acid residues of human GnRHR1 are conserved in the *C. elegans* homologue. The highest degree of conservation was observed in those amino acids involved in the receptor activation site (83 %), the G protein (G_{q11}) coupling site (75 %) and in amino acids involved in formation of the binding pocket (59 %). Lesser conservation was observed in the G protein (G_s) coupling site (50 %), PKC phosphorylation sites (50 %) while the ligand binding site was least conserved (36 %), indicating the possibility that the ligand for the *C. elegans* GnRHR1 is significantly different to that of human GnRHR1. We also identified a pituitary gene candidate, a previously reported leucine-rich GPCR (LGR; NM_073147), which had 47.6 % nucleotide similarity to human follicle-stimulating hormone. Phylogenetic analysis based on the alignment of reported 339 residue GnRH receptor variants yielded six most parsimonious trees, the strict consensus of which resulted in the collapse of relatively few nodes. The presence of two GnRH receptor variants in *Caenorhabditis,* and the close relationship between the GnRH receptor and the GPCR adipokinetic hormone receptor in *Drosophila,* support an early origin of GnRH and GnRH receptor and are suggestive of multiple episodes of gene duplication. These analyses suggest the potential for an evolutionarily conserved, primordial neuroendocrine (HPG) system in *C. elegans*.

Poster Session

Alumni Lounge
12:10-1:10

Abstracts 1-16

Abstract 1

Pregnancy Enhanced eNOS Activation in Uterine Artery Endothelial Cells Shows Altered Sensitivity to Ca^{2+}, U0126 and Wortmannin but not LY294002 – Evidence that Pregnancy Adaptation of eNOS Activation Occurs at Multiple Levels of Cell Signaling

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During pregnancy, vascular remodeling and vasoactive agents such as nitric oxide (NO) increase blood flow to the uteroplacental unit. Using our Uterine Artery Endothelial Cell culture model, based on cells from pregnant (P-UAEC) and nonpregnant (NP-UAEC) ewes, we investigate the relative physiologic roles of Ca^{2+} vs kinase in the regulation of eNOS activity. When Ca^{2+} mobilization is fully inhibited using inhibitors of PLC (U73122) and the IP3 receptor (2-APB), significant residual eNOS activity in both P and NP-UAEC remains. No change in ATP stimulated ERK2, Akt or eNOS phosphorylation with U73122 (0.01-1 μM) or 2-APB (1-50 μM) is observed. The MEK 1/2 inhibitor U0126 (10 μM) did not alter ATP stimulated eNOS activity in P-UAEC, but potentiated the ATP response in NP-UAEC. Using two PI3K inhibitors, we observed no effect with LY294002 (10 μM) on eNOS activity in P and NP-UAEC, but wortmannin (10 μM) inhibited both P and NP-UAEC eNOS activation. Expression of constitutively active Akt (ca-Akt) in UAEC resulted in slight elevation of basal eNOS activity, but relative ATP stimulated eNOS activation was not altered by ca-Akt. Wortmannin continued to inhibit eNOS activation by ATP in the presence of ca-Akt; LY294002 still had no inhibitory effect. Our data indicate both $[Ca^{2+}]i$ and multiple kinases are involved in the regulation of eNOS activity in our model. We report pregnancy adaptation of eNOS activation includes the reduced sensitivity to ERK mediated attenuation of eNOS activity and enhanced stimulation of eNOS activity though a wortmannin sensitive, LY294002 insensitive, Akt independent mechanism. *Supported by Grants USDA 0002159 (IMB), NIH HL64601 (IMB), NIH HD 38843 (IMB), and AHA predoctoral fellowship 0010154Z (JAS)*

Abstract 2

Cryopreservation of Large Equine Embryos

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[1]Endocrinology Reproductive Physiology Program, University of Wisconsin-Madison Madison, WI, USA

Cryopreservation of embryos is a very useful technique in many species; it allows the storage of the specimen until a proper recipient, time and location is selected to perform the embryo transfer. Through the years many research groups have attempted to cryopreserve equine embryos, being only successful when the diameter of the embryo was 300 µm or smaller. Small horse embryos are hard to obtain because the conceptus enters the uterus as late morula or early blastocyst at day six after ovulation. The diameter of embryos recovered at this day ranges from 132 to 756 µm, and therefore many embryos will not be suitable for standard cryopreservation procedures. The objective of this experiment is to evaluate the effect of artificial reduction of blastocoelic cavity in the cryopreservation of large (>500µm) equine embryos.

Twenty one embryos were recovered 7.5 to 9 days after ovulation from 8 light-horse mares. Control group embryos (n=10) were vitrified as follows: exposure to 5 M Ethylene Glycol in TCM 199 + 20 % FCS (Sol-1) for 3 min followed by 7 M Ethylene Glycol in TCM 199 + 20 % FCS with 0.5 M Galactose and 6% PVP-40 (Sol-2) for 45 seconds; loaded in ¼ cc straws and direct plunged into liquid nitrogen. In the treatment group (n=11) the artificial reduction of blastocoelic cavity took place after the first minute of exposure in Sol-1 by aspiration of the blastocoelic fluid with a glass micro-needle. Aspiration was completed before the 3 min of exposure and the collapsed embryos were treated as the control group.

We have not warmed any of the vitrified embryos, but the proposed warming protocol for both groups is as follows: warming of the straw at 38ºC for 20 seconds; "in-straw" dilution of Sol-2 with 0.5 M Galactose in TCM 199 + 20 % FCS. Further dilution of cryoprotectant by transfer to 0.5, 0.25, and 0.125 M Galactose in TCM 199 + 20 % FCS for 3 min each.

Evaluation points: re-expansion of the embryos at 12, 24 and 48 h of culture in DMEM/F12 +10 % FCS at 38.5 ºC, 5 % CO_2 in air. Estimation of the percentage of life cells by a non-lethal cell viability assay.

Abstract 3

Effect of Erythropoietin on Lung after Oxygen Exposure in Newborn Rats

Noah D. Grams, Sharon E. Blohowiak, David P. Carlton, Pamela J. Kling
Department of Pediatrics, University of Wisconsin - Madison

Background: Erythropoietin, the primary hormone regulating erythropoiesis, is present in amniotic fluid. Data support that fetal swallowing of Epo stimulates growth of fetal intestinal epithelium and vasculogenesis in mesenteric vascular endothelium. Due to fetal breathing, Epo may play a similar role in fetal and premature newborn lung. Rat lung development at term is comparable to premature human. We hypothesized that Epo stimulates lung growth and protects lung development after hyperoxic exposure.

Methods: In the hyperoxia experiment, P1-P4 suckling rats were exposed to 80% oxygen ± 425 U/kg SQ Epo and compared to P1-P4 rats in room air ± 425 U/kg SQ Epo. Body weights, lung wet weights and simple lung dry weights were determined. Lung weights were expressed in mg/g of rat weight. Hematocrit was determined. Tissue histology was examined. To examine Epo dosing over a longer period, we examined additional P4-P12 suckling rats in room air ± 425 U/kg/d SQ Epo.

Results: P4 rats in 80% oxygen weighed less (8.81 ± 0.23 g) than those in room air (10.11 ± 0.18), $p<0.0001$ and Epo did not affect body weight. When expressing lung weight as a ratio to body weight, P4 lung wet weights (19.23-19.92 mg/g rat) and lung dry weights (15.71-15.98 mg/g rat) did not differ between the 4 groups. Marked differences in microscopic lung architecture were observed. Lungs exposed to hyperoxia without Epo exhibited thickened airspace walls and decreased alveolar crests. Lungs exposed to Hyperoxia +Epo more closely resembled room air groups. Epo exerted other physiologic effects; hematocrit percentages in room air +Epo (31.9 ±0.9%) and hyperoxia +Epo (31.9 ± 0.7%), higher than room air control (27.2 ±1.4%) and hyperoxia alone (27.8 ±0.8%), $p<0.004$. In the longer experiment, mean body weight in P12 rats was similar, but mean lung wet weight in +Epo group trended greater (20.5±1.1 mg/g rat) than control (18.4±0.8 mg/g rat), $p=0.06$. Dry weight did not differ between control group (3.63 ± 0.38 mg/g rat) and +Epo group (3.841 ± 0.32 mg/g rat). Hematocrit values were higher in the +Epo group (42.8 ±1.2%) than control group (31.6 ± 1.2%), $p<0.0001$.

Conclusion: Erythropoietic effects were observed in both experiments, supporting a physiologic response to Epo. In hyperoxia, Epo may normalize disturbed lung architecture. Architectural changes are not reflected in differences in lung wet or dry weight in the 4-day treatments. Animals treated with Epo for longer duration may exhibit a difference in lung wet, but no difference in dry weight. This may be explained by increased vascular growth with longer treatment, as Epo stimulates vasculogenesis. We will now quantitate vascular surface area on histologic slides and measure hemoglobin content of lungs to estimate vascular contribution to lung wet weights.

Abstract 4

Effect of Estradiol and Progesterone Removal on Steroid Metabolism in Dairy Cows.

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High steroid metabolism decreases circulating steroid concentrations and may adversely affect reproductive efficiency in dairy cows. Liver blood flow (LBF) is increased in lactating dairy cows in response to an elevated demand of feed comsumption. An increase in LBF favors steroid metabolism since more hormone would pass through the liver, the major site of progesterone (P4) and especially estradiol (E2) metabolism. Although some studies have already related steroid metabolism with LBF and feed intake in dairy cows, it remains unclear how such metabolism happens in the bovine liver. In order to elucidate the mechanisms of regulation of hepatic metabolism of E2 and P4 in dairy cows, we hypothesized that the removal of E2 and P4 by ovariectomy would lower steroid break down.

Non-lactating Holstein cows had ovarian function synchronized (GNRH+ CIDR7d, PGF2α d8 and GnRH d10). Five d after the last GnRH, cows underwent liver biopsy and catether placement in both jugular veins. Also, all follicles larger than 6 mm were aspirated, by using transvaginal ultrasound. On the following day, cows received iv infusion containing E2-17β (2.1mg/ml) and P4 (10mg/ml) for 6 h, followed by infusion of bromosulphothalein (BSP) (2.4mg/ml) for 4 h. Blood samples were collected every hour during infusions. After 3 weeks, cows were ovariectomized and assigned in a complete ramdomized design with 2 treatments, as follows: control group (n=4) received an ear implant that delivered continuously E2-17β and two vaginal devices (CIDR) that delivered P4 continously; treatment group (n=4) no hormone replacement. After 28 d of treatment, the animals underwent liver biopsy and catheter placement. On the following day, steroid and BSP iv infusions and blood sampling were performed as described previously. Cows were fed to meet maintenance requirements from a week before the beginning of trial untill the last infusion period. The circulating concentration at steady state was used to calculate the metabolic clearance rate (MCR) of E2, P4 and BSP (used to calculate LBF).

Overall, there was no significant difference ($p>0.05$) between groups on MCR of BSP, which means there was no changes in LBF in response to lack of steroid exposure. MCR of E2 after 3h of infusion (at steady state) showed no difference between groups ($p>0.05$). Although we could not evaluate MCR of P4 (since it did not reach steady state during the infusion), we could not detect a difference in P4 concentration between groups ($p>0.05$). We will now quantitate mRNA of steroid metabolism enzymes in liver biopsy samples by qPCR to evaluate the changes in the expression of metabolizing enzymes.

Abstract 5

Mechanism of Rapid Action of Estrogen in LHRH Neurons: A Possible Role of G-Protein Coupled Receptor 30 in GT1-7 Cells

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Mechanism of estrogen (E_2) action in luteinizing hormone releasing hormone (LHRH) neurons is still unclear. Previously, we have shown that E_2 causes rapid effects in LHRH neurons derived from monkey embryos and immortalized LHRH neurons (GT1-7 cells): E_2 increased the frequency of firing activity as well as the frequency and synchronization of intracellular calcium ($[Ca^{2+}]_i$) oscillations in primate LHRH neurons and increased the frequency of ($[Ca^{2+}]_i$) oscillations in GT1-7 cells with a short latency. However, this rapid E_2 action was neither blocked by the pure estrogen receptor blocker ICI 182,780 nor by the ER selective antagonist RR-THC. Because GPR30, an orphan G protein-coupled receptor, has been shown to mediate E_2 action in several breast cancer cells, in the present study we examined the possible role of GPR30 in the rapid E_2 action in primate LHRH neurons as well as in GT1-7 cells. RT-PCR analysis indicates that GPR30 mRNA was expressed in primate LHRH neurons and GT1-7 cells. Immunocytochemical staining with a specific antibody to the 2nd extracellular loop as well as the C-terminus region of GPR30 indicates that primate LHRH neurons also expressed GPR30 peptide. To assess the possible involvement of GPR30 in E_2 action, we further investigated the effects of pertussis toxin, which inhibits the G_i subunit of adenylate cyclase, on the E_2-induced $[Ca^{2+}]_i$ changes in primate LHRH neurons and GT1-7 cells. Preliminary data suggested that the effects of E_2 on $[Ca^{2+}]_i$ oscillations were consistently blocked by pertussis toxin. These results support our hypothesis that GPR30 plays a role in E_2 action in LHRH neurons. Supported by NIH grants HD15344, HD11533, and RR00167.

Abstract 6

Cumulus Removal Technique has Molecular Impact on Bovine IVP Embryo

Amy Reeder[1], Jyoti Watters[2], Rick Monson[3], Janelle Wiebelhaus-Finger[3], Jack Rutledge[3]

[1] Endocrinology and Reproductive Physiology Program, [2] Comparative Biosciences Department and [3] Animal Sciences at the University of Wisconsin, Madison

The method of cumulus cell removal from a bovine zygote for *in vitro* embryo production (IVP) influences further embryonic development. Although mechanical cumulus cell removal methods vary between laboratories and may include vortexing or hand-stripping, methods that can more gently remove cumulus cells and that more closely mimic *in vivo* processes are continuously being sought. Microfluidics (μF) is a newer technology that utilizes unique fluid dynamic profiles at the nano-liter level to remove cumulus cell masses from the zygote. Our laboratory had previously shown that this μF method, when compared to vortexing and hand-stripping, at 24 hours post fertilization, increased embryonic developmental rates to be more consistent with those observed *in vivo*. In the present studies, we tested the hypothesis that μF cumulus removal results in lower mRNA expression levels of stress- and apoptotic-related genes compared to the hand-stripping method typically used in human IVP laboratories. Quantitative PCR was employed using a panel of developmentally relevant genes including the heat-shock cognate protein 70 (HSC70) and BAX1, a pro-apoptotic gene. Holstein embryos from 4 cows were pooled according to cumulus removal method at 24 hours post cumulus removal. We observed a down-regulation of both HSC70 and BAX1 in μF treated embryos when compared to their hand-stripped counterparts. This is consistent with the idea that the down-regulation of apoptotic and stress-activated genes may be involved in the lack of developmental delay observed in embryos following μF cumulus removal.

Abstract 7

Effect of Erythropoietin on Ion Transport in the Respiratory Epithelium

PM DeYoung, AK Kapur, PJ Kling, and DP Carlton
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Erythropoietin has a wide range of nonhematopoietic effects in the body, including the regulation of certain enzymes in the lung. Clinically, its administration has been associated with a decrease in the number of days in oxygen for sick premature infants. If erythropoietin has a favorable effect on respiratory illness, one mechanism could be a reduction in pulmonary edema. Therefore, we hypothesized that erythropoietin modifies the ion transport characteristics of the respiratory epithelium in ways linked to the clearance of fluid from the distal airspace. A necessary first step in ion transport-linked fluid clearance is the uptake of sodium into the cell, in particular through channels inhibited by amiloride. We exposed A549 cells, an immortal adenocarcinoma cell line with features similar to Type II cells, to 35 U/ml of erythropoietin for 24h and measured the uptake of ^{22}Na, a radioisotope tracer. Erythropoietin increased total sodium uptake on average by 30% (n = 10, control: 214 ± 128 vs erythropoietin 278 ± 169 nmol/mg protein, $p<0.05$), an effect that was predominately due to transport through amiloride-sensitive sodium channels (n = 10, control: 180 ± 122 nmol/mg protein vs erythropoietin 238 ± 159, $p<0.05$). To better understand these findings, we studied the effect of erythropoietin on the Na-K-ATPase, an enzyme important in driving this movement of sodium. To accomplish this goal, we measured the uptake of $^{86}Rb^{+}$ (a mimic of K^{+}) in the presence and absence of ouabain, an inhibitor of Na-K-ATPase. We found that after 24h, erythropoietin (35 U/ml) increased ouabain-sensitive Rb uptake on average by 25% (n=8, control: 1.6 ± 0.6 vs erythropoietin: 2.0 ± 0.8 nmol Rb/million cells/min, $p<0.05$), indicating an increase in Na-K-ATPase activity. To confirm this finding, we disrupted the cell membrane and assessed Na-K-ATPase activity by an alternative method in which we measured the release of inorganic phosphate in the presence and absence of ouabain. Similar to our results in intact cells, erythropoietin (24h, 35 U/ml) increased the activity of the Na-K-ATPase in this cell free system (n=7, control: 0.9 ± 0.4 vs erythropoietin: 2.1 ± 1.3 mg phosphate/mg protein, $p<0.05$). In additional studies, we grew A549 cells on a semipermeable membrane and measured fluid movement across the monolayer by measuring the change in protein concentration in the supernatant as a reflection of fluid loss. After 24 h erythropoietin increased by nearly 25% the volume of fluid lost from the supernatant (n=6, control: 132 ± 26 vs erythropoietin: 164 ± 34 μL/24 h, $p<0.05$). We then added amiloride (1mM) to monolayers treated with erythropoietin and found that fluid loss from the supernatant decreased to that of control cells (n=6, erythropoietin/amiloride: 126 ± 26 μL/24 h). Our findings show that erythropoietin increases sodium uptake, Na-K-ATPase activity and transepithelial liquid movement in vitro. We speculate that if erythropoietin were to increase sodium transport and Na-K-ATPase activity in vivo, then lung water balance and respiratory difficulty might improve as well.

Abstract 8

Mesothelin-MUC16 Binding is a High Affinity, N-glycan Dependent Interaction that Facilitates Peritoneal Metastasis of Ovarian Tumors

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The mucin MUC16 and the glycosylphosphatidylinositol anchored glycoprotein mesothelin likely facilitate the peritoneal metastasis of ovarian tumors. The biochemical basis and the kinetics of the binding between these two glycoproteins are not clearly understood. Here we have addressed this deficit and provide further evidence supporting the role of the MUC16-mesothelin interaction in facilitating cell-cell binding under conditions that mimic the peritoneal environment.

In this study we utilize recombinant-Fc tagged human mesothelin to measure the binding kinetics of this glycoprotein to MUC16 expressed on the ovarian tumor cell line OVCAR-3. OVCAR-3 derived sublines that did not express MUC16 showed no affinity for mesothelin. In a flow cytometry-based assay mesothelin binds with very high affinity to the MUC16 on the OVCAR-3 cells with an apparent K_d of 5-10 nM. Maximum interaction occurs within 5 mins of incubation of the recombinant mesothelin with the OVCAR-3 cells and significant binding is observed even after 10 sec. A five-fold molar excess of soluble MUC16 was unable to completely inhibit the binding of mesothelin to the OVCAR-3 cells. Oxidation of the MUC16 glycans, removal of its N-linked oligosaccharides, and treatment of the mucin with wheat germ agglutinin and erythroagglutinating phytohemagglutinin abrogates its binding to mesothelin. These observations suggest that at least a subset of the MUC16-asscociated N-glycans is required for binding to mesothelin. We also demonstrate that MUC16 positive ovarian tumor cells exhibit increased adherence to A431 cells transfected with mesothelin (A431-Meso$^+$). Only minimal adhesion is observed between MUC16 knock-down cells and A431-Meso$^+$ cells. The binding between the MUC16 expressing ovarian tumor cells and the A431-Meso$^+$ cells occurs even in the presence of ascites from patients with ovarian cancer.

The strong binding kinetics of the mesothelin-MUC16 interaction and the cell adhesion between ovarian tumor cells and A431-Meso+ even in the presence of peritoneal fluid strongly support the importance of these two glycoproteins in the peritoneal metastasis of ovarian tumors. The demonstration that N-linked glycans are essential for mediating mesothlein-MUC16 binding may lead to novel therapeutic targets to control the spread of ovarian carcinoma.

A Novel Three-Dimensional In Vitro Implantation Model to Study Primate Embryo Development and Early Pregnancy

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Objective: To develop a model to study implantation and placenta formation in vitro with rhesus monkey embryos.

Hypothesis: A novel nonhuman primate in vitro 3-D system can provide cues for implantation and interaction with the extracellular environment not available in 2-D planar models.

Methods: We developed an in vitro 3-D implantation model utilizing blastocyst stage rhesus monkey embryos embedded in 3-D Matrigel explants, coupled with different feeder cell microenvironments.

Results: Signs of implantation including enlargement of embryo mass, invasion and proliferation of trophectoderm cell layers, cystic formation, and cellular outgrowths derived from the embryo, initiated within the first week post embedding. Trophoblast structures with protrusion and branches growing from the surface of embryo implants were observed. Rapid proliferation and differentiation of the trophoblast structures provided evidence of interactions between the embryo and the 3-D environment up to 45 days in culture. Immunohistochemical staining for CG and other biomarkers, combined with immunoassays for CG and progesterone showed secretion curves similar to early pregnancy, indicated positive characteristics of trophoblastic cell lineages. In addition, our study found morphological factors to predict successful establishment and prolonged embryo development, as well as an optimized culture microenvironment of media and feeder cells.

Conclusions: We have established a 3-D in vitro system showing the potential to model implantation initiation in vitro, and revealed the capability of the embryo to interact with the extracellular matrix. Continuing studies will accelerate our understanding of nonhuman primate embryo development, with potential for insights into early pregnancy loss and related pathologies.

Abstract 10

Synergy between PKA and PKC Stimulation of StAR Expression: Evidence for Distinct Nuclear Pathways Controlled by Acetylation/De-acetylation

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Previous work has shown that mouse StAR transcription in MA10 cells is stimulated synergistically by activation of PKC and PKA pathways (1). Other work has implicated histone acetylation as a key regulatory mechanism for StAR in granulosa cells (2) and MA10 cells (3). Chromatin immunoprecipitation has shown that histone acetylation is enhanced by PKA stimulation of StAR along with binding of specific regulatory factors (4). Histone de-acetylases (HDACs) inhibit StAR by binding to a sin3 complex in the proximal promoter. In these experiments we examine the synergy between PMA and Br-cAMP in two types of adrenal cell, a novel primary culture generated from temperature – sensitive large T antigen mice and early passage Y-1 cells. We show that protein acetylation plays very different roles in the PKA and PKC stimulated processes and that their synergy surprisingly shows characteristics that closely match the PKC pathway. We provide the first analysis of Type 1 nuclear HDACs and of Type 2 HDACs which exchange between the nucleus and cytoplasm. We find that StAR expression is sensitive to HDAC inhibition in both adrenal and MA10 cells but in opposite ways depending on the mode of stimulation. We show initial ChIP analyses of Histone acetylation under conditions of Br-cAMP stimulation and synergistic stimulation.

During an Experimental Rhinovirus (RV) Infection, Human Bronchoalveolar (BAL) Cells Secrete Increased Amounts of Monocyte Chemoattractant Protein 1 (MCP-1)

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Rationale: Alveolar macrophages are critical cells orchestrating the innate and adaptive immune response in the airway. Furthermore, it is believed that the cytokine dysregulation that accompanies virus-induced exacerbations of asthma is regulated, in part, by macrophage interaction with virus in the airway. This study tested the hypothesis that during an active RV infection, macrophages demonstrate altered elaboration of the chemokine MCP-1.

Methods: Patients (4 normal, 11 allergic asthmatic) underwent BAL prior to (baseline phase), and 4 days following (inoculation phase), inoculation with live human RV-16. Recovered BAL cells (mean 90% macrophages/monocytes) were cultured for 24h and the release of MCP-1 in the medium was measured by ELISA.

Results: 1) During the baseline phase of the study, *in vitro* stimulation of BAL cells with RV-16 significantly increased MCP-1 elaboration and *in vitro* stimulation with lipopolysaccharide significantly decreased MCP-1 release ($p<0.05$). 2) During the inoculation phase, BAL cells from normal and asthmatic patients showed greater production of MCP-1 ($p < 0.05$) than cells acquired during the baseline phase.

Conclusions: These data suggest that that the inflammatory microenvironment in the lower airway during an active RV infection alters the phenotypic properties of alveolar macrophages increasing their production of MCP-1. The consequence may be an increase in monocytic inflammation/recruitment associated with worsening of asthma symptoms.

These studies were supported by NIH grant AI50500 and NRSA grant T32-HD041921-01.

Abstract 12

Co-culture Effects on Trophoblast Differentiation from Human Embryonic Stem Cells

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Objective: Our laboratory has recently shown that when human embryonic stem cells (hESC) are allowed to differentiate under specific conditions in culture, they will differentiate into trophoblasts and secrete high levels of placental hormones. We therefore propose a three-dimensional, co-culture system to achieve the goal of utilizing various effector cell types to drive differentiation.

Methods: We used GFP-expressing H1 human embryonic stem cells (H1EGFP hESC) in combination with red Cell Tracker-labeled human term placental fibroblasts (TPF) and chorionic villus sampling fibroblasts (CVS) to form embryoid bodies (EBs). These EBs were kept in culture, in suspension for 30 days with media collection and replenishing done daily on half the media.

Results: Levels of human chorionic gonadotropin (hCG) in combination (TPF/H1EGFP) EBs in suspension increased 3-fold by day 20, peaked by day 25 (244.5 ng/ml) and dropped again by day 30, compared with hESC-only EBs, that maintained a low level of secretion (<19.1 ng/ml). CVS/H1EGFP combination EBs increased earlier at day 15, dropped by day 20 and then peaked at day 30. Immunohistochemistry of both combination EB types confirmed trophoblast differentiation by cytokeratin and hCG staining.

Conclusions: Effector cells can facilitate trophoblast differentiation through cell to cell contact with hESC within aggregated EBs. Furthermore, the three-dimensional, suspension environment fosters a greater hCG secretion than is seen in hESC-only EBs.

This work was supported by NIH grants HD046919 and HD038843.

Abstract 13

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. Nucleoporin 153 (NUP153) is a part of the nuclear pore and is important in mRNA export. Previous research in our lab identified this gene as one of leptin's possible targets. We treated normal murine mammary gland (NMuMG) cells with leptin over a period of 0-12 hours and analyzed NUP153 mRNA using reverse transcription followed by quantitative real time PCR (qPCR). Western blot analysis was done to confirm our qPCR results. A study utilizing cells grown on a permeable membrane was performed to determine differences between the proteins in the apical and basal media. 2D gel electrophoresis showed that the 6 hour time point had differences in proteins between the apical and basal media, while the other time points showed fewer differences. From our time course studies, we concluded that the 6 hour time point yields the changes that we want to investigate further. A dose response study treating cells with 0-100 ng/mL leptin and analyzing NUP153 mRNA using qPCR and western blotting has been completed. It appears that 3 ng/mL of leptin for 6 hours produces the greatest increase in NUP153 mRNA.

Abstract 14

Molecular and Functional Markers of Developmental Toxicity in a Human Embryonic Stem Cell-based *In Vitro* Model

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Birth defects are the largest cause of infant morbidity and mortality in the United States. Exposure to chemicals during human development plays a critical role on the onset and progression of congenital disorders. Animal models have a limited ability to predict human developmental toxicity in response to therapeutic or environmental compounds. Approximately 10% of experimental animals in toxicology research are required for teratogenic evaluation of compounds. Human embryonic stem cell technology is an innovative and robust alternative to predict developmental toxicity of chemicals during human pregnancy. Importantly, hESC technology should reveal developmental pathways that are particularly sensitive to detrimental effects of chemicals. Valproic acid (VPA) is a histone deacetylase inhibitor with therapeutic indications for epilepsy and bipolar disorder. VPA is a known teratogen that determines multiple birth defects, namely spina bifida, skeletal abnormalities and cognitive disorders such as autism. The present study examines the ability of human embryonic stem cell technology to predict toxic outcomes of valproic acid during early human development. Biomarkers of toxic response were identified using a systems biology approach that integrated molecular and functional cellular responses. Strikingly, VPA toxicity in this human in vitro model was consistent with in vivo animal studies at the molecular level. Our preliminary studies with VPA provide proof of concept for future application and use of hESC technology in predictive developmental toxicity of chemicals.

Abstract 15

Initial Characterization of Murine Muc16

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CA125 is a peptide epitope contained in the high molecular weight transmembrane mucin MUC16, which is over expressed in epithelial ovarian cancer (EOC). EOC is the most common type of ovarian cancer, the deadliest of gynecological cancers. CA125 is the human serum EOC marker used to monitor the progression of EOC. While the value of the mucin MUC16 has thus far been only diagnostic, it is implicated in the pathogenesis of EOC.

Recently, several mouse models of EOC in immunocompetent mice have been developed. In one prominent model, transgenic mice expressing the SV40 T-antigen under the direct influence of the Mullerian inhibitory substance (an ovary-specific gene) were developed. These mice spontaneously developed ovarian cancers and may therefore serve as excellent models to study the biological marker MUC16. From these mice were cultured several ovarian cancer cell lines (designated as MOVCAR). The expression of Muc16 in the MOVCAR cells has not been demonstrated, however (note murine Muc16 vs. human MUC16 nomenclature). Here we report the initial characterization of Muc16 expressed by the MOVCAR cells.

We have verified Muc16 mRNA expression in the MOVCAR cells with RT-PCR. We have partially purified soluble murine Muc16 produced by the MOVCAR cell lines using size exclusion and affinity chromatography, and the chromatography profiles are similar to those for human MUC16 isolated from human ovarian cancer cells. Using SDS-Page and Western blotting with a panel of monoclonal antibodies against human MUC16, we have probed for the presence of soluble Muc16. We consistently observe murine Muc16 bands around 250 kDa, which is significantly smaller than human MUC16 (~3 million Da). Performing flow cytometry with these mAbs, we have demonstrated both cell surface and intracellular expression of Muc16 in the MOVCAR cells.

We have also shown binding of murine Muc16 and human mesothelin by flow cytometry and Western blotting. Mesothelin, a GPI-anchored protein normally expressed in the mesothelial lining of the peritoneal cavity and also over expressed in ovarian cancer cells, is a specific binding partner for human MUC16. The interaction of mesothelin and MUC16 contributes to the metastasis of ovarian cancer within the peritoneal cavity. The murine Muc16-human mesothelin binding provides additional evidence of Muc16 expression by the MOVCAR cells.

Given this initial evidence of murine Muc16 expression by MOVCAR cells, we will further explore similarities between murine and human Muc16 in hopes of contributing to the understanding of the biological roles of Muc16.

Abstract 16

Generation of antigen-specific antibody panels for diagnostic use in the detection of cancer markers.

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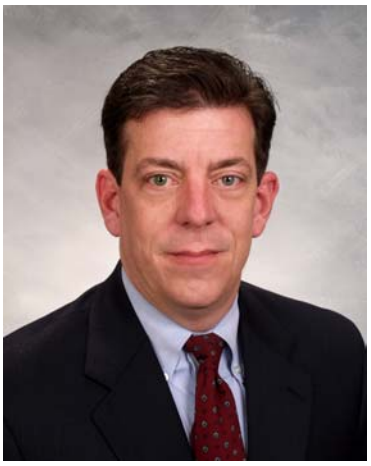
NeoClone, 1202 Ann Street, Madison, WI 53714.

NeoClone develops monoclonal antibodies (mAb) using a novel retroviral technology. Our process targets antigen-specific B cells and transforms them into stable plasmacytomas, which secrete high levels of immunoglobulins. Since our process does not require cell fusions or multiple rounds of selection for specificity and stability, we can rapidly and efficiently produce large numbers of antigen-specific antibody producing cell lines. This efficient process is ideal for the generation of antibodies to be used in diagnostic applications where high clonal diversity is important for selecting antibodies based on characteristics such as affinity and specificity as well as stability and compatibility with different detection platforms. As an example, in partnership with investigators at the EDRN (Early Detection Research Network- NCI), we have generated a panel of antibodies against CA125, a well-known cancer antigen used for diagnosis of ovarian and other cancers. Over 20 antibodies were developed and initial screening was completed within 4 months. We are currently evaluating mAb pairs for use in a ³Sandwich² ELISA and have identified several candidates with suitable sensitivity for diagnostic use.

Biosketch for Dr. Richard Bowen, Keynote Speaker



Dr. Richard Bowen received his B.S. at the University of South Carolina in 1979, and continued on to medical school at the Medical University of South Carolina in Charleston. He completed his family medicine residency in 1988 at Spartanburg Regional Medical Center in South Carolina. He was an emergency department physician for four years, then switched to hospital based family practice in 1995. Clinical research then caught his interest, and he began an outpatient practice specializing in the treatment and clinical research of obesity. Due to his involvement with the withdrawal of Fen-Phen, the anti-obesity drug, he was awarded a large grant from Wyeth to evaluate the long-term effects of exposure to fenfluramine. In April of 1996, he formulated a new hypothesis regarding the etiology, possible prevention, and treatment of Alzheimer's disease. A patent application was filed in November of 1997 which was eventually awarded in June of 2001. In March of 2001 he and a colleague founded Voyager Pharmaceutical Corp. He is now a part of O.T.B. Research. He has authored and co-authored over 27 publications on Alzheimer's, obesity, and sex hormones in journals such as JAMA, Journal of Biochemistry, Journal of Neuroendocrinology, and Endocrine.

The Implications of Living and Dying for Sex

Richard L. Bowen
OTB Research, Raleigh, NC

Although numerous theories of aging have been postulated, a comprehensive theory of aging that explains our progression through life ('aging') has yet to be formulated. We recently put forth a new theory ('The Reproductive-Cell Cycle Theory of Aging') which introduces a new definition of aging that has facilitated the conceptualization of why and how we age at the evolutionary, physiological and molecular levels (Bowen and Atwood, 2004). This theory proposes that aging can be defined by the rate of change in an organism over time. This definition not only includes changes that result in the loss of function (senescence) but also changes that result in a gain of function (growth and development). Most of the major changes that occur in an organism over time can be attributed to changes in cell proliferation, differentiation and death. The Reproductive-Cell Cycle Theory holds that these changes are primarily regulated by the hormones of the HPG axis (reproductive cell cycle-signaling factors). Specifically, that these reproductive hormones act in an antagonistic pleiotrophic manner via cell cycle signaling, promoting growth and development early in life in order to achieve reproduction, but later in life, in a futile attempt to maintain reproduction, become dysregulated and drive senescence. The Reproductive-Cell Cycle Theory of Aging is able to explain (1) the simultaneous regulation of the rate of aging and reproduction as evidenced by the fact that environmental conditions and experimental interventions known to extend longevity are associated with decreased reproductive cell cycle signaling, thereby slowing aging and preserving fertility in hostile reproductive environments; (2) how differing rates of reproduction between species are associated with differences in their lifespan; (3) the apparent paradox that size is directly proportional to lifespan and inversely proportional to fertility between species but vice versa within a species; and (4) provides a credible reason for why and how aging occurs at the evolutionary, physiological and molecular levels. The theory also is able to explain many phenomena associated with aging including what determines the rate of growth and development and the ultimate size of an organism, what regulates the initiation of puberty, why organisms with predator evading attributes such as birds and turtles live so long and how caloric restriction extends longevity, i.e. very fundamental, yet unanswered questions. The theory has immediate and practical implications for extending longevity and delaying/preventing age-related diseases. For example, interventions that regulate serum HPG hormones are predicted to extend longevity and delay age-related diseases. With respect to this latter point, we have demonstrated that suppression of serum gonadotropins using a GnRH superagonist stabilizes cognition in individuals with mild-moderate Alzheimer's disease who were taking acetylcholinesterase inhibitors concurrently over a 1 year period in a Phase II clinical trial. Future clinical trials will determine the utility of such hormonal manipulations in offsetting age-related diseases.

Session III: Techniques Workshop: Speaker Biosketches

Lewis Sheffield received his B.S. and M.S. at Clemson University, and his Ph.D in Dairy Science in 1983 at the University of Missouri. His postdoctoral work was performed at Michigan State University. Dr. Sheffield has served on the UW-Madison faculty since 1986, and is a Professor in the Dairy Science department. His research focuses on mammary development, lactation, and breast cancer.

Mark Scalf received a B.S. in Chemistry/Biochemistry from Virginia Tech in 1995 and a Ph.D. in Analytical Chemistry from UW-Madison in 2000. His graduate work was performed in Lloyd Smith's laboratory, with a thesis titled "DNA and Protein Analysis using Matrix Assisted Laser Desorption/Ionization and Electrospray Ionization Mass Spectrometry". He worked for three years in the biotechnology industry as a research scientist performing mass spectrometry-based proteomics studies. Dr. Scalf is currently a research scientist in the laboratory of Lloyd Smith at UW-Madison and is involved in both technology development and applications of mass spectrometric proteomics.

Dr. Anne Griep received her B.A in Molecular Biology and Music from Wellesley College and her Ph.D. in Biochemistry from the University of Wisconsin-Madison in 1985. She conducted postdoctoral training in Developmental Biology at the National Institutes of Health where she became an expert in the generation of and use of transgenic and mutant mice in biomedical research. She returned to the University of Wisconsin in 1990 to become the Director of the Transgenic Animal Facility (TAF) in the University of Wisconsin Biotechnology Center and in 1991 joined the Anatomy Department as an Assistant Professor and was promoted to Associate Professor with Tenure in 1997, She has been full Professor in the Anatomy Department since 2002. Dr. Griep's research group in the Anatomy Department is interested in understanding the role of cellular tumor suppressor genes in normal development and cancer.

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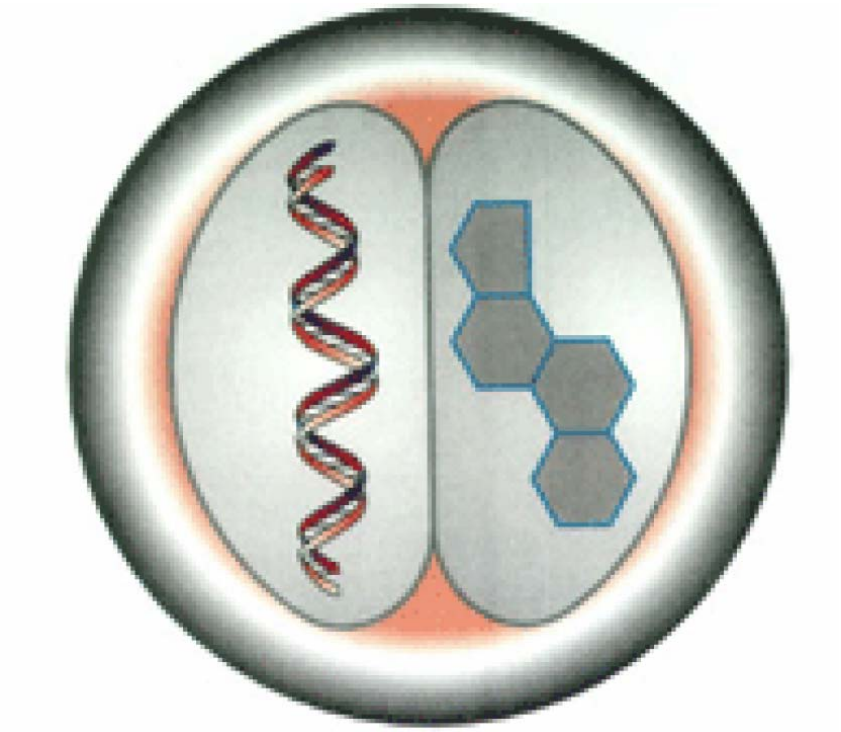
Notes

Please join us next year for the 2007 Annual Endocrinology - Reproductive Physiology Research Symposium



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