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ABSTRACTS

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001

ORIGINS OF DNA DAMAGE IN SPERMATOZOA

R. J. Aitken

Biological Sciences, University of Newcastle, Callaghan, NSW, Australia

DNA damage is frequently encountered in the spermatozoa of sub-fertile male mammals and is correlated with a range of adverse clinical outcomes including impaired fertilization, disrupted embryonic development, increased rates of miscarriage and an enhanced risk of disease in the progeny. The etiology of DNA fragmentation in human spermatozoa is closely correlated with the appearance of oxidative base adducts and evidence of impaired chromatin remodelling during spermiogenesis. In light of these associations we propose a two step hypothesis for the origins of DNA damage in spermatozoa. In Step 1, a variety of intrinsic (diabetes, varicocele, testicular torsion, obesity) and extrinsic (radiofrequency electromagnetic radiation, heat, cigarette smoke, diet, environmental toxicants) factors collude to generate a state of oxidative stress in the testes. This stress impedes spermiogenesis resulting in the generation of spermatozoa with poorly remodelled chromatin. These defective cells readily default to an apoptotic pathway comprising motility loss, caspase activation, phosphatidylserine exteriorization and the production of reactive oxygen species (ROS) by the mitochondria. In Step 2, these mitochondrial ROS attack the spermatozoa inducing lipid peroxidation and oxidative DNA damage, which then leads to DNA strand breakage and cell death. Nucleases activated and released during the apoptotic process are denied access to the sperm nucleus because the unique physical architecture of this cell prevents it. For this reason, a majority of the DNA damage encountered in human spermatozoa is oxidative. Given the importance of oxidative stress in the etiology of DNA damage, there should be a significant therapeutic role for antioxidants in the treatment of this condition. Furthermore, if oxidative DNA damage in spermatozoa is providing a sensitive readout of systemic oxidative stress, the implications of these findings could stretch beyond our immediate goal of trying to minimize DNA damage in spermatozoa as a prelude to assisted conception therapy.

002

PIG SPERM EGG INTERACTION AND FORMATION OF A ZONA PELLUCIDA BINDING COMPLEX

B. M. Gadella

Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Netherlands

In order to achieve fertilization sperm cells first need to successfully interact with the zona pellucida. Before reaching the zona pellucida the sperm cell undergoes extensive remodeling both in the male and female genital tract. These changes serve to mediate optimal recognition of the zona pellucida in the oviduct (primary zona pellucida binding). Optimal sperm-zona interactions are crucial for porcine oocyte fertilization: The zona pellucida- attached sperm cell is triggered to undergo the acrosome reaction and will also become hypermotile. Together these two responses allow the sperm cell to drill through the zona pellucida (secondary zona pellucida binding) this coincides with local sperm zona drilling so that a few sperm can reach the perivitellin space. This delaying strategy allows only one sperm cell in a given time-point to bind and fuse with the oocyte (fertilization) and thus minimizes the risk of polyspermy. The polyspermy block is essentially executed by the fertilized oocyte that immediately secretes its cortical granule content into the perivitellin space. This content blocks sperm-oocyte fusion either by sticking to the oolemma or by the induction of a biochemical reaction of the zona pellucida (zona pellucida hardening). The cortical reaction thus blocks sperm-zona pellucida binding and/or sperm-zona pellucida drilling. Note that zona pellcuida interactions under pig IVF conditions relatively frequently result in polyspermy. It is not clear whether this is also the case in vivo after natural mating. More importantly we do not know how other artificial reproductive technologies affect polyspermy rates. This is especially relevant for new sperm treatments and insemination technologies in which sperm are activated by capacitation media essentially mimicking the IVF media. Therefore, better understanding of sperm activation and of the arrangement of proteins involved in zona pellucida interactions are relevant for designing strategies to further improve pig reproduction

003

OXIDATIVE STRESS, OSMOTIC STRESS AND APOPTOTIC CHANGES: EFFECTS ON EQUINE SPERMATOZOA

B. A. Ball, S. Meyers

School of Veterinary Medicine, University of California Davis, Davis, CA, United States

Although considerable progress has been made over the past few years in liquid or frozen storage of equine spermatozoa, there remains a large inter-individual difference in the success of semen preservation for the stallion. Equine spermatozoa undergoing low-temperature storage undergo oxidative damage to membrane phospholipids, proteins and chromatin. Osmotic stress also leads to damage to the plasma membrane and alteration in sperm metabolism. Furthermore, evidence from a number of species suggests that ejaculated spermatozoa undergo apoptotic-like changes as a consequence of cryopreservation. It appears likely that these three processes are interlinked and may impact various compartments in the sperm cell via similar pathways. Therefore, an understanding of these processes and their common metabolic pathways may be important in attempts to obviate adverse affects on equine spermatozoa during storage. Further research should evaluate the molecular pathways which may represent convergence of these stresses on the sperm cell with an aim to reducing their net detrimental effect on sperm during preservation. Supported by the John P. Hughes Endowment, the UC Davis Center for Equine Health, and the National Center for Research Resources, National Institutes of Health.

004

A NEW ERA IN CONTRACEPTIVE DEVELOPMENT: NON-HORMONAL OPTIONS THAT ALSO TARGET SEXUALLY TRANSMITTED INFECTIONS

E. Dimitriadis

Prince Henry's Institute of Medical Research, Melbourne, VIC, Australia

Despite huge increases in access to contraceptives globally more than 700,000 maternal deaths related to unintended pregnancies occurred between 1995 and 2000 mostly in developing countries. Over 80 million women have unintended or unwanted pregnancies annually. Remarkably, there have been no new methods of contraceptives developed in the last 50 years. The extremely high incidence of sexually transmitted infections (STIs) indicates that it is desirable to develop contraceptives that also target STIs. Two interleukin (L) 6-type cytokines, leukemia inhibitory factor (LIF) and IL11, are obligatory for implantation in mice and are dysregulated in endometrium of some women with infertility. Both LIF and IL11 are also thought to have roles in Chlamydia-induced inflammation which can lead to a multitude of pathologies. LIF and IL11 antagonists were produced and their contraceptive efficacy tested in mice. Polyethylene glycol (PEG) was conjugated to LIF antagonist (LA) or IL11 antagonist (IL11A) to increase their serum half-life. PEGLA injected during the peri-implantation period blocked LIF action in the endometrium and totally prevented embryo implantation while having no embryo toxic effects[1]. Similarly, injection of PEGIL11A blocked decidua formation resulting in pregnancy failure[2]. In women, vaginally administered drugs preferentially localise to the uterus suggesting that vaginal administration of PEGLA is an appropriate delivery method for contraceptive purposes. Further, vaginally administered PEGLA may be useful as a 'dual-role' contraceptive to also block STIs. PEGLA administered via vaginal gel was shown to prevent implantation having minimal effects on non-uterine LIF targets. This is the first study to show the contraceptive efficacy of a PEGylated compound delivered vaginally. It further indicates that PEGLA may be useful as a dual-role contraceptive. Contraceptive trials in non-human primates are currently underway to determine the effect of PEGLA on implantation. If effective, this will offer new opportunities as pharmacological, non-hormonal dual-role contraceptives for women.

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(2) Menkhorst E, Salamonsen LA, Robb L, Dimitriadis E (2009) Biol of Reprod 80: 920–927.

006

HUMAN GERM CELL FORMATION AND DIFFERENTIATION FROM PLURIPOTENT STEM CELLS

R. A. ReijoPiera

Center for Human Embryonic Stem Cell Research and Education, School of Medicine, Stanford University, Stanford, Australia

Human embryo development begins with the fusion of egg and sperm, followed by reprogramming of the DNA, a series of cell divisions and activation of the embryo's genome. As development continues, the germ cells (egg and sperm) must be set aside from other cell types. A major cause of infertility in men and women is quantitative and qualitative defects in human germ cell (oocyte and sperm) development. Yet, it has been difficult to study human germ cell development, especially features that are unique relative to model organisms. We have developed a system to differentiate human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) to germ cells and to quantitate and isolate primordial germ cells (PGCs) derived from both XX- and XY-bearing hESCs and iPSCs. This allowed silencing and overexpression of genes that encode germ cell-specific cytoplasmic RNA-binding proteins (not transcription factors) and resulted in the modulation of human male and female germ cell formation and developmental progression. We observed that human DAZL (Deleted in AZoospermia-Like) functions in female and male PGC formation and maintenance, whereas closely-related family members, BOULE and DAZ, promote entry into meiosis and development of haploid gametes with sperm-specific methylation patterns at imprinted loci in the male. We also conducted critical proof-of-concept studies in mice that showed that phenotypes observed in germ cell development in vitro from wildtype, heterozygous, and Dazl-/- mutation-carrying mouse ESCs (mESCs) mirrored the phenotypes that were observed in vivo. Furthermore, transplantation of XX mESC-derived oocytes resulted in recruitment of somatic cells to form follicles. These studies comprised the first direct experimental analysis of the genetics of human germ cell development and set the stage for extensive exploration of complex genetic variants linked to infertility. Results are significant to the generation of gametes for developmental genetic studies and potential clinical applications.

010

PERICONCEPTIONAL UNDERNUTRITION: LIFE-LONG EFFECTS FOR THE OFFSPRING

F. H. Bloomfield[1,2], M. H. Oliver[1,2], A. L. Jaquiery[1,2], C. Hernandez[1,2], J. R.G. Challis[4], A. White[3], J. E. Harding[1]

[1]Liggins Institute, University of Auckland, Auckland, New Zealand

[2]National Research Centre for Growth and Development, New Zealand

[3]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

[4]Physiology, University of Toronto, Toronto, Ontario, Canada

Poor nutrition in women of child-bearing age is common, even in Western countries. It has been estimated that approximately 30% of women of child-bearing age in affluent cities such as Sydney and Southampton are either actively dieting or have a nutritional intake that does not meet daily recommended requirements for all nutrients. We have investigated the effect of reduced maternal nutrition before and around the time of conception on fetal growth and development, and have followed offspring through to adulthood. In this paradigm, ewes were fed to lose 10-15% of their body weight and then to gain weight according to conceptus mass. Control ewes were well fed throughout. Different timing and duration of undernutrition in the periconceptional period were utilised to investigate the most critical window for fetal development. Periconceptional undernutrition resulted in accelerated development of the fetal hypothalamic-pituitary-adrenal (HPA) and glucose-insulin axes in late gestation, and preterm birth. Offspring of periconceptionally undernourished ewes demonstrated altered laterality and an altered response to isolation stress; HPA axis function was also suppressed. As offspring aged, glucose tolerance decreased and became significantly impaired by young adulthood compared with control offspring. The effects of maternal undernutrition on offspring were modified by offspring sex and also by being one of a twin pair. Interestingly, our data also demonstrate that conception as a twin, regardless of maternal nutritional status, also affects all these outcomes but in a different way to maternal undernutrition. Preliminary data suggest that epigenetic changes in feeding centres of the hypothalamus may play a role in the mechanism behind some of these effects. These studies suggest that even moderate maternal undernutrition in very early pregnancy has life-long effects. Should this also be true in humans, then health care messages for women may need to be targeted prior to pregnancy.

011

GESTATIONAL DIABETES – COMPLICATIONS, MANAGEMENT, OUTCOMES

J. A. Rowan

National Women's Health, Auckland City Hospital, Grafton, New Zealand

Gestational diabetes (GDM) is associated with increased maternal risks of hypertensive complications, caesarean section and later diabetes. The fetus is exposed to an excess nutrient load and risks of macrosomia, trauma at delivery, neonatal complications and later obesity and associated metabolic consequences. Perinatal outcomes are improved by treating GDM, but the effect on longer term outcomes is not clear. Can we improve pregnancy and later outcomes further by considering choice of medication and treatment targets? The metformin in gestational diabetes (MiG) trial demonstrated that pregnancy outcomes were not different between women randomized to metformin compared to insulin. A composite of neonatal complications was seen in 32.0% and 32.2% respectively, RR 0.99 (95% CI 0.80–1.23). Examining glucose control during treatment in tertiles, women who achieved a mean fasting capillary glucose level <4.9 mmol/L had the lowest risk of neonatal complications. Those achieving a postprandial capillary glucose mean <6.5mmol/L had lower rates of preeclampsia and birth weight >90th centile. Obesity was not a significant factor predicting outcomes (unlike diet-alone treated women). Preliminary analyses from the follow up of two year old children from the MiG trial, the offspring follow up (TOFU) show body composition, diet and activity assessments are similar, with small differences between the metformin and insulin groups, respectively in biceps (6.1 vs 5.6 mm P = 0.04) and subscapular skin folds (6.38 vs 6.10 mm P = 0.03) and upper arm circumference (17.3 vs 16.7 cm P = 0.003). Ratios of central to peripheral fat as measured by waist: hip circumference, suscapular : triceps skin folds and abdominal : thigh fat by DEXA were no different. Further analyses will be performed when the final data entries are completed and details of these will be presented.

020

HORMONAL REGULATION OF SPERMATOGENESIS

D. Handelsman

ANZAC Research Institute, The University of Sydney, NSW, Australia

Spermatogenesis is a spatially and temporally co-ordinated proliferation of the germinal epithelium in the semeniferous tubules. The germ cells are embedded in a scafolding formed by adjacent Sertoli cells linked tightly by intercellular junctions and with each germ cell enshrouded by elongations of Sertoli cell cytoplasm. Spermatogenesis comprises serial stages from the mitotic replication of the stem and early germ cells, followed by meiosis, the reductive division producing haploid, amorphous gametes which subsequently undergo spermiogenesis, the metamorphosis into terminally differentiated and functional spermatozoa. Although long known that all but the earliest stages are hormonally regulated by pituitary secretion of LH and FSH, it has remained difficult to separate gonadotrophin effects by classical endocrine ablate-replace methods as these two heterodimeric hormones have identical α and homologous β subunits, are secreted from the same pituitary gonadotrophs to target cognate receptors expressed on adjacent testicular cells as equally homologous, heptahelical G-coupled protein receptors. Over two decades our laboratory has developed a variety of complementary genetic and pharmacological approaches to dissect the individual and co-operative effects of LH, its main effector testosterone and FSH on spermatogenesis. Using the gonadotrophin and testosterone deficient *hpg* mouse, double transgenic human FSH secreting mouse and the androgen receptor knockout mouse lines together with steroidal depot homone delivery, we have explored systematically annd defined the individual primary actions of FSH and testosterone and their interactions in the regulation of testis growth, spermatogenesis and ultimately male fertility.

031

THE ORIGIN OF ANEUPLOIDY IN HUMANS: WHERE WE'VE BEEN, WHERE WE'RE GOING

T. Hassold

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

With the advent of the human genome project in the 1990s, DNA markers became available, allowing us to determine the parent and meiotic stage of origin of human aneuploid conditions. This approach has been extensively used to study the origin of trisomies, with one over-arching conclusion: the vast majority of trisomies derive from errors in the development of the egg and, in particular, from nondisjunction occurring in the first maternal meiotic division (MI). However, against this general background, it has also become apparent that there is considerable chromosome-to-chromosome variation. For example, certain aneuploidies (e.g., the XXY condition, or Klinefelter syndrome) are commonly paternal in origin, while others (e.g., trisomy 16, the most common human trisomy) almost always originate from maternal MI errors. Thus, we now know that individual chromosomes behave differently with respect to mechanisms of meiotic nondisjunction. In this presentation, we will summarize results of these parental origin studies of aneuploidy and discuss the involvement of the first identified molecular correlate of nondisjunction – aberrant meiotic recombination – in the genesis of these abnormalities. We will also look forward, discussing the development of mouse models of human aneuploidy, and recent advances in molecular cytogenetics that make it possible to directly analyze human meiosis "as it happens" in fetal oocytes and in spermatocytes

032

CDH1: A CELL CYCLE PROTEIN INVOLVED IN FEMALE MEIOSIS AND PREVENTION OF ANEUPLOIDY

K. T. Jones

School of Biomedical Sciences, University of Newcastle, Callaghan, NSW, Australia

Mammalian oocytes are arrested at the dictyate stage of prophase I in the ovary. In growing follicles, oocytes can become responsive to Luteinising Hormone and will undergo meiotic resumption just before ovulation. During the first meiotic division, homologous chromosomes are segregated, a process that is very error prone in human oocytes. By ovulation the oocyte has extruded its first polar body and has re-arrested at metaphase of the first meiotic division. Recent work from our lab has established that the protein Cdh1 is involved uniquely in both in the process of prophase I arrest and the correct segregation of homologs in meiosis I. Thus in cultured oocytes, in vitro antisense knockdown of Cdh1 induces both meiotic resumption and high rates of aneuploidy as a result of non-disjunction during first meiosis. Cdh1 causes prophase I arrest by inducing cyclin B1 degradation and maintaining low levels of the kinase CDK1, whose activity induces meiotic resumption. Cdh1 is an activator of the Anaphase-Promoting Complex (APC), a ubiquitin ligase that earmarks proteins such as cyclin B1 for proteolysis. Cdh1 prevents aneuploidy by causing the degradation of Cdc20, a protein that is responsible for activating the APC once all homologs are correctly aligned at metaphase. Thus loss of Cdh1 seems to prematurely activate APC(Cdc20) activity. It is interesting that a single protein can affect two important meiotic transitions in oocytes. However to explore its functions more fully, and confirm that an in vitro knockdown is faithfully replicated by in vivo loss, a targeted knockout of Cdh1 is needed. Therefore we have generated an oocyte specific Cdh1 knockout by ZP3 promoter driven Cre- recombinase activity in oocytes carrying loxP insertions in the single copy Cdh1 gene. This talk will therefore focus on the effects of an in vivo Cdh1 knockout.

033

IMPORTIN A2 MEDIATES SUBNUCLEAR TARGETING OF THE CAJAL BODY COMPONENT COILIN; A KEY ROLE IN SPERMATOGENESIS?

J. D. Ly-Huynh[1,2], A. Efthymiadis[2], P. Whiley[1,3], E. Richards[3], H. Lescesen[1,2], I. Meier[4], G. A. Matera[4], K. L. Loveland[1,3], D. A. Jans[1,2]

[1]ARC Centre of Excellence in Biotechnology and Development, Australia

[2]Department of Biochemistry and Molecular Biology, Nuclear Signalling Lab, Monash University, Melbourne, VIC, Australia

[3]Departments of Biochemistry and Molecular Biology/Anatomy and Developmental Biol, Monash University, Melbourne, VIC, Australia

[4]Departments of Biology/Genetics, University of North Carolina, Chapel Hill, United States

Spermatogenesis, the progressive maturation of immature germ cells to form spermatozoa, requires nucleocytoplasmic shuttling of nuclear factors to implement changes in gene transcription, as well as the storage and alternative splicing of mRNA transcripts in the nucleus that is vital for fertility. The key cellular mediators of nuclear entry are members of the importin (IMP) superfamily, of which the five different IMPα proteins in mouse testis are expressed dynamically throughout spermatogenesis, consistent with roles in transporting distinct, specific cargoes critical to gamete maturation. We identified the central Cajal body (CB) component Coilin as a specific testicular binding partner of IMPα2 in a yeast 2-hybrid screen and confirmed this interaction by coimmunoprecipitation from testis lysates. CBs are small nuclear inclusions that can associate with histone genes and facilitate histone pre-mRNA processing by recruiting RNA processing factors; intriguingly, expression of IMPα2 but not other IMPαs can regulate the number and size of CBs, as shown in cell culture experiments. Immunohistochemical analysis revealed that Coilin is predominantly in spermatocytes and in round and elongating spermatids in the rodent testis. The physiological importance of its role is indicated by the fact that Coilin knockout mice have reduced fertility, smaller testes and aberrant spermatogenesis. Our future work will focus on the testicular functions of Coilin and IMPα2 during spermatogenesis, and their roles in coordinating the pre-assembly, storage and targeting of transcription complexes to RNA processing machinery in the nucleus.

040

ARE ENVIRONMENTAL EXPOSURES AFFECTING HUMAN REPRODUCTIVE HEALTH?

P. Hunt

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

The concern that human reproductive health may be affected by chemicals in our daily environment has grown in recent years with the recognition that: (1) some countries have seen a recognizable decline in sperm counts and an increase in urogenital tract abnormalities among newborn males, (2) the incidence of some cancers has increased precipitously, and (3) the number of infertile couples has increased markedly in many countries. Our laboratory focuses on the oocyte and the factors that cause the production of chromosomally abnormal eggs. We know that the risk of a chromosomally abnormal pregnancy is strongly influenced by maternal age, but there is growing concern that environmental exposures may influence the ability of both the male and female to produce normal gametes. Our laboratory has focused on the effect of exposures to a ubiquitous chemical to which humans are exposed daily, bisphenol A (BPA). BPA is used in a wide variety of consumer products from plastics and resin coatings to eyeglasses and pressure printed receipts. Our studies in mice demonstrate that BPA exposure during fetal development adversely affects female fertility because BPA influences several significant stages of egg development. In the male mouse, we and others have found that prenatal, perinatal, and adult exposures can affect the function of the testis. In current studies we are attempting to determine if effects seen in the mouse are also a feature of BPA exposed primates. We are using a rhesus monkey model to determine how BPA is metabolized in the pregnant and nonpregnant female and how BPA exposure influences the developing fetus. Lastly, in human studies we are evaluating BPA levels in the developing fetus and assessing their effect on the developing fetal ovary.

041

ENDOCRINE DISRUPTING IMPACTS IN RECEIVING WATERS OF THE SYDNEY BASIN

R. Lim

Department of Environmental Sciences, University of Technology Sydney, Ultimo, NSW, Australia

Water reuse for a number of activities including potable water and replacement of environmental flows is becoming more significant due to the prolonged drought Australia has recently experienced. There is also much debate regarding potential impacts of compounds such as steroid endocrine disrupting chemicals (EDCs), pharmaceuticals & personal care products (PPCPs), and persistent organic pollutants (POPs) to environmental and human health. This paper presents an overview of findings on some EDCs in the Sydney Basin to assess the environmental risk they pose. A tiered approach, using a suite of endpoints spanning *in vitro* (e.g., estrogen receptor binding assay, the 2-hybrid yeast test) to *in vivo* (using the mosquitofish (*Gambusia holbrooki*) to assess vitellogenin induction, and morphological and behavioural changes) studies was conducted on aquatic systems receiving urban and treated sewage effluents. *In vitro* bioassays suggest low levels of estrogenicity in sewage contaminated waterways. Both estradiol (E2) and estrone (E1) were identified in all river water samples, suggesting that sewage contamination is widespread. The synthetic hormone, ethynylestradiol (EE2), was below detection limits in all samples tested. Results indicate that the STPs were not the only source of EDCs in aquatic systems within the Sydney area. Improvements in treatment technologies in STPs have substantially reduced EDC levels in final effluent as indicated by a reduction in endocrine disrupting effects on the mosquitofish over several years of study. In addition, advanced tertiary treatment technology removed EDCs to levels below that measurable by *in vitro* assays and *in vivo* fish testing. This tiered weight of evidence approach provided insights to the risks EDCs in sewage effluent produced from current treatment technologies have on the environment.

042

THE EFFECT OF THE INSECTICIDE PYRIPROXYFEN ON OVARY SYNTHESIS IN THE CHRISTMAS ISLAND RED CRAB, *GECARCOIDEA NATALIS*; A POSSIBLE CASE OF ENDOCRINE DISRUPTION?

S. M. Linton[1], L. Barrow[2], C. Davies[3], L. Harman[1]

[1]School of Life and Environmental Sciences, Deakin University, VIC, Australia
[2]King Island Natural Resource Management Group, King Island, TAS, Australia
[3]Marine and Atmospheric Research, CSIRO, Brisbane, QLD, Australia

The yellow crazy ant, Anopholepis gracilipes is an invasive species on Christmas Island, Indian Ocean whose population needs to be controlled before there is irrevocable environmental damage. The insecticide, pyriproxyfen, a compound which mimics juvenile hormone of insects, has been proposed to do this. Before it can be used in the field, its effects on non target species such as the endemic red crab, Gecarcoidea natalis, need to be investigated. Land crabs, like all decapods, may utilise a similar hormone called methyl farnesoate which is thought to be involved in controlling early ovary development. Pyriproxyfen may also mimic methyl farnesoate and thus disrupt this process. The effect of pyriproxyfen on early ovary synthesis in G. natalis was investigated by feeding crabs a mixture of leaf litter and bait containing 0.5% pyriproxyfen (experimental groups) or a mixture of leaf litter and bait containing no pyriproxyfen (control groups) at simulated baiting doses (2 kg ha^{-1} and 4 kg ha^{-1}). Two additional groups of crabs were fed ad libitum, either bait containing 0.5% pyriproxyfen or the control bait. Experiments were conducted from July to September of 2007. Red crabs synthesise their ovaries annually over two months (July to September) in the dry season. This situation of high nitrogen demand is funded from small excesses of nitrogen assimilated from a mainly leaf litter diet. Pyriproxyfen affected early ovary development. Ovaries from crabs in the experimental groups at all baiting levels had a higher total nitrogen content and dry mass than that of the control animals. The ovaries from the experimental animals were also more mature; they contained more previtellogenic and early vitellogenic oocytes, of a larger diameter, than ovaries of the control animals. Significant amounts of pyriproxyfen were accumulated in the target tissues, the midgut gland and ovary. Minor amounts of pyriproxyfen were accumulated in muscle, a non-target tissue. By mimicking methyl farnesoate, pyriproxyfen may have caused endocrine disruption in G. natalis. In particular it may have stimulated early ovary development and synthesis of yolk protein.

054

TROPHOBLAST, PLACENTA AND EARLY EMBRYO: HOW THE MARSUPIAL DEVELOPS

M. B. Renfree, S. R. Frankenberg, C. Freyer

ARC Centre of Excellence for Kangaroo Genomics, Department of Zoology, The University of Melbourne, VIC, Australia

In marsupials, the blastocyst forms as a single cell layer of cells. The marsupial blastocyst has no inner cell mass, so the 80–100 cell tammar embryo remains in diapause as a unilaminar blastocyst. All marsupials have a unilaminar stage, but what is unusual is that in the tammar the total cessation of cell division and cell metabolism lasts for 11 months each year. Marsupials are placental mammals. The yolk sac forms the definitive placenta up to birth. Only very few marsupials, such as the bandicoot, have a chorio-allantoic placenta, which supplements the placental functions of the yolk sac. However, the understanding how the unilaminar layer of trophoblast cells of the diapausing blastocyst become specified into placental and embryonic tissues has been an ongoing puzzle. To identify genes that do become differentially expressed in tammar development, we targeted the stage of the earliest appearance of the embryonic disc, at which the remainder of the blastocyst is then defined as trophoblast, as well as early cleavage stages. Intriguingly, we found no evidence for early differential expression of the canonical pluripotency genes POU5F1, SOX2 and NANOG, or of CDX2. By contrast, we found overt differential expression of GATA3, the closely related gene GATA2, and FGF4. This expression profile suggests that in the tammar, mechanisms regulating trophoblast- and pluriblast-specific expression of POU5F1, SOX2, NANOG and CDX2 are temporally secondary to those regulating GATA2 & -3 and FGF4 expression. Together, our results may signify the evolution of alternative mechanisms of early lineage specification in marsupials, or alternatively reveal a general hierarchy of signalling mechanisms that are masked in the relatively rapid and "compressed" development of mice. The results of our ongoing study have important implications for understanding not only marsupial stem cells but the early development of all therian mammals.

055

EVEN REPTILES DO IT, THE STRUCTURE AND FUNCTION OF PLACENTAE IN VIVIPAROUS LIZARDS

M. Thompson

School of Biological Sciences, University of Sydney, NSW, Australia

Live birth (viviparity) has evolved independently more than 100 times in squamate reptiles (lizards and snakes). Most viviparous lineages are characterised by a simple placenta with squamous epithelia on both maternal and embryonic sides, and the remnants of an eggshell persist for some, if not all, of pregnancy. The embryos are predominantly lecithotrophic, with maternal-embryonic exchanges being limited mostly to inorganic ions, water and respiratory gases. Nevertheless, there is differentiation of a chorioallantoic placenta and a yolk sac (omphalo) placenta in all species. Complex placentae have evolved in the Squamata in only four or five lineages, all in the lizard family Scincidae (skinks). In species with complex placentation, the uterus differentiates to allow different functions in association with each embryonic membrane type. Species with complex placentae are characterised by a hypertrophy of maternal and embryonic cells, elaboration of the maternal surface, a reduction in yolk with a concomitant increase in placentotrophy and, in some, regional differentiation of the choriallantois into a placentome and a paraplacentome. Both the placentome and omphaloplacenta are organs of embryonic nutrition, but they transport nutrients by different mechanisms; the paraplacentome is a specialised gas exchange organ. The most placentotrophic species are in the South American genus Mabuya, where the females produce micro-lecithal eggs and the placenta is more complex than in any mammal, with four specialised structures for nutrient exchange and one for gas exchange. The number of independent origins of viviparity and the range of placental complexities exhibited by skinks enables us to infer the evolutionary trajectories that have resulted in microlecithal eggs and an almost complete reliance of placentotrophy from oviparous ancestors.

056

EUTHERIAN MAMMALS DO IT DIFFERENTLY: PLACENTAL ENDOCRINE STRATEGIES FOR THE MAINTENANCE OF PREGNANCY IN RODENTS AND PRIMATES

B. J. Waddell

School of Anatomy & Human Biology, The University of Western Australia, Nedlands, Australia

The placenta of rats and humans share important anatomical similarities, each with a chorio-allantoic, single discoid, haemochorial structure that facilitates highly efficient nutrient transport. Importantly, however, these similarities reflect convergent evolution and conceal markedly different developmental trajectories and endocrine functions. Placental endocrine signals are essential to drive maternal adaptations that facilitate fetal development and ultimately successful birth. Central to these adaptations is a sustained increase in production of the sex steroids progesterone and oestrogen, each driven by very different placental signalling in rodents and primates. Specifically, while the rat placenta supplies androgen precursors for ovarian (luteal) oestrogen synthesis, in humans and closely-related primates the fetal adrenal cortex supplies androgen precursors for placental oestrogen synthesis. In both cases the resultant increase in oestrogen provides a local stimulus to ovarian (rat) and placental (primate) progesterone synthesis. This shift from a placental-ovarian to a feto-placental unit for oestrogen synthesis in primates may have evolved to ensure greater fetal influence over maternal adaptations. Placental regulation of maternal physiology is also mediated via a third steroid group, the glucocorticoids, which promote a successful pregnancy outcome via effects on maternal metabolism and fetal organ maturation. Glucocorticoids are produced within the HPA axis, activity of which is enhanced by the placenta (eg, via oestrogen in rodents and CRH in primates). Moreover, the placenta regulates access of maternal glucocorticoids to the fetus via expression of the 11 b -HSD enzymes which constitute the placental glucocorticoid barrier. Intriguingly, this barrier effectively disappears during late fetal life in rodents but increases markedly in primates (notably baboons and humans). We hypothesise that this opposite developmental change is due in part to the evolution of the feto-placental unit for oestrogen synthesis in these primate species, and the associated need to prevent suppression of the fetal HPA axis by maternal glucocorticoids in late gestation.

058

GESTATIONAL DIABETES AND TYPE 2 DIABETES IN PREGNANCY IN AUSTRALIA

N. W. Cheung

Centre for Diabetes & Endocrinology Research, Westmead Hospital, Westmead, NSW, Australia

In the last 30 years, there has been a dramatic increase in the incidence of gestational diabetes (GDM) in Australia. GDM has become a significant population health issue and Australia has been at the forefront of international research into its significance and management. More recently, the tsunami of GDM has been followed by a growing wave of type 2 diabetes in pregnancy. Type 2 diabetes is becoming more prevalent than type 1 diabetes in pregnancy, and adverse pregnancy outcomes are more common. However, diabetes itself is but one factor influencing outcomes in this group of women, with obesity, cultural issues and socioeconomic disadvantage being other significant issues. The research of our group has focused on examining traditional and non-traditional risk factors for GDM, and for the progression from GDM to type 2 diabetes in Australia. Our research has also been directed towards breaking the nexus between GDM and type 2 diabetes. The identification of women with GDM is an opportunity to institute interventions to prevent both GDM and type 2 diabetes. Unfortunately there are numerous barriers to improving lifestyle and reducing diabetes risk in this population. The National Diabetes Services Scheme has provided the opportunity to start translating some of our research into health promotion activities. The NDSS has greatly aided the management of diabetes and GDM by providing subsidised diabetes related products. It has also been established to provide information and services to people with diabetes. As part of this charter, the NDSS has recently started health promotion activities in the area of diabetes in pregnancy. It will underpin a national recall and screening program for diabetes after GDM, and forms the basis for other public health initiatives such as providing information to women with diabetes in pregnancy, facilitating the prevention of diabetes after GDM.

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THE RELATIONSHIP BETWEEN SERTOLI CELL STATUS AND IDIOPATHIC MALE INFERTILITY

J. T. Haverfield[1,2], P. G. Stanton[1,3], S. J. Meachem[1,2]

[1]Prince Henry's Institute of Medical Research, Clayton, VIC, Australia
[2]Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC, Australia
[3]Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

The cornerstone of normal adult testicular function is a mature Sertoli cell population. The maturational switch for Sertoli cells occurs at puberty, where immature Sertoli cells differentiate into a mature population that hold the necessary architectural and functional characteristics to regulate spermatogenesis (1). Data from rodent models (2, 3) suggest a relationship between Sertoli cell immaturity and infertility, however clinical data confirming this relationship is limited. We postulate that adult Sertoli cells in the infertile human testis display an immature status, with more severe disruptions of spermatogenesis associating with a greater extent of Sertoli cell immaturity. Using testicular biopsy samples obtained from fertile men (n = 3) and infertile patients (n = 6/group) displaying meiotic arrest (MA) and Sertoli cell only (SCO) syndrome, we sought to survey the status of Sertoli cell populations. All samples were immunofluorescently probed for three hallmark features of adult Sertoli cell maturation; organisation of the inter-Sertoli tight junctions, expression of the androgen receptor and proliferative ability. Differences between groups were quantified using stereology. The results show that the majority of infertile patients display highly disorganised tight junctions, a feature not seen in fertile men, however surprisingly no difference in the extent of tight junction disruption was observed between MA and SCO. Preliminary data also show that some component of the Sertoli cell population in MA and SCO patients was non-functional and proliferative. These results suggest that the Sertoli cell population in men suffering from idiopathic infertility present an abnormal maturational status that is independent of the extent of spermatogenic disruption. Moreover, this study supports the growing body of evidence proposing that the adult Sertoli cell population is not a homogenous, terminally differentiated population, and suggests that the failure of Sertoli cells to reach or maintain their mature status may be the cornerstone of abnormal adult testicular function.

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CHARACTERISATION OF THE GTPASE DYNAMIN THROUGHOUT MURINE SPERM MATURATION

A. T. Reid[1], S. D. Roman[1,2], R. Aitken[1,2], B. Nixon[1]

[1]Reproductive Science Group, School of Environmental and Life Sciences, University of Newcastle, Callaghan, NSW, Australia
[2]ARC Centre of Excellence in Biotechnology and Development, School of Environment, University of Newcastle, Callaghan, NSW, Australia

Throughout sperm maturation distinct remodelling events occur that imbue the cells with both the ability to bind the zona pellucida and undergo the acrosome reaction. Of long standing interest to our laboratory is the elucidation of the molecular mechanisms that underpin the attainment of these key functional attributes. This process begins with a complex range of morphological changes that accompany spermatogenesis, and is continued through post-testicular phases of maturation in both the male (epididymal maturation) and female (capacitation) reproductive tracts. However, among these changes only those occurring during the initial stages of spermatogenesis are intrinsically driven. The fact that the majority of sperm remodelling is extrinsically stimulated, and occurs in the absence of new protein synthesis, highlights the potential importance of processes such as intracellular protein trafficking. This has directed our focus towards the dynamin family of protein traffickers. The GTPase dynamin exists in three isoforms, namely Dnm1, Dnm2 and Dnm3 and is an integral part of the molecular machinery required for vesicle mediated protein translocation. Recent research from our laboratory has demonstrated the presence of these three isoforms in distinct, cell-specific locations during murine spermatogenesis. Immunofluorescence on mouse testis revealed that both Dnm1 and 2 are present within a region corresponding to the developing acrosome in maturing sperm, whilst Dnm3 appears to reside solely within pre-meiotic germ cells. Interestingly, Dnm1 and Dnm2 are both retained within the peri-acrosomal region of the sperm head in mature spermatozoa. Additionally, upon the induction of capacitation in vitro, staining for both Dnm1 and 2 becomes significantly reduced. Collectively these data support the novel hypothesis that dynamin not only participates in sperm remodelling events during spermatogenesis but may also have a previously unappreciated role in capacitation-associated priming of the sperm surface for interaction with the oocyte.

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THE EFFECT OF PATERNAL OBESITY IN MICE ON REPRODUCTIVE AND METABOLIC FITNESS OF F1 MALE OFFSPRING

M. Mitchell[1], T. Fullston[1], N. O. Palmer[1], H. W. Bakos[1], J. A. Owens[1], M. Lane[1,2]

[1]School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia
[2]Repromed, Adelaide, SA, Australia

We know relatively little of the consequences of male obesity for reproductive success compared to female obesity. Conflicting evidence exists in both humans and rodents regarding whether paternal obesity alters sperm motility and concentration. However, we have described impaired embryo and fetal development, and implantation, in rodents as a consequence of paternal obesity. This study investigated whether founder male obesity influenced the reproductive and metabolic fitness of males of the subsequent F1 generation. C57BL/6 founder male mice were fed a standard chow (CD) or a high-fat diet (HF) for 8wks. This increases adiposity in the absence of changes in fasting glucose levels. Males were mated to female C57BL/6 mice, and subsequent m ale F1 offspring from HF (HF-F1) or CD (CD-F1) founders were weighed weekly and maintained on standard chow. At 8wks and 14wks glucose tolerance tests were performed and following euthanasia, tissues and sperm collected. Sperm reactive oxygen species (ROS) and DNA damage levels were determined, and various organs weighed. HF-F1 males pups were significantly heavier relative to CD-F1 males ($P < 0.05$) although adult bodyweight did not differ significantly. Despite this, liver, pancreas, testes and epididymis weight was significantly elevated for HF-F1 males at 17wks of age ($P < 0.05$). At both 8wks and 14wks of age HF-F1 males were hypoglycaemic and had impaired glucose metabolism. Sperm analysis of HF-F1 males indicated a significant increase in ROS levels ($P < 0.05$), DNA damage ($P < 0.05$) and a decrease in fertilization rates in vitro ($P < 0.05$). This data indicates significant physiological changes and perturbed sperm parameters in F1 males as a consequence of founder male obesity. It supports further interrogation of male and female F1 offspring, and warrants examination of potential effects for a subsequent F2 population.

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LRGUK – A NOVEL GENE INVOLVED IN MALE FERTILITY

K. Deboer[1], C. Borg[1], D. Jamsai[1], R. Prawer[1], V. Adams[2], M. O'Bryan[1]

[1]Deptartment of Anatomy and Developmental Biology, Monash University, Melbourne, VIC, Australia
[2]The Australia Phenomics Facility, The Australian National University, Canberra, ACT, Australia

Infertility affects a large number of Australian men. The causative factor in many of these cases is likely to be genetic in origin. As such, the identification and characterisation of novel genes involved in male fertility represents an important area of research. We have undertaken an N-ethyl-nitrosourea (ENU) mutagenesis screen to identify novel genes involved in male fertility. From this screen we have identified a mouse line that we have designated "Kaos." Male mice that are homozygous for the Kaos mutation are infertile as a result of severely disrupted spermatogenesis, with Kaos homozygote males containing only 20% of the normal number of elongated spermatids in their testes, when examined by nuclear resistance to Triton X-100 solubilisation. In contrast, Kaos homozygote females are fertile. To identify the Kaos causal mutation, we used a combination of DNA linkage analysis and candidate gene sequencing. A point mutation was identified in exon 14 of the Leucine-rich Repeats and Guanylate Kinase domain containing (Lrguk) gene, which introduces a pre-mature stop codon into the Lrguk coding sequence. The function of the LRGUK protein is currently unknown. However preliminary expression and phenotype analysis suggests that Lrguk may have a role in spermiogenesis (i.e. haploid germ cell development). The deduced LRGUK protein contains several potentially important domains including a Guanylate Kinase-like (GK) domain and a number of leucine-rich repeats. The GK-like domain has some homology to guanylate kinase, a metabolic enzyme involved in purine nucleotide metabolism. Studies are currently underway to determine whether the LRGUK protein possesses guanylate kinase enzyme activity. In other proteins, the GK-like domain has evolved into mediating protein-protein interactions (1), and current studies are also aimed at examining this possibility. These studies should provide insight into the function of LRGUK and its role in spermatogenesis.

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MEIOTIC ACROBATS: MONOTREME SEX CHROMOSOME ORGANISATION DURING SPERMATOGENESIS

F. Grutzner, A. Casey, T. Daish

School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, SA, Australia

Monotremes feature an extraordinarily complex sex chromosome system which shares extensive homology with bird sex chromosomes but no homology to sex chromosomes of other mammals (1,2,3). At meiotic prophase I the ten sex chromosomes in platypus (nine in echidna) assemble in a sex chromosome chain. We previously identified the multiple sex chromosomes in platypus and echidna that form the meiotic chain in males (1,2,4). We showed that sex chromosomes assembly in the chain in a specific order (5) and that they segregate alternately (1). In secondary spermatocytes we observed clustering of X and Y chromosomes in sperm (6). Our current research investigates the formation of the synaptonemal complex, recombination and meiotic silencing of monotreme sex chromosomes. Meiotic sex chromosome inactivation (MSCI) has been observed in eutherian mammals, marsupials and birds but has so far not been investigated experimentally in monotremes. We found that during pachytene the X5Y5 end of the chain closely associates with the nucleolus and accumulates repressive chromatin marks (e.g. histone variant mH2A). In contrast to the differential accumulation of mH2A we observe extensive loading of the cohesin SMC3 on sex chromosomes in particular during the pachytene stage of meiotic prophase I. We have also used markers of active transcription and gene expression analysis to investigate gene activity in platypus meiotic cells. I will discuss how these findings contribute to our current understanding of the meiotic organisation of monotreme sex chromosomes and the evolution of MSCI in birds and mammals.

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NUCLEOSOME RETENTION DURING CHROMATIN PACKAGING IN HUMAN SPERMATOZOA

S. D. Roman[1,2], A. T. Reid[1,2], K. McEwan[1], D. M. Campbell[1,2], D. A. Jans[2,3]

[1]Biology, University of Newcastle, Callaghan, Australia
[2]ARC Centre of Excellence in Biotechnology & Development, NSW, Australia
[3]Dept of Biochemistry & Molecular Biology, Monash University, Clayton, VIC, Australia

In contrast to the histone packaging of somatic cells, spermatozoa are predominantly packaged by the protamine proteins. However, human spermatozoa retain ~15% histone packaging. Regions that are left nucleosome bound could be: either genes that are active shortly after fertilisation or genes that are transcribed late during spermatogenesis. DNA damage at histone bound loci would be of consequence to spermatogenesis and/or to a resulting embyro post-fertilisation. Western blot analysis confirmed the presence of acetylated H3 and H4 in sperm. Interestingly, we identified the presence of these modified histones in isolated good quality sperm considered to have complete packaging. This indicates that histone retention is not a consequence of aberrant chromatin remodelling. Using a combination of ChIP (chromatin immunoprecipitation) and tiling arrays we have identified regions bound by acetylated histone 3 (H3). ChIP-PCR confirmed histone retention at several of these loci. For the previously identified loci in exon 1 of the TNP-2 gene we were able to narrow the region bound to the size of one nucleosome only. Using a modified form of ChIP known as carrier ChIP we examined histone retention in individual ejaculates. We demonstrate that humans consistently retain acetylated H3 at the same loci. Retention of one nucleosome at the TNP-2 loci was maintained across ejaculates and donors. In conclusion, retention of acetylated H3 is consistently maintained during packaging of the genome in spermiogenesis.

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RESPONSES OF THE PREIMPLANTATION EMBRYO TO DEFINED GENOTOXIC STRESSES

C. O'Neill, X. Mu, M. Farnham, X. L. Jin

Sydney Centre for Developmental and Regenerative Medicine, University of Sydney, Sydney, NSW, Australia

Sydney Centre for Developmental and Regenerative Medicine , Kolling Medical Research Institute, Sydney Medical School, University of Sydney, Royal North Shore Hospital, St Leonards, NSW 2065

Genotoxic cellular stressors induce damage to the structure of DNA. This potentially compromises the genetic integrity of the cell. Such damage is particularly dangerous in the early embryo since transmission of defects can affect most cells in the body. Canonical responses of cells to such stress include delaying or blocking mitosis to allow DNA repair to occur or induction of cell death (apoptosis) to ensure that damage is not propagated. To date there have been only limited studies of the response of the preimplantation embryo to genotoxic stress. Ultra violet (UV) irradiation typically induces single strand DNA breaks while drugs such as the chemotherapeutic cisplatin commonly induce double strand breaks. ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and rad3 related) are checkpoint kinase that mediate early responses of the cell to DNA damage. ATM primarily responds to DNA double strand breaks while ATR typically responds to single strand breaks and stalled replication forks. However, ATM and ATR may have partially overlapping and complementary functions. Both kinases can exert P53 dependent and independent responses. ATR protein was detected by immunofluorescence in all preimplantation stages up to the morulae stage. ATM protein was detected in oocytes and all stages. Cisplatin or UV-irradiation at the 2-cell stage caused increased nuclear staining of both ATM and ATR. UV-irradiation of 2-cell embryos induced irreversible, ATR-dependent, P53-independent cell-cycle block without apoptosis. Cisplatin allowed cell-cycle progression with progressive ATR and ATM-dependent, P53-independent apoptosis over subsequent cell-cycles. UV-irradiation of morula caused an ATR and ATM-dependent, P53-independent block of blastocyst formation while the block caused by cisplatin was ATR and ATM-dependent and at least partially P53-dependent. The results demonstrate complexity and maturation of the cellular responses of the embryo to defined genotoxic stressors and help to define the nature of embryopathy under these circumstances.

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INTERSPECIES SOMATIC CELL NUCLEAR TRANSFER IS DEPENDENT ON COMPATIBLE CYTOPLASMIC FACTORS AND MITOCHONDRIAL DNA

Y. Jiang[2], R. Kelly[1,2], A. Peters[2], H. Fulka[2], D. A. Mitchell[1], J. C. St. John[1,2]

[1]Centre for Reproduction and Development, Monash Institute of Medical Research, Clayton, VIC, Australia
[2]Clinical Sciences Research Institute, University of Warwick, Coventry, United Kingdom

Interspecies somatic cell nuclear transfer (iSCNT) offers significant opportunities to analyze and understand nuclear-cytoplasmic interactions. Using a murine-porcine interspecies model, we investigated the importance of nuclear-cytoplasmic compatibility, specifically mitochondrial DNA (mtDNA), on successful development. Transfer of somatic murine fetal fibroblasts into enucleated porcine oocytes resulted in extremely low blastocyst rates (0.4%); increased DNA strand breaks; deficient nuclear pore complex arrangements and increased aberrant karyokinesis than observed in porcine-porcine SCNT embryos. Using allele specific-PCR analysis, murine mtDNA was detected at ever-decreasing levels to the blastocyst stage, with peak levels being 0.14 ± 0.055% in 2-cell embryos. Furthermore, these embryos reduced total mtDNA copy number during preimplantation development in a manner similar to porcine embryos. Injecting mouse embryonic stem cell extract and mitochondria along with the murine donor cell into a mitochondria depleted porcine oocyte, increased blastocyst zona pellucida thinning and blastocyst rates significantly (0.4% vs 3.42%) compared to the non-supplemented iSCNT group. They also had significantly more murine mtDNA at the 2-cell stage than the non-supplemented embryos, which was maintained throughout preimplantation development. At later stages of preimplantation development, they possessed 48.00% ± 17.38% murine mtDNA and exhibited a mtDNA copy number profile similar to murine embryos. Overall, these data demonstrate that the addition of species compatible cytoplasmic factors and mitochondrial DNA improve developmental competence of iSCNT embryos.

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AGGREGATING CLONED WITH *IN VITRO* FERTILISED EMBRYOS RESULTS IN CHIMAERAS AND IMPROVED FETAL SUVIVAL IN CATTLE

F. C. Oback, T. Delaney, J. E. Oliver, M. C. Berg, R. S.F. Lee, D. N. Wells

Reproductive Technologies, AgResearch Limited, Hamilton, New Zealand

Cloning cattle by somatic cell nuclear transfer (NT) results in low survival and high frequencies of abnormal placentation and fetal development. We postulate that such anomalies may be overcome by complementing NT embryos with in vitro fertilised (IVF) embryos to form chimaeras. The gender and germline composition of chimaeras can be experimentally manipulated. Using embryological methods, we aim to produce chimaeric fetuses that are functionally male and produce sperm derived from the somatic NT embryo. Provided sufficient contribution from the IVF embryo, such chimeras should develop more normally than clones. At the 12- to 16-cell stage, individual male NT embryos were aggregated with female IVF embryos derived from X-sorted sperm. Following aggregation, there were no significant difference in blastocyst development between NT/IVF aggregates and disaggregated and re-aggregated IVF and NT controls (86/183 = 47% v. 77/233 = 33% v. 47/109 = 43%, respectively). Suitable quality embryos were transferred individually into synchronised recipient animals. Pregnancy establishment at Day (D) 35 was not significantly different between aggregate, IVF and NT groups (18/57 = 32% v. 11/45 = 24% v. 6/31 = 19%, respectively). Whilst there was no difference in survival between aggregates and IVF controls to ~D100, aggregates survived significantly better than NT controls (16% v. 18% v. 0%; respectively; $P < 0.05$). In the aggregate group, 7/8 fetuses recovered were phenotypically male. Using RT-PCR, expression of the female-specific mRNA for Xist was detected in 4/5 liver samples, indicating chimaerism. Despite improved survival to ~D100 compared to NT, 3/7 fetuses in the aggregate group still displayed evidence of abnormalities, such as fetal overgrowth. Further studies will explore alternative aggregation strategies and germline transmission of the NT-derived genome in chimaeras.

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DIMETHYL FORMAMIDE IMPROVES THE DNA INTEGRITY AND MOTILITY OF SEX-SORTED CRYOPRESERVED STALLION SPERMATOZOA

Z. Gibb[1], L. H.A. Morris[2], W. M.C. Maxwell[1], C. G. Grupen[1]

[1]Faculty of Veterinary Science, The University of Sydney, Camperdown, NSW, Australia
[2]EquiBreed NZ Ltd., Cambridge, New Zealand

The fertility of flow-cytometrically sex-sorted stallion spermatozoa is compromised by the additional stress of cryopreservation, a step which is essential for the wider application of this technology. The aim of this study was to compare the cryoprotective effects of dimethyl formamide (DMF) and glycerol at different concentrations. Sex-sorted and control spermatozoa were cryopreserved in the presence of 2, 3 or 4% DMF or glycerol. Post-thaw total motility (TM) was assessed using computer assisted sperm assessment and viability was assessed using Syto-16/propidium iodide staining and flow cytometry at 0, 45 and 90 min post-thaw. The DNA integrity (%DFI) was determined using the sperm chromatin structure assay immediately post-thaw. Compared to controls, se x-sorting caused a significant decrease in TM (34.6 vs. 9.4%) and viability (30.0 vs. 12.5), and a significant increase in %DFI (11.3 vs. 63.2%). The TM of control and sex-sorted spermatozoa did not differ at 0 min (29.2 vs. 22.0%), but were significantly different at 45 and 90 min (37.4 vs. 5.6% and 37.2 vs. 0.5% respectively). The viability of sex-sorted spermatozoa decreased significantly at each time point (20.9, 9.6 and 6.9% at 0, 45 and 90 min) though the viability of control spermatozoa did not decrease over time. DMF resulted in lower %DFI values than glycerol for sex-sorted, but not control spermatozoa (59.6 vs. 66.0). However, DMF reduced the viability of sex-sorted spermatozoa compared with glycerol (11.0 vs. 13.9%). DMF resulted in higher TM than glycerol for control spermatozoa only (37.9 vs. 31.3%). Increasing the cryoprotectant concentration caused a significant decrease to the viability of control spermatozoa (33.4, 30.8 and 25.9% for 2, 3 and 4% respectively), but had no effect on sex-sorted spermatozoa. The results of this study indicate that the cryoprotective effect of DMF on sex-sorted stallion spermatozoa was superior to that of glycerol.

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THE ROLE OF L-PROLINE IN PREIMPLANTATION MOUSE EMBRYO DEVELOPMENT IN VITRO

S. Ozsoy, M. B. Morris, M. L. Day

Physiology and Bosch Institute, University of Sydney, Camperdown, NSW, Australia

Amino acids are known to play important roles in preimplantation embryo development, including regulation of cell volume and metabolism. Inclusion of L-glutamine, glycine and betaine in embryo culture medium has been shown to improve development in vitro by acting as organic osmolytes, thereby regulating cell volume. The purpose of the present study was to examine the effect of L-proline on preimplantation mouse embryo development in vitro. One-cell stage embryos were cultured in modified HTF, at low density (1 embryo/ 100 μL) and high density (1 embryo/μL) in the presence and absence of amino acids. Development of the embryos was scored every 24 h until the blastocyst stage. At low density, L-proline significantly increased the proportion of embryos developing to the blastocyst stage. This effect was abolished by culture at high density, suggesting that L-proline was activating a pathway similar to that involved in autocrine signalling by trophic factors in the preimplantation embryo. The improvement in development observed in the presence of L-proline was not due to its action as an organic osmolyte since the osmolality of the medium was 270 mOsm. Furthermore, glycine and betaine, which are known to act as osmolytes in embryos, had no effect on blastocyst development. In embryonic stem cells L-proline is taken up by an amino acid transporter and is involved in the regulation of growth and differentiation (1). The present data suggest that L-proline may have a similar, important role in preimplantation development.

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FETAL CALF SERUM AFFECTS HESC METABOLISM AND GENE EXPRESSION LEADING TO DIFFERENTIATION IN CULTURE

C. X. Yeo, J. Rathjen, D. K. Gardner

Zoology, The University of Melbourne, Parkville, VIC, Australia

Fetal calf serum (FCS) has conventionally been used to support the growth and maintenance of human embryonic stem cells (hESCs). FCS however, is an undefined complex mixture containing factors which potentially alter the functionality of hESCs. Inclusion of FCS during embryo culture negatively impacts embryo metabolism and viability but comparative studies on hESCs have been hindered by the lack of serum and feeder independent culture systems. Using a recently available defined culture system, the effects of FCS on hESC metabolism and pluripotentcy were investigated. Mel2 hESCs were grown at 37°C in 5% CO2 for 3 days on matrigel (hESC-qualified) coated tissue culture wells in mTeSR1 medium. hESCs were then cultured in mTeSR1 (control) or in mTeSR1 supplemented with 20% FCS from different manufacturers or knockout serum replacement (KOSR), for 96 hours. Media was renewed daily. At the end of the culture period, spent media was collected and cells were trypsinised and counted and/or collected for gene expression analysis. FCS decreased cell survival and altered hESC morphology from densely packed colonies with distinct borders into non-uniform heterogeneous populations comprising of hESC-like cells and fibroblastic-like cells with high cytoplasmic to nuclear ratios. Media analysis revealed altered cell metabolism with increased glucose consumption rates per cell ($P < 0.01$) with FCS supplementation, compared to cells cultured in mTeSR1 alone. Gene expression analysis revealed that FCS, regardless of its manufacturer, decreased the expression of some pluripotent markers and increased differentiation markers. A decrease in pluripotent gene expression was also observed in hESCs cultured with KOSR compared to mTeSR1 alone. Maintenance of homogeneity in hESC populations is crucial for the advancement of hESC clinical therapies. This study demonstrates that FCS promotes heterogeneity and impacts the metabolic function and gene expression of hESCs thereby supporting the need for serum-free culture systems as standard practice in hESC culture.

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VASCULAR ENDOTHELIAL GROWTH FACTOR GENE POLYMORPHISMS IN PLACENTAL IMPAIRMENT AND SMALL FOR GESTATIONAL AGE BIRTH

P. H. Andraweera[1], G. A. Dekker[1,2], R. C. Nowak[1], S. D. Thompson[1], L. M.E. McCowan[3], R. A. North[1], C. T. Roberts[1]

[1]Disciplne of Obstetrics and Gynaecology, Research Centre for Reproductive Health, University of Adelaide, Adelaide, SA, Australia

[2]Women's and Children's Division, Lyell McEwin Hospital, Elizabeth Vale, SA, Australia

[3]Department of Obstetrics and Gynaecology, University of Auckland, Auckland, New Zealand

Impaired placental angiogenesis is implicated in the pathophysiology of small for gestational age (SGA) infants. Placental expression of vascular endothelial growth factor (VEGF), a potent angiogenic factor, is reduced in SGA pregnancies. We aimed to evaluate the association of two single nucleotide polymorphisms (SNPs, VEGF-2578C/A and VEGF+936C/T) in VEGF gene which reduce VEGF expression, in SGA pregnancies and examine their effects on first trimester placental VEGF expression. 3196 nulliparous pregnant women, their partners and babies were recruited in Adelaide and Auckland to a prospective multicentre cohort study (SCOPE Study). Amongst 2123 Caucasian women, 216 (11.9%) delivered a SGA infant defined as <10th customised centile. Uncomplicated Caucasian pregnancies served as controls (n = 1176). Uterine and umbilical artery Doppler velocimetry was performed at 20 weeks gestation. DNA extracted from peripheral blood from couples and cord blood from babies was genotyped using Sequenom MassARRAY. 74 first trimester placentae collected from elective terminations of pregnancy were genotyped for the same SNPs and the VEGF expression determined by RT-PCR. Neonatal VEGF+936 CT+TT genotypes associate with SGA (OR 1.6, 95%CI 1.1–2.3), lower birthweight (P = 0.005), customised birthweight centile (P = 0.03), lower placental weight (P = 0.04) and an increased uterine artery resistance index (RI, P = 0.004). Maternal VEGF+936 CT+TT associate with bilateral notching of the uterine artery waveform (OR 1.4, 95%CI 1.0–1.8) and an increased umbilical artery RI (OR 1.5, 95%CI 1.1–2.1). VEGF+936 CT first trimester placentae have lower VEGF expression compared to CC (P = 0.045). Neonatal *VEGF-2578* AA associates with bilateral uterine artery notching (OR 1.5, 95%CI 1.1–2.2) and increased umbilical artery RI (OR 1.6, 95%CI 1.0–2.6). Maternal VEGF-2578 CA+AA associate with increased umbilical artery RI (OR 1.5, 95%CI 1.0–2.2). VEGF polymorphisms reduce first trimester VEGF expression and associate with increased resistance in the placental circulation suggesting impaired placental function. VEGF+936 SNP confers increased risk for SGA.

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HYPERTENSIVE DISORDERS OF PREGNANCY ARE ASSOCIATED WITH IMMUNOREGULATORY GENE POLYMORPHISMS

A. Highet, S. Thompson, D. Furness, V. Zhang, G. DEKKER, C. Roberts

Discipline of Obstetrics and Gynaecology, Robinson Institute Research Centre for Reproductive Health, University of Adelai, Adelaide, SA, Australia

Pregnancy is a controlled state of inflammation. Deregulation of cytokine networks can lead to adverse pregnancy outcomes including preeclampsia (PE). We aimed to identify single nucleotide polymorphisms in immunoregulatory genes that signify an increased risk of the gestational hypertensive disorders PE and gestational hypertension (GH). 1169 nulliparous pregnant women and their partners were recruited prospectively for the Adelaide SCOPE study. PE and GH were classified using strict guidelines. Uncomplicated pregnancies served as controls. Peripheral blood from couples and cord blood from neonates were collected. DNA was extracted and genotyped for Interleukin (IL)-6 rs1800795, IL-4 rs2243250, IL-10 rs1800896 and rs1800871, mannose binding lectin (MBL) rs1800450, transforming growth factor beta 1 (TGFβ-1) rs1800469 and cyclooxygenase (COX)-2 rs20417 & rs5275 and inducible nitric oxide synthase (NOS2A) rs1137933 using the Sequenom MassARRAY system. Genotypes for Caucasian PE (n = 75) and GH (n = 102) were compared with controls (n = 422) and analysed using Chi-Square. In neonates IL-6 G allele carriage was associated with PE (P = 0.011, OR = 2.0, 95% CI =1.2–3.7) and the CC genotype associated with GH (P = 0.002). Neonatal IL-10 RS180071 AA genotype associated with PE (P = 0.041) and IL-10 RS1800896 AA associated with GH (P = 0.022). Paternal NOS2A C allele was more frequent in PE (P = 0.03, OR = 2.1, 95% CI =1.1–4.5), and maternal NOS2A CC more frequent in GH (P = 0.018). Increased neonatal carriage of MBL rs1800450 AA+GA genotypes associated with GH (P = 0.03, OR = 2.2, 95% CI =1.1–4.5). No associations were observed between TGFβ-1 or COX2 genotypes and PE or GH. Associations between neonatal IL-6 G, which confers high placental IL-6 expression, and PE suggest a possible mechanism by which PE is a pro-inflammatory exacerbation of placental origin. Since placental IL-10 is important for maternal tolerance of the fetus, genotypes predisposing to low IL-10 expression in the neonate which associate with both PE and GH, suggest a role for decreased placental IL-10 in these disorders.

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DEVELOPMENT OF POTENT AND STABLE PC6 INHIBITORS TO BLOCK EMBRYO IMPLANTATION FOR FEMALE CONTRACEPTION AND PREVENTION OF HIV

H. Ho[1,2], G. Nie[1,2]

[1]Implantation and Placental Development, Prince Henry's Institute of Medical Research, Clayton, VIC, Australia
[2]Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

Proprotein convertase (PC) 6, a member of the PC family that activate precursor proteins into their active forms, is a critical endometrial factor for embryo implantation. Blocking PC6 production in mice inhibits decidualisation (a critical process of implantation) and inhibits implantation. PCs including PC6 also play a critical role in HIV infection through cleaving HIV envelope precursor protein gp160 into functional gp120 and gp41. PC inhibitors are demonstrated to inhibit HIV transmission via blocking gp160 cleavage. We hypothesised that PC6 is a potential target for the development of female contraception that could also provide protection from HIV infection. One key requirement to prove this concept in an animal model is a potent PC6 inhibitor that is stable in serum. Polyarginine peptide (polyR) is published to be a potent PC6 inhibitor that also inhibits HIV. We have confirmed that polyR inhibits PC6 in vivo and completely blocks decidualisation of human endometrial stromal cells in culture. However, polyR has short serum half-life. The aim of this current study was to generate polyR derivatives that are potent PC6 inhibitors, with increased serum stability. We modified polyR by either PEGylation with different sized PEG (polyethylene glycol) or cyclization, and tested their potency, stability and utility in vivo. Modifications at both terminals of polyR dramatically reduced its PC6 inhibitory potency. PEGylation at the C-terminal, regardless of the PEG size, had no effect. In silico docking experiments showed that N-terminal PEGylation or cyclization affected the binding of polyR to the PC6 active site, but not C-terminal PEGylation. One of the polyR derivatives, C-30kDa-PEG polyR was confirmed to be as potent as the parental peptide but much more stable in serum. Studies are currently in place to inhibit embryo implantation in mice using C-30kDa-PEG polyR and to determine its ability to inhibit HIV.

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TROPHOBLAST ANTIPHOSPHOLIPID ANTIBODY INTERNALISATION BY A B_2 GLYCOPROTEIN I-ANIONIC PHOSPHOLIPID-MEGALIN COMPLEX

C. A. Viall[1], L. W. Chamley[1], Q. Chen[1,2]

[1]Department of Obstetrics and Gynaecology, The University of Auckland, New Zealand
[2]Hospital of Obstetrics and Gynaecology, Fudan University, Fudan, China

Women with antiphospholipid antibodies (aPL) are at an increased risk of preeclampsia, recurrent miscarriage, stillbirth and intrauterine growth restriction. Antiphospholipid antibodies may predispose to these pathologies by damaging the placenta, although exactly how is not understood. Recently, a novel pathogenic mechanism was suggested by work which showed that aPL are specifically internalised by placental trophoblasts where they caused aberrant trophoblast death. Internalisation may occur via an endocytic receptor called megalin in a process that seems to involve at least one of the two components of the antigen for aPL, the anionic phospholipid-binding protein β_2 glycoprotein I (β_2GPI). However, whether internalisation is also dependent upon anionic phospholipids is unknown. Identifying the receptor pathway responsible for aPL internalisation may provide insight into the pathogenesis of aPL in the placenta. To investigate the process of aPL internalisation, first trimester placental explants were cultured with fluorescently-labeled monoclonal aPL, or a control antibody and/or β_2GPI or acetylated β_2GPI, which can not bind anionic phospholipids. The explants were then sectioned and the localisation of the aPL, β_2GPI, or acetylated β_2GPI was determined by confocal microscopy. The localisation of megalin expression in placental explants was determined by immunohistochemistry. Megalin was expressed throughout the syncytiotrophoblast but more strongly in some regions. After an overnight incubation, both aPL and β_2GPI, but not control antibodies were co-localised in the cytoplasm of the syncytiotrophoblast. Acetylated β_2GPI was not internalised and partially blocked aPL uptake. These results suggest that aPL are internalised into the synctiotrophoblast by a receptor-dependent mechanism involving β_2GPI, anionic phospholipids and megalin. This work forms the first step to understanding how aPL are internalised by trophoblasts. Further investigation of this mechanism and the subsequent intracellular effects of aPL may lead to a new therapeutic strategy for aPL-positive pregnant women by preventing the pathogenic effect of aPL on the placenta.

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THE ROLE OF FOCAL ADHESION PROTEINS AND THEIR HORMONAL REGULATION IN RAT UTERINE EPITHELIAL CELLS DURING EARLY PREGNANCY

Y. Kaneko[1], M. Day[2], C. R. Murphy[1]

[1]Anatomy and Histology, The University of Sydney, Sydney, NSW, Australia
[2]Physiology, The University of Sydney, Sydney, NSW, Australia

Uterine epithelial cells (UECs) undergo extensive alteration during early pregnancy followed by their removal in order for the implanting blastocyst to penetrate into the underlying endometrium. Focal adhesions (FAs) may play a role during this process as FAs provide adhesion between the cell and its underlying basal lamina. The present study investigated the distribution and expression of FA proteins in rat UECs at the time of implantation and their hormonal control as well as their expression in blastocyst stage embryos. Immunofluorescence microscopy showed that the principal focal adhesion proteins, talin and paxillin, were localised along the basal cell surface of UECs on day 1 of pregnancy, however they were markedly reduced from the site of FAs at the time of implantation. This is thought to be a critical process in the removal of UECs, which allows the invasion of the blastocyst into the underlying endometrium. Hormone treatments in ovariectomised rats showed that disassembly of talin and paxillin from the site of FAs were predominantly under the control of progesterone. Formation of FAs is initiated by the clustering of specific integrin subunits and both integrin beta1 and beta3 colocalised and interacted with talin at the site of FAs on day 1 of pregnancy. Integrin beta1 and beta3 disassembled from the site of FAs at the time of implantation, however integrin beta3 significantly increased along the apical membrane at this time suggesting a role in embryo attachment. In the rat blastocyst, integrin beta3 was concentrated around the nuclei of the trophoblast cells and once the blastocyst was placed onto a receptive endometrial cell line, Ishikawa cell line, integrin beta3 relocated to the apical membrane of the trophoblast cells. Taken together, our results show that disassembly of FA proteins plays a pivotal role in the removal of UECs in order to establish successful implantation and is tightly regulated by ovarian hormones.

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THE PRORENIN RECEPTOR/PLZF PATHWAY IN HUMAN AMNION

K. G. Pringle[1], A. L. Conquest[1], C. M. Mitchell[1], T. Zakar[1,2,3], E. R. Lumbers[1]

[1]School of Biomedical Sciences and The Mothers and Babies Research Centre, University of Newcastle, Newcastle, NSW, Australia
[2]Obstetrics and Gynaecology, John Hunter Hospital, Newcastle, NSW, Australia
[3]School of Medical Practice and Public Health, University of Newcastle, Newcastle, NSW, Australia

Prorenin, despite being inactive, is the major form of renin found in amniotic fluid and reproductive tissues. Prorenin becomes active if it binds to the novel prorenin receptor (ATP6AP2). The prorenin-ATP6AP2 complex has been found to stimulate translocation of Promyelocytic Zinc Finger (PLZF) protein to the nucleus where it increases expression of the p85α subunit of PI3 kinase (PI3K-p85α) and represses the expression of ATP6AP2[1]. Progesterone and glucocorticoids have also been shown to stimulate PLZF[2, 3]. We aimed to find out if PLZF and the prorenin-ATP6AP2 pathway interact in human reproductive tissues. Human amnion was cultured for 24 h in media containing vehicle, dexamethasone, amniotic fluid or recombinant human (rh) prorenin. Total RNA was extracted using TRIZol® and converted to cDNA for quantitative real-time PCR using SuperScript III and random hexamers. mRNA abundances for PLZF, PI3K-p85α and ATP6AP2 were calculated relative to Alien RNA using the ΔΔCT method. Our preliminary data show that exposure of amnion explants to dexamethasone upregulates PLZF and PI3K-p85α mRNA but has no effect on ATP6AP2. Culture of amnion explants with amniotic fluid also increases PLZF but does not change PI3K or ATP6AP2. In contrast, culture of amnion explants with (rh) prorenin increases PI3K mRNA but not PLZF or ATP6AP2. As expected, dexamethasone affects PLZF expression, however in amnion there is no interaction with the ATP6AP2 pathway. In addition, we believe we have identified a novel prorenin/ATP6AP2 signalling pathway which acts on PI3K-p85α independent of PLZF. In contrast to these data, amniotic fluid increases PLZF but not PI3K-p85α mRNA levels suggesting that amniotic fluid contains other factors that oppose prorenin and glucocorticoid effects on PI3K-p85α.

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IDENTIFICATION AND CHARACTERISATION OF SURFACE PROTEIN COMPLEXES IN HUMAN SPERMATOZOA

K. A. Redgrove[1], B. Nixon[1], E. A. McLaughlin[1], M. K. O'Bryan[2], R. J. Aitken[1]

[1]Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

[2]Department of Anatomy and Developmental Biology, Monash University, Melbourne, VIC, Australia

A unique characteristic of mammalian spermatozoa is that upon ejaculation, they are unable to recognise and bind to an ovulated oocyte. These functional attributes are only realised following the sperms ascent of the female reproductive tract whereupon they undergo a myriad of biochemical and biophysical changes collectively referred to as 'capacitation'. Since spermatozoa are both transcriptionally and translationally quiescent cells, this functional transformation must be engineered by a combination of post-translational modification and spatial reorganisation of existing sperm proteins. Indeed, evidence from our laboratory suggests that a key attribute of capacitation is the remodeling of the sperm surface architecture leading to the assembly and/or presentation of multimeric sperm-oocyte receptor complex(es). Through the novel application of Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE), we have secured the first direct evidence that human spermatozoa express a number of these protein complexes on their surface. Furthermore, we have demonstrated that a subset of these complexes harbour putative zona adhesion proteins and display strong affinity for solubilised zona pellucidae. In this study, we have extended our findings through the characterisation of one such complex containing arylsulfatase A (ASA), a protein with recognised affinity for sulfated ligands present within the zona pellucida. Through the application of immunohistochemistry and flow cytometry we revealed that ASA undergoes a capacitation-associated translocation to become expressed on the apical region of the human sperm head, a location compatible with a role in the mediation of sperm-zona pellucida interactions. This dramatic relocation was completely abolished by incubation of capacitating spermatozoa in exogenous cholesterol, suggesting that it may be driven in part by alteration in the membrane fluidity characteristics. Our current research is focused on confirming the role of ASA in human sperm-zona pellucida adhesion and elucidating the precise cellular mechanisms that underpin the proteins translocation to the cell surface.

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SPRASA A POTENTIAL CONTRACEPTIVE VACCINE TARGET?

L. W. Chamley, A. Wagner, A. N. Shelling

Obstetrics and Gynaecology, University of Auckland, Auckland, New Zealand

A number of attempts have been made to develop immunocontraceptive vaccines for use in humans or animals. We have reported the discovery of a sperm-specific protein, SPRASA, as the antigen for antisperm antibodies in some infertile men [1]. More recently we found that SPRASA is also expressed by oocytes. The expression of SPRASA by both sperm and oocytes makes it a potential target for an immunocontraceptive vaccine that might function in both males and females. We undertook this study to investigate whether immunising mice against SRPASA would affect fertility. Ethical approval was obtained from the Animal Ethics Committee. Two groups of five female CD1 mice were immunised, up to four times, with recombinant human SPRASA or with keyhole limpet haemocyanin (KLH, controls). After immunisation, the females were date-mated with males of proven fertility and monitored for coital plugs. Weights were monitored for 12 days post mating to confirm pregnancy or lack of pregnancy. Mice that were not pregnant were again dated-mated. Mice that were pregnant were euthanased and their litters examined. Ovaries from three SPRASA and three control immunised mice were sectioned to completion and examined for follicles of various stages. All of the control KLH immunised female mice became pregnant within two matings. In contrast, the SPRASA-immunised females were profoundly infertile and none of them became pregnant despite being mated no fewer than five times each. There were no significant differences in the numbers of primary, early antral, antral or pre-ovulatory follicles between the SPRASA and control mice but there were fewer primordial in the SPRASA immunised females ($P = 0.0028$). Antisperm (and antioocyte) antibodies have long been known to be associated with infertility. Our results show SPRASA appears to be crucial for fertility and which may be a useful target for a contraceptive vaccine.

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SHORT TERM XENOBIOTIC EXPOSURE COMPROMISES LONG TERM OOCYTE VIABILITY

A. P. Sobinoff[1], V. Pye[1], B. Nixon[1,2], S. D. Roman[1,2], E. A. McLaughlin[1,2]

[1]Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

[2]The ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Mammalian females are born with a finite number of non-renewing primordial follicles, the majority of which remain in a quiescent state for many years. These follicles serve as the primary source of all developing oocytes in the ovary, and cannot be regenerated post fetal development. Due to their non-renewing nature, these "resting" oocytes are particularly vulnerable to environmental and toxic insults, especially to those which are capable of inducing oxidative stress. Recent evidence suggests that certain synthetic chemical compounds, known as xenobiotics, have the potential to generate oxidative stress through the production of free oxygen radicals (ROS) as a byproduct of the cell's detoxification process. Given the redox sensitive nature of the mammalian oocyte, we hypothesise that xenobiotic exposure may have adverse effects on long term oocyte viability. In this study, we attempted to identify the effects of short term xenobiotic exposure on long term oocyte viability. Female Swiss neonatal mice (day 4) were administered 7 daily consecutive doses of 4-Vinylcyclohexene diepoxide (40mg/kg/daily; 80mg/kg/daily) Methoxychlor (50mg/kg/daily; 100mg/kg/daily) or Menadione (7.5mg/kg/daily; 15mg/kg/daily). Mice were then superovulated at 6wks and their oocytes collected for analysis. Sperm-egg fusion assays revealed a significant decrease ($P < 0.01$) in sperm egg binding (1.4–7 fold) and fusion (4–20 fold) in a dose dependent manner for all three xenobiotic treatments in vivo, signifying a decrease in oocyte membrane fluidity. Follow-up lipid peroxidation analysis on xenobiotic cultured oocytes also showed a significant ($P < 0.01$) dose dependent increase (1.3–2.5 fold) in membrane lipid peroxidation for each xenobiotic compared to the control. These results provide some of the first evidence of short term xenobiotic exposure causing long term oocyte dysfunction, possibly interfering with the fluidity and/or elasticity of the oocyte plasma membrane through xenobiotic ROS induced lipid peroxidation.

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PRODUCTION OF EMBRYOS IN SUPEROVULATED EWES USING FROZEN-THAWED, SEX-SORTED AND REFROZEN-THAWED SPERM

K. H. Beilby, Y. B. Kaurivi, W. M.C. Maxwell, G. Evans, S. P. De Graaf, C. G. Grupen

Veterinary Science, The University of Sydney, Camperdown, NSW, Australia

Flow cytometric sex-sorting of sperm that has previously been cryopreserved allows sex-sorting technology to be applied more widely. While offspring have been produced following artificial insemination of synchronised ewes with frozen-thawed, sex-sorted and refrozen-thawed (FSF) sperm (1), the fertility of FSF-sperm in superovulated ewes has not been reported. The aim of this study was to determine the effect of cryopreservation prior to sperm sex-sorting and freezing on embryo production in superovulated ewes. Several ejaculates from 2 rams were either frozen-thawed, then sex-sorted and re-frozen (FSF X- and Y-chromosome enriched sperm), or immediately sex-sorted before freezing (SF X- and Y-chromosome enriched sperm). A portion of each ejaculate was also cryopreserved without sex-sorting (control). Thirty-one ewes were superovulated and inseminated (15×10^6 sperm per insemination dose) with either SF X, SF Y, FSF X, FSF Y or control sperm as previously described (2). Embryos were recovered 6 d after insemination and assessed. The superovulatory response (mean number of corpora lutea per ewe: 11.8 ± 1.3) and the embryo recovery rates (72.0 ± 5.9%) did not differ significantly among the groups. The fertilisation rates tended to differ ($P = 0.068$) as a result of sperm treatment (control: 33%; SF: 54%; FSF: 18%) and were unaffected by sperm sex (X: 33%; Y: 37%). Of the embryos that were recovered, those derived from FSF-sperm were predominantly at the blastocyst stage (65%), whereas those derived from SF-sperm were evenly distributed among the blastocyst (30%), morula (38%) and arrested (32%) stages, suggesting that fertilisation lifespan of SF-sperm was greater than that of FSF-sperm. A greater proportion of embryos derived from Y-sperm were at the blastocyst stage compared with embryos derived from X-sperm (53% vs. 26%; $P < 0.05$). This study is the first to demonstrate that FSF-sperm is capable of fertilising oocytes of superovulated ewes.

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(2) de Graaf et al (2007) Theriogenology 67: 550–5.

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THE EFFECT OF PATERNAL DIET INDUCED OBESITY ON SPERM CAPACITATION, ACROSOME REACTION, BINDING AND FERTILISATION IN MOUSE MODEL

H. W. Bakos[1,2], N. O. Palmer[1], M. Lane[1,2]

[1]Obstetrics and Gynaecology, The University of Adelaide, Adelaide, SA, Australia
[2]Repromed, Adelaide, SA, Australia

While the effects of obesity on male fertility are emerging, the direct effects on sperm function are less clear. The aim of this study was to determine the effects of diet-induced obesity on sperm capacitation, acrosome reaction, oocyte binding and fertilisation. C57/Bl6 male mice (n = 12) were randomly allocated to two groups; group 1 received a control diet (6% fat) while group 2 received a high fat diet (HFD, 22% fat) for up to 14 weeks. Mice were sacrificed and spermatozoa obtained. Capacitation and acrosome reaction were measured using Arachis hypogaea (peanut) agglutinin. Sperm binding to oocytes was assessed by co-incubation of sperm with superovulated cumulus-enclosed oocytes for 4 hrs. Fertilisation rates were expressed as the percentage of oocytes with 2 pronuclei from the total number inseminated 6 hrs post insemination. The percentage of non-capacitated sperm in males fed a high fat diet was significantly lower compared to males fed a control diet (12.3% vs 21.1%; $P < 0.01$). The percentage of acrosome reacted sperm did not differ between the groups. Following 4 hrs of co-incubation with cumulus-enclosed oocytes, the number of sperm bound to each oocyte was significantly lower in the HFD group compared to controls (41.14±2.5 vs 58.39±2.4; $P < 0.01$). Moreover, the percentage of fertilized oocytes was significantly lower in the HFD group compared to controls (25.9% vs 43.9; $P < 0.01$). This study demonstrates that males fed a HFD to induce obesity have impaired spermatozoa as evidenced by lower levels of capacitation and reduced ability to bind and fertilise an oocyte. These data therefore provide direct evidence that the metabolic health of the male can have a significant impact on sperm function parameters that are associated with infertility.

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THE ROLE OF CALCIUM ACTIVATED CHLORIDE CHANNELS AT FERTILISATION

S. Dalati, M. L. Day

Physiology and Bosch Institute, University of Sydney, Camperdown, NSW, Australia

Sperm entry into the oocyte triggers a signal transduction pathway resulting in intracellular calcium [Ca^{2+}] oscillations that coincide with hyperpolarisations in membrane potential (Em). Ca^{2+} oscillations have been previously described and found to be important for embryo development, yet Em hyperpolarisations and their importance at fertilisation still remains unclear. Thimerosal, a sulfhydryl reagent, has been shown to mimic the physiological changes caused by sperm following fertilisation. It does this by direct sensitisation of the inositol 1,4,5-triphosphate receptor-1 to basal levels of inositol 1,4,5-triphopshate. Previous patch clamp analysis of unfertilised mouse oocytes has shown that thimerosal elicits simultaneous Em hyperpolarisations and Ca^{2+} oscillations. These results have lead us to hypothesise that hyperpolarisations in Em may be due to the activation of a Ca^{2+} activated Cl^- channel (CaCC) present in the membrane of mouse oocytes. The present study aims to identify this CaCC and assess its role in early development following fertilisation. Hyperpolarisations induced by thimerosal were inhibited by niflumic acid, a selective blocker of CaCC's. The inhibition of Em hyperpolarisations suggests that a CaCC is present and plays an active role in initiating hyperpolarisations. To identify the function of the CaCC at fertilisation, in vitro fertilisation was performed in the presence of niflumic acid. Niflumic acid inhibited polar body extrusion and pronuclei formation; two events that are indicators of fertilisation. Furthermore, Ca^{2+} imaging experiments with the calcium sensitive dye fura 2-AM, demonstrated that in the presence of niflumic acid, Ca^{2+} oscillations induced by thimerosal are reduced in size, number and duration. Taken together these data suggest that the activation of a Ca^{2+} activated Cl^- channel in the mouse oocyte may play an important role in the events occurring at fertilisation.

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ELEVATED FSH INCREASES PRIMORDIAL FOLLICLE RESERVE WITHOUT INCREASING PRIMORDIAL FOLLICLE FORMATION OR DECREASING OOCYTE QUALITY

C. M. Allan, K. J. McTavish, S. Lamb, K. A. Walters, D. J. Handelsman

ANZAC Research Institute/University of Sydney, Concord, NSW, Australia

Declining ovarian follicle reserve and oocyte quality with age can dictate the mammalian female reproductive lifespan. Elevated circulating levels of follicle-stimulating hormone (FSH) coincides with reproductive ageing in women, and is predicted to accelerate the depletion of ovarian follicle reserve by increasing the recruitment of remaining follicles. Unexpectedly, we found that transgenic expression of human FSH (TgFSH) increased primordial follicle reserve in mature GnRH-deficient hypogonadal (hpg) female mice functionally lacking endogenous gonadotrophin secretion[1]. Advancing our finding of increased primordial reserve in TgFSH-hpg mice, we find that total primordial follicle numbers is significantly increased (60%) in mature 26 week old non-hpg TgFSH females compared to WT controls. Thus, the FSH-induced increase in primordial reserve is maintained despite increased ovulation rate[2] in mature TgFSH females. Embryo transfer experiments showed that embryos derived from 26 week old TgFSH females developed normally in recipient WT females, indicating no significant reduction in the viability of oocytes or early embryos produced by TgFSH mice. In addition, equal primordial numbers in 5 day old TgFSH versus WT ovaries suggested that FSH-induced changes to ovarian reserve was not due to an increased initial primordial follicle pool. Initial analysis of postnatal gene expression (qPCR) found elevated expression of granulosa cell markers (Kit-L, inhibin β) in TgFSH-hpg vs non-Tg hpg ovaries, indicating FSH actions during early follicle development. Therefore, contrary to a classical view that preantral follicles are gonadotrophin-independent, we provide in vivo evidence demonstrating FSH-induced changes to early follicle populations, as well as elevated FSH activity increasing or maintaining primordial reserve without compromising oocyte quality.

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JAK/STAT SIGNALLING IN FOLLICULOGENESIS

J. M. Sutherland[1], R. Keightley[1], R. L. Robker[2], D. L. Russell[2], E. A. McLaughlin[1]

[1]ARC Centre of Excellence in Biotechnology and Development, School of Environmental & Life Sciences, The University of Newcastle, NSW, Australia

[2]Robinson Institute Research Centre for Reproductive Health, School of Paediatrics and Reproductive Health, The University of Adelaide, SA, Australia

Primordial follicle activation marks the first stage of pre-pubertal ovarian folliculogenesis, and is therefore fundamental to female fertility. Entry into development is initiated by a group of pleiotropic cytokines and growth factors, originating in and acting upon both the oocyte and granulosa support cells of the ovarian follicle through the Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) signalling pathway. Pivotal to this process is the transcriptional regulation of target genes via STAT complexes and negative regulation by the Suppressors of Cytokine Signalling (SOCS) family of proteins. Preliminary evidence indicates that STAT3 facilitates the activation of primordial follicles, while SOCS4 counterbalances the activity of STAT3, mediating the controlled release of primordial follicles into the growing pool throughout reproductive life. Leukemia Inhibitory Factor (LIF) has been previously demonstrated as a key granulosa cell derived cytokine involved in inducing primordial follicle activation. Through both quantitative gene expression (qPCR) and immunoblotting we have demonstrated that LIF can significantly upregulate STAT3 mRNA production (~2-fold) as well as increase STAT3 protein phosphorylation within neonatal mouse ovarian explants culture. Furthermore, through the generation of a recombinant SOCS4 protein construct, and its use in subsequent protein-protein pull-downs, we were able to multiple targets involved in oocyte maturation, STAT3 interactions, and JAK/STAT signaling. These targets were also found to be significantly upregulated via qPCR analysis in neonatal mouse ovaries treated with LIF. These results support our current model for the involvement of STAT3 and SOCS4 in a basic negative feedback loop within the JAK/STAT signalling pathway that results in the regulation of primordial follicle activation and development.

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PUMA MEDIATES GERM CELL DEATH DURING OVARIAN DEVELOPMENT AND DETERMINES INITIAL PRIMORDIAL FOLLICLE NUMBER IN MICE

F. Morgan[1,2], **K. J. Hutt**[1], **C. L. Scott**[3,4], **M. Cook**[3], **A. Strasser**[3,4], **J. K. Findlay**[1], **J. B. Kerr**[2]

[1]***Ovarian Biology Lab, Prince Henry's Institute, Clayton, VIC, Australia***

[2]***Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC, Australia***

[3]***The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia***

[4]***Department of Medical Biology, Melbourne University, Parkville, VIC, Australia***

The proteins that control the number of primordial follicles initially established within the ovary are largely unknown. Here we investigated the hypothesis that PUMA, a pro-apoptotic protein belonging to the Bcl-2 family, regulates germ cell death during ovarian development and thereby determines the number of primordial follicles that make up the ovarian reserve. Ovaries were obtained from embryonic day 17.5 (E17.5) and post-natal day 10 (PN10) wild-type (wt) and puma$^{-/-}$ mice and subjected to morphological, molecular and stereological characterisation (n = 3–6 mice/genotype/age). At E17.5, ovaries were densely populated with germ cells and early meiotic oocytes. Immunostaining for MVH and PCNA confirmed the identity of germ cells and proliferating germ cells, respectively. Pyknotic nuclei and TUNEL positive germ cells were rarely detected, suggesting that cell death was uncommon at this age. At PN10, primordial follicle assembly was complete for both genotypes, as confirmed morphologically and by immunostaining for oocyte markers GCNA and MSY2. The number of germ cells in E17.5 wt and puma$^{-/-}$ ovaries was comparable (P = 0.81, See Table 1). However, PN10 puma$^{-/-}$ ovaries contained significantly more primordial follicles than wt ovaries (P < 0.001, See Table 1), revealing an over-endowment of primordial follicles in the absence of PUMA. These data show that PUMA regulates the developmentally programmed death of germ cells between E17.5 and PN10 in the mouse and thereby determines the number of primordial follicles that make up the initial ovarian reserve.

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Table 1. Germ cell density per $10^4 \mu m^2$ (mean ± s.e.m.)

	E17.5	PN10
wt	18.2 ± 4.4	0.79 ± 0.12
puma$^{-/-}$	17.0 ± 1.4	1.63 ± 0.14
P value	0.81	<0.001

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STRUCTURAL ANALYSIS OF GDF-9 MUTATIONS ASSOCIATED WITH PREMATURE OVARIAN FAILURE AND TWINNING

C. M. Simpson[1,2], K. L. Walton[1], P. G. Stanton[1,2], C. A. Harrison[1,2]

[1]Prince Henry's Institute of Medical Research, Clayton, VIC, Australia

[2]Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

Premature Ovarian Failure (POF) is a disorder associated with female infertility affecting 1% of women under the age of 40 years [1]. It is characterised by amenorrhea associated with an increase in plasma gonadotrophins and decreased estrogen [2, 3]. The major consequence of POF is the loss of fertility, however women also have increased risk of osteoporosis [4] and cardiovascular disease [5]. The causes of POF are unknown; in recent times genetic factors have been considered as etiological components. Genetic screening of women with POF has identified three mutations in Growth and Differentiation Factor-9 (GDF-9), an oocyte-secreted factor critical for folliculogenesis. These are S186Y, V216M and T238A, which are all located within the GDF-9 prodomain [2]. Mutations in GDF-9 may also contribute to the earlier menopause observed in mothers of dizygotic twins, suggestive of a direct relationship to women with POF [6]. The GDF-9 mutations associated with dizygote twinning (DZ) are; P103S located in the prodomain, and P374L and R454C located within the mature domain [6, 7]. The aim of this study is to understand the consequence of these mutations on the synthesis, secretion and biological activity of GDF-9. Utilising site-directed mutagenesis, all six mutations were individually introduced into GDF-9 cDNA and the impact on protein synthesis and secretion was assessed following transfection in HEK293T cells. Mutants S186Y and T238A appeared to have no affect on mature GDF-9 secretion; mutant R454C resulted in misfolded GDF-9; while mutants V216M, P103S and P374L resulted in a significant decrease of mature GDF-9. As the prodomain is necessary for correct folding, dimerisation and secretion of the biologically active mature protein [8], this decrease is most likely due to the impaired posttranslational processing of the prodomain. In vitro biological activity of these mutants is currently being assessed using a HEK293T cell luciferase reporter assay. Preliminary data suggests that mutants V216M and T238A lead to increased biological activity. It is concluded that GDF9 mutations associated with POF and DZ alter synthesis, secretion and bioactivity of GDF-9. It is anticipated that this study will provide structural basis for the impact of heterozygous GDF-9 mutations on ovarian insufficiency in humans.

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OVARIAN PHENOTYPE OF THE IKK CONDITIONAL KNOCKOUT MOUSE

A. E. Drummond, I. Kuyznierewicz, P. J. Fuller

Prince Henry's Institute of Medical Research, Clayton, VIC, Australia

Nuclear factor-κB (NFκB) designates a family of transcription factors that has been shown to modulate antiviral, inflammatory and immune responses and promote tumorigenesis. Activation of NFκB is dependent on IKKβ, a component of the IκB kinase (IKK) complex which promotes degradation of IκB inhibitory proteins and allows nuclear translocation of NFκB. Little is known about the role of NFκB signalling in the ovary. We created a gonadal specific IKKβ conditional knockout mouse to explore NFκB action in the ovary. A transgenic mouse line containing floxed IKKβ alleles (M Karin, UCSD) was crossed with a transgenic mouse line expressing cre-recombinase under the control of the anti-Müllerian hormone receptor (AMHR) promoter (M Matzuk, BCM). Female mice arising from this breeding regime will not express IKKβ in granulosa cells and cannot activate NFκB signalling. Ovaries and serum were collected from mice at 7 and 15 weeks of age. Histological analyses were undertaken and the gonadotrophic hormones, follicle stimulating hormone (FSH) and luteinising hormone (LH) were measured. The ovaries of 7 week old IKKβ null mice contained follicles of all developmental stages although corpora lutea were absent indicating that these mice were infertile. Some follicle subtypes may be under-represented and apoptosis may be enhanced; these studies are ongoing. Serum FSH and LH levels were elevated compared to littermate controls. By 15 weeks of age corpora lutea were present. The fertility of the IKKβ conditional knockout is currently being assessed. In summary, IKKβ conditional knockout mice exhibit a reproductive phenotype which includes delayed ovulation. These results validate the hypothesis that ovulation is an inflammatory-like response. This model will be a valuable tool for reproductive research; the subtlety of the phenotype allowing us to tease out the underlying mechanisms and role of NFκB signalling in ovarian function.

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CONDITIONAL TARGETED DELETIONS OF STAT3 TO IDENTIFY ITS ROLE IN OOCYTES AND GRANULOSA CELLS

L. N. Watson[1], R. L. Robker[1], K. R. Dunning[1], E. A. McLaughlin[2], D. L. Russell[1]

[1]Obstetrics and Gynaecology, Adelaide University, Adelaide, SA, Australia

[2]Environmental and Life Sciences, University of Newcastle, Sydney, NSW, Australia

Signal transducers and activators of transcription-3 (STAT3) is a transcription factor activated by JAK kinases after cytokine-receptor binding. We have identified active phospho-STAT3 in granulosa cells of ovarian follicles from the very early activated stage. Abundant STAT3 has also been identified by us and others in oocytes, cumulus and granulosa cells of mature preovulatory follicles. Further, we have found the STAT responsive gene product SOCS4 is present in granulosa cells of early activated and growing follicles (1). To determine the role of STAT3 in follicle growth and ovulation we have generated three unique lines of conditional STAT3 null mice with STAT3 deletion in granulosa cells or oocytes. Mice with STAT3 gene sequenced flanked by LoxP elements ($STAT3^{fl/fl}$) were crossed with lines expressing Cre-recombinase in granulosa cells (AmhR2-Cre, FSHR-Cre) or oocytes (ZP3-Cre). Fertility analysis of $STAT3^{fl/fl}$;Zp3-Cre females crossed with wildtype males showed no effect on fertility from STAT3 deletion in the oocyte. In contrast, both FSHR-Cre and AmhR2-Cre mediated granulosa deficient STAT3 lines had significantly reduced litter sizes; 1.4-fold and 1.9-fold lower respectively compared to control littermates ($STAT3^{fl/+}$;Cre^{+} or $STAT3^{fl/fl}$;Cre-). Furthermore AmhR2-Cre mediated STAT3 deletion resulted in a significant 1.9-fold reduction in litters per month and an increased time to first litter. Surprisingly we did not find any difference in ovulation rate after eCG+hCG stimulated superovulation, nor in naturally cylcing mice. Zygotes flushed the morning after mating and placed into culture showed no deficit in cleavage of 2-cell embryos or development to blastocyst. A potential uterine deficiency was investigated and gross morphological observations indicate a defect in the uteri which is consistent with the recent report of Cre-recombinase expression in uterine cells derived from the Mullerian duct mesenchyme (2). Thus STAT3 deficiency in oocytes does not lead to infertility while AmhR2-Cre and FSHR-Cre mediated STAT3 deficiency leads to a sub-fertile phenotype.

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SIRT3 IN OVARIAN CELLS IS ALTERED BY MATERNAL AGE AND OVARIAN RESERVE

L. Pacella[1], D. Zander-Fox[1,2], T. Hussein[1,2], T. Fullston[1], M. Lane[1,2]

[1]Obstetrics and Gynaecology, Adelaide University, Adelaide, SA, Australia

[2]Embryology, Repromed, Dulwich, SA, Australia

Maternal age and reduced AMH levels affect the follicular environment and consequently oocyte viability. The Sirtuin family of protein deacetylases are able to regulate various cellular functions involved in the ageing process in other tissues. In particular, SIRT3 is related to longevity in several cell types and regulates mitochondrial function, however, its presence and role in ovarian cells remains unknown. This study therefore, investigated the presence of SIRT3 in granulosa and cumulus cells, from patients undergoing IVF, and determined the impact of maternal age and low AMH on SIRT3 levels. Granulosa and cumulus cells were collected from women (n = 36), after informed consent, and classified into 3 groups; A (<35 years, normal AMH), B (>40 years (advanced maternal age), normal AMH) and C (<35 years, low AMH). The presence of SIRT3 was determined by q-PCR (expressed as fold-change) or immunohistochemistry. SIRT3 was present in the ovarian cells of all patients analysed. SIRT3 gene expression was reduced in granulosa cells from women with low AMH (0.67±0.17) compared to women with normal AMH (1.00±0.23; $P < 0.05$). In cumulus cells, levels were reduced with advanced maternal age (0.81±0.08) compared to women <35 years (1.00±0.22; $P < 0.05$). SIRT3 protein co-localised with mitochondria in the ovarian cells, confirming previous findings for other cell types. In comparison to women <35 years with normal AMH, image analysis determined that SIRT3 protein levels were significantly reduced in the granulosa and cumulus cells from women of advanced maternal age by 21.4% and 31.8% and in women with low AMH by 34.1% and 47.2% respectively. This is the first study to demonstrate SIRT3 presence in human ovarian cells. The observation that SIRT3 levels are altered by advanced maternal age or low AMH (reduced ovarian reserve) implicate its role in ovarian ageing and plausibly in the decrease in oocyte viability observed in these women.

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ACTIVIN A HAS A STIMULATORY EFFECT *IN VITRO* ON EARLY FOLLICLE DEVELOPMENT IN RAT OVARIES

D. A. Cossigny[1,2], J. K. Findlay[1,2], A. E. Drummond[1,3]

[1]Prince Henry's Institute of Medical Research, Clayton, Australia
[2]Obstetrics & Gynaecology, Monash University, Clayton, VIC, Australia
[3]Biochemistry, Monash University, Clayton, VIC, Australia

Activins are dimers of inhibin β subunits and are growth and differentiation factors belonging to the transforming growth factor-β (TGF-β) superfamily (1). Both βA and βB subunits are highly expressed in rat granulosa cells, while theca cells express little or no β subunit mRNAs (2). Oocytes lack expression of either subunit (3, 4). Activin is suggested to facilitate the responsiveness of granulosa cells to FSH (5). We hypothesized that activin, with or without FSH, could enhance the transition from the primordial to later preantral stages of follicle development. In two independent experiments, day 4 rat ovaries (n = 3 from different rats per treatment) were randomly assigned and cultured (6, 7) for 10 days in DMEM/Hams F-12 media with either no additives, FSH (100ng/mL), activin A (50ng/mL), or both. Day 4 fresh ovaries were also used as controls. Media and treatments were refreshed every alternate day. Ovaries were fixed and sectioned, or placed into Ultraspec for RNA extraction and real-time PCR analysis. Follicle numbers were counted as described previously (7). The proportion of atretic follicles (TUNEL staining) was determined in 3 randomly selected sections per ovary. Primordial follicles in all treatment groups were approximately 20% of those in Day 4 fresh ovaries. Primary follicles increased significantly ($P < 0.05$) only in the combined treatment group, where preantral follicles increased significantly ($P < 0.0001$) only when treated with activin A alone. Activin A alone decreased the proportion of atretic follicles in the primary and preantral classes, where the combined treatment increased the proportion of atretic preantral follicles. Real-time analysis revealed that expression levels of follistatin, FSH receptor and activin βA and βB subunits were all expressed at significantly higher levels in the Activin A-only treated group ($P < 0.05$). In summary, there was no effect on primordial follicle activation by any treatment. Activin alone had a stimulatory effect *in vitro* on subsequent folliculogenesis, but in the presence of FSH its effect was counteracted shown by an increase in atresia. Reasons for an increase in atretic preantral follicles in the combined treatment group are unclear. These studies support a stimulatory role for activin A in early follicle development and confirm the *in vivo* effects of activin on folliculogenesis (4).

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LESION WEIGHT AND GLANDULAR DEVELOPMENT ARE SUPPRESSED IN A TGFB1 DEFICIENT MOUSE MODEL OF ENDOMETRIOSIS

M. Z. Johan, W. V. Ingman, S. A. Robertson, M. L. Hull

Obstetrics and Gynaecology, Research Centre for Reproductive Health, Adelaide, SA, Australia

Endometriosis causes subfertility, pelvic pain and dysmenorrhea, and affects 10% of women of reproductive age globally. The pathology of endometriosis is still poorly understood; however microarray data from a mouse model revealed transforming growth factor beta 1 (TGFB1) as central component in molecular pathways that promote tissue remodelling of ectopic endometrial tissues [1]. We hypothesised that a host deficiency of TGFB inhibits the growth of endometriotic lesions and changes the cellular composition of the tissues. To test this hypothesis, human eutopic endometrial tissue was implanted into Tgfb1 –/– mice with Tgfb1+/+ wildtype mice were used as controls (n = 8 and 19 respectively). All mice were on a background of severe combined immunodeficiency to prevent graft rejection. The weight and volume fraction of the glandular and stromal compartments of the resulting lesions were evaluated and the sections were stained with BrdU as a marker of proliferating cells. Sixty percent of mice developed ectopic endometrial lesions in both groups. The median weight of the xenografts from Tgfb1+/+ wildtype mice was 11-fold higher than the Tgfb1–/– mice (Mann-Whitney U test, P = 0.0275). The glandular volume fraction in endometriosis-like lesion from Tgfb1–/– mice was 0.35 and was 33% lower than in lesions from the control mice (volume fraction of glands = 0.52) (independent t test, P = 0.0415). These studies show that TGFB1 is critical for normal endometriosis-like lesion development and a host deficiency of TGFB1 is associated with reduced weight and glandular volume fraction of xenografts. Targeted suppression of TGFB1 in the host response could be a successful therapeutic strategy for women with this disease.

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DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TO MULLERIAN TISSUE

L. YE[1], R. Mayberry[2], E. Stanley[2], A. Elefanty[2], C. Gargett[1]

[1]O&G, MIMR, Monash University, Melbourne, VIC, Australia

[2]MISCL, Monash University, Melbourne, VIC, Australia

The human uterus develops from the distal Mullerian Duct, a derivative of the mesoderm germ layer. Unlike other mammalian species (eg. mouse) the endometrium of the human uterus develops prenatally during gestation. Little is known about the developmental process involved. A better understanding of human endometrial development may shed light on the mechanisms involved in endometrial regeneration and pathogenesis of adult proliferative endometrial diseases. Mouse neonatal uterine mesenchyme (mNUM) is inductive and can maintain the phenotype of normal adult human endometrial epithelial cells [1]. Both adult human endometrial stroma and neonatal mouse endometrial mesenchyme secrete growth factors of the TGF-beta family including BMPs which have been shown to play an important role in differentiation of human embryonic stem cells (HESC) [2, 3]. Hypothesis: mNUM will direct differentiation of HESC to form Mullerian Duct-like epithelium. Aim: to investigate the role of mNUM in differentiating HESC in vitro and in vivo using A tissue recombination technique. Method: Embryoid bodies (EB) were formed from GFP labelled HESC (ENVY) and GFP-MIXL1 HESC reporter line [4, 5] and recombined with 2 × 0.5mm pieces of day 1 epithelial cell-free mNUM. Recombinant tissues were either harvested for gene expression analysis or grafted under the kidney capsule of NOD/SCID mice. Results: We found by qRT-PCR that mNUM induces HESC to form mesendoderm/mesoderm progenitors in vitro, obligate intermediates of the developing Mullerian Duct. After further incubation in vivo under the guidance of mNUM, HESC differentiated to form duct-like structures comprising mesoepithelial cells that co-expressed several key developmental proteins of the Mullerian Duct including Emx2, Pax2, Hoxa10, CA125, and also intermediate filament markers such as CK8/18, Vimentin, (n = 8). Conclusion: Our study demonstrated for the first time that mNUM can direct HESC to form a mesodermally derived epithelium that is Mullerian Duct-like, providing a novel model for studying human uterine development.

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GENOME-WIDE ASSOCIATION STUDY IDENTIFIES A LOCUS AT 7P15.2 ASSOCIATED WITH THE DEVELOPMENT OF MODERATE-SEVERE ENDOMETRIOSIS

G. W. Montgomery[1], J. N. Painter[1], C. A. Anderson[2,3], D. R. Nyholt[1], S. Macgregor[1], S. H. Lee[1], P. M. Visscher[1], P. Kraft[4,6], N. G. Martin[1], A. P. Morris[2], S. A. Treloar[1,5], S. H. Kennedy[7], S. A. Missmer[4,6], K. T. Zondervan[2,7]

[1]Queensland Institute of Medical Research, Brisbane, QLD, Australia
[2]Wellcome Trust Centre for Human Genetics, University of Oxford, Oxford, United Kingdom
[3]Wellcome Trust Sanger Institute, Hinxton, United Kingdom
[4]Harvard School of Public Health, Boston, MA, United States
[5]Centre for Military and Veterans' Health, University of Queensland, Bsribane, QLD, Australia
[6]Brigham and Women's Hospital, Boston, MA, United States
[7]Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, United Kingdom

Endometriosis is a common gynaecological disease associated with severe pelvic pain and sub-fertility. There is considerable debate whether different endometriosis stages represent disease progression, or whether moderate-severe (rAFS III/IV) disease is pathological and minimal-mild (rAFS I/II) an epiphenomenon. We conducted a genome-wide association study using 540 082 SNPs in 3194 surgically confirmed endometriosis cases and 7060 controls from Australia and the UK. We used novel statistical methods to estimate the proportion of common variation explained by all markers and performed polygenic predictive modelling for disease stage, both showing significantly increased genetic loading among the 42% of cases with moderate-severe endometriosis. The strongest signals of association were also observed for moderate-severe disease. We subsequently genotyped 72 SNPs in an independent US dataset comprising 2392 endometriosis cases and 1646 controls. An association with rs7798431 on 7p15.2 for moderate-severe endometriosis ($P = 6.0 \times 10^{-8}$, OR = 1.34 (1.21–1.49)) was replicated, reaching combined genome-wide significance ($P = 1.7 \times 10^{-9}$; OR = 1.26 (1.17–1.35)). The implicated inter-genic region involves a 48 kb segment of high LD upstream of plausible candidate genes NFE2L3 and HOXA10. This locus is the first to be robustly implicated in the aetiology of endometriosis, with evidence of association limited to moderate-severe disease.

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PROGESTERONE RECEPTOR-REGULATED GENES IN THE PREOVULATORY OVARIAN FOLLICLE AND OVIDUCT

L. Akison, D. Russell, R. Robker

The Robinson Institute, Research Centre for Reproductive Health, University of Adelaide, Adelaide, SA, Australia

Ovulation is a highly regulated and precisely timed reproductive process but the underlying molecular mechanisms are not well understood. Progesterone receptor (PGR) is a transcription factor highly yet transiently expressed in granulosa cells (GCs) of preovulatory follicles; has low expression in cumulus-oocyte-complexes (COCs); and is abundantly expressed in the oviduct. $PR^{-/-}$ mice validate its essential role in ovulation as they are anovulatory, despite normal growth and development of ovarian follicles and oocytes. Our aim was to use microarray to identify differentially expressed genes in GCs, COCs and oviducts from $PR^{-/-}$ and $PR^{+/-}$ mice, specifically genes potentially involved in oocyte release and transport. GCs, COCs and oviducts were collected from 21d-old mice ($n = 5$; 3 mice/replicate) at 8h post-hCG/44h post-eCG. Extracted RNA samples were hybridized to Affymetrix Mouse Gene 1.0 ST Arrays and post-experiment processing/analysis performed using Partek Genomics Suite. Gene ontology analysis was performed using Ingenuity Pathway Analysis (IPA). In GCs, 296 genes were differentially expressed ($P < 0.05$); 78% down-regulated in $PR^{-/-}$. IPA identified genes involved in cancer migration/invasion, chemotaxis, and adhesion; the chemokine receptor Cxcr4, was >3-fold down-regulated in $PR^{-/-}$. Proteases were also decreased; Adam8 (3.5-fold) and Adamts1 (2.6-fold) in $PR^{-/-}$. In oviducts, 1003 genes were differentially expressed at $P < 0.05$ and 266 genes at $P < 0.01$; 93% were down-regulated in $PR^{-/-}$. IPA identified genes involved in cell adhesion, movement/migration, invasion and chemotaxis as well as muscle contraction and vasoconstriction. The most highly down-regulated was Itga8 (>9-fold), one of 11 integrins, well known cellular adhesion receptors, differentially expressed. In COCs, 44 genes were differentially expressed ($P < 0.05$); 52% down-regulated in $PR^{-/-}$. IPA identified 18 genes (41%) involved in cancer invasion/migration or adhesion. Thus, this study has identified novel gene targets for PGR regulation, which may have essential roles in the molecular control of oocyte release into the oviduct at ovulation.

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GENOMIC IMPRINTING IN THE MARSUPIAL MAMMARY GLAND

J. M. Stringer[1], G. Shaw[1], A. Pask[2], M. B. Renfree[1]

[1]Zoology, The University of Melbourne, Parkville, Australia

[2]Molecular and Cellular Biology, The University of Connecticut, Storrs, Connecticut, United States

Genomic imprinting is an epigenetic mechanism that differentially regulates the expression of certain genes, resulting in expression from only one parental allele. In mammals, genomic imprinting occurs in the placenta of both eutherians and marsupials, and plays an important role in regulating nutrition and growth of the developing fetus. The mammary gland also provides a critical source of nutrition for the neonate in all mammals, but there are few imprinting studies of this organ. Marsupials deliver tiny, altricial young that complete development during an extended lactation. INS (insulin) is paternally expressed in the eutherian and marsupial yolk sac and curiously is the only gene that is solely imprinted in this organ (1, 2). Insulin regulates carbohydrate metabolism, protein synthesis and cell growth. Insulin, (plus cortisol and prolactin) is required for the onset of lactation and the synthesis of milk (3). We characterised INS expression and examined its imprint status in the mammary gland of the tammar wallaby. INS mRNA is expressed in the mammary gland of the tammar from birth and throughout of lactation with highest expression at the initiation of lactation (Phase 1-2a) and around Phase 3 of lactation. Direct sequencing of 7 individuals at various stages of lactation confirmed that INS is imprinted in the mammary gland. Surprisingly, INS may also be imprinted in several other organs in the adult and juvenile wallaby. Preliminary bisulfite sequencing suggests there is a differentially methylated region located upstream of INS which may help to regulate INS expression. This is the first study to identify INS imprinting outside the yolk sac. As INS is critical for lactation, this is also the first indication that genomic imprinting may regulate lactation, suggesting that imprinting in the mammary gland may be as critical for post-natal survival as placental imprinting is for pre-natal development.

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GENOME-WIDE LINKAGE SCAN FOR FAMILIAL DIZYGOTIC TWINNING

J. N. Painter[1], G. Willemsen[2], D. R. Nyholt[1], C. Hoekstra[2], D. Duffy[1], A. Henders[1], L. Wallace[1], S. Healy[1], L. A. Cannon-Albright[3], M. Skolnick[3], N. G. Martin[1], D. I. Boomsma[2], G. W. Montgomery[1]

[1]Molecular Epidemiology, Queensland Institute of Medical Research, Herston, QLD, Australia

[2]2. Department of Biological Psychology, VU University, Amsterdam, Netherlands

[3]Department of Internal Medicine, University of Utah, Salt Lake City, United States

The tendency to conceive dizygotic (DZ) twins is a complex trait influenced by genetic and environmental factors. To search for new candidate loci for twinning we have conducted a genome-wide linkage scan in 525 families using microsatellite and single nucleotide polymorphism (SNP) marker panels. Non-parametric linkage analyses including 523 families containing a total of 1115 mothers of DZ twins (MODZT) from Australia and New Zealand (ANZ) and The Netherlands (NL) produced four linkage peaks above the threshold for suggestive linkage, including a highly suggestive peak at the extreme telomeric end of chromosome 6 with an exponential (exp)LOD score of 2.813 (P = 0.0002). Since the DZ twinning rate increases steeply with maternal age independent of genetic effects, we also investigated linkage including only families where at least one MODZT gave birth to her first set of twins before the age of 30. These analyses produced a maximum expLOD score of 2.718 (P = 0.0002), largely due to linkage signal from the ANZ cohort, however, ordered subset analyses indicated this result is most likely a chance finding in the combined dataset. Linkage analyses were also performed for two large DZ twinning families from the USA, one of which produced a peak on chromosome 2 in the region of two potential candidate genes. Sequencing of FSHR and FIGLA, along with INHBB in MODZTs from two large NL families with family-specific linkage peaks directly over this gene, revealed a potentially functional variant in the 5' untranslated region of FSHR that segregated with the DZ twinning phenotype in the UT family. Work is continuing screening candidate genes. Our data provide further evidence for complex inheritance of familial DZ twinning.

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PLACENTAL EXPRESSION OF MICRORNAS ALTERS WITH GESTATION IN THE GUINEA PIG

P. A. Grant[1], K. L. Kind[2], A. Sohlstrom[3], K. Gatford[1], C. T. Roberts[1], J. A. Owens[1]

[1]School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia
[2]School of Animal and Veterinary Sciences, University of Adelaide, Adelaide, SA, Australia
[3]Biomedicine and Surgery, Division of Nutrition, Linkoping University, Linkoping, SA, Australia

Functional development of the placenta ensures an adequate supply of nutrients for fetal growth throughout gestation. Placental nutrient transport capacity increases during gestation, through alterations in structure and abundance of its molecular determinants, including expression of Slc2a1 (glucose transporter type-1) and Slc38a2 (system A amino acid transporter), as well as Igf1and Igf2. Each of these genes are predicted targets of microRNAs. Non-coding RNAs can down-regulate, as well as activate translation, by interacting with complementary regions in the promoter, coding, or 3'UTR of target mRNAs(1). MicroRNAs are present in the mammalian placenta(2), but little is known about developmental changes in their expression and actions. We hypothesised that placental expression of microRNAs which target molecular mediators of nutrient transport changes during gestation. Expression of microRNAs in the guinea pig placenta was examined at D30 (n = 7) and D60 (n = 7) of gestation (term = D70) by Exiqon microarray. Gene expression was measured by real-time PCR. Predicted gene targets were identified using miRecords and networks and pathways by Ingenuity Pathway Analysis. Placental expression of 119 microRNAs was upregulated (P < 0.05), and that of 40 was down-regulated (P < 0.05), at late compared to early gestation. Of the 20 most abundant differentially up- or down-regulated microRNAs, 11 are predicted to target members of solute carrier families. This includes has-miR-26a (↑2X), which is predicted to target Slc38a2 mRNA and expression of has-miR-26a and Slc38a2 was positively correlated (P < 0.02). Alternate predicted targets of has-miR-26a include networks involving amino acid metabolism, molecular transport and small molecular biochemistry. These findings support the hypotheses that gestational changes in microRNA expression act to regulate functional development of the placenta, including expression of genes that mediate nutrient transport.

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GENES CONTROLLING PHALLUS DEVELOPMENT

K. Chew[1], A. Pask[1,2], G. Shaw[1], M. B. Renfree[1]

[1]Zoology, The University of Melbourne, Parkville, VIC, Australia
[2]Department of Molecular and Cellular Biology, The University of Connecticut, Storrs, Connecticut, United States

Abnormalities of the limb and genital urinary tract are amongst the most prevalent congenital birth defects. The phallus and limb are both appendages controlled by similar patterning mechanisms during the early stages of differentiation. However, the phallus later undergoes an androgen dependent masculinisation phase whilst the limb does not. Marsupials deliver altricial young that complete their development after birth so are ideal models to investigate appendage development. In the tammar, development of the phallus occurs after birth during a period when the testicular androgen is low while the young is in the pouch, stages that normally occur in utero in mice. Exposure to androgens during development, but before differentiation of the phallus can induce sex reversal of the female phallus and hypospadias.[1] However, the genes controlling development of the phallus and how they interact with the changing androgen environment have not been well studied as yet in any mammal. We are investigating the expression of several key genes, namely SHH, FGF8, BMP4 and DLX5, using RT-PCR, immunohistochemistry and in situ hybridization throughout the differentiation of the phallus and after treatment with androgens. Endogenous application of the potent androgen androstanediol to the phallus resulted in the downregulation of SHH, FGF8, BMP4 and DLX5. These genes are crucial for the correct development of both limb and phallus. However, the limb does not have an androgen dependant phase and it is not sexually dimorphic at these stages. SHH protein was localised in the epithelium of the phallus and in the urethral groove. The expression of the genes patterning the limb and phallus in the tammar therefore appear to be broadly conserved with those of the mouse. However, this is the first identification of androgen-regulated gene expression in the developing mammalian phallus.

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METHYLATION OF GENES OF THE RENIN ANGIOTENSIN SYSTEM (RAS) IN EARLY HUMAN AMNION

C. M. Mitchell[1], T. Zakar[1,2,3], S. D. Sykes[1], K. G. Pringle[1], E. R. Lumbers[1]

[1]School of Biomedical Sciences and The Mothers and Babies Research Centre, University of Newcastle, Newcastle, NSW, Australia

[2]Obstetrics and Gynaecology, John Hunter Hospital, Newcastle, NSW, Australia

[3]School of Medical Practice and Public Health, University of Newcastle, Newcastle, NSW, Australia

The renin angiotensin system (RAS) genes angiotensin converting enzyme (ACE), angiotensin II type 1 receptor (AGTR1) and prorenin receptor (ATP6AP2) have CpG islands at their promoters, so these genes may be regulated by CpG island methylation as may the expression of two proteases implicated in prorenin activation (cathepsin D {CTSD} and kallikrein 1 {KLK1}). We measured CpG island methylation of 3 RAS genes and of CTSD and KLK1 in amnion using the Methyl-Profiler assay (SA Biosciences), which discriminates methyl-CpG density between hypermethylated, intermediately methylated and unmethylated CpG islands. DNA from human amnion collected between 10–17.8 weeks gestation, at elective caesarean section and after labour at term was analysed. The bulk (>80%) of CpG islands in all genes examined except for KLK1 was unmethylated and without intermediate methylation throughout gestation, while the rest was hypermethylated. There was no change in methylation density with labour (Table 1). In early gestation KLK1 methylation was greater than ACE, AGTR1 and ATP6AP2 ($P < 0.05$). KLKI methylation shifted towards the unmethylated state after labour ($P < 0.05$). There were correlations between methylation of ACE, AGTR1 and ATP6AP2 ($P < 0.02$) but not between RAS genes and KLKI. This suggests that the 3 RAS genes in amnion are not controlled by CpG island methylation. It is likely, however, that KLK1 is silenced in early gestation amnion partially by CpG island methylation, which is reduced by term. Since Kallikrein 1 can activate prorenin, the methylation status of this gene may regulate RAS activity in amnion during gestation.

Table 1. Methylation of RAS genes and proteases in amnion at different stages of human gestation

	Early gestation	Term	After labour
ACE	0.5 ± 0.1 (7)	2.7 ± 1.2 (8)	8.2 ± 4.3 (8)
AGTR1	5.4 ± 4.9 (8)	1.7 ± 0.7 (8)	6.2 ± 2.6 (7)
ATP6AP2	2.6 ± 1.0 (8)	6.9 ± 2.6 (7)	17.4 ± 10.3 (4)
CTSD	6.3 ± 3.8 (8)	7.2 ± 6.2 (6)	2.8 ± 1.9 (6)
KLK1	39.8 ± 5.3 (7)	33.8 ± 4.3 (3)	4.8 ± 2.0 (3)*

Mean % hypermethylated ± s.e.m. (*n*). *$P<0.05$ vs early gestation.

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MICRORNA AND EARLY MALE GERM CELLS

S. C. McIver[1,2,3], S. D. Roman[1,2,3], B. Nixon[1,2,3], E. A. McLaughlin[1,2,3]

[1]ARC Centre of Excelence in Biotechnology and Development, Australia

[2]Reproductive Science Group, Australia

[3]Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia

MicroRNAs are short regulatory noncoding RNA molecules that bind to the 3' untranslated region of mRNA targets to control translation therefore influencing the abundance of many different protein molecules. Aberrant expression of miRNA is linked to many diseases and developmental abnormalities. Testicular Germ Cell Tumours (TGCT) develop from Carcinoma in Situ cells which have been identified as dysfunctional gonocytes. Due to the continual rise in the rate of testicular cancer in the developed world – the molecular mechanisms underlying the failure of gonocytes to differentiate into spermatogonia is of great interest. Gonocytes from post natal day 1 testes and spermatogonia from day 7–9 mice were enriched by 2–4% BSA gradient sedimentation. Total RNA including microRNA was extracted and analysed in by Illumina miRNA microarray. Total RNA was also reverse transcribed using specific primers and analysed by qPCR. Three biological replicates were performed in both the microarray and qPCR experiments. Bioinformatic analysis with SAM (Significance Analysis of Microarrays) identified seven significantly different miRNA molecules between spermatogonia and gonocytes. qPCR analysis confirmed two miRNAs were significantly upregulated in spermatogonia (743a, 463*) and three miRNAs were significantly down regulated in spermatogonia (293, 290-5p, 291a-5p). Several miRNA molecules were selected for further study (293, 290-5p, 136, and 146a) and overexpression assays first in the P19 cell line, then in isolated spermatogonia will help determine their function. In the future the role of these molecules in human seminoma will be analysed using overexpression within a seminoma cell line. It is hypothesised that these miRNA molecules control genes involved in male development and differentiation, such as stella, nanog and oct3/4, and may also play a role in tumour development. In conclusion miRNA expression is significantly different between gonocytes and spermatogonia and we propose that this results in the initiation of differentiation and commencement of spermatogenesis.

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DIFFERENCES IN GENE EXPRESSION BETWEEN APICAL AND BASAL CELLS OF THE MEMBRANA GRANULOSA

H. F. Irving-Rodgers[1], S. T. Lee[1], N. Hatzirodos[1], K. Hummitzsch[1], T. R. Sullivan[2], R. J. Rodgers[1]

[1]Obstetrics and Gynaecology, The University of Adelaide, Adelaide, SA, Australia

[2]Public Health, The University of Adelaide, Adelaide, SA, Australia

Granulosa cells constitute the ovarian follicular epithelium which at the beginning of folliculogenesis forms a single layer of flattened cells. As the follicle matures the cells acquire a cuboidal morphology, proliferate and differentiate into the cumulus cells surrounding the oocyte, and the mural granulosa cells forming the inner layer of the follicle (the membrana granulosa). Mural granulosa cells may further differ in their functionality depending on whether they are situated apically or basally within the stratified membrana granulosa. Late in folliculogenesis granulosa cells develop the ability to produce oestradiol, and also a specialised extracellular matrix (focimatrix) which is more abundant between apical cells. In order to investigate possible differences between granulosa cells, the expression of genes for oestradiol synthesis (CYP11A1, CYP19A1), focimatrix components (LAMB2, COL4A1, HSPG2), FSH and LH receptors, and cell cycle genes (CCND2, CCNE1, CCNE2, CDKN1B, CDKN2D) were examined in apical and basal granulosa cells from large healthy bovine follicles [n = 18, 14.3 ± 0.3 mm (mean + SEM)] using quantitative RT-PCR. Apical granulosa cells were collected by flushing the follicle with balanced salt solution. The remaining cells were detached from the follicular basal lamina by gently scraping; these are the basal granulosa cells. This collection method resulted in equivalent cell yields of apical and basal cells. Expression for all genes was significantly higher in basal cells in comparison to apical cells ($P < 0.05$), except for the cycle genes CCND2 and CDKN2D, which did not differ between cell populations. These results suggest that functional heterogeneity exists within the membrana granulosa. How differences between apical and basal cells are established is unknown but may be due to the proximity of the basal cells to the follicular basal lamina. The relevance of this aspect of follicle maturation to the endocrine function of granulosa cells has yet to be determined.

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MAELSTROM – A PROTEIN THAT IS ESSENTIAL FOR SPERMATOGENESIS AND TRANSPOSABLE REPRESSION IS EXPRESSED IN ADULT OVARY OF MAMMALS AND BIRDS

S. Lim[1], E. Tsend-Ayush[1], R. Kortschak[1], C. Ricciardelli[2], M. Oehler[2], F. Grutzner[1]

[1]Genetics, University of Adelaide, Adelaide, 5005, Australia

[2]Obstetrics and Gynaecology, University of Adelaide, Adelaide, SA, Australia

Maelstrom (MAEL) is a highly evolutionarily conserved protein located at the perinuclear structure of animal germ cells called nuage. The MAEL protein contains HMG and Tudor domains and associates with components of the piRNA and RNAi pathways and chromatin remodelling factors. Recent work has shown that MAEL is required for the differentiation of the germ-line stem cell lineage and for the retroposon repression. In mouse, Mael is expressed in male germ cells and is essential for spermatogenesis and retroposon suppression. We have investigated the evolution of the Mael gene in mammals and birds. As expected the gene is highly conserved in all three mammalian lineages and in chicken. Interestingly, the platypus MAEL has exclusive changes in the DnaQ-H 3'-5' exonuclease domain and computational modelling suggested that these changes may affect the folding of the protein. Expression analysis revealed that the Mael gene is transcribed in testis but also in adult ovaries of chicken, platypus, mouse and human. In situ hybridisation of the Mael transcript on ovary sections of mouse and platypus shows that gene expression is found in pre-antral and antral follicles. The data so far also showed some differences in the expression pattern between mouse and platypus. In mouse, we detected transcript in oocyte, granulosa cells and cumulus cells whereas in the platypus we only observed expression in oocyte. Earlier work demonstrated that Drosophila Mael mutant ovaries had mislocalisation of the RNAi pathway proteins, Dicer and Argonaute2. It is well known that RNAi pathyway is involved in the repression of transposon in the testis and ovary across animal kingdom. As a key component of the RNAi pathway, MAEL is reported to co-localise and interact with MILI and MIWI proteins. These finding may suggest a role of MAEL in retroposon control in ovary and folliculogenesis.

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WINGLESS (WNT)3A INDUCES TROPHOBLAST MIGRATION AND MATRIX METALLOPROTEINASE-2 SECRETION THROUGH CANONICAL WNT SIGNALLING AND PROTEIN KINASE B/AKT ACTIVATION

S. Sonderegger[1,2], P. Haslinger[2], A. Sabri[2], J. V. Otten[2], C. Leisser[2], C. Fiala[3], M. Knofler[2]

[1]Embryo Implantation Laboratory, Prince Henry's Institute of Medical Research, Clayton, VIC, Australia
[2]Department of Obstetrics and Fetal Maternal-Medicine, Reproductive Biology Unit, Medical University of Vienna, Vienna, 1090, Austria
[3]Gynmed Clinic, Vienna, 1150, Austria

Human placenta and trophoblasts express WNT ligands and WNT receptors suggesting a role for WNT-signalling in placental development. Indeed, a recombinant WNT ligand was recently shown to promote trophoblast migration/invasion, however, the involved signalling cascades and their target genes have not been elucidated. The aim was to investigate signal transduction via canonical WNT-signalling or phosphatidylinositide 3-kinase (PI3K)/AKT-signalling, their cross-talk as well as trophoblast-specific protease expression in trophoblastic SGHPL-5 cells and primary 1st trimester extravillous trophoblasts (EVT). WNT3A-dependent activation/phosphorylation of AKT (pAKT) and the down-stream kinase glycogen synthase kinase (GSK)-3β were determined by Western blotting (WB). WNT3A-induced canonical WNT-signalling was analysed by luciferase reporter assay (TOPFlash) and nuclear recruitment of β-catenin. Trophoblast migration was studied using transwell assays and villous explant cultures. MMP2 expression/activation was investigated by qRT-PCR and WB/gelatine-zymography of supernatants. All experiments were performed +/– inhibitors of AKT-signalling or canonical WNT-signalling using LY294002 (PI3K inhibitor) or recombinant Dickkopf-1 (DKK1). WNT3A induced pAKT, luciferase expression of the canonical WNT reporter ($P < 0.05$) as well as accumulation of nuclear β-catenin. Inhibition of PI3K abolished WNT-dependent pAKT, pGSK-3β and cell migration, but did not affect TOPFlash activity or appearance of nuclear β-catenin. Inhibition through DKK1 did not influence pAKT and pGSK-3β, but decreased WNT reporter activity, nuclear β-catenin and cell migration. Both inhibitors decreased WNT3A-induced MMP2 expression in SGHPL-5 cells and pure EVTs ($P < 0.05$). WNT3A activates PI3K/AKT as well as canonical WNT-signalling through distinct receptors in invasive trophoblasts, since DKK1 did not activate the particular kinase. Although cross-talk between PI3K/AKT and canonical WNT-signalling has been observed in some cell types, these pathways seem to act independently in trophoblasts. However, both pathways promote Wnt-dependent migration and expression of MMP2. This is the 1st study identifying MMP2 as a novel target gene of canonical WNT-signalling in trophoblast.

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MOLECULAR FILTRATION PROPERTIES OF THE EXPANDED CUMULUS MATRIX: CONTROLLED SUPPLY OF METABOLITES AND EXTRACELLULAR SIGNALS TO CUMULUS CELLS AND THE OOCYTE.

K. R. Dunning, L. N. Watson, J. G. Thompson, R. L. Robker, D. L. Russell

The Robinson Institute, The University of Adelaide, Adelaide, SA, Australia

Cumulus matrix genes are positively correlated with oocyte competence [1]. Formation of the expanded cumulus matrix during oocyte maturation is well described; however its function remains elusive. We investigated whether cumulus matrix acts as a molecular filter, based on recognised filtration properties of analogous matrices. We found that cumulus matrix controls metabolite supply to the oocyte and retains prostaglandin E2 (PGE_2), which is critical in oocyte maturation. The uptake of fluorescently labelled hydrophilic and hydrophobic metabolites showed that cumulus matrix formation significantly impeded diffusion to the oocyte. Expanded in vivo matured cumulus oocyte complexes (COCs, eCG+hCG16h) resisted uptake of glucose and cholesterol compared to unexpanded (eCG44h, $P < 0.05$), as assessed by confocal microscopy and spatial quantitation of fluorescence ($P < 0.05$). In vitro maturation (IVM) results in pronounced compositional deficiency of cumulus matrix proteins [2] and poor oocyte quality. Glucose and cholesterol were transported more readily into cumulus cells and the oocyte of IVM COCs (matured in αMEM/5% FCS/50 mIU/mL FSH, 16 h) compared to in vivo matured COCs ($P < 0.05$ and $P = 0.08$, respectively). Taking the inverse approach we found that PGE_2 synthesised by cumulus cells is retained within the matrix compartment of in vivo matured COCs but IVM COCs did not retain PGE_2 and secreted 4.3-fold more into the media. The relationship of retained to secreted PGE_2 was significantly higher after in vivo maturation vs IVM COCs ($P < 0.0001$). This property of the COC matrix reveals a potential mechanism whereby the prostaglandin signal intensifies through a physicochemical mechanism rather than gene regulation. This is the first demonstration that cumulus matrix regulates diffusion toward and secretion from the COC, thus excluding glucose, known to negatively affect oocyte quality, and trapping factors, including PGE_2, with critical roles in oocyte maturation and fertilisation. Thus, IVM may reduce oocyte quality due to poor trafficking of metabolites and signalling molecules.

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MOLECULAR CHARACTERIZATION OF RENIN-ANGIOTENSIN SYSTEM COMPONENTS IN HUMAN INTRAUTERINE TISSUES AND FETAL MEMBRANES FROM VAGINAL DELIVERY AND CAESAREAN SECTION

F. Z. Marques[1], K. G. Pringle[2], M. Markus[1], A. Conquest[2], J. J. Hirst[2], M. Sarris[3], T. Zakar[2], B. J. Morris[1], E. R. Lumbers[2,3]

[1]School of Medical Sciences and Bosch Institute, University of Sydney, Sydney, NSW, Australia
[2]School of Biomedical Sciences & Mothers & Babies Research Centre, University of Newcastle & Hunter Medical Research Institute, Newcastle, NSW, Australia
[3]School of Medical Sciences, University of New South Wales, Sydney, NSW, Australia

The expression of the (pro)renin receptor (ATP6AP2) in late gestational human tissues suggests that the prorenin-angiotensin system (RAS) might influence pregnancy outcome. Here w e characterized the RAS in term fetal membranes (amnion and chorion), decidua and placenta (n = 38) from women undergoing elective cesarean section (non-labouring) or following spontaneous delivery (after labour), and myometrium (n=16) from elective or emergency cesarean (labouring) deliveries. RT-qPCR was used to quantify prorenin (REN), AGT, ACE, ACE2, AGTR1, AGTR2, ATP6AP2 and MAS1 mRNAs, and immunohistochemistry was used to localize prorenin, AGT, ACE, ACE2 and AGTR1 proteins. In myometrium, mRNAs for downstream signalling proteins (ZBTB16, TGFB1 and PTGS2) were also measured. ACE and AGT mRNA levels were higher in labouring myometrium ($P < 0.05$), consistent with elevated production of angiotensin II (Ang II), which, by the upregulation of PTGS2 occurring in labour ($P = 0.022$), could influence labour. In amnion, expression of all RAS component mRNAs, except ATP6AP2, was low. After labour amnion showed lower ACE ($P = 0.014$) and higher AGTR2 ($P = 0.01$) mRNA levels. In decidua, RAS components other than AGTR1 and AGTR2 were abundant. Amnion and chorion exhibited higher immunostaining of AGT and prorenin than expected from their low mRNA levels, suggesting that these proteins could have been originated from decidua, where the cognate genes are more active. In placenta, prorenin and AGT were localized to syncytiotrophoblasts and ACE was localized to fetal capillary endothelial cells, while ACE2 distribution was diffuse. AGTR1 mRNA and protein expression was high in the placenta. We propose that ACE in fetal vessels could contribute Ang II to the fetus, while ACE2 in syncytiotrophoblasts might convert placental or maternal circulating Ang II to angiotensin-(1–7), which might then be supplied to the maternal bloodstream. In conclusion, the abundance and distribution of intrauterine RAS components suggest diverse roles for this local RAS in pregnancy.

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HORMONALLY REGULATED MIRNAS TARGET THE TUBULOBULBAR COMPLEX IN THE TESTIS

P. K. Nicholls[1,2], P. G. Stanton[1,2], K. L. Walton[1], R. I. McLachlan[1], L. O'Donnell[1], C. A. Harrison[1,2]

[1]Prince Henry's Institute of Medical Research, Clayton, VIC, Australia
[2]Department of Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

Spermatogenesis is absolutely dependent on follicle stimulating hormone (FSH) and androgens; acute suppression of these hormones inhibits germ cell development and thus sperm production. The removal of intercellular junctions and release of spermatids by the Sertoli cell, a process known as spermiation, is particularly sensitive to acute hormone suppression(1). To define the molecular mechanisms that mediate FSH and androgen effects in the testis, we investigated the expression and hormonal regulation of micro-RNAs (miRNA), small non-coding RNAs that regulate protein translation and modify cellular responses. By array analysis, we identified 23 miRNAs that were upregulated >2-fold in stage VIII seminiferous tubules following hormone suppression, and in vitro in primary Sertoli cells. We subsequently validated the expression and hormonal regulation of several miRNAs, including miR-23b, -30d and -690 by quantitative PCR in primary Sertoli cells. Bioinformatic analysis of potential targets of hormonally-suppressed miRNAs identified genes associated with Focal adhesions (54 genes, $P = -\ln(17.97)$) and the Regulation of the actin cytoskeleton (52 genes, $P = -\ln(10.16)$), processes known to be intimately associated with adhesion of spermatids to Sertoli cells(2, 3). Furthermore, this analysis identified numerous components of the testicular tubulobulbar complex (TBC) as being targets of hormonally sensitive miRNAs. The TBC is a podosome-like structure between Sertoli and adjacent spermatids in the testis, which internalises intact inter-cellular junctions by endocytotic mechanisms prior to spermiation(4). We then demonstrate the hormonal regulation of predicted miRNA target proteins, and validate novel inhibitory miRNA interactions with Pten, nWASP, Eps15 and Picalm by luciferase knockdown in vitro. We hypothesise that hormonally suppressed miRNAs inhibit TBC function, and subsequently, endocytosis of intercellular junctions. In conclusion, we have demonstrated that hormonal suppression in the testis stimulates the expression of a subset of Sertoli cell miRNAs that are likely regulators of cell adhesion protein networks involved in spermiation.

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TRANSLATIONAL CONTROL IN FOLLICULOGENESIS AND OOCYTE DEVELOPMENT: A ROLE FOR RNA-BINDING PROTEIN MUSASHI-1

K. M. Gunter[1], B. A. Fraser[1], A. P. Sobinoff[1], V. Pye[1], N. A. Siddall[2], G. R. Hime[2], E. A. McLaughlin[1]

[1]Environmental and Life Sciences, The University of Newcastle, Callaghan, NSW, Australia
[2]Department of Anatomy and Cell Biology, University of Melbourne, Parkville, VIC, Australia

Control of the maternal mRNA pool during oocyte maturation is crucial to the correct temporal and spatial expression of proteins, particularly during oocyte transcriptional quiescence. We have identified Musashi-1 as being present within the oocyte/ovary, where this RNA-binding protein is believed to act as a translational repressor of target mRNAs. Recent studies in mammalian neural and intestinal systems have identified a number of cell cycle regulators as potential targets of Msi-1. Using Msi-1 protein-RNA immunoprecipitation, we have also identified musashi-2 (msi-2) and c-mos as putative targets in the mouse oocyte. To further study these targets, a transgenic mouse was produced to overexpress Msi-1 exclusively in the oocyte. QPCR analysis, performed on intact ovaries of wild type (WT) and Tg mice, confirmed a 1.5-fold increase in msi-1 expression in tgMsi-1/+ ovaries in excess of WT ovary expression. QPCR analysis of Msi-1 target expression, performed on intact WT and Tg ovaries, in conjunction with transcript obtained from the Msi-1 protein-RNA immunoprecipitation, revealed an overall increase in expression in the tgMsi-1/+ and Msi-1 IP samples, respectively, of $p21^{WAF-1}$ (~2.5-fold; undetected), cdkn2a (~2-fold; undetected), notch1 (~3-fold;undetected), c-mos (no difference; ~41-fold) and msi-2 (~7-fold; ~10-fold). Immunohistochemical analysis of Msi-2 protein expression in transgenic juvenile mouse ovaries, demonstrated a decrease in expression of Msi-2 in tgMsi-1/+ ovaries, when compared to WT ovary expression, suggesting that Msi-2 mRNA is translationally repressed by Msi-1. Therefore, preliminary analysis suggests that Msi-1 may play a role in regulating transcripts of genes necessary for processes characteristic of meiotic progression and oocyte development.

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IN VITRO CHARACTERISATION OF BIOFILM FORMATION IN HUMAN FOLLICULAR FLUID

E. S. Pelzer[1], J. A. Allan[2], J. M. Allan[2], T. Launchbury[2], C. L. Knox[1]

[1]QUT, Institute of Health and Biomedical Innovation, Kelvin Grove, QLD, Australia
[2]The Wesley Hospital, Wesley Monash IVF, Brisbane, QLD, Australia

Previous studies have detected bacteria in follicular fluid collected at the time of trans-vaginal oocyte retrieval but this was not shown to adversely affect ART outcomes. The antimicrobial properties of follicular fluid have also been investigated, and follicular fluid was reported to be inhibitory to Gram positive bacteria after four days, but supported the growth of Gram negative bacteria. In this study 36 follicular fluid specimens collected from assisted reproductive technology (ART) patients were tested for the presence of bacteria by culture and 16S rRNA PCR assays. Follicular fluid aliquots (24) were incubated and subcultured for 28 weeks and the ability of these follicular fluids to form biofilms in vitro was assessed. A further 12 follicular fluid specimens were inoculated into broth media with and without the addition of estradiol and progesterone at concentrations reported in follicular fluid of hyperstimulated women. Bacteria in polymicrobial (22%) or singles species populations (78%) were detected in the 24 follicular fluid specimens tested. The most prevalent bacteria detected were Lactobacillus species (18/35 total bacterial isolates, 51%) and Propionibacterium spp. (5/35, 14%). All follicular fluids supported the growth of bacteria in vitro for up to 28 weeks. Bacteria in 18/24 (75%) of follicular fluid specimens, formed biofilms in vitro. The majority of biofilms formed were grade IV 7/18 (39%) or grade III biofilms 7/18 (39%). Lactobacilli were also the most prevalent cultivable species within mature biofilms (51% of total isolates). Whilst high levels of steroid hormones were required for the growth of Bifidobacterium spp. in vitro, the growth of both Streptococcus agalactiae and Escherichia coli was inhibited by these hormones. Follicular fluid is not sterile and is an excellent growth media for bacteria, capable of supporting survival for long periods of time. Bacteria which can survive within follicular fluid over time can form mature biofilms, which enables persistence. Women undergoing ART cycles have elevated concentrations of steroid hormones within their follicular fluid and this can modulate the ability of some bacterial species to proliferate. The impact of these bacteria on the developing oocyte and ART pregnancy outcomes requires further investigation.

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PLACENTAL HTRA3 IS REGULATED BY OXYGEN TENSION AND SERUM LEVELS ARE ALTERED DURING EARLY PREGNANCY IN WOMEN DESTINED TO DEVELOP PREECLAMPSIA

G. Nie, Y. Li, M. Puryer, L. Salamonsen

Uterine Biology, Prince Henry's Institute of Medical Research, Melbourne, VIC, Australia

The pathogenic origin of preeclampsia is defective placental development (placentation) and function. Preeclampsia is not diagnosed until later in pregnancy and reliable early detection is highly desirable. HtrA3 is a recently cloned gene with high expression during placentation in the mouse, rhesus monkey and human. In human 1st trimester placenta, HtrA3 is highly expressed in maternal decidual cells and in certain trophoblast cell types. Placental HtrA3 is secreted into the maternal circulation and clearly detectable in serum of pregnant women in the 1st trimester. The present study examined placental production and serum profile of HtrA3 across gestation in women, the potential molecular mechanisms regulating HtrA3 production, and association between maternal HtrA3 serum levels and preeclampsia. Immunohistochemistry determined HtrA3 expression pattern and cellular localization in 1st, 2nd and 3rd trimester placenta. Maternal serum HtrA3 levels were analysed by Western blotting. Regulation of placental HtrA3 production and secretion by oxygen tension was investigated in 1st trimester placental explants and trophoblast cells. Placental HtrA3 protein was maximally produced in the 1st trimester, then dramatically down-regulated, especially in the syncytiotrophoblast. HtrA3 was secreted into the maternal circulation with a serum profile reflecting placental production. Oxygen tension regulated HtrA3; low oxygen enhanced, while transition from low-to-high oxygen decreased, HtrA3 protein production in syncytiotrophoblast. Maternal serum HtrA3 levels at ~13–14 weeks of gestation were significantly higher in women who subsequently developed preeclampsia. It appeared that HtrA3 down-regulation was delayed in preeclamptic pregnancies. In conclusion, HtrA3 protein production is closely associated with oxygen tension in the placenta. The decline in HtrA3 at the end of 1st trimester may reflect the placental low-to-high oxygen switch. Abnormally high levels of serum HtrA3 at the end of 1st trimester is associated with preeclampsia.

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DEVELOPMENT OF A VAGINALLY APPLIED, NON-HORMONAL CONTRACEPTIVE: THE CONTRACEPTIVE EFFICACY AND IMPACT ON BONE TURNOVER OF PEGLA, A LONG-ACTING LIF ANTAGONIST

E. M. Menkhorst[1], J. G. Zhang[2], P. O. Morgan[2], I. J. Poulton[3], D. Metcalf[2], L. A. Salamonsen[1], N. A. Sims[3], N. A. Nicola[2], E. Dimitriadis[1]

[1]Prince Henry's Institute, Clayton, VIC, Australia
[2]Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia
[3]St Vincent's Institute of Medical Research, Melbourne, VIC, Australia

The WHO has called for the urgent development of pharmacological, non-hormonal contraceptives. Leukaemia inhibitory factor (LIF) is obligatory for embryo implantation in mice and associated with infertility in women. Injection of a long-acting LIF antagonist (PEGLA) blocks uterine LIF, preventing implantation in mice, making PEGLA a promising non-hormonal contraceptive. LIF and LIFR null mice show decreased bone volume associated with increased osteoclast number and size, suggesting PEGLA may target bone. Vaginally administered PEGLA could be a 'dual-role' contraceptive: delivered in a microbicide which blocks the vaginal transmission of sexually transmitted infections. We aimed to establish the contraceptive efficacy of vaginally administered PEGLA and identify non-uterine targets of PEGLA in mice. PEGLA was administered to mated female mice by intraperitoneal (IP) injection or vaginally (n = 4/group) during the peri-implantation period to determine its effect on implantation and bone turnover. The tissue and blood accumulation of ^{125}I-PEGLA or control was identified at various time-points following IP injection (≤120 h) or vaginal administration (≤24 h) (n = 3/group). PEGLA administered via vaginal gel blocked implantation (0.0+0.0 vs 8.5+0.5) at a lower dose (500 μg) than IP injection (1500 μg). PEGLA administered by IP injection resulted in fewer (4.0+0.3% vs 7.7+1.5%; $P < 0.05$) but larger (20.9+0.9 μm vs 18.1+0.5 μm; $P < 0.05$) osteoclasts and increased trabecular bone volume (6.8+0.9% vs 3.1+1.1%; $P < 0.05$) but vaginally administered PEGLA had no effect on bone ($P > 0.05$). ^{125}I-PEGLA accumulated more quickly (10 min vs 30 min) and was retained longer (96 h vs 24 h) in blood and tissue following IP injection compared to vaginal administration. This is the first study to show the contraceptive efficacy of a PEGylated compound following vaginal delivery. Local delivery of PEGLA decreased the required dose and eliminated the effect on bone, suggesting that local administration would minimise the non-target effects of PEGLA. Contraceptive trials are now required in non-human primates to progress PEGLA towards human clinical trials.

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COMBINATION METHOTREXATE AND EPIDERMAL GROWTH FACTOR RECEPTOR INHIBITION AS A NOVEL MEDICATION-BASED CURE OF ECTOPIC PREGNANCIES

U. Nilsson[1], T. G. Johns[2], T. Wilmann[1], Y. Gao[1], C. Whitehead[1], E. Dimitriadis[3], E. Menkhorst[3], B. Saglam[1], S. Greenall[2], A. Horne[4], S. Tong[1]

[1]Ritchie Centre, Monash Institute of Medical Research, Translational Obstetrics Group, Clayton, VIC, Australia
[2]Monash Institute of Medical Research, Centre for Cancer Research, Clayton, VIC, Australia
[3]Prince Henrys Medical Research Institute, Uterine Biology Group, Clayton, VIC, Australia
[4]The Queen's Medical Research Institute, University of Edinburgh, Centre for Reproductive Biology, Edinburgh, United Kingdom

Ectopic pregnancies are serious gynaecological emergencies that can cause fatal haemorrhage. Most are treated surgically. Given the placenta is heavily reliant on Epidermal Growth Factor Receptor (EGFR) signaling, we set out to develop a medication-based treatment of ectopics using combination gefitinib (EGFR inhibitor) and methotrexate (folate antagonist). Both drugs are well tolerated and available in tablet form. We used in vitro and in vivo approaches to test the ability of gefitinib and methotrexate to regress placental tissue, and to explore molecular mechanisms. In vitro assays included immunohistochemistry (EGFR staining) western blot (EGFR phosphorylation), cell viability assays (Cell-Titre Blue, LDH cytotoxicity assay, xCELLigence system), apoptosis assays (PCR, FACS of M30 antibody) and The Bioplex Platform (phosphorylation of the EGFR pathway). JEG-3 xenografts were used to assess regression of placental tissue in vivo, where serum hCG was also measured (ELISA). EGFR was highly expressed in placentae from ectopic pregnancies. Combination treatment was supra-additive in inducing cell death of placental tissue in vitro, with >70% cell death by 48 hours (Syncytialised BeWos, JEG-3 and 1st trimester trophoblast). This supra-additive effect was demonstrated in both end point assays (cell viability) and regular monitoring using The xCELLigence system. Gefitinib potently blocks EGFR phosphorylation in placental tissues in vitro (Western blot). Both drugs may be converging to inhibit Akt phosphorylation (Bioplex analysis). Combination treatment increases apoptosis (FACS of M30 antibody). In vivo, Gefitinib or MTX as single agents induced significant decrease in xenograft tumour volume in a dose dependent manner ($n \leq 5$ mice per treatment). However, combining these drugs was supra-additive in decreasing xenograph tumour volume and weight. Serum hCG in mice was lowest with combination treatment. Combination gefitinib and methotrexate potently regresses placental tissue. It may be a novel therapeutic approach to cure ectopic pregnancies, potentially replacing surgery with tablets.

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EXTRACELLULAR CALRETICULIN ALTERS ENDOTHELIAL CELL AND TROPHOBLAST CELL FUNCTIONS *IN VITRO* CONSISTENT WITH PRE-ECLAMPSIA

N. M. Gude[1,2], K. E. Crawford[1,2], J. L. Stevenson[1], S. P. Brennecke[1,2]

[1]Perinatal Medicine, Royal Women's Hospital, Parkville, VIC, Australia
[2]Obstetrics and Gynaecology, University of Melbourne, Parkville, VIC, Australia

Pre-eclampsia is a multisystem disorder of human pregnancy that involves abnormal placentation via insufficient trophoblast cell invasion of the maternal spiral arteries and widespread maternal endothelial cell dysfunction. Factors in plasma of pre-eclamptic women affect both trophoblast and endothelial cell functions during in vitro culture (1). The calcium-binding protein calreticulin is elevated in peripheral blood with pre-eclampsia compared to normotensive pregnancy (2). The aim of this study was to determine the effects of exogenous calreticulin at concentrations relevant to normotensive pregnancy (2 μg/mL) and to pre-eclampsia (5 μg/mL) on human trophoblast cell (HTR8) and microvascular endothelial cell (myometrial) numbers and migratory activity. Cell migration was measured by scratch assay; changes in cell number were measured by MTS assay (Promega). The results showed that calreticulin at 5μg/ml did not affect HTR8 cell number (control 68 044+24 542 cells, with calreticulin 72 810+30 673 cells, $n = 3$, $P > 0.05$) after 48 hours, but significantly inhibited migration of the cells by 48+11% compared to the control at 26 hours ($n = 4$, $P < 0.02$). Calreticulin at 5 μg/mL and under conditions that did not change cell number significantly increased migration of the myometrial endothelial cells by 39+7% ($n = 4$, $P < 0.01$) at 20 hours. Calreticulin at 5 μg/mL, however, significantly reduced endothelial cell numbers after 3–5 days (control 6213+1937 cells, with calreticulin 1937+728 cells, $n = 6$, $P < 0.05$). There was no significant change to the functions of either cell type with 2 μg/mL of calreticulin. In conclusion, exogenous calreticulin at a concentration consistent with that found in maternal blood with pre-eclampsia was shown to alter trophoblast and endothelial cell migratory activity and reduce endothelial cell numbers during in vitro culture. These results indicate that elevated circulating calreticulin may contribute to the cellular mechanisms that underlie the development of pre-eclampsia.

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UNDERSTANDING THE CROSSTALK IN THE HUMAN UTERINE CAVITY: ROLES FOR SOLUBLE MEDIATORS DURING EMBRYO IMPLANTATION

N. Hannan[1,2], P. Paiva[2], K. L. Meehan[2], C. Hincks[2], L. J.F. Rombauts[3], D. K. Gardner[1], L. A. Salamonsen[2]

[1]Zoology, University of Melbourne, Parkville, VIC, Australia

[2]Uterine Biology, Prince Henry's Institute, Clayton, VIC, Australia

[3]Monash IVF, Clayton, VIC, Australia

Embryo implantation requires synchronized dialogue between a receptive endometrium and an activated blastocyst via locally produced soluble mediators. During the mid-secretory (MS) phase of the menstrual cycle there is increased glandular secretion into the uterine lumen. These secretions contain important mediators that modulate the endometrium and support the conceptus during implantation. Analysis of the composition of uterine fluid across the menstrual cycle and in fertile and infertile women will, therefore, provide new insights into uterine receptivity. We hypothesized that multiplex platform analysis of human uterine lavages would identify soluble mediators important for the establishment of pregnancy in humans. Lavages were collected (by flushing the uterine cavity with 5 mL of saline) from fertile and infertile women during the MS phase and from fertile women during the mid-proliferative (MP) phase of the menstrual cycle. Comparison of lavages from the three cohorts was performed using quantitative MilliplexTM Luminex® cytokine/chemokine assays containing 42-analytes. Luminex analysis detected a number of cytokines in uterine fluid, revealing 8 soluble mediators previously unknown in the endometrium and present in human uterine fluid including, PDGF-AA, TNFβ, sIL-2Rα, Flt-3 ligand, sCD40L, IL-7, IFNα2 and GRO. Furthermore comparison of the three cohorts revealed VEGF levels were significantly higher in the fertile (MS) fluid when compared to infertile. Functional studies demonstrated that rhVEGF treatment significantly increased the adhesive properties in cells present at the maternal-fetal interface. These findings suggest VEGF plays a role in regulating embryo implantation. Furthermore identifying the soluble mediators in uterine fluid may provide potential markers of endometrial receptivity, insight into the unique microenvironment essential for pregnancy and a profile of maternal factors that influence the implanting blastocyst.

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IDENTIFICATION OF SCAFFOLDING PROTEINS AS PC6 SUBSTRATES IN THE HUMAN ENDOMETRIAL EPITHELIAL CELLS FOR EMBRYO IMPLANTATION

S. Heng, Y. Li, A. N. Stephens, A. Rainczuk, G. Nie

Prince Henry's Institute of Medical Research, Clayton, VIC, Australia

Successful embryo implantation is an important step in establishing pregnancy, which requires a healthy embryo and a receptive endometrium. Establishment of endometrial receptivity involves morphological and physiological changes initially in the endometrial epithelium, however the underlying molecular mechanisms are not fully understood. We have previously demonstrated that proprotein convertase 5/6 (PC6), a member of the proprotein convertase (PC) family, is up-regulated in the endometrium specifically at implantation in association with epithelial differentiation in the human and monkey. PCs convert a range of precursor proteins of important functions into their bioactive forms, they are thus regarded as critical "master switch" molecules. The aim of this study was to identify target proteins of PC6 in the endometrial epithelial cells important for implantation. We used a HEC1A cell line in which PC6 was stably knocked down by siRNA approach (HEC1A-PC6). HEC1A cells that were similarly transfected with a scrambled siRNA sequence (HEC1A-control) were used as the control. Previous study confirmed that HEC1A-PC6 cells had much reduced capacity to adhere to blastocyst. A proteomic comparison between HEC1A-PC6 treated with or without human recombinant PC6 identified ezrin as a potential PC6 substrate. Ezrin is a cytoplasmic protein which is known to bind to ezrin-radixin-moesin-binding phosphoprotein 50 (EBP50) thereby translocating to the plasma membrane. This complex has been associated with cytoskeletal re-organisation and changes in cell polarity. Co-immunoprecipitation of ezrin and EBP50 showed that knockdown of PC6 allowed the binding of ezrin to the C-terminus of EBP50 in HEC1A-PC6, whereas PC6 cleavage of EBP50 in HEC1A-control prevented the binding. This was also confirmed by immunofluorescence showing that ezrin and EBP50 were co-localized to the plasma membrane in HEC1A-PC6. This study thus identified that PC6 regulates scaffolding proteins such as EBP50 and ezrin in the endometrium for embryo implantation.

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LYMPHATICS IN THE HUMAN PLACENTAL BED AND SURROUNDING THE SPIRAL ARTERIOLES DISAPPEAR DURING ENDOMETRIAL DECIDUALISATION

M. Volchek[2], J. E. Girling[1], G. E. Lash[3], L. Cann[1], B. Kumar[2], S. C. Robson[3], J. N. Bulmar[3], P. A.W. Rogers[1]

[1]Obstetrics & Gynaecology, Monash University, Clayton, VIC, Australia

[2]Anatomical Pathology, Monash Medical Centre, Clayton, VIC, Australia

[3]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom

The mammalian placenta plays central roles in maternal tolerance of the semi-allogeneic fetus and fluid balance between maternal and fetal compartments. The lymphatics play a role in both immune function and fluid balance. The aim of this study was to describe the distribution of lymphatic vessels in human placental bed and decidua, with particular focus on the lymphatics that surround the remodelling spiral arteries during decidualisation and trophoblast invasion. Placental bed, non-placental bed and decidual biopsies were obtained from women undergoing elective pregnancy termination (6–18 weeks gestational age) and from women undergoing elective caesarean section at term. Double immunohistochemical labeling was performed on serial sections to identify lymphatic vessels in conjunction with blood vessels, smooth muscle, epithelial and trophoblast cells, or proliferating cells. Using representative photomicrographs, descriptive findings of the organisation of the human placental bed lymphatics were made. Lymphatic vessels positive for podoplanin (D2-40) were abundant in hypersecretory endometrium (lacking stromal decidualisation) at all stages of gestation. By contrast, the decidua was nearly always devoid of lymphatics. In some samples, structures that appeared to be regressing lymphatics were observed at the boundary between hypersecretory and decidual tissues. Lymphatic vessels were notably absent from the vicinity of spiral arteries that were surrounded by decidualised stromal cells. Lymphatic vessels in hypersecretory endometrium appeared larger and more elongated as gestation progressed. Proliferating lymphatic vascular endothelial cells were identified in both large vessels, and in streaks of D2-40 positive cells that could have been newly forming lymphatic vessels. In conclusion endometrial stromal cell decidualisation results in a loss of lymphatics, with this phenomenon being particularly apparent around the spiral arterioles; the functional consequences of this loss have yet to be elucidated.

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THE UNFOLDED PROTEIN RESPONSE MAY CONTRIBUTE TO GLUCOCORTICOID-INDUCED PLACENTAL GROWTH RESTRICTION IN THE RAT VIA INCREASED PLACENTAL EXPRESSION OF HEAT SHOCK PROTEIN 70

P. J. Mark, J. L. Lewis, M. L. Jones, B. J. Waddell

School of Anatomy & Human Biology, The University of Western Australia, Nedlands, WA, Australia

Perturbations of normal endoplasmic reticulum (ER) physiology occur in a number of pathological conditions, including diabetes and preeclampsia. These pathologies are associated with elevated levels of inappropriately folded proteins and induction of ER stress. Accumulation of misfolded proteins induces the unfolded protein response which increases ER protein folding capacity and promotes ER-associated degradation of unfolded proteins. Glucocorticoids are essential for maturation of fetal organs, however excess exposure during pregnancy retards fetal and placental growth. Glucocorticoids also induce ER stress within macrophages, which reside within the placenta, and activate immune responses which can lead to oxidative stress and subsequent placental dysfunction. We hypothesised that excess glucocorticoid exposure would induce ER stress within the placenta and contribute to restriction of fetal and placental growth. This study compared placentas (n = 6/group) for control (Con) and dexamethasone-exposed pregnancies (Dex; 0.75 μg/mL drinking water from day 13 of gestation) at days 16 and 22 of gestation in the rat (term = 23 days). Placentas were dissected into junctional (JZ) and labyrinth (LZ) zones for separate analysis. Quantitative PCR was used to determine expression of mRNA for markers of ER stress, including heat shock factors (HSF-1 and HSF-2), heat shock proteins (HSP-70 and HSP-90) and C/EBP homologous protein (CHOP10). HSF-1 expression increased 2- to 4-fold from day 16 to 22 in both placental zones, but was not increased by glucocorticoids. Dex-exposure increased HSP-70 expression 2- to 3-fold in the LZ at both days of gestation, indicative of an ER stress response. Similar patterns for JZ expression of HSP-70 were observed. JZ expression of HSP-90 was also upregulated by Dex at day 22 but not day 16. CHOP10 was not induced by Dex-administration in either zone at either gestational time, which suggests that rather than activation of the ATF6/PERK pathway, the activation of ER stress is likely to be via XBP1 induction.

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EXTRACELLULAR MATRIX DYNAMICS IN SCAR-FREE ENDOMETRIAL REPAIR

J. Evans[1], T. Kaitu'u-Lino[2], L. A. Salamonsen[1]

[1]Prince Henry's Institute of Medical Research, Clayton, VIC, Australia
[2]Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia

Tissue destruction and repair occur simultaneously within the menstruating uterus. Whilst the factors that control menstruation are increasingly understood (1) the endometrial milieu which governs repair remains elusive. The extracellular-matrix (ECM) plays a dynamic role within the repairing endometrium, with roles suggested for fibronectin and certain integrins (2). We here use both an in vivo mouse model of breakdown and repair (3) and an in vitro model of re-epithelialisation to determine factors involved in repair of this tissue. Murine uterine horns were subjected to laser-capture microdissection of the repairing luminal epithelium (LE) and immediate sub-luminal stroma. Extracted RNA was processed for pathway-focused array that revealed expression of epithelial and leukocyte expressed integrins along with their ligands, proteases and protease inhibitors and basement membrane proteins as elevated in repairing uterine horns. Immunohistochemistry confirmed expression and localisation of integrins a 5 and b 1 in addition to integrin ligands VCAM-1 and fibronectin. Additionally, we demonstrated intimate association of neutrophils with repairing/recently repaired luminal epithelium. To confirm the relevance of these ECM interactions in a human model, human endometrial luminal epithelial cell (ECC-1) monolayers were wounded and treated with inhibitors of MMPs (doxycycline, GM6001) or integrin-fibronectin interactions (RGDS). Significant inhibition of in vitro re-epithelialisation was seen following treatment of wounded ECC-1 monolayers with RGDS, doxycycline or GM6001, suggesting a requirement for these ECM components in endometrial re-epithelialisation. We subsequently demonstrated that menstrual blood derived factors may play a role in the initial endometrial re-epithelialisation. Menstrual blood and peripheral blood were collected on the same day from volunteers. Treatment of wounded ECC-1 monolayers with menstrual blood plasma enhanced the rate of repair compared with peripheral blood plasma from the same woman. Enhanced understanding of the mechanisms governing scar-free repair in the endometrium may help resolve pathological endometrial bleeding disorders and may be applied to aid healing in other tissues.

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THE ROLE OF PHAGOCYTOSIS OF APOPTOTIC SYNCYTIAL KNOTS IN THE PREVENTION OF ENDOTHELIAL CELL ACTIVATION: AN IMPORTANT ADAPTATION FOR NORMAL PREGNANCY

Q. Chen[1], H. Jin[2], P. Stone[1], L. Chamley[1]

[1]Obstetrics & Gynaecology, The University of Auckland, Auckland, New Zealand
[2]The Obstetrics & Gynaecology Hospital, Fudan University, Shanghai, China

Preeclampsia is characterised by an exaggerated inflammatory response and maternal endothelial cell activation. Syncytial knots, dead multinucleated fetal cells shed from the placenta in large numbers during all pregnancies, may be phagocytosed by maternal endothelial cells. Our previous studies showed that phagocytosis of necrotic but not apoptotic syncytial knots led to endothelial cell activation. It is known that phagocytosis of apoptotic cells leads to active tolerance of immune responses and in this study we questioned whether phagocytosis of apoptotic syncytial knots leads to suppression of the endothelial cells ability to be activated. Syncytial knots were harvested from 1st trimester placental explants. Monolayers of endothelial cells were pre-treated with apoptotic syncytial knots for 24 hours. After washing, the endothelial cells were treated with the endothelial cell activators LPS, PMA, IL-6, or necrotic syncytial knots for 24 hours. In some experiments the inhibitor of phagocytosis, cytochalasin D, was added into the cultures along with apoptotic syncytial knots. Endothelial cell-surface ICAM-1 was measured using cell based ELISAs. Expression of ICAM-1 by endothelial cells that had phagocytosed apoptotic syncytial knots prior to treatment with LPS, PMA, IL-6, or necrotic syncytial knots was significantly (P =/<0.003) reduced, compared to control endothelial cells that had not phagocytosed apoptotic syncytial knots. Inhibiting phagocytosis of apoptotic syncytial knots with cytochalasin D abolished this protective effect. Our data suggest phagocytosis of apoptotic syncytial knots results in the suppression of the ability of endothelial cells to be activated by a number of potent chemical activators, as well as by the physiologically relevant activator, necrotic syncytial knots. This work suggests that the release of apoptotic syncytial knots from the placenta during normal pregnancy may be a mechanism by which the fetus attempts to protect the maternal vasculature against activation.

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PREDICTING GESTATIONAL HYPERTENSION AND PREECLAMPSIA FROM MATERNAL ANGIOTENSIN II AND ANGIOTENSIN 1–7 LEVELS AT 15 WEEKS GESTATION

S. D. Sykes[1], E. R. Lumbers[1], K. G. Pringle[1], T. Zakar[1], G. A. Dekker[2,3], C. T. Roberts[3]

[1]Endocrinology, Mothers and Babies Research Centre, Hunter Medical Research Institute, John Hunt, New Lambton, Australia

[2]Lyell McEwin Hospital, Adelaide, SA, Australia

[3]Robinson Institute, University of Adelaide, Adelaide, SA, Australia

Angiotensin II (AngII) is the main effector peptide of the renin angiotensin system (RAS). The RAS is also involved in the aetiology of hypertension. Angiotensin 1–7 (Ang1–7) acting on the Mas receptor may counteract AngII effects. RAS activity is increased in early gestation. We wanted to determine if maternal plasma AngII and Ang1–7 levels in early gestation predict the onset of hypertension in late gestation. Circulating AngII and Ang1–7 have been measured by RIA (D Casley, Prosearch Pty. Ltd.) in EDTA treated plasma from healthy nulliparous pregnant women at 15 weeks gestation from the Adelaide SCOPE cohort for preeclampsia (PE) (n = 50) and gestational hypertension (GHT) (n = 50), with 131 controls. Log transformation and linear regression showed an inverse, weak (R^2=0.068), statistically significant relationship (P = 0.003) between AngII and Ang1–7. The predictive capability of these peptides for pregnancy outcome was determined by logistic regression and area under the curve after ROC analysis (AROC). The summaries of these analyses are shown in Table 1. AngII did not increase the predictive strength of either model and was omitted. These models show for the first time that circulating Ang1–7 levels are highly statistically significant predictors of hypertensive diseases of pregnancy as early as 15 weeks gestation, well before diagnosis of disease.

Table 1. Variables predictive of PE and GHT and the subsequent AROC

sBP, dBP = systolic and diastolic blood pressure, respectively; Pr/Cr = urinary protein/creatitine ratio

Preeclamsia model			Gestational hypertension model		
Variable	Odds ratio	95% CI	Variable	Odds ratio	95% CI
Age	0.92	0.85-1.00	Cigarettes	0.96	0.91-1.02
Cigarettes	0.91	0.85-0.98	sBP	1.07	1.02-1.11
sBP	1.05	1.01-1.09	dBP	1.04	0.99-1.10
Pr/Cr	0.91	0.83-1.00	Pr/Cr	0.96	0.88-1.05
Log (Ang 1-7)	3.52	1.70-7.29	Log (Ang 1-7)	2.38	1.21-4.69
AROC	0.75		AROC	0.75	

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FRIEND OR FOE? THE ROLES OF PAF AND P53 DURING EMBRYONIC DIAPAUSE

J. C. Fenelon[1,2], C. O'Neill[3], G. Shaw[1,2], M. B. Renfree[1,2]

[1]ARC Centre of Excellence for Kangaroo Genomics, Australia

[2]Zoology, University of Melbourne, Parkville, VIC, Australia

[3]Royal North Shore Hospital, The University of Sydney, Sydney, NSW, Australia

In the tammar wallaby, *Macropus eugenii*, the blastocyst normally remains in embryonic diapause for 11 months without cell division or apoptosis occurring. Progesterone regulates reactivation by inducing active secretion from the endometrium, but the molecular cross-talk between the endometrium and blastocyst is unknown. This process may involve the phospholipid paf. Paf is an embryotrophin that acts as a trophic/survival factor in the early embryo, partly by inactivating (via the PI3K/Akt pathway) the expression of p53, a cell cycle arrest factor (1,2). *In vitro,* paf production from the tammar endometrium increases after diapause (3). This study examined the expression of the paf receptor (pafr) and p53 in the tammar endometrium and embryo at entry into, during and reactivation from diapause. Both *pafr* and *p53* mRNA were expressed in the endometrium at all stages. However there was no quantitative change in pafr expression. In the endometrium, pafr protein is present on the membrane of the glandular epithelium at all stages examined, but p53 was not expressed in the endometrial nuclei at any stage and hence does not appear to be active. Both *pafr* and *p53* mRNA were also expressed in the embryo from the early cleavage stages, during diapause and in the reactivated blastocyst. Pafr protein was present in the embryo both before and after diapause, but levels were greatly reduced during diapause, indicating it may be necessary for active growth. Unexpectedly, the expression of p53 in the embryo does not appear to depend on the presence or absence of pafr. p53 was expressed in the nuclei of the cleavage stage embryonic cells before diapause, but not during or after diapause. These results suggest that paf and pafr may participate in the molecular control of embryonic diapause in the tammar independent of p53.

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POTENTIAL MARKERS FOR THE PROSPECTIVE ISOLATION OF HUMAN ENDOMETRIAL EPITHELIAL STEM/PROGENITOR CELLS

C. E. Gargett[1,2], C. S.C. Tan[1,2], H. Buhring[3]

[1]The Ritchie Centre, Monash Institute of Medical Research, Clayton, Australia
[2]Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia
[3]CSO Facility for Flow Cytometry and Monoclonal Antibodies, University Clinic of Tubingen, Tubingen, Germany

The human endometrium regenerates each month following menstruation, parturition and in post-menopausal women taking hormone replacement therapy. Adult stem/progenitor cells discovered residing in human and mouse endometrium may be responsible for this regenerative capacity. However, assays used to identify these stem/progenitor cells are retrospective. The aim of this study is to identify surface markers for the prospective isolation of human endometrial epithelial progenitor cells using a panel of 22 antibodies. Flow cytometry and was used in the initial screen and immunohistochemistry was used to reveal the location of marker expression. Multi-colour FACS protocols were developed with promising markers in conjunction with EpCAM (epithelial cell marker) and to exclude endothelial ($CD31^+$), leukocytes ($CD45^+$) and stromal ($CD90^+$) cells. Sorted subpopulations were assessed for clonogenicity and self-renewal activity using in vitro cloning assays. Six antibodies were short-listed. 2D1D12 enriched for progenitor cells that formed epithelial clones in culture (n = 2). The $2D1D12^+EpCAM^+$ fraction produced very few colony-forming units (CFU). $2D1D12^-EpCAM^+$ fraction gave rise to small CFU. However the $2D1D12^+EpCAM^-$ population showed the greatest progenitor activity, producing many large CFU that could be serially cloned twice, indicating self renewal activity. This preliminary data suggests that $2D1D12^+EpCAM^-CD90^-CD31^-CD45^-$ population may enrich for human endometrial epithelial stem/progenitor cells. Importantly, large CFU have previously been reported to exhibit stem cell properties of self-renewal, differentiation and high proliferative potential (1). Future studies will focus on xenografting this population to assess tissue reconstitution ability in vivo. The identification of endometrial epithelial stem/progenitor cell marker(s) will enable their prospective isolation for further characterisation and will assist in the investigation of their potential role in endometrial proliferative disorders such as endometriosis and endometrial cancer.

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INTEGRIN CLEAVAGE IS MEDIATED BY PROPROTEIN CONVERTASE 6 IN HUMAN ENDOMETRIAL EPITHELIAL CELLS FOR ENDOMETRIAL RECEPTIVITY AND IMPLANTATION

S. G. Paule, G. Nie

Implantation and Placental Development, Prince Henrys Institute of Medical Research, Clayton, VIC, Australia

Integrins are transmembrane glycoproteins composed of non-covalently associated α and β chains. Integrins participates in cell–cell interaction and binding to components of the extracellular matrix. Integrin expression changes during the establishment of receptivity for implantation. Integrins $\alpha_1\beta_1$, $\alpha_4\beta1$ and $\alpha_v\beta_3$ are expressed in the endometrium during the window of implantation [1, 2] and deficiency in integrin $\alpha_v\beta_3$ is associated with idiopathic infertility and delayed endometrial maturation [1, 3, 4]. Proprotein convertases (PCs) cleave proproteins at the basic amino acids consensus motif (K/R)-(X)n-(K/R)↓(where n = 0, 2, 4 or 6 and X is any aa) for activation. Integrins require post-translational cleavage for activation and are known to be cleaved by PCs. PC6 plays a critical role in the establishment of endometrial receptivity. We hypothesized that PC6 cleaves integrins for its activation in the endometrial epithelium for implantation. The aim of this study was to investigate the functional importance of PC6 in integrin cleavage, using a stable PC6-knockdown in HEC1A cells (HEC1A-HP) by siRNA technology. Cells transfected with a scrambled siRNA sequence (HEC1A-control) were used as the control. To determine the amount of functional integrins on the cell surface, HEC1A-PC6 and HEC1A-control were subjected to an integrin monoclonal antibody array. The amount of functional integrins α_1, α_3, α_5, α_v, α_v, β_3, β_1, β_3, β_4 and $\alpha_5\beta_1$ on the cell surface was much less in HEC1A-HP than HEC1A-control cells. Western blot analysis confirmed that cleavage of pro-integrin α_5 into disulfide-linked heavy chain (110kDa) and light chain (35kDa) was greater in HEC1A-control compared to HEC1A-HP cells, suggesting that knockdown of PC6 affects integrin cleavage. Our studies imply that integrin cleavage is mediated by PC6 in endometrial epithelial cells for the establishment of receptivity for embryo implantation.

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LOCATION OF ACTIVE TGFB1 IN THE MAMMARY GLAND DURING DIFFERENT STAGES OF DEVELOPMENT

X. Sun[1,2], S. A. Robertson[1,2], W. V. Ingman[1,2]

[1]Obstetrics and Gynaecology, University of Adelaide, Adelaide, SA, Australia
[2]Research Centre for Reproductive Health, Robinson Institute, Adelaide, SA, Australia

Development of the mammary gland involves complex interactions between epithelial and stromal cells under the influence of hormones and cytokines. Transforming growth factor beta 1 (TGFB1) is a multi-functional cytokine that we have reported to be essential for normal mammary gland development. TGFB1 is produced and secreted as part of a latent complex, and requires activation at the site of action to have biological effects. In situ hybridisation studies have shown mRNA encoding *Tgfb1* is mainly expressed by mammary epithelium; however, the expression pattern of active TGFB1 in the mammary gland during different developmental stages is still unclear. Mammary gland tissue was collected from mice at puberty (5 weeks old), adult diestrus and late pregnancy (day 18 pc). Frozen sections were stained with antibody specifically reactive with active TGFB1 protein (not latent TGFB1 or other TGFB isoforms) for immunofluorescent analysis. Qualitative assessment of the staining revealed different patterns of active TGFB1 localisation depending on developmental stage. The strongest expression of active TGFB1 was observed in the mammary gland at diestrus compared to puberty and pregnancy. At diestrus, active TGFB1 was located around the surface of mammary epithelial cells. The staining was heterogeneous, with distinct zones of active TGFB1 accumulated around some but not all epithelial cells. During puberty, active TGFB1 was observed only within the lumen of the ducts. During late pregnancy, TGFB1 was homogenously distributed within the alveolar epithelium. The different patterns of active TGFB1 observed during puberty, diestrus and pregnancy suggest that TGFB1 has different roles in the mammary gland dependent on developmental stage.

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EMBRYO IMPLANTATION IS ASSOCIATED WITH SPECIFIC EXPRESSION OF PROPROTEIN CONVERTASE 6 IN THE RABBIT UTERUS

Y. Li[1], P. K. Nicholls[1], S. Heng[1], Z. Sun[2], J. Wang[2], G. Nie[1]

[1]Uterine Biology, Prince Henry's Institute of Medical Research, Melbourne, VIC, Australia
[2]Shanghai Institute of Planned Parenthood Research, Shanghai, China

Proprotein convertase 5/6 (PC6) is a member of the proprotein convertase family that endoproteolytically cleave latent precursor proteins into their biologically active state. We have previously demonstrated that endometrial PC6 is critical for embryo implantation in mice and primates, including human. PC6 regulates the endometrial physiology specifically at implantation in association with epithelial differentiation during the establishment of endometrial receptivity (in human and monkey) and stromal cell decidualization (in the mouse, human and monkey). PC6 was further confirmed to be a unique PC member that is tightly regulated in the endometrium in relation to implantation. Our further studies (unpublished) suggest that PC6 regulates adhesion molecules in the endometrial epithelium for implantation in women. It is known that between the mouse and human, the endometrial stroma-mediated responses are similar whereas the epithelial cells behave differently. Because PC6 regulates primarily the stromal component (decidualization) in the mouse, in vivo mouse models are critical to investigate the roles of PC6 in decidualization. To address the function of PC6 in endometrial epithelium, non-mouse models relevant to human implantation are required. The rabbit is regarded as an excellent model to study the molecular events of embryo adhesion and attachment. The current study aimed to determine the expression pattern and localisation of PC6 in the rabbit uterus during early pregnancy. Quantitative RT-PCR analysis showed that PC6 mRNA expression was dynamically up-regulated in the rabbit uterus immediately prior to implantation. Western blotting and immunohistochemical analyses demonstrated that PC6 protein was predominantly localised to the basal glands throughout pregnancy, and up-regulated specifically in the epithelium at the embryo attachment site. These findings suggest that PC6 may play an essential role in rabbit implantation, and that the rabbit is a useful animal model to investigate the function of PC6 during embryo attachment.

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MUSASHI FAMILY OF RNA BINDING PROTEINS: CELL CYCLE REGULATORS IN SPERMATOGENESIS

E. A. McLaughlin[1,3], B. A. Fraser[1,3], V. Pye[1,3], M. Bigland[1,3], N. A. Siddall[2,3], G. R. Hime[2,3]

[1]Environmental & Life Sciences, University of Newcastle, Callaghan, NSW, Australia
[2]Anatomy and Developmental Biology, University of Melbourne, Parkville, VIC, Australia
[3]ARC Centre of Excellence in Biotechnology & Development, Australia

Mammalian meiosis is a tightly regulated process involving specialized cell cycle progression and morphogenetic changes. We have demonstrated that the Musashi family of RNA binding proteins is implicated in the regulation of spermatogonial stem self renewal and germ cell differentiation. Here we describe the novel mechanism by which the Musashi family proteins, Msi1 and Msi2, act to control exit from spermatogonial mitotic amplification and normal entry into meiosis. Gene and protein analysis indicated overlapping Msi1 and Msi2 profiles in enriched populations of isolated germ cells and reciprocal subcellular expression patterns in spermatogonia and pachytene spermatocytes/ round spermatids in testes sections. Recombinant Msi1 protein-RNA pulldown and microarray analysis coupled with in vitro shRNA knockdown studies in spermatogonial culture and subsequent immunoprecipitation and qPCR established that Msi1 targeted Msi2 mRNA for post transcriptional translational repression. Immunoprecipitation of Msi2 target mRNA and subsequent qPCR together with in vitro shRNA knockdown studies in round spermatid culture identified a cell cycle inhibitor protein CDKN1C ($p57^{kip2}$) as the principal target of Msi2 translational inhibition. Immunolocalisation of CDKN1C protein indicated that expression of this cell cycle regulator coincided with the nuclear import of Msi1 and the appearance of cytoplasmic Msi2 expression in early pachytene spermatocytes. Using a transgenic Msi1 overexpression mouse model in conjunction with quantitative gene and protein expression, we confirmed Msi1 targeting of Msi2 and subsequent Msi2 targeting of CDKN1C for translational repression in vivo. Ectopic overexpression of Msi1 in germ cells induces substantial Msi2 downregulation and aberrant CDKN1C expression, resulting in abnormal spermatogenic differentiation, germ cell apoptosis/arrest and sterility. In conclusion, our results indicate a sophisticated molecular switch encompassing cell cycle protein regulation by Musashi family proteins, is required for normal exit from mitotic division, entry into meiosis and post meiotic germ cell differentiation.

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TGFB 2-BETAGLYCAN REGULATE FOETAL TESTIS DEVELOPMENT *IN VITRO*

M. A. Sarraj, A. Umbers, J. K. Findlay, K. L. Stenvers

Reproductive Development, Prince Henry's Institute, Clayton, VIC, Australia

Betaglycan is a co-receptor for the TGFβ superfamily, known to modulate TGFβ binding in target cells. We have previously found that betaglycan null murine testes at 12.5–13.5 dpc display poorly delineated seminiferous cords and disrupted Leydig cell development (1). Both TGFβs and inhibins are expressed by the fetal testis and it is currently unclear which regulate its development. We tested the hypothesis that loss of betaglycan compromises the functions of TGFβ2 in the differentiating fetal testis as TGFβ2 is known to bind poorly to its type II receptor in the absence of betaglycan. We tested the effect of TGFβ2 on betaglycan wildtype and null foetal gonad/mesonephros complexes using hanging drop or agar block culture methods. From each embryo, one gonad acted as a control; the other was treated. Gonads were cultured in the presence or absence of TGFβ2 (2.5–5 ng/mL) for 48 hours (n =3 pairs). In both culture methods, development in the absence of exogenous growth factor recapitulated normal cord development in wildtype testis and the disrupted cord phenotype in null testes. TGFβ2-treated cultures, 13.5 dpc wildtype mouse testes displayed a 14–35% reduction in total area compared to untreated cultures. Null testes exhibited significantly smaller reductions in gonadal area (2–13%; $P < 0.01$), indicating that betaglycan null testes exhibit reduced sensitivity to TGFβ2-mediated growth inhibition. However, preliminary observations suggest that TGFβ2 treatment partly rescued cord formation in two of three betaglycan knockout testes in vitro, with testis morphology confirmed by laminin and AMH immunostaining. These data support the notion that TGFβ2 acts via betaglycan to regulate cord development during foetal testis development.

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(1) Sarraj et al., 2010. Biol Reprod; 82(1): 153–62.

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THE MECHANISM OF SPERMATID MATURATION – A LINK TO TUMOUR SUPPRESSION

D. Jamsai[1,2], S. J. Smith[1,2], A. E. O'Connor[1,2], D. J. Merriner[1,2], C. Borg[1,2], B. Clark[1], V. Adams[3], C. J. Ormandy[4], M. K. O'Bryan[1,2]

[1]Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC, Australia
[2]The ARC Centre of Excellence in Biotechnology and Development, Monash University, Clayton, VIC, Australia
[3]The Australian Phenomics Facility, The Australian National University, Canberra, ACT, Australia
[4]The Garvan Institute of Medical Research, Sydney, NSW, Australia

To comprehensively uncover novel male fertility regulators, we utilised an unbiased forward genetic screen, ENU mutagenesis. Using this approach, we have identified several novel infertile mouse lines including a male-specific infertile line that we designated "Joey". The mutant Joey mice produced no sperm due to an arrest of male germ cells at the round spermatid stage. The mutation was identified in the RNA binding motif 5 (Rbm5) gene that resulted in an arginine to proline substitution within a highly conserved RNA recognition motif of the protein. The substitution of proline is likely to interfere with RNA binding and/or recognition. In humans, the RBM5 gene maps to a region that is frequently deleted in lung cancers. Ex vivo studies have suggested that RBM5 is a tumour suppressor, apoptosis modulator and RNA splicing regulator. To date, the role of Rbm5 has never been liked to male fertility and the Joey line is the only mouse model of Rbm5 dysfunction. Using our RBM5-specific antibody, we showed that RBM5 is expressed in pachytene spermatocytes and round spermatids. Based on the protein localisation, the proposed role of RBM5 in mRNA processing, the onset of the Joey phenotype, and the site of the identified mutation, we hypothesise that the Rbm5 mutant allele results in a hypomorphic protein, and that RBM5 has an essential role in regulating male germ cell mRNA storage, transport and/or translational regulation of mRNAs that are critical for spermatid maturation. Further, we generated mice compound heterozygous of the Joey Rbm5 mutation and Rbm5 null alleles. We showed that the compound heterozygous males are infertile due to spermatid maturation arrest resembling the Joey mutant males. This result further confirmed the identification of the Rbm5 mutation as a cause of infertility in the Joey mice and a crucial role of Rbm5 in male fertility.

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THE ROLE OF MEGALIN IN PROSTATE DEVELOPMENT OF THE MOUSE

M. Gamat, G. Shaw, M. B. Renfree

Department of Zoology, The University of Melbourne, Parkville, VIC, Australia

Prostatic development is dependent on androgens; but the precise mechanism by which androgens mediate their effect is still unclear. Megalin, a cell membrane transporter, may shuttle sex steroids into cells to regulate androgen-responsive genes responsible for prostatic bud induction in the urogenital sinus (UGS). In megalin knockout mice, testicular descent fails and the vagina fails to open in females, both of which are dependent on sex steroid signalling (Hammes et al. 2005). In this megalin-mediated pathway, SHBG-bound sex steroids bind to megalin, which is internalised. The SHBG-sex steroid complex is released, and the sex steroid is released from SHBG where it can bind to the androgen receptor to regulate androgen responsive genes. Receptor-Associated Protein (RAP) is a molecular chaperone protein that protects newly synthesised megalin from binding to potential ligands in the cytoplasm prior to insertion into the cell membrane. We hypothesised that megalin may shuttle SHBG-bound androgens across the cell membrane. This study characterised the expression and evaluated a possible role for megalin in the development of the mouse prostate. Megalin, SHBG and RAP transcripts were detected in the developing male and female UGS of the mouse from day E14.5 to day E18.5 (when prostatic buds start to form) and in the adult prostate. Megalin, SHBG and RAP protein were localised in the urogenital epithelium. To assess the role of megalin in prostatic development, UGS tissues were incubated with androgens in the presence and absence of RAP. Incubating UGS tissues with RAP did NOT inhibit prostatic bud initiation. Furthermore, in the UGS of megalin knockout mice, prostatic bud formation appeared to be identical to those of wild-type littermates. These results demonstrate that megalin is not involved in prostatic bud initiation. However, the ubiquitous expression of megalin suggests that its role is redundant in the prostate.

(1) Hammes A et al. (2005) Role of endocytosis in cellular uptake of sex steroids. Cell 122(5), 751–62.

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PARENTAL OBESITY RETARDS EARLY EMBRYONIC DEVELOPMENT AND ALTERS CARBOHYDRATE UTILISATION

N. K. Binder[1], M. Mitchell[1,2], D. K. Gardner[1]

[1]Zoology, The University of Melbourne, Parkville, VIC, Australia

[2]School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia

Parental obesity impacts reproductive success and often results in gestational complications. In this study the effects of maternal and paternal obesity on preimplantation embryo quality were investigated through analysis of cell cycle length and carbohydrate utilisation. Zygotes derived from matings of lean or obese C57BL/6J mice were used to evaluate separately maternal and paternal obesity. Embryos were cultured individually, and development monitored with high temporal time-lapse microscopy (every 15 min). After 78 h of culture, glucose consumption and lactate production by expanded blastocysts was determined using ultramicrofluorimetry. Maternal obesity was associated with a significant delay ($P < 0.01$) in pre-compaction cell cycle length of approximately 1.5 h. Post-compaction there was a significant increase ($P < 0.05$) in glucose consumption by embryos from obese mothers compared to control embryos, while the glycolytic rate was unchanged. Paternal obesity was associated with a significant cell cycle delay ($P < 0.05$) of approximately 1 h from the second cleavage stage onwards. Resultant blastocysts showed disproportionate changes in carbohydrate metabolism, with a significantly increased ($P < 0.05$) glycolytic rate compared to control embryos. Metabolic changes were still permissive to blastocyst formation, however cell numbers were significantly reduced ($P < 0.001$) with both maternal (lean: 54.2 ± 0.8 vs obese: 48.4 ± 1.0) and paternal (lean: 60.5 ± 0.09 vs obese: 50.9 ± 0.09) obesity. These data will help to determine the impact of parental obesity on preimplantation embryo physiology. Slow embryonic development and high glycolytic rate have been linked to reduced implantation rates and are general indicators of compromised embryo quality.

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A MODEST INFLAMMATORY INSULT IN THE PRE-IMPLANTATION PERIOD ALTERS OVIDUCT CYTOKINE EXPRESSION AND PROGRAMS FETAL DEVELOPMENT

P. Y. Chin, J. G. Thompson, S. A. Robertson

Obstetrics and Gynaecology, University of Adelaide, Adelaide, SA, Australia

The cytokine milieu surrounding the pre-implantation embryo contributes to programming optimal embryo development. Perturbations to the maternal environment such as infection and inflammation during the pre-implantation period can influence cytokine synthesis and may cause changes in embryo development that compromise pregnancy outcome. We aimed to investigate the effect of an inflammatory insult with LPS during the pre-implantation period on later fetal development and the role of oviduct cytokine expression in mediating this response. LPS (at various doses of 0.5–62.5 μg) was administered i.p. to C57Bl/6 mice on both day 3 and day 4 post coitum (pc). The effects of treatment on day 4 blastocysts and day 18 fetal development were analysed. Blastocysts from LPS-treated mothers showed reduced viability and smaller total cell number, but were only significant when doses of >12.5 μg LPS were administered. This was not a direct effect of LPS, as no effect of LPS on embryo development in vitro was seen, even at very high LPS concentrations (25 μg/mL). At day 18 pc, pregnancy rates and the number of viable fetuses, as well as fetal and placenta weights were all reduced after low dose LPS treatment (0.5 μg) compared to control PBS-treated females. qPCR analysis of day 4 oviduct tissue revealed that administration of 12.5 μg of LPS resulted in increased mRNA expression of cytokines including IL6, TNFA, IL1B, IFNG, IL10 and LIF. Our findings show that a modest pro-inflammatory insult with LPS in the pre-implantation period programs the embryo for later adverse effects on fetal and placental development. The effects of LPS appear to be mediated indirectly via the maternal tract, and altered oviduct cytokine expression which impairs pre-implantation embryo development is implicated as the underlying mechanism. This model will now be utilised to investigate the potential role of specific inflammatory cytokines TNFA and IFNG in mediating this response.

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TRP53-DEPENDENT EXPRESSION OF GENES REGULATING EMBRYO VIABILITY

L. Ganeshan, C. O'Neill

Sydney Centre for Developmental and Regenerative Medicine, University of Sydney, Sydney, NSW, Australia

Embryos from inbred strains are sensitive to the stresses of culture in vitro compared to hybrid strains. This difference provides a powerful model for identifying genetic regulators of embryo viability in vitro. We showed that culture stress can cause the activation of the TRP53 stress response pathway, culminating in poor embryo viability.[1] Directed genomic analysis of potential target genes was undertaken by comparing blastocysts cultured from zygotes of C57BL6.Trp53$^{+/+}$, C57BL6.Trp53$^{-/-}$ and B6CBF2 genetic backgrounds. This comparison allowed genes with altered expression due to culture in susceptible strains to be identified and determined whether their changed expression was primarily due to the actions of TRP53. Culture had no effect on the expression of a number of house-keeping genes tested. Of known TRP53 target genes tested, only Bax showed a negative association with embryo viability. Interestingly, this was only partially reduced in C57BL6.Trp53$^{-/-}$, indicating that other factors account for much of its up-regulation. Pluripotency genes showed a complex picture. Nanog was strongly negatively correlated with viability yet NANOG protein was strongly positively associated, indicating complex regulatory control; Oct4 showed no association yet OCT4 was strongly positively associated; Utf1 was the only pluripotency gene tested that showed Trp53 dependent down-regulation in cultured C57BL6.Trp53$^{+/+}$. Markers of differentiation, Cdx2 (trophectodermal), Gata6 (endodermal) and Brachyury (mesodermal) did not differ between strains. The results identify Trp53-dependent alterations in gene expression in regulators of cell survival (Bax) and pluripotency (Utf1) that may account for the reduction in the capacity of susceptible blastocysts to form a proliferating inner cell mass. The study also identifies for the first time the possibility of complex post-transcriptional regulation of other pluripotency genes (Nanog and Oct4) that may shed future light on the regulation of pluripotency.

(1) Li A, et al. Biol. Reprod. 2007; 76: 362–367.

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ATMOSPHERIC OXYGEN ALTERS THE EMBRYONIC METABOLOME AS QUANTIFIED BY CARBOHYDRATE UPTAKE AND AMINO ACID UTILISATION

P. L. Wale, D. K. Gardner

Zoology, University of Melbourne, Parkville, Australia

Oxygen regulates embryo development at both the cleavage and post compaction stages. In this study we investigated the effects of atmospheric oxygen during the preimplantation stages, on the post-compaction embryonic metabolome through quantification of glucose consumption and amino acid utilization. Zygotes obtained from F1 hybrid mice (C57BLxCBA) were randomly allocated to either 5% or 20% oxygen. In the first experiment, following the first 48 h of culture, embryos were cultured individually in 1 μL drops of modified G2 medium (0.5 mM glucose) and moved to fresh drops of medium every 24 h. The glucose concentration in the spent media samples, including controls containing no embryo, was determined by microfluorimetry. In the second experiment, embryos which had developed to the early blastocyst stage after 72 h were cultured for a further 24 h in groups of 10 in 2 μL drops of G2. Analysis of amino acid utilization was performed using liquid chromatography-triple quadrupole mass spectrometry. Glucose consumption by embryos cultured in 5% oxygen was significantly greater on day 4 and day 5 (4.89 ± 0.29 and 6.13 ± 0.41 pmol/embryo/h) compared to embryos cultured in 20% oxygen (2.59 ± 0.40 and 5.09 ± 0.28 pmol/embryo/h; $P < 0.05$). In contrast amino acid utilisation by embryos cultured in 5% oxygen was significantly less than embryos cultured in 20% oxygen ($P < 0.05$). The data generated will help to determine the aetiology of oxygen toxicity to the preimplantation embryo. Higher glucose utilisation by embryos in 5% oxygen is consistent with their improved development. Conversely, the increased utilisation of amino acids by blastocysts in 20% oxygen may reflect an adaptation to increased oxidative stress as a result of culture in a non-physiological oxygen concentration. This study demonstrates that atmospheric oxygen during the preimplantation period perturbs the embryonic metabolome which results in a compensatory increase in amino acid utilisation.

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EFFECTS OF SPECIES DIFFERENCES ON OOCYTE REGULATION OF GRANULOSA CELL PROLIFERATION

J. Lin[1], J. Juengel[2], K. McNatty[1]

[1]School of Biological Sciences, Victorica University of Wellington, Wellington, New Zealand
[2]Invermay Agriculture Centre, AgResearch, Dunedin, New Zealand

The role of oocytes in regulating ovulation quota between species is not fully understood. In sheep, the oocyte-derived growth factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) have profound effects on ovarian follicular development and ovulation-quota. The aim of these studies was to compare the ability of oocytes from sheep (low ovulation-rate) and rat (poly-ovulator) to stimulate radiolabelled thymidine uptake by granulosa cells (GC) both within and between the two species. For these experiments, 32 oocytes denuded of cumulus-cells were co-incubated with 20 000 GC from either species. Rat or sheep oocytes stimulated thymidine uptake by GC from the same species ($P < 0.005$). Sheep oocytes also stimulated thymidine uptake by rat GC ($P < 0.001$) but not vice versa. To investigate this further, oocytes and GC were co-incubated with 3.2 μg/mL or 7.6 mg/mL monoclonal antibodies specific to GDF9 or BMP15 respectively and to a hydatids antigen (control). Both sheep and rat oocyte stimulation of thymidine uptake by GC was inhibited with the GDF9 antibody ($P < 0.05$) but not control, irrespective of species of GC. Sheep oocyte stimulation of rat GC was also inhibited using an antibody to BMP15 ($P < 0.05$). However, when using the BMP15 antibody to block the effects of rat oocytes on rat GC, the inhibition of thymidine uptake was modest (i.e. ~10%) albeit significant ($P < 0.05$). The molecular forms of GDF9 and BMP15 in spent media were examined by Western blotting under reducing conditions. For both species, oocyte-secreted GDF9 was present in the mature form. For sheep oocytes, secreted BMP15 was present as promature and monomeric mature forms whereas from rats, trace amounts of mature form was sometimes, but not always, detected. Thus, in sheep both BMP15 and GDF9 are essential for regulating GC proliferation whereas in rats, although responsive to BMP15, GC proliferation is likely regulated primarily by GDF9.

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INHIBITION OF PORCINE OOCYTE NUCLEAR MATURATION *IN VITRO* USING A PHOSPHODIESTERASE INHIBITOR AND AN ADENYLATE CYCLASE ACTIVATOR

M. Bertoldo, T. Sellens, C. G. Grupen

Faculty of Veterinary Science, University of Sydney, Camden, NSW, Australia

Asynchronous nuclear and cytoplasmic maturation is thought to contribute to poor embryo production in vitro. Nuclear arrest is mediated by cAMP and can be maintained within the oocyte using non-specific phosphodiesterase inhibitors (3-isobutyl-1-methylxanthine ; IBMX) and the adenylate cyclase activator forskolin (FSK) (1). The aim of this study was to investigate the effect of IBMX and FSK supplementation on porcine oocyte nuclear maturation during COC recovery and IVM using a defined culture system. In all experiments, cAMP modulators were added to Hepes-buffered media held in collection tubes. COCs recovered from 3-5mm diameter follicles of prepubertal ovaries were cultured in basic maturation media in the absence of FSH. Nuclear maturation was assessed using orcein dye. In Experiment 1, IVM media was supplemented with 0, 50 or 500 μM IBMX. In Experiment 2, IVM media was supplemented with 0, 5, 10, 50 and 100 μM FSK. In Experiment 3, IVM medium was supplemented with combinations of IBMX and FSK to give the treatments; control, 50IBMX/50FSK, 50IBMX/100FSK, 500IBMX/50FSK and 500IBMX/100FSK. Nuclear maturation was assessed at 0, 2, 4 and 18 h after the onset of IVM. At 18 h of culture, there were no differences in the proportion of oocytes supplemented with 0, 50 or 500 μM IBMX reaching MII. Incubation with 10, 50 or 100 μM FSK resulted in 8–16% of oocytes at MII at 18 h compared to the other groups (25–29%; $P < 0.001$). The combinations of IBMX and FSK resulted in greater proportions (86–98%) of oocytes remaining at the GV stage at 18 h compared to the control (16%; $P < 0.001$). There were no differences in the proportion of oocytes remaining at the GV stage at the earlier time points ($P > 0.05$). The results demonstrate that these cAMP modulators, in combination, are highly effective in maintaining porcine oocyte meiotic arrest in vitro for an extended period.

(1) Albuz FK et al., Proceedings of the 25th Annual Meeting of ESHRE, Amsterdam, The Netherlands, 2009.

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ANTIBODIES AGAINST BMPR-IB UNCOVERS A PARACRINE FUNCTION FOR THE RECEPTOR IN MALE MOUSE LEYDIG CELL TESTOSTERONE PRODUCTION *IN VITRO*

I. M. Ciller, U. A. Ciller, J. R. McFarlane

Centre for Bioactive Discovery in Health and Ageing, School of Science and Tech., University of New England, Armidale, NSW, Australia

The male reproductive system is regulated by pituitary gonadotrophins and local testicular factors including the transforming growth factor-β superfamily members which up-regulate and modulate testosterone production respectively. Type I and type II bone morphogenetic protein (BMP) receptors have been identified in both steroidogenic and non-steroidogenic cells in the testis and have been reported to impact steroid production via their effect on steroidogenic enzymes. In this study we investigated if BMPR-IB had an autocrine or paracine role in testosterone production using BMPR-IB antibodies in testis tissue and Leydig cell culture in vitro. Immature (21d) and mature (60d) mice were sacrificed by CO2 asphyxiation and the testis removed, decapsulated and cultured in basal and equine chorionic gonadotrophin (eCG)-conditioned media for three hours at 32°C in 5% CO2, in the presence and absence of anti-BMPR-IB. Additionally, Leydig cells were Percoll purified from adult mouse testicular interstitial cells and cultured for three hours with and without anti-BMPR-IB under human chorionic gonadotrophin (hCG) stimulated or unstimulated conditions. After three hours of incubation the culture media was aspirated into labeled vials and assayed for testosterone using a radioimmunoassay. In adult testicular slice culture, treatment with anti-BMPR-IB resulted in a significant decrease in basal and eCG-stimulated testosterone production by 37% and 41% respectively, while having no significant effect on basal or eCG-stimulated testosterone production by the immature testis. In purified Leydig cell culture from adult male mice BMPR-IB immunization had no effect on testosterone production in basal or hCG-stimulated conditions. In conclusion, anti-BMPR-IB significantly reduced testosterone production in adult testicular slice culture but not in cell culture, demonstrating that BMP paracrine signalling from the seminiferous tubules is likely to be important in modulating testosterone production by Leydig cells. Additionally, the paracrine signalling appears to be developmentally regulated only occurring in the adult testis.

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THE CHAPERONIN CONTAINING TCP-1 (CCT/TRIC) MULTISUBUNIT COMPLEX IS INVOLVED IN MEDIATING SPERM-OOCYTE INTERACTIONS

M. D. Dun[1,2], R. Aitken[1,2], B. Nixon[1]

[1]Reproductive Science Group, School of Environmental and Life Sciences, Discipline of Biological Sciences, Callaghan, NSW, Australia

[2]ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Mammalian spermatozoa only express their capacity for fertilization following capacitation, a process characterized by a suite of biophysical and biochemical changes that occurs as the cells ascend the female reproductive tract. A key event associated with the attainment of a capacitated state is a dramatic reorganization of the sperm surface architecture to render these cells competent to bind to the protective matrix of the oocyte, the zona pellucida. Our previous analysis of these remodeling events has provided compelling evidence that they include the assembly and/or presentation of multimeric protein complexes on the sperm surface. In addition, we have demonstrated that at least two of these complexes possess strong affinity for solubilized zona pellucida. In our current study we have utilised mass spectrometry analysis to reveal that one of these complexes comprises the eight subunits that form a composite, multimeric structure known as the chaperonin containing TCP-1 (CCT/TRiC) complex. Our collective data suggest that this complex participates indirectly in zona pellucida interaction, possibly through the conveyance of key zona adhesion molecules to the sperm surface during capacitation. Consistent with this notion, we were able to demonstrate that the sperm CCT/TRiC complex releases its bound substrates upon exposure to ATP, and this treatment induced a significant, concomitant reduction in the ability of capacitated sperm to bind to the zona pellucida. Furthermore, the use of immunoprecipitation assays confirmed the interaction of the CCT/TRiC complex with at least one putative zona pellucida receptor candidate, namely zona pellucida binding protein 2 (ZPBP2). Future work is now aimed at identifying additional zona receptors that may reside within this complex and the pathways that regulate its functional assembly.

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PURIFICATION AND CHARACTERISATION OF MOUSE TESTICULAR MACROPHAGES: GENE EXPRESSION RESPONSE TO LIPOPOLYSACCHARIDE ACTIVATION INDICATES AN IMMUNOSUPPRESSIVE PHENOTYPE

W. R. Winnall[1], J. Gould[2], J. A. Muir[1], P. Hertzog[2], M. P. Hedger[1]

[1]Centre for Reproduction and Development, Monash Institute of Medical Research, Clayton, VIC, Australia
[2]Centre for Innate Immunity and Infectious Diseases, Monash Institute of Medical Research, Clayton, VIC, Australia

Studies on rat testicular macrophages (TMs) have indicated that these cells play an important role in testis function by supporting the immunosuppressive environment that protects developing germ cells and by responding to pathogens. By comparison, mouse TMs are essentially uncharacterised due to difficulties in isolating sufficient cells for study. We have established a technique for isolating 95% pure TMs from adult mice by differential adherence. Mouse TMs were cultured for 3 h with saline, 10 or 100 ng/mL lipopolysaccharide (LPS) and compared with resident peritoneal macrophages (PMs) and bone marrow-derived macrophages (BMMs). Expression of inflammatory regulators was determined using real-time Q-PCR and Agilent™ microarray analysis. Microarray analysis indicated that each macrophage type displayed very distinct gene expression profiles. There were 526 genes uniquely expressed in TMs at basal levels compared with the other macrophages and 268 genes uniquely expressed by TMs after LPS treatment. Q-PCR determined that LPS induced expression of the anti-inflammatory cytokine interleukin (IL)-10 in each of the macrophage types, with BMMs the strongest responders. LPS stimulated IL-10 mRNA approximately 100-fold in TMs, but only 20-fold in PMs. The anti-inflammatory transforming growth factor-β1 was not significantly induced at this time-point in any macrophage type. In terms of pro-inflammatory mediators, the TM response to LPS was always lower compared to the BMMs. Compared to PMs, the responses of TMs were similar for the hallmark pro-inflammatory cytokine tumour necrosis factor- a, but 40% less for IL-1β. TMs were also deficient in production of IL-6 and cyclooxygenase-2 and IL-12. TMs were therefore relatively strong responders to LPS in terms of IL-10, but weak responders in terms of pro-inflammatory mediators, indicating an immunosuppressive phenotype. The isolation and gene measurement methods established in this study will allow us to use knockout and transgenic mouse models to determine the role for TMs in testicular inflammation/fibrosis models.

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THE CONSEQUENCES OF ACRYLAMIDE EXPOSURE ON THE MALE GERMLINE

B. J. Nixon[1,2], B. Nixon[1], S. D. Roman[1,2]

[1]The Reproductive Science Group, School of Environmental and Life Sciences, Discipline of Biological Sciences, Callaghan, NSW, Australia
[2]ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia

Acrylamide is a common industrial compound that has recently been identified in cooked, carbohydrate-rich foods such as potato chips, breads and cereals. Acrylamide has been found to be a reproductive toxin in rodents, eliciting male infertility and transgenerational toxicity through the male germline; thus dietary exposure to the compound may have consequences for male fertility and reproduction in humans. The aim of this project was to elucidate the mechanisms of acrylamide toxicity in male germ cells of mice. Freshly isolated early male germ cells were exposed to acrylamide and assessed for cell viability and aberrant morphology. DNA damage in these cells was also investigated using a modified version of the Comet Assay, which allows for adduct specificity. Significant increases in cell death or aberrant morphology were not observed following acrylamide exposure (1 μM, 18 hours). However, a significant increase in DNA damage (125% increase in mean tail DNA assessed by Comet) was identified; which may originate from either the metabolic conversion of acrylamide to glycidamide, leading to glycidamide adducts, or from oxidative stress. Additionally, the regulation of cytochrome P450 gene expression was measured using real time PCR and early male germ cells were found to upregulate gene expression of cytochrome P450 enzymes in response to acrylamide exposure. Collectively, these results support a genotoxic mode of action of acrylamide toxicity, in addition to potential oxidative damage in male germ cells. However, the mechanism by which acrylamide elicits toxicity in germ cells requires further investigation. Future outcomes of this research may provide insight into factors necessary for the healthy development of offspring and aid in the risk assessment of dietary acrylamide exposure in humans.

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SIRT6 PROTEIN IS REDUCED IN TESTES AND SPERM FROM OBESE MALE MICE

N. O. Palmer[1], T. Fullston[1], M. Mitchell[1], M. Lane[1,2]

[1]School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia
[2]Repromed, Dulwich, SA, Australia

Obesity in males is associated with altered hormone levels, reduced sperm function and increased sperm DNA damage. However, the underlying molecular mechanism has not been identified. Mammalian SIRT6 protein exhibits caloric intake dependant DNA damage repair in other tissue types. However, a possible role for SIRT6 in male obesity and subfertility has not been considered. Therefore, the aim of this study was to assess the effects of male obesity on SIRT6 in testes and mature sperm. Five week old C57BL6 male mice (n = 10 per diet) were fed either a control diet (CD) (6% fat) or a high fat diet (HFD) (21% fat) for 16 weeks before collection of sperm and testes. There was no difference in Sirt6 mRNA levels as determined by qPCR in testes from HFD males. Immunohistochemistry showed SIRT6 localised to the nucleus of transitioning spermatids from late round spermatids until early elongating spermatids. SIRT6 relative fluorescence of these positive cell types was significantly decreased by 22% in males fed the HFD compared to CD (P < 0.05). This was confirmed by a decrease in total SIRT6 protein in testes from HFD males as detected by an immunoabsorbance assay (P < 0.05). Surprisingly, SIRT6 was only present in the acrosome of mature sperm. Acrosomal localisation was confirmed by the loss of SIRT6 staining after an induced acrosome reaction. SIRT6 levels in the acrosome of mature sperm was decreased by 11% in males fed the HFD (P < 0.05). This is the first study to show that SIRT6 is located to the acrosome of mature sperm, specific cells within the testes and is reduced in an obese state. Furthermore, this study suggests a possible role for SIRT6 in the acrosomal reaction and therefore potentially fertilisation, processes which are known to be reduced by male obesity.

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ROLE OF CRISP4 IN ION CHANNEL REGULATION AND MALE REPRODUCTION

A. J. Koppers[1], G. M. Gibbs[1], T. Reddy[1], P. McIntyre[2], M. K. O'Bryan[1]

[1]Department of Anatomy and Developmental Biology, Monash University, Melbourne, VIC, Australia
[2]Department of Pharmacology, University of Melbourne, Melbourne, VIC, Australia

CRISPs are a group of 3 proteins found in mammals (4 in the mouse) which show a strong expression bias in the male reproductive organs. Whilst the function of most CRISPs are yet to be elucidated, mouse CRISP2 is a known regulator of the ion channel, ryanodine receptor. CRISP4 is most abundantly produced by the principal cells of the epididymis and are secreted into the lumen, where they adhere to sperm during epididymal transit. In this study we examined the role of CRISP4 ion channel regulation in mouse spermatozoa through cell assays and mouse models. The identification of the Transient Receptor Potential (TRP) ion channel, TRPM8 to interact with CRISP4 was confirmed using stably-transfected CHO cell lines. Production of CRISP4 KO mouse model, whilst males are fertile, they exhibit a subtle infertility phenotype characterized by a reduced ability to capacitate and undergo the acrosome reaction. This data is further emphasized by the ability of TRPM8 agonists, icillin and menthol, to inhibit the acrosome reaction in mouse spermatozoa that could be prevented by the addition of recombinant CRISP4 crisp domain. Corresponding to these data, CRISP4 is localized to the tail and head of mouse spermatozoa. In conclusion, we have demonstrated that CRISP4 is a regulator of TRPM8 in mouse spermatozoa, and due to its expression and localization pattern is an important protein in sperm epididymal maturation.

SRB POSTERS

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LEUKEMIA INHIBITORY FACTOR IS A CRITICAL REGULATOR OF DECIDUALIZATION OF ENDOMETRIAL STROMAL CELLS IN HUMANS AND MICE

L. Lin[1,2], E. M. Menkhorst[1], E. Dimitriadis[1]

[1]Embryo Implantation Laboratory, Prince Henry's Institute of Medical Research, Clayton, VIC, Australia
[2]Department of Anatomy and Developmental Biology, Monash University, Melbourne, VIC, Australia

Decidualization is the differentiation of endometrial stromal cells into decidual cells. It is a critical process in embryo implantation, placentation and the establishment of pregnancy. Inadequate decidualization can lead to infertility, abnormal placentation and recurrent miscarriage. Endometrial leukemia inhibitory factor (LIF) is indispensible in blastocyst implantation in mice and dysregulated in infertile women. LIF is produced by 1st trimester decidual cells but its role in decidualization is not known. This study aimed to examine the role of LIF in human and mouse decidualization. Primary human endometrial stomal cells (HESC) were isolated and decidualized (D) by treatment with estradiol (E) +medroxyprogesterone acetate (MPA) for 14 days. HESC were also treated with E+MPA+/–LIF (0.5, 5, 50, 100 and 200 ng/mL) for 14 days. Prolactin secretion was used to assess the extent of decidualization (n = 6). D and non-D HESC were also treated with LIF (0.5, 5, 50, 100 and 200 ng/mL +/– LIF inhibitor) for 15 min and the phosphorylation (p) of signal transducer and activator of transcription (STAT)3/STAT3 abundance was detected by Western blot (n = 4). RNA was isolated for analysis of LIF and LIF receptor (R) mRNA expression during decidualization (n = 4). HESC treated with E+MPA+LIF (50, 100 and 200 ng/mL) secreted more prolactin compared to cells treated with E+MPA alone ($P < 0.05$). LIF increased pSTAT3/STAT3 abundance in D and non-D cells while LIF+LIF inhibitor abolished pSTAT3/STAT3. LIF mRNA was downregulated while LIF-R mRNA increased during decidualization. In vivo, mated mice (n = 5) were injected intraperitoneally with a unique long acting LIF inhibitor post-implantation at day 4.5 of pregnancy and resulted in reduced decidualization compared to control. This is the first study to demonstrate that LIF promoted decidualization of HESC possibly via pSTAT3. It further suggested that LIF regulated decidualization in mice demonstrating a newly identified critical role for LIF in the establishment of pregnancy.

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FIBROID ASSOCIATED HEAVY MENSTRUAL BLEEDING: CORRELATION OF CLINICAL SYMPTOMS, DOPPLER ULTRASOUND ASSESSMENT OF VASCULATURE AND TISSUE GENE EXPRESSION PROFILES

S. Tsiligiannis[1], M. Zaitseva[1], P. Coombs[2], P. Shekleton[2], B. Vollenhoven[1], M. Hickey[3], P. Rogers[1]

[1]Centre for Women's Health Research, Department of Obstetrics and Gynaecology, Monash University, Clayton, VIC, Australia
[2]Department of Diagnostic Imaging, Monash Medical Centre, Clayton, VIC, Australia
[3]Department of Obstetrics and Gynaecology, Melbourne University, Parkville, VIC, Australia

Understanding of the mechanisms that cause fibroid associated heavy menstrual bleeding (HMB) is limited. Despite many fibroids having a highly vascular peri-fibroid myometrial (PFM) zone, angiogenic gene expression in this area has never been investigated. The aim of this study was to correlate clinical symptoms, ultrasound appearances and tissue gene expression profiles in women scheduled for hysterectomy due to symptomatic fibroids. We hypothesised that fibroid heterogeneity, colour flow and spectral Doppler resistive indices would correlate with differences in gene expression profiles. It was thought and that increased peri-fibroid gene expression of key angiogenic genes would correlate with increased peri-fibroid vascularity. N=6 patients underwent B-mode, colour and spectral Doppler ultrasound assessment. Following hysterectomy tissue samples collected from three areas – fibroid, PFM and distant myometrium (DM) were analysed using quantitative RT-PCR and a customised angiogenesis PCR array. A higher mean peak systolic velocity (PSV) in the PFM region when compared to mean PSV within the fibroid ($P < 0.001$) was seen. Differences in angiogenic gene expression were consistent with the heterogeneity of the clinical data collected. One fibroid sample showed dissimilar gene expression to all other fibroids; at ultrasound and sample collection significant degenerative features were observed. Fibroid heterogeneity within a single uterus was also demonstrated, with two fibroids from the one uterus having significantly dissimilar gene profiles and ultrasound appearances. No differences in gene expression were found between PFM and DM. Despite this, gene interaction maps showed different interaction of genes between fibroid and PFM regions compared to genes between the fibroid and the DM. These are the first molecular data demonstrating that the PFM region may be functionally distinct from distant myometrium.

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NUTRIENT INTAKE OF *BOS INDICUS* HEIFERS DURING EARLY AND MID-GESTATION CAUSES CHANGES TO PLASMA CONCENTRATIONS OF TRIIODOTHYRONINE (T3) AND THYROXINE (T4) OF THEIR PROGENY

G. C. Micke[2], T. M. Sullivan[2], V. E.A. Perry[1]

[1]QSML & RRRC, Goondiwindi, QLD, Australia

[2]Veterinary school, University of Queensland, QLD, Australia

Fetal and postnatal growth are mediated by thyroid hormones (TH). Maternal nutrient intake during gestation can program postnatal TH concentrations. This may have significant economic implications for beef cattle production. We investigated the effect of feeding beef heifers high (H = 240%) and low (L = 70%) levels of recommended daily crude protein intake during the first and second trimesters of gestation in a two-by-two factorial design on progeny (n = 68) plasma concentrations of free and total triiodothyronine (FT3 and TT3) and free and total thyroxine (FT4 and TT4) from birth until weaning at 6 mo of age. Exposure to low compared to high protein diets during the second trimester resulted in increased plasma FT3 concentrations relative to TT3 (P = 0.04) at birth. For male progeny, exposure to low compared to high protein diets during the first trimester resulted in greater plasma FT4 concentrations from birth until weaning (P = 0.02). Also for males from birth until weaning, LH had greater plasma TT3 concentrations than HH (P < 0.01). For female progeny, HH had greater plasma TT3 concentrations relative to TT4 than HL from birth until weaning (P = 0.03). Plasma concentrations of FT3 were positively associated with average daily gain relative to birth weight at 1 (r = 0.41; P < 0.01) and 3 mo FT3 (r = 0.41; P < 0.01). Heifer protein intake during the first and second trimesters of gestation has a permanent effect to progeny plasma TH concentrations and these changes are associated with the postnatal growth pathway.

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DECIDUAL HTRA3 NEGATIVELY REGULATES TROPHOBLAST INVASION

H. Singh[1], S. Makino[2,3], Y. Endo[2], G. Nie[1]

[1]Prince Henry's Institute of Medical Research, Clayton, VIC, Australia

[2]Cell-Free Science and Technology Research Center, Ehime University, Matsuyama, Japan

[3]Center for Eukaryotic Structural Genomics, University of Wisconsin-Madison, Madison, United States

Controlled trophoblast invasion cell into the maternal decidua (interstitial invasion) is important for placental development. Abnormalities in the invasion process may lead to pregnancy complications. Decidua secrets many factors to control trophoblast invasion. Serine protease HtrA3 is highly expressed in the decidual cells in the late secretory phase of the menstrual cycle and throughout pregnancy. It is highly expressed in first trimester in most trophoblast cell types, but not in the invading interstitial trophoblast. HtrA3 and its family members are down-regulated in a number of cancers and are proposed as tumor suppressors. We hypothesized that HtrA3 is an inhibitor of trophoblast invasion. The current study aimed to investigate whether HtrA3 secreted by decidual cells regulates trophoblast invasion. Human endometrial stromal cells (HESC) were decidualised with estradial, medroxyprogesterone acetate and cyclic AMP. Real-time RT-PCR, western blotting and immunocytochemistry demonstrated that decidualisation increased HtrA3 mRNA and protein expression. HtrA3 was also detected by western blotting in the conditioned media (CM) of decidualised HESC (96 h), confirming its secretory nature. For functional studies, wild type and protease inactive mutant HtrA3 were produced using wheat germ cell-free technology. The mutant has negligible protease activity and significantly inhibited the wild type protease activity, supporting its dominant-negative inhibition and utility as a specific inhibitor of the wild type protein. CM of decidualised HESC suppressed invasion of trophoblast HTR-8 cells, whereas inhibition of HtrA3 in the decidual HESC CM by exogeneous addition of HtrA3 mutant increased trophoblast HTR-8 cell invasion. These results strongly support our hypothesis that decidual HtrA3 negatively regulates trophobalst invasion.

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OPPOSING ROLES FOR MUCINS 13 AND 15 IN EARLY PREGNANCY

C. E. Poon[1], M. Day[2], C. R. Murphy[1]

[1]Discipline of Anatomy & Histology, The University of Sydney, Sydney, NSW, Australia
[2]Discipline of Physiology, The University of Sydney, Sydney, NSW, Australia

Successful pregnancy is dependent on the cumulation of numerous changes in both the uterine luminal epithelium (ULE) and the invading blastocyst prior to implantation. The apical surface of the ULE constitutes the first point of contact with the blastocyst; thus forming an important player in the implantation event. A constituent of this surface comprises the Mucin family of proteins, which is suggested to play an important role in implantation. Prior studies have found that some Mucins form an anti-adhesive surface on the uterine epithelium to prevent implantation of the blastocyst. The formation of this anti-adhesive surface relates to their large extracellular domains which act to 'mask' adhesive receptors present on the ULE from interaction with corresponding ligands present on the blastocyst surface. This process of contact inhibition has been termed 'steric hindrance'. This study examined the localisation of previously uncharacterised Mucins 13 and 15 in the uterus and blastocyst during early pregnancy to investigate their role in the implantation event. Western blotting, cell fractionation and immunofluorescence techniques were utilised in this study. It was found that Mucin 13 localised to the apical cell surface of the ULE at time of implantation while conversely Mucin 15 was lost from the apical cell surface. In the blastocyst, both proteins were localised to the trophoblast cells and the inner cell mass, with Mucin 15 additionally present in the zona pellucida. Further, the changes in localisation of these proteins in the ULE corresponded to changes in their glycosylation profiles from pre-implantation to time of implantation. These results demonstrate that Mucins 13 and 15 participate in contrasting roles in implantation. Mucin 13 may promote adhesion between the uterus and blastocyst to facilitate implantation while Mucin 15 may prevent adhesion through the mechanism of steric hindrance, given its absence from the apical cell surface at time of implantation.

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PREDICTING GESTATIONAL DIABETES FROM MATERNAL ANGIOTENSIN II AND ANGIOTENSIN 1–7 LEVELS AT 15 WEEKS GESTATION

S. D. Sykes[1], E. R. Lumbers[1], K. G. Pringle[1], T. Zakar[1], G. A. Dekker[2,3], C. T. Roberts[3]

[1]Endocrinology, Mothers and Babies Research Centre, Hunter Medical Research Institute, John Hunt, New Lambton, Australia

[2]Lyell McEwin Hospital, Adelaide, SA, Australia

[3]Robinson Institute, University of Adelaide, Adelaide, SA, Australia

Gestational diabetes (GD), a pregnancy complication defined by glucose intolerance with onset during pregnancy, is a condition affecting 5.5%–8.8% of Australian pregnancies[1]. Untreated GD increases perinatal mortality and babies from GD pregnancies have an increased risk of diabetes and obesity, whilst mothers have an increased risk of type II diabetes later in life[1]. Circulating levels of angiotensin 1–7 (Ang1–7), a peptide of the renin angiotensin system, have been reported to be reduced in third trimester pregnancies with GD[2]. The effects of Ang1–7 generally oppose those of angiotensin II (AngII) and it is possible that in early gestation pro-angiogenic functions of AngII are counterbalanced by Ang1–7, causing placental insufficiency and pregnancy complications. We wanted to determine the predictive capability of AngII and Ang1–7, in early gestation, for GD. Healthy nulliparous pregnant women from the Adelaide SCOPE cohort with GD (n = 36) or serving as controls (n = 131) had both peptides measured at 15 weeks using an RIA (D. Casley, Prosearch Pty. Ltd.) on EDTA treated plasma. A predictive model was constructed using logistic regression and area under the curve after ROC analysis (AROC, Table 1). AngII did not change the model and was omitted. This model shows that for every one unit increase of Log (Ang1–7 pg/mL) peptide levels the odds of acquiring GD increase five times, suggesting that Ang1–7 levels in early gestation may be a better disease marker than those seen at late pregnancy.

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(2) Nogueira AI, Santos RAS, Simoes e Silva AC, et al., 2007. The pregnancy-induced increase of plasma angiotensin-(1-7) is blunted in gestational diabetes. Regul. Pep., 141, 55–60.

Table 1. The logistic regression model and subsequent AROC for GD model

sBP = systolic blood pressure; Pr/Cr = urinary protein/creatitine ratio

Variable	Odds ratio	95% CI
Age	1.18	1.07-1.31
Cigarettes	0.94	0.85-1.04
sBP	1.02	0.98-1.07
Weight	1.01	0.99-1.04
Height	0.93	0.86-1.00
Pr/Cr	0.87	0.76-0.99
Log (Ang1-7)	5.45	2.04-14.55
AROC	0.85	

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MICRORNAS ARE DIFFERENTIALLY EXPRESSED BETWEEN MID AND LATE GESTATION AND IN DIFFERENT FUNCTIONAL ZONES OF THE RAT PLACENTA

W. Kong[1], S. Moretta[1], P. A. Grant[1], M. Dziadek[2], J. A. Owens[1]

[1]Obstetrics and Gynaecology, University of Adelaide, Adelaide, SA, Australia

[2]Garvan Institute of Medical Research, Sydney, NSW, Australia

MicroRNAs (miRNAs) are short, single-stranded, non-coding RNAs that regulate translation, by interacting with complementary sites in the 3'UTRs of target mRNAs. Each can target multiple mRNAs and each mRNA can be targeted by many different miRNAs to form regulatory networks. Placental development is characterised by dynamic, co-ordinated changes in expression of regulatory and functional genes that drive invasion, differentiation and growth. These changes may involve miRNAs, but placental miRNA expression during gestation has been little studied in any species to date. MiRNA expression was examined (by microarray) in the rat in mid (day 16) and late (day 20) gestation (term ~22 days) and the two major zones of the placenta, the labyrinth and junctional zones, at and between each gestational age. At day 16, 107 miRNAs were differentially expressed between the labyrinth and junctional zone ($P < 0.05$); 47% being more highly expressed and the remainder less expressed in the labyrinth compared to the junctional zone. At day 20, 78 miRNAs were differentially expressed between the two zones ($p<0.05$); 53% being higher and the remainder lower in the labyrinth compared to the junctional zone. 216 miRNAs were differentially expressed in the labyrinth zone between mid and late gestation; 73% of the differentially expressed labyrinth zone miRNAs were upregulated at day 20, many from a cluster on chromosome 6, a feature of the late gestation mouse placenta as well (Kong et al., unpublished). In the mouse, these same clustered miRNAs are located on chromosome 12 in an imprinted region, with the miRNAs expressed from the maternal allele. Disruption of their expression impairs placental vascular, labyrinth and junctional zone development [1].Together these findings imply that these clustered miRNAs are likely to play a role in labyrinth zone development and further study is warranted into which placental transcripts are targeted by these imprinted miRNAs.

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FOLLISTATIN SPLICE VARIANTS FST288 AND FST315 INCREASE DURING EARLY MOUSE PREGNANCY: REGULATION BY PROGESTERONE?

R. G. Craythorn[1,2], W. R. Winnall[2], M. P. Hedger[2], P. A.W. Rogers[1], D. M. De Kretser[2], J. E. Girling[1]

[1]Centre for Women's Health Research, Monash University Department of Obstetrics and Gynaecology and Monash Institute, Melbourne, VIC, Australia

[2]Centre for Reproduction and Development, Monash Institute of Medical Research, Melbourne, VIC, Australia

Follistatin acts by binding and neutralising the activity of activin-A, which has important regulatory roles in development, reproduction and inflammation. There are two isoforms of follistatin comprising 288 and 315 amino acids (FST288 and FST315), resulting from alternative gene splicing. FST288 binds spontaneously to heparan sulphate and is largely bound to cell surface proteoglycans. FST315 is the predominant circulating form and can only bind to heparan sulphate after binding activin-A. The regulation of these splice variants in the female reproductive tract have not been investigated in detail. In this study, our aim was to quantify the expression of FST288 and FST315 mRNA in the mouse uterus during early pregnancy (days 1–4, pre-implantation), and in response to exogenous oestradiol-17b (100 ng × single s.c. injection, dissection after 24 h) and progesterone (1 mg × three daily s.c. injections, dissection 24 h after last injection) in ovariectomised mice. Gene expression was analysed using quantitative RT-PCR. Primers amplifying a product from exon 5 to 6a (unique to FST288) or from exon 5 to 6b (unique to FST315) were used to discriminate the isoforms. In early pregnancy, expression of both FST288 and FST315 increased significantly (approximately 35-fold and 100-fold, respectively) on days 3–5, relative to days 1–2, corresponding with the increase in circulating progesterone levels that occurs at day 3. A significant increase in FST288 and FST315 mRNA expression (both approximately 35-fold) was also observed in ovariectomised mice in response to exogenous progesterone, but there was no increase in response to oestradiol-17β. In contrast to the similar rate of increase in response to exogenous progesterone, FST315 mRNA expression increased more rapidly than FS288 in early pregnancy, indicating that differential regulation of the two isoforms also occurs. We conclude that progesterone regulates both FST288 and FST315 mRNA expression during early pregnancy in the mouse uterus.

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DEVELOPMENT AND VALIDATION OF AN ELISA FOR HIGH TEMPERATURE REQUIREMENT FACTOR A3 (HTRA3): EARLY DETECTION OF PREECLAMPSIA

K. Dynon, G. Nie

Implantation and Placental Development Group, Prince Henry's Institute of Medical Research, Clayton, VIC, Australia

Preeclampsia (PE) is a multisystemic condition in pregnant women that can be life threatening for both mother and baby. PE is a hypertensive disorder that develops concurrently with proteinuria after 20 weeks of gestation. Abnormal placental development during early pregnancy precedes the onset of PE later in gestation. Early diagnosis of PE is essential to reduce PE-related mortality and morbidity. To date there is no clinically useful biochemical diagnostic method that can detect PE during early pregnancy. Our laboratory discovered and cloned the serine protease HtrA3 and has shown that HtrA3 protein levels are intimately involved in placentation (1). Persistently high serum levels of HtrA3 are detected at the end of the first trimester in pregnant women who subsequently develop PE, suggesting that monitoring HtrA3 in maternal blood during early pregnancy may identify women at risk for PE. To develop monoclonal antibodies specific for HtrA3; and to develop an enzyme-linked immunosorbent assay (ELISA) to detect HtrA3 in human sera. Monoclonal antibodies were generated against full length human HtrA3 and small HtrA3 peptides and tested on recombinant HtrA3, human sera and first trimester decidual and villous tissues using western blot, immunoprecipitation and Amplified luminescent proximity homogeneous assay (Alpha)LISA technology. Three antibody pairs were identified that detected either short and/or long isoforms of HtrA3 in sera and placental tissues. Recombinant HtrA3 was detected by AlphaLISA and higher levels of HtrA3 were detected in serum of PE women compared to gestation-matched controls in preliminary testing. These antibody pairs can now be used for the development of specific and high throughput assays. The AlphaLISA will then be used to validate that abnormal serum HtrA3 levels during early pregnancy can predict preeclampsia.

(1) Nie et al, (2006) Biol Reprod 74, 366–374.

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CAVEOLIN 1 AND 2 IN THE UTERUS DURING EARLY PREGNANCY

R. J. Madawala, C. R. Murphy

Anatomy and Histology, The University of Sydney, Sydney, NSW, Australia

Rat uterine epithelial cells undergo many changes during early pregnancy in order to become receptive to blastocyst implantation. These changes include basolateral folding and the presence of vesicles of various sizes which are at their greatest number during the pre-implantation period. The present study investigated the possible role that caveolin 1 and 2 plays in this remodelling specifically days 1, 3, 6, 7, and 9 of pregnancy. Caveolin is a major protein in omega shaped invaginations of the plasma membrane called caveolae that are considered to be specialised plasma membrane subdomains. Caveolae are rich in cholesterol, glycosphingolipids, and GPI anchored proteins and are involved in endocytosis and membrane curvature. Immunofluorescence microscopy has shown caveolin 1 and 2 on day 1 of pregnancy are localised to the cytoplasm of luminal uterine epithelial cells, and by day 6 of pregnancy (the time of implantation), it concentrates basally. By day 9 of pregnancy, expression of both caveolin 1 and 2 in luminal uterine epithelia is cytoplasmic as seen on day 1 of pregnancy. A corresponding increase in protein expression of caveolin 1 on day 6 of pregnancy in luminal uterine epithelia was observed. Interestingly however, caveolin 2 protein expression decreases at the time of implantation as found by western blot analysis. Both caveolin 1 and 2 were localised to blood vessels within the endometrium and myometrium and also the muscle of the myometrium in all days of pregnancy studied. In addition, both caveolin 1 and 2 were absent from glandular epithelium, which is interesting considering that they do not undergo the plasma membrane transformation. The localisation and expression of caveolin 1 and 2 in rat luminal uterine epithelium at the time of implantation suggest possible roles in trafficking of cholesterol and/or various proteins for either degradation or relocation. Caveolins may contribute to the morphology of the basolateral membrane seen on day 6 of pregnancy. All of which may play an important role during successful blastocyst implantation.

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REGULATION OF THE RENIN ANGIOTENSIN SYSTEM (RAS) IN A TROPHOBLAST CELL LINE BY CYCLIC ADENOSINE MONOPHOSPHATE (CAMP) AND 5'-AZA-2'-DEOXYCYTIDINE (AZA)

Y. Wang, K. G. Pringle, Y. Chen, T. Zakar, E. R. Lumbers

School of Biomedical Sciences and The Mothers and Babies Research Centre, University of Newcastle, Newcastle, NSW, Australia

Renin and renin-like activities have been detected in early gestation placentae[1,2]. To explore what regulates RAS expression in trophoblasts we studied the effects of cAMP and AZA (DNA demethylating agent) on the expression of mRNAs for prorenin receptor (ATP6AP2), prorenin (REN), angiotensinogen (AGT), angiotensin converting enzyme (ACE), ACE2, angiotensin II type 1 receptor (AGTR1), and a downstream target of the prorenin receptor, cyclooxygenase-2 (PTGS2) in the early human trophoblast cell line HTR-8/SVneo. Cells were cultured for 24 or 48 h with vehicle, 0.5 mM cAMP or 15 mM AZA. Messenger RNA abundances were measured relative to Alien RNA using real-time qRT-PCR. All mRNAs were detected except for ACE and ACE2. REN mRNA abundance was increased by cAMP and AZA ($P < 0.0001$). However, cAMP was more effective than AZA at both 24 and 48 h ($P < 0.0001$; $P < 0.05$) at the concentrations employed. AZA significantly increased AGT mRNA levels at 24 h ($P = 0.001$) and AGTR1 mRNA levels at 48 h ($P < 0.0001$), while cAMP had no effect. P TGS2 mRNA expression was increased by cAMP at 24 h ($P < 0.0001$) and by AZA at 48 h ($P < 0.0001$). cAMP and AZA had no effect on ATP6AP2 abundance. Thus, prorenin may function directly through the prorenin receptor, since cAMP upregulated both REN and its downstream target PTGS2 in HTR-8/SVneo cells, especially considering that ACE and ACE2 were not expressed. Our findings also suggest that expression of the RAS in trophoblast is regulated by DNA methylation as there was sustained over expression of REN, AGT, AGTR1 and PTGS2 with AZA. The ATP6AP2 promoter possesses CpG islands; however, its expression was not affected by AZA. This may suggest that either DNA methylation is not involved in the regulation of this gene, or that the over expressed prorenin binding to its receptor is causing suppression of ATP6AP2 abundance via a previously identified negative feedback pathway.

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THE DISTRIBUTION OF PROMININ-1 IN THE RAT UTERUS DURING EARLY PREGNANCY

S. N. Dowland, L. A. Lindsay, C. R. Murphy

School of Medical Sciences (Anatomy and Histology), The University of Sydney, NSW, Australia

Prominin-1 is a recently discovered pentaspan membrane protein present in characteristic cholesterol-based vesicles and associated with microvilli. These vesicles are used to deliver prominin-1 to the apical plasma membrane in a number of cell types. Previous work on uterine epithelial cells has demonstrated a loss of microvilli and the presence of large, cholesterol-based vesicles at the time of implantation. Thus this study aims to determine a role for prominin-1 in rat uterine epithelial cells during early pregnancy. Immunofluorescence microscopy reveals punctate and diffuse prominin-1 staining below the apical plasma membrane on day 1 of pregnancy. At the time of blastocyst implantation (day 6) however, prominin-1 appears concentrated at the apical surface of the cell. Western blotting of isolated uterine epithelial cell lysate revealed a change in prominin-1 glycosylation during early pregnancy. Prominin-1 was determined to be glycosylated on day 1 of pregnancy, but these carbohydrate side chains were lost by the time of attachment. Results seen in the present study indicate that prominin-containing vesicles may be prevented from reaching the apical plasma membrane by the terminal web on day 1 of pregnancy. On day 6, the loss of the terminal web may allow the vesicles to approach and incorporate into the apical plasma membrane, as seen with other uterine vesicles. The deglycosylation of prominin-1 at this time is suggested to allow the protein to bind its ligand and activate downstream signalling pathways that permit implantation. This study constitutes the first reported observation of prominin in endometrial lumenal epithelial cells. These preliminary results, in consideration with previous reports of prominin expression in trophoblast cells, suggest an important role for this protein in early pregnancy.

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EPIGENETIC REGULATION OF THE CRH GENE PROMOTER INVOLVES SPECIFIC CPG DE-METHYLATION

X. Pan[1,2], C. Abou-Seif[1], M. Allars[1], Y. Chen[1], R. C. Nicholson[1]

[1]Mothers and Babies Research Centre, Hunter Medical Research Institute, Newcastle, NSW, Australia
[2]School of Medicine and Public Health, The University of Newcastle, Callaghan, NSW, Australia

Corticotropin Releasing Hormone (CRH), is expressed in many regions of the central nervous system and in some peripheral tissues, and plays an important role in determining gestational length. In placenta, a cAMP regulatory site (CRE) is crucial for CRH gene regulation. The promoter of CRH gene has 9 CpG sites, which should be the targets of epigenetic regulation by DNA methylation. The BeWo cell line, derived from human gestational choriocarcinoma, has been widely used as an in vitro model for the placenta. BeWo cells only produce CRH after exposure to cAMP. The DNA methyl transferase (DNMT) inhibitor 5-aza-cytidine stimulates CRH expression 5-fold in camp treated BeWo cells, indicating the CRH promoter as a target of DNMTs. To evaluate methylation differences of the 9 CpG sites in CRH gene promoter in BeWo cells after treatment with cAMP. Genomic DNA was extracted from BeWo cells treated or not with cAMP. Sodium bisulfite conversion was used to modify the genomic DNA. PCR was used to amplify the CRH promoter region with primers that did not contain CpG sites. The PCR products were cloned and sequenced. The CpG methylation status of each sample was obtained by comparing the sequencing results with the original sequence. In non-stimulated cells (control) CpG -4 was methylated in 50% of the clones and CpG -6 was methylated in 75% of the clones, but the other 7 sites were methylated in every clone. In the cAMP treated cells however there was 100% methylation at CpG sites 6 through 9, but only partial methylation at CpG-1 and 3 (60%), CpG-4 and 5 (40%). Most interestingly, there was no methylation found at CpG-2 in any of the clones from cAMP treated cells, indicating that specific CpG de-methylation around the CRE is required for CRH gene expression.

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PREECLAMPSIA AND SMALL FOR GESTATIONAL AGE BABIES ARE ASSOCIATED WITH DEFECTS IN THE FOLATE-HOMOCYSTEINE METABOLIC PATHWAY

D. L.F. Furness, S. D. Thompson, G. A. Dekker, C. T. Roberts

The Robinson Institute, Obstetrics and Gynaecology, University of Adelaide, Adelaide, Australia

The folate-homocysteine metabolic pathway plays a critical role in determining the availability of folate and methionine which are essential for placental and fetal development. Defects in this pathway can increase circulating homocysteine concentration and impair DNA synthesis. The purpose of this study was to investigate genetic polymorphisms involved in folate-homocysteine metabolism in association with preeclampsia (PE) and small for gestational age (SGA) babies. The Adelaide SCOPE (Screening for Pregnancy Outcomes) was a prospective study including 1169 nulliparous pregnant couples. Adelaide samples and patient information were collected by SCOPE research midwives. Genotyping was performed by Sequenom MassARRAY. Non-Caucasian couples were excluded from analyses. Pregnancy outcomes were strictly classified: PE (n = 71), SGA (n = 94) PE+SGA (n = 20), controls (n = 450). Chi Square and univariate with post-hoc analyses were performed. Women who developed PE and SGA had a mean BMI of 30.1 and 27.8 respectively, compared to women with healthy pregnancies 25.7 (P <0.001 and P = 0.002). Maternal and fetal MTHFD1 were associated with reduced customised birthweight centiles (P = 0.034 and P = 0.028 respectively). Paternal MTR 2756 genotype was associated with reduced customised birthweight centiles (P = 0.016) and with PE+SGA compared to healthy pregnancies P = 0.006. Neonatal MTR 2756 showed a similar trend with the G allele increased in PE+SGA P = 0.070. In addition, the paternal MTHFR 677 TT genotype was associated with PE+SG (P = 0.026). Variant polymorphisms within folate-homocysteine metabolism genes can slow enzyme activity increasing plasma homocysteine leading to maternal and placental vascular damage associated with both PE and SGA. This study builds on previous work in our laboratory that focused on maternal genotype and circulating factors shown to be associated with PE and SGA including serum folate, vitamin B12 and plasma homocysteine.

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HOMEOBOX GENES ARE DIFFERENTIALLY EXPRESSED IN PRIMARY VILLOUS AND EXTRAVILLOUS TROPHOBLAST CELL LINEAGES DURING EARLY PREGNANCY

P. Murthi[1], N. A. Pathirage[1], R. Keogh[1], M. Cocquebert[2], N. Segond[2], S. P. Brennecke[1], T. Fournier[2], D. Evain-Brion[2], B. Kalionis[1]

[1]Department of Perinatal Medicine and Dept of Obsterics and Gynaecology, Royal Women's Hospital and University of Melbourne, Parkville, VIC, Australia

[2]INSERM 767, Universite Paris Des Cartes, Paris, France

During human placental development trophoblast cells differentiate along either the villous cytotrophoblast (VCT) lineage to form the syncytiotrophoblast (ST) or the invasive extravillous cytotrophoblast (EVCT) lineage (1). Abnormalities in early differentiation processes are characteristic of poor placentation, which is associated with fetal growth restriction (FGR) and pre-eclampsia (PE), the major clinical complications of human pregnancy (2). A large family of homeobox gene transcription factors controls "cell-fate decisions" during development (3), but the expression profile and role of homeobox genes in the human trophoblast cell lineages is not well understood. The aim of the study was to determine homeobox gene expression in primary cultures of mononuclear VCT (2 h) and EVCT (2 h) obtained from first trimester human chorionic villi of 8–12 weeks of gestation and in vitro differentiated ST (72 h) and invasive EVCT (48 h), respectively. The isolation and characterization of freshly isolated VCT, EVCT and in vitro differentiated ST and invasive EVCT were performed as described previously (1,4). The homeobox gene mRNA profile was performed using PCR arrays in a pooled sample of VCT and EVCT (n = 6 in each group) and further validated by real-time PCR. Homeobox gene expression studies revealed MSX2 mRNA levels were the highest in VCT (2 h) but undetectable in EVCT (2 h). Further comparisons of homeobox gene expression in in vitro differentiated ST to invasive EVCT showed marked increase in MSX2, DLX3, DLX4 and MEIS1 mRNA levels in ST, which are regulators of cellular differentiation in many studies. Homeobox genes HLX and HHEX, which are implicated in regulating cellular proliferation showed decreased mRNA levels in ST compared to invasive EVCT. Our results demonstrated several known placental and novel homeobox genes are differentially expressed in trophoblast cell lineages. Functional studies of these candidate genes will provide a better understanding of the molecular mechanisms of early placental development.

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MATERNAL DIETARY ARGININE SUPPLEMENTATION DURING LATE GESTATION IMPROVES REPRODUCTIVE EFFICIENCY IN PIGS

M. J. De Blasio[1], C. T. Roberts[1], K. Kind[1], R. Smits[2], M. Nottle[1], J. A. Owens[1]

[1]Robinson Institute & School of Paediatrics and Reproductive Health, The University of Adelaide, Adelaide, SA, Australia

[2]Rivalea Pty Ltd, Corowa, NSW, Australia

Arginine (a non-essential amino acid) and its conversion to nitric oxide (NO) can promote formation of new blood vessels and cause vasodilation. This may reduce resistance and increase blood flow to the uterus and placenta, and the delivery of nutrients for fetal growth and survival. In pregnant rats, dietary arginine deficiency causes IUGR and increases fetal death and perinatal mortality, whereas dietary arginine supplementation reverses this. Human IUGR is associated with impaired NO synthesis, and eNOS activity in umbilical vein endothelial cells, but maternal arginine supplements have produced inconclusive results. We hypothesised that maternal arginine supplementation (MAS) in the pig (a species with naturally occurring IUGR), during late gestation, when placental angiogenesis and vascularity increase, would increase birth and placental weights. Large White (LW) and Landrace (LR) gilts (n = 285) and sows (N=326), were fed either a control or arginine supplemented (+25g/d arginine, Nutreco Progenos premix) diet (2.5kg/d) in late gestation (d75-term at ~114 days). Number born, born alive, still born and mummified, birth weight and d10 weight of progeny were measured. Data were analysed using Univariate ANOVA. MAS in late gestation in gilts and sows reduced the number of still born (Con: 1.17 ± 0.13 piglets/litter; Arg: 0.84 ± 0.09 piglets/litter; P = 0.046). In LW gilts, MAS increased birth weight (Con: 1.21 ± 0.05 kg; Arg: 1.34 ± 0.05 kg; P < 0.05), and litter birth weight (Con: 13.38 ± 0.72 kg; Arg: 15.27 ± 0.73 kg; P < 0.05). MAS also increased birth weight in LW (Con: 1.17 ± 0.06 kg; Arg: 1.30 ± 0.06 kg; P < 0.05) and LR (Con: 1.47 ± 0.05 kg; Arg: 1.60 ± 0.05 kg; P < 0.05) sows, and reduced still borns in LW sows (Con: 1.12 ± 0.14 piglets/litter; Arg: 0.77 ± 0.09 piglets/litter; P < 0.05). MAS in late gestation improves pregnancy outcomes in terms of piglet survival and birth weight, in LW and LR gilts and sows. MAS during critical periods of placental development may enhance placental-fetal blood flow and nutrient transfer, thereby improving fetal growth and survival.

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DIFFERENTIAL EFFECTS OF CAMP AND CRH IN CELL VIABILITY AND CELL FUSION OF CYTOTROPHOBLASTIC CELLS

Y. Chen, M. Allars, C. Abou-Seif, R. Smith, R. C. Nicholson

Mothers and Babies Research Centre, John Hunter Hospital, The University of Newcastle, Newcastle, NSW, Australia

Cell fusion of cytotrophoblasts is critical for a successful pregnancy, and is marked biochemically by proteins such as human chorionic gonadotrophin (hCG) and syncytin, and morphologically as large multi-nucleated cells. Placental corticotrophin releasing hormone (CRH) plays diverse roles during pregnancy and has been linked to the length of gestation. It is well accepted that CRH expression could be upregulated by some hormones, such as hCG via cellular cAMP, and that trophoblastic cell fusion can be induced by Forskolin (increases cAMP). We have shown, by reporter gene assay in human primary placental cells, that 8-Br-cAMP increased CRH gene expression. Thus, it is of interest to determine the roles of 8-Br-cAMP and CRH on trophoblast cell viability and cell fusion. Treatment with 8-Br-cAMP for 72 h significantly inhibited BeWo cell viability (MTT assay) by 29.7% (50 μM) and 54.8% (100 μM), and increased both apoptotic and necrotic cells (FACS analysis). 8-Br-cAMP (100 μM, 48 h) resulted in the appearance of nuclear fragments (DAPI stain) and Fas ligand gene expression by 6.8-fold (real-time RT-PCR). Syncytin 1 mRNA increased by 3.2-fold (real-time RT-PCR) and hCG secretion increased by 4-fold (ELISA assay). The formation of syncytium (CellMask and Hoechst co-stain) could clearly be seen by 72h. CRH mRNA increased with 8-Br-cAMP treatment of BeWo cells (15-fold at 48 h). Treatment with CRH (100 nM) had a mild inhibitory effect on cell growth (~18%) but no effect on cell viability, up-regulated the expression of syncytin 1 mRNA (2.3-fold at 48 h) and induced cell fusion (72 h), but had no effect on hCG secretion. In summary, we show that 8-Br-cAMP and CRH are both potential inducers of cytotrophoblast cell fusion, and there is a dissociation between morphological and biochemical differentiation. Our data also indicates that the process of cell fusion can be associated with both apoptotic and non-apoptotic events.

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POLYURIA IN PREGNANCY – CHALLENGES OF DIAGNOSIS!

A. SAKTHIVEL[1], A. J. Nankervis[1], J. Conn[1], J. Oats[1], S. Chitturi[2]

[1]Endocrinology Services, The Royal Women's Hospital, Parkville, VIC, Australia
[2]Endocrinology, Royal Darwin Hospital, Darwin, NT, Australia

Increased urinary frequency is a widely experienced symptom in pregnancy; true polyuria is less common and can have a number of causes. It is essential, however that it should be recognized and appropriately investigated, as it may be potentially life threatening to mother and fetus. Diabetes insipidus (DI) is a rare complication of pregnancy. It can be the first presentation of pre-gestational DI, gestational DI or DI associated with acute fatty liver of pregnancy. The latter conditions are illustrated by the following two clinical cases. Osmotic Homeostasis can be altered in pregnancy due to resetting of the osmostat and degradation of vasopressin by vasopressinase secreted by the placenta in increasing quantities in the 2nd and 3rd trimesters. Liver failure results in the availability of higher circulating levels of vasopressinase. These cases highlight the difficulties and importance of diagnosis and treatment. Pregnant women often report polyuria during the 3rd trimester. It is important to have a high level of clinical suspicion for the pathological causes, despite the non-specific presentation as underlying conditions can have catastrophic sequelae.

Table 1.

Case description	Gestational DI	DI associated with fatty liver of pregnancy
Patient details	31 years old Primi	36 years old G2P1 MCDA Twins
Polyuria (L)	6	7
Blood pressure	100/70	160/100
Serum Na (mmol)	142	142
Serum osmolality (mosm/L)	298	298
Urine osmolality (mosm/L)	118	112
Renal function	Normal	Creatinine 0.18 mmol/L
Oral glucose tolerance test	Normal	Normal
Urine microscopy	Normal	Normal
Coagulapathy	INR 1.1	INR 1.5
Liver function test	Normal	ALT 490 IU/L, ALP 653 IU/L, AST 741 IU/L, GGT 105 IU/L
Full blood exam	Normal	Thrombocytopenia
Mode of delivery	Normal vaginal delivery	Emergency lower segmental caesarian section
Treatment	DDAVP	DDAVP and intravenous fluids
Resolution	Postpartum Day 5	Postpartum Day 7

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RELAXIN REGULATES AQUAPORIN EXPRESSION IN THE CERVIX OF LATE PREGNANT MICE

Y. Soh, L. J. Parry

Zoology, The University of Melbourne, Parkville, VIC, Australia

Aquaporins (AQPs) have been implicated in the regulation of fluid balance in the cervix during pregnancy to promote hydration, a characteristic of cervical ripening in late gestation. There are four AQPs in the cervix; AQP3, 4, 5 and 8. Cervical fluid balance involves AQP5 and 8 in early pregnancy and AQP3 and 4 in late pregnancy [1]. However, the factors involved in the regulation of cervical AQPs are unknown. We propose that the ovarian peptide hormone relaxin regulates cervical AQPs because high circulating levels of relaxin correspond to changes in AQPs and it is involved in cervical ripening. To test this hypothesis, expression of aqp3, 5 and 8 was compared in the cervices of relaxin wildtype (Rln+/+) and relaxin knockout (Rln–/–) at various stages of pregnancy (day 14.5, 16.5 and 18.5 pregnancy) by quantitative PCR. In the Rln–/– mice, aqp3 expression was significantly lower on day 18.5 gestation compared to Rln+/+ littermates. Aqp5 and 8 expression did not change significantly between genotypes. To determine whether relaxin could restore the Rln+/+ phenotype, Rln–/– mice were implanted with Alzet osmotic minipumps on day 12.5 pregnancy to infuse either recombinant H2 human relaxin (200 μg/mL; Corthera Inc) or 0.9% saline as a control. Cervices were collected after 4 or 6 days of infusion for gene expression analysis. Relaxin infusion in pregnant Rln–/– mice increased cervical aqp3, and also decreased aqp5 expression compared with saline-controls in the 6-day infusion group. Additionally, relaxin treatment caused a 6-fold increase in cervix wet weight, dispersal of collagen fibres and a decrease in relaxin receptor (Rxfp1) expression. These data suggest that relaxin promotes cervical hydration through an action on AQPs.

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THE ROLE OF FIZZY RELATED 1 IN MALE MEIOSIS

L. Hopkins[1], V. Pye[1], B. Fraser[1], J. Holt[2], K. Jones[2], E. McLaughlin[1]

[1]Reproductive Science Group, The University of Newcastle, Callaghan, NSW, Australia

[2]Egg to Embryo Research Group, The University of Newcastle, Callaghan, NSW, Australia

Accurate chromosome segregation during mitosis and meiosis is facilitated by a regulatory complex known as the Anaphase Promoting Cyclosome (APC), an ubiquitin ligase complex that tags proteins with ubiquitin. Subsequently targeted proteins are recognised by the 26S proteosome and degraded. In mammalian cells, two temporally regulated co-activators are required for the APC to function; fizzy and fzr1. In studies of female oocyte development fzr1 has been demonstrated to play an important role in maintaining G2 arrest during meiosis by controlling spatial levels of the cell cycle protein Cyclin B1 but the role of Fzr1 in spermatogenesis remains unknown. Germ cell specific conditional knockout fzr1mice were generated using the DDX4-Cre and flox/flox fzr1 mouse lines and initial gross morphological analysis indicated that at 7 weeks of age null mice possessed significantly smaller testes (21.81 mg ± 0.23 mg) when compared to heterozygote (99.86 mg ± 1.58 mg) and wildtype littermates (93.06 mg ± 1.16 mg) $n = 3$ $P < 0.0001$. Quantitative gene expression analysis confirmed almost complete absence of fzr1 transcript in testes (20-fold decrease) in comparison to wild-type. Immunoblotting and immunohistochemistry revealed no expression of Fzr1 protein in meiotic and post meiotic germ cells when compared to heterozygote and wild type littermates. Histomorphological analysis of testes tissue sections revealed Fzr1 null males exhibited spermatogenic arrest and a complete absence of round spermatids with concomitant apoptosis in the residual spermatocytes. Epididymal examination confirmed a complete lack of mature spermatozoa in the cauda epididymis of null males. In contrast, both wild type and heterozygote mice displayed normal spermatogenesis and epididymal sperm analysis indicated no distinguishable differences in seminal characteristics with normal motility, morphology and sperm-zona binding capacity. Based on these observations we hypothesise that Fzr1 plays a significant role in the establishment and maintenance of meiosis possibly through regulation of key cell cycle proteins.

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HEDGEHOG SIGNALLING COMPONENTS IN DEVELOPING RAT TESTIS

Z. Sahin[1], A. Szczepny[2,3], I. Ustunel[1], K. Loveland[3,4]

[1]Histology and Embryology, Akdeniz University, Antalya, Turkey
[2]Monash Institute of Medical Research, Monash University, Melbourne, VIC, Australia
[3]Australian Research Council Centre of Excellence in Biotechnology and Developme, Australia
[4]Biochemistry & Molecular Biology and Anatomy & Developmental Biology, Monash University, Melbourne, VIC, Australia

Hedgehog (Hh) signalling regulates normal development of many tissues and is upregulated in some cancers. Mice which lack the testicular desert hedgehog (Dhh) ligand exhibit disrupted embryonic gonad formation and male infertility in adulthood. However, the roles and sites of Hedgehog (Hh) signalling activity in the developing rat testis are unknown. Transcripts encoding Hh pathway components in embryonic and juvenile rat testes were localised by in situ hybridization with DIG-labelled cRNA probes. On embryonic day (E) 17.5, Sertoli cells contained transcripts encoding Dhh and both gonocytes and Sertoli cells contained the Ptc2 and Smo receptor transcripts. The cytoplasmic regulators (fused [Fu], suppressor of fused [SuFu]) and the three Gli transcriptional mediators were also detected in gonocytes. On E21.5 and postnatal day 4, all transcripts were present in Sertoli cells, but not gonocytes. To test the function of Hh signalling at the onset of rodent spermatogenesis, the newborn (day 1) mouse testis was cultured in hanging drop cultures. Addition of the selective Hh signalling inhibitor, cyclopamine, yielded a significantly lower level of the Hh target, Gli1 mRNA, as measured by real time PCR compared to vehicle controls. This confirms Hh pathway activity and inhibition in this system. Meiotic markers, SYCP3 and Stra8, were consistently upregulated (n = 6) following cyclopamine addition, however the fold-change varied between experiments. Overall, the cellular expression data indicate that Hh signals are active in both embryonic and juvenile rodent testes. The finding that Hh genes analysed in this study are expressed in both Sertoli and germ cells shows that both cell types are potential Hh targets, depending on the developmental stage of testis. The upregulation of candidates for Dhh target genes in the juvenile mouse testis in vitro suggests that Hedgehog signaling downregulates the expression of meiotic genes in gonocytes via paracrine mechanisms.

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PROTEOMIC AND FUNCTIONAL ANALYSIS OF HUMAN SPERM DETERGENT RESISTANT MEMBRANES

A. L. Anderson[1], L. Mitchell[1,2], E. A. McLaughlin[1,2], M. K. O'Bryan[2,3], R. J. Aitken[1,2], B. Nixon[1]

[1]Reproductive Science Group, The University of Newcastle, Callaghan, NSW, Australia
[2]ARC Centre of Excellence in Biotechnology and Development, The University of Newcastle, Callaghan, NSW, Australia
[3]Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC, Australia

Mammalian spermatozoa attain the ability to fertilize an oocyte as they negotiate the female reproductive tract. This acquisition of functional competence is preceded by an intricate cascade of biochemical and functional changes collectively known as 'capacitation'. Among the universal correlates of the capacitation process is a remarkable remodeling of the lipid and protein architecture of the sperm plasma membrane. While the fundamental mechanisms that underpin this dynamic reorganization remain enigmatic, emerging evidence has raised the prospect that it may be coordinated, at least in part, by specialized membrane microdomains, or rafts. In the studies described herein we have demonstrated that human spermatozoa express recognized markers of membrane rafts. Further, upon depletion of cellular cholesterol through either physiological (capacitation) or pharmacological (methyl-β-cyclodextrin) intervention, these membrane rafts appear to undergo a polarized redistribution to the peri-acrosomal region sperm head. The polarized targeting of membrane rafts to the sperm head encourages speculation that they represent platforms for the organization of proteins involved in sperm-oocyte interactions. Support for this notion rests with the demonstration that membrane rafts isolated on the basis of their biochemical composition in the form of detergent resistant membranes (DRMs), possess the ability to adhere to homologous zona pellucida. Furthermore a comprehensive proteomic analysis of the DRMs identified a number of proteins known for their affinity for the zona pellucida in addition to other candidates putatively involved in the mediation of downstream binding and/or fusion with the oolemma. Collectively these data afford novel insights into the sub-cellular localization and potential functions of membrane rafts in human spermatozoa

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MEASURING CHANGES IN TESTICULAR CELL POPULATIONS USING FLOW CYTOMETRY

G. Morin[1], K. Loveland[1,2]

[1]Biochemistry & Molecular Biology, Monash University, Clayton, VIC, Australia
[2]Anatomy & Developmental Biology, Monash University, Clayton, VIC, Australia

Spermatogenesis is first established during the first two weeks postpartum by the transition of undifferentiated (Kit-) into differentiated spermatogonia (Kit+). We recently showed that changes in the level of the growth factor activin alters the proportion of spermatogonial subtypes (1). However, detection of this transition by histology is unreliable. This project objective is to develop methods to efficiently measure changes in somatic and germ cell populations at the onset of spermatogenesis. Using surface (Kit receptor) and internal (mouse vasa homologue {MVH}) markers, we evaluated the proportion of differentiating germ cells in wild type Swiss mice by flow cytometry. Whole testes of mice at 7, 10, 14 days postpartum (dpp) were enzymatically dissociated and single cell suspensions were labelled with anti-Kit receptor antibody to detect Leydig cells and differentiating spermatogonia. These suspensions were then fixed and permeabilized in order to detect MVH, allowing spermatogonia to be distinguished from Leydig cells. Our present results show that combined Kit and MVH labelling is effective for evaluating the proportion of undifferentiating and differentiating germ cells. Our preliminary observations identified an elevation in the proportion of Kit+MVH+ cells between 7 and 10 days from 0.37 to 18%, indicating that spermatogonial differentiation advances dramatically between these ages. At day 14, the proportion of Kit+MVH+ cells decreased to 11%, as the emerging spermatocytes dilute spermatogonial numbers. These findings agree with published data (2). We have also used surface markers to discriminate between spermatogonia and Leydig cells without fixation or permeabilization, allowing us to isolate these cells for molecular and proteomics analysis. This will facilitate comparative profiling of germ cells with different levels of Kit, including those in mice with altered levels of growth factors (2) and hormones that govern the progression of testis development.

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SLX4, A KEY REGULATOR OF MEIOSIS AND DNA REPAIR IN THE MALE GERMLINE

I. R. Bernstein[1,2], E. A. McLaughlin[1,2], M. K. O'Bryan[2,3]

[1]Environmental and Life Sciences, The University of Newcastle, Callaghan, 2308, Australia
[2]ARC Centre of Excellence in Biotechnology and Development, Australia
[3]Monash University, Department of Anatomy and Developmental Biology, Clayton, VIC, Australia

Genome integrity relies on the ability of DNA repair proteins to open, cleave and recombine DNA. Slx4 (synthetic lethal of unknown function) or BTBd12 (BTBD12 domain-containing protein 12) the human homologue is a DNA repair protein which has multiple independent cellular roles. Importantly, it is a component in Holliday junction homologous recombination (HR) where it interacts with other proteins to repair double strand breaks (DSB). SLX4 forms a heterodimer complex with SLX1, an endonuclease, in response to DNA damage. This complex works by recruiting several associated proteins including XPF- ERCC1 a 3' flap endonuclease, to DSB allowing proper single strand annealing repair of the DNA. An essential part of meiosis in the male and female germ cell formation is DNA recombination which is initiated by DSB. Meiotic error at this stage can cause aneuploidy and infertility. As SLX4 acts as a key regulator of recombination we proposed that loss of this protein would cause dysfunction in germ cell development. Breeding pairs of $SLX4^{+/-}$ heterozygous mice were obtained from EUCOMM and germline transmission of the null allele confirmed. Adult mice were normal and healthy but analysis of the male reproductive tract confirmed abnormally small testes (wt ± vs testes). Further investigation demonstrated hypospermatogenesis with major loss of meiotic spermatocytes and post meiotic and spermatids. Epididymal sperm retrieval confirmed that the $SLX4^{-/-}$ male does produce poorly motile sperm with significant acrosomal abnormalities and associated failure of sperm-zona pellucida binding (2.5 ± 1.6 vs 13.6 ± 3.6 sperm bound) when compared to heterozygote or wild type littermates. Interestingly the female SLX4 null mouse also has small ovaries compared with het and wt littermates (wt vs null). Histomorphological analysis reveals a significant reduction in the ovarian follicle pool compared to wild type littermates with concomitant increased levels of apoptosis. Early breeding trials confirm significant loss of fertility in null males and females probably due to deficient sperm development and oocyte dysfunction. Our study now aims to further examine the role of SLX4 in germ cell development in the testes and ovary and its impact on infertility.

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INVESTIGATION INTO THE EXPRESSION OF HUMAN FRIENDLY BEHAVIOUR IN THE OSTRICH (*STRUTHIO CAMELUS*) – EFFECT OF IMPRINTING

I. A. Malecki, P. K. Rybnik-Trzaskowska

Animal Biology, University of Western Australia, Crawley, WA, Australia

In birds, filial imprinting to humans occurs after artificial hatching, which may lead to human following and sexual imprinting. We investigated the effect of imprinting on expression of favourable to human behaviours in juvenile ostriches. For the first seven days from hatching, the birds were exposed to a dummy female. At 12 months of age, a year before ostriches reach sexual maturity, the males (n = 25) were given testosterone intramuscularly in five every second day injections to induce precocial sexual behaviour. Additional six males, not exposed to the dummy, received the vehicle. Two weeks after the first injection, behavioural data collection commenced and, a range of behaviours were scored in five times over the period of 2 weeks from the last injection. The behaviours were given a score of 1 (behaviour present) or 0 (behaviour not present). The males exposed to a dummy female (n = 11) scored less for friendly to human behaviours than those not exposed to a dummy. No courtship type behaviour was observed in all males. Plasma testosterone concentrations increased and reached maximum levels by day 6 post-injection in exposed and not exposed to a dummy males but the levels were not different from the control (vehicle). We conclude that exposure to a dummy led to less friendly to human behaviour, while absence of the dummy led to more friendly behaviour towards humans. Testosterone injections failed to induce precocial sexual behaviour possibly because the amount given was insufficient or the birds could not yet express sexual behaviour to humans. The levels of testosterone in blood plasma occurred coincidently as they were probably associated with pre-pubertal development.

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SPIF – A NOVEL TESTIS-SPECIFIC GENE AND ITS INTERACTION WITH PKA

S. J. Tannock[1,2], E. A. McLaughlin[1,2], R. J. Aitken[1,2], S. D. Roman[1,2]

[1]ARC Centre of Excellence in Biotechnology and Development, Australia

[2]Environmental and Life Sciences, The University of Newcastle, Callaghan, Australia

The activation of protein kinase A (PKA) is strongly implicated in capacitation and sperm motility. However, the full pathway is yet to be elucidated. To identify potential PKA binding partners in sperm, a yeast two-hybrid assay was performed using the testis specific catalytic subunit (Cs) of PKA as the 'bait' to screen a mouse testis cDNA library. A novel cDNA clone termed Sperm PKA Interacting Factor (SPIF) was identified from the screen on three separate occasions. The interaction was confirmed by a protein pull-down using a C-terminal recombinant protein to SPIF and a PKA_{Cs} antibody. During cloning and sequence analysis, SPIF was found to contain two isoforms; a full length (4770 bp) and a truncated form (2784 bp) with alternate start sites and an identical 3' end, with only the full length isoform containing the PKA binding motif. SPIF was found to be testis specific using PCR and Northern Blotting with high expression levels in round spermatids and adult testis. The interaction between SPIF and PKA was further demonstrated with protein co-localisation in round spermatids and in the midpiece and flagellum of mouse sperm. In summary, we have identified a novel testis specific gene that in concert with PKA could prove to be an essential link in the incomplete capacitation pathway

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EMU SEMINAL PLASMA PROTEINS AND BETWEEN-MALE VARIATION

A. Tawang[1], I. A. Malecki[1], J. Cummins[2], G. B. Martin[1]

[1]Animal Biology, University of Western Australia, Crawley, WA, Australia
[2]School of Veterinary & Biomedical Sciences, Murdoch University, Murdoch, WA, Australia

Seminal plasma proteins may have a few functions in semen but their role in the emu (*Dromaius novaehollandiae*) has not been studied. We used ejaculates of six males collected during the peak of the breeding season to determine the protein make up of their seminal plasma. Following collection and sperm analysis semen was centrifuged to separate the sperm from seminal plasma. The concentration of proteins in seminal plasma was estimated and 20 μg/mL was found optimal for one-dimensional electrophoresis. Samples were separated with SDS-PAGE, using 12.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. Molecular weights and density of individual bands were estimated by 1D Total Lab gel analysis software. We identified 12 protein bands although they were not present in every male. One male was found to contain 12, one 10, three males had 9 bands and one had only 8 bands. Protein bands were in the range from 135 kDa to 7 kDa. Four bands (108–91 kDa, 67–78, 29–36 and 7 kDa) were found in all males. The 10 kDa band was present in all but one male. The intensity of those bands was similar between males except for one male with 29–36 kDa band that had less intensity. Further investigation is under way to further characterize those proteins and their relationship to ejaculate quality.

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CLAUDIN-8 IS EXPRESSED IN BOVINE TESTIS GERM CELLS

M. Rookledge, M. Colgrave, S. Stockwell, S. Schmoelzl

Livestock Industries and Food Futures Flagship, CSIRO, Armidale and St Lucia, NSW/QLD, Australia

Germline stem cells in the testis allow for the continued production of spermatozoa throughout a male's life. These cells are capable of self-renewal and have the ability to colonise testis tissue and give rise to spermatocytes after transplantation. Identification of germ cells and other cell types within testis tissue is important for increasing understanding of germ cell biology. Work in this lab is focused on the bovine testis, and we use both germ and non-germ cell markers for cell identification and germ cell enrichment. Such markers are also used for monitoring physiological and pathological changes in testis tissue after treatments such as irradiation. At present cell type markers for germ cells are limited, particularly in livestock species. This study has therefore investigated candidate marker genes for expression in testis cells. Here, we present quantitative gene expression data for Claudin-8 (CLDN8) in testis tissue. Claudin-8 is a membrane protein involved in the formation of tight junctions, such as are formed by germ cells in the testis. We used qRT-PCR to examine the expression of CLDN8 and other candidate genes in germ cell enriched and non-enriched testis cell fractions. Our qRT-PCR analysis shows that CLDN8 is preferentially expressed in germ cell enriched fractions. Testis cell fractions were also analysed for expression of established germ cell markers such as PLZF (ZBTB16) and VASA (DDX4), as well as for Sertoli cell marker GATA4. Our analysis shows a positive correlation between expression of CLDN8 and established germ cell markers, and a negative correlation between expression of CLDN8 and established Sertoli cells markers.

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IS THERE VARIATION IN THE LEVEL OF INPUT OF KISSPEPTIN TERMINALS ONTO GNRH NEURONS ACROSS THE EQUINE OESTROUS CYCLE?

C. J. Scott[1], C. A. Setterfield[1], A. Caraty[2], S. T. Norman[1]

[1]E H Graham Centre for Agricultural Innovation (NSW I & I) and Charles Sturt University, Wagga Wagga, NSW, Australia

[2]Unité de Physiologie de la Reproduction et des Comportements, INRA-CNRS-Université François Rabelais de Tours-Haras Nationaux, Nouzilly, 37380, France

Kisspeptin (KP) plays a key role in reproductive function including the regulation of gonadotrophin releasing hormone (GnRH) and luteinising hormone (LH) secretion in many species but little is known about its role in the mare. In this study, we examined the location of KP-producing neurons in the brain of the mare, their potential interactions with GnRH neurons, and temporal changes in their expression across the oestrous cycle. Mares (n = 3/group) were killed at oestrus (just prior to ovulation), mid-dioestrus, and late dioestrus and the head was perfusion fixed with paraformaldehyde, and hypothalamus collected. Coronal sections (40 µm) were used for dual-label immuno-stained for KP & GnRH. The majority of KP-immunoreactive (-ir) neurons were located in the arcuate nucleus/median eminence (especially mid and caudal regions), and periventricular nucleus. There was a trend (P = 0.09) towards increasing numbers of KP-ir neurons across the cycle. GnRH-ir neurons, located primarily in the arcuate nucleus (especially mid arcuate), as well as the preoptic area, did not change in number across the cycle. Numerous interactions between KP and GnRH neurons were observed, primarily in the arcuate nucleus; KP fibres interacting with GnRH cell bodies, fibre-fibre interactions between KP and GnRH, and GnRH fibres interacting with KP cell bodies. Overall we found KP inputs to 32% of GnRH-ir cells, but the number of these interactions did not vary across the oestrous cycle. This study has confirmed the reciprocal innervation between KP & GnRH neurons in the mare. Although we did not detect variation in the degree across the oestrous cycle this may reflect the sample size issues inherent to equine research.

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SEASONAL EFFECTS ON OVARIAN DYNAMICS AND OOCYTE DEVELOPMENTAL COMPETENCE IN SOWS EXPERIENCING EARLY-PREGNANCY LOSS

M. Bertoldo[1], P. K. Holyoake[2], G. Evans[1], C. G. Grupen[1]

[1]Faculty of Veterinary Science, University of Sydney, Camden, Australia

[2]Wagga Wagga Agricultural Institute, Industry and Investment NSW, Wagga Wagga, NSW, Australia

Seasonal infertility in the domestic sow has a considerable economic impact on the pig industry. Reduced oocyte quality and increased wean-to-service interval (WSI) have been observed during the seasonal infertility period (1, 2). The aim of this study was to determine the relationship between season, WSI, ovarian dynamics and oocyte quality in sows experiencing early-pregnancy loss. Sows that experienced early-pregnancy loss were grouped according to their previous WSI (≤ 6 or > 6 d) and the presence (CL) or absence (NCL) of corpora lutea on their ovaries at the time of slaughter. Groups were WSI≤6d/CL, WSI≤6d/NCL, WSI>6d/CL and WSI>6d/NCL. Cumulus-oocyte complexes were recovered from antral follicles (3–8 mm) and subjected to parthenogenetic activation following in vitro maturation in summer (n = 742) and late-spring (n = 594). Data were analysed using a generalised linear mixed-model in GenStat release 10. The mean number of CL/sow was greater (11.57 ± 3.30) in late-spring compared to summer (9.26 ± 0.99; P < 0.05). There were no effects of WSI on oocyte developmental potential. A greater proportion of oocytes developed to the blastocyst stage in summer (55.94 ± 5.17%) compared to late-spring (31.18 ± 6.38%; P < 0.05). Blastocysts derived from oocytes collected in summer had a greater number of cells (46.60 ± 1.17) than blastocysts derived from oocytes collected in spring (43.2 ± 0.95; P < 0.05). Blastocysts derived from oocytes of CL ovaries had more cells (47.08 ± 1.13) than those derived from oocytes of NCL ovaries (42.91 ± 0.75; P < 0.05). It appears that sows experiencing early-pregnancy loss have inherently poor oocyte quality. The greater developmental potential of oocytes in summer suggests a proportion of sows culled for early-pregnancy loss in summer have good oocyte quality, but fail to conceive and/or initiate pregnancy due to other summer-autumn related factors, such as heat stress.

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INVESTIGATING THE ROLE OF CYCLIN A2 DURING OOCYTE MEIOSIS

P. C. Jennings, K. T. Jones

School of Biomedical Sciences, University of Newcastle, Callaghan, NSW, Australia

Aneuploidy is often derived from chromosomal segregation errors in oocytes, leading to Down Syndrome and early embryo loss. Thus it is important to understand the molecular control of female meiosis. Cyclins and cyclin-dependent kinases (CDKs), particular those contributing to Maturation Promoting Factor (MPF, or CDK1) activity, play key roles in regulating meiosis. While cyclin B1 is classically regarded as the regulatory component of MPF, cyclin A2 can also bind and activate CDK1, and in mammalian somatic cells it is known to promote both G1/S and G2/M transitions. There is an absolute requirement for cyclin A2 during development and differentiation as its deficiency results in early embryonic lethality. As such, cyclin A2 has not been extensively studied, particularly as to its role in meiosis.

To examine the role cyclin A2 plays during mammalian female meiosis, we carried out knockdown experiments. Microinjection into mouse oocytes of cyclin A2 siRNA induced a ~70% knockdown. Cyclin A2 knockdown did not inhibit germinal vesicle breakdown but did act to delay it. Extrusion of the first polar body was significantly reduced ($P = 0.002$) in comparison to non-injected controls and those injected with a negative control siRNA. Furthermore, microinjection of cyclin A2 can stimulate entry into meiosis. Thus it seems possible that cyclin A2/CDK can exhibit MPF activity. In conclusion, our data suggest that cyclin A2 can regulate meiotic entry in oocytes and also plays an important role in successful passage through the first meiotic division.

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GYCOMIC ANALYSES OF THE GRANULOSA AND THECA OF BOVINE OVARIAN FOLLICLES

N. Hatzirodos[1], J. Nigro[2,3], A. V. Vashi[2], H. F. Irving-Rodgers[1], B. Caterson[4], T. R. Sullivan[5], J. A. Ramshaw[2], J. A. Werkmeister[2], R. J. Rodgers[1]

[1]Research Centre for Reproductive Health, Robinson Institute, University of Adelaide, Adelaide, SA, Australia
[2]Molecular and Health Technologies, CSIRO, Clayton, VIC, Australia
[3]Department of Anatomy and Developmental Biology, Monash University, Clayton, VIC, Australia
[4]Connective Tissue Biology Laboratories,Cardiff School of Biosciences, Cardiff University, Cardiff, United Kingdom
[5]Data Management and Analysis Centre, Discipline of Public Health, University of Adelaide, Adelaide, SA, Australia

Development of ovarian follicles involves changes in cell function and remodelling of the follicular wall. Remodelling necessitates changes in the extracellular matrix, including proteoglycans (PGs). PGs contain glycosaminoglycans (GAGs) covalently bound to a protein core. The length of GAG chains in PGs and the degree and pattern of sulphation differs between cell types and change as cells alter their phenotype. PGs that have been identified in follicles include the chondroitin sulphate (CS) PG, versican and inter-a trypsin inhibitor, and the heparan sulphate (HS) PGs, perlecan and type XVIII collagen. The latter two are found in focimatrix, the follicular and the thecal subendothelial basal laminas. To examine GAGs composition in follicles, bovine antral follicles of various sizes were collected. Follicles were dissected and a biopsy taken for histological classification of health. Theca layers and granulosa cells were collected separately and analysed by fluorophore-assisted carbohydrate (FACE) analysis of GAGs following digestion to disaccharides with chondroitinase ABC, hyaluronidase, heparinase, and heparitinases I and II. Four non GAG sugars and 12 different GAG derived disaccharides were identified and quantitated on a per DNA basis. Healthy versus atretic follicles for each cell type were compared and correlation analyses were also undertaken. Immunohistochemistry using CS specific antibodies was also conducted. There was no effect of size on the GAG content for either granulosa or theca cells. The 4- and 6- sulphated CS sugars were the most abundant following digestion in all tissues. Theca had higher levels than granulosa cells of HS derived disaccharides and also of un- or 4- or 6- N-sulphated CS derived disaccharides. Some sulphated CS moieties localised uniquely to the stroma surrounding blood vessels in the theca externa. Atretic follicles had lower amounts of disaccharides derived from HS in both granulosa and thecal cells, suggesting that heparanase may be activated upon atresia.

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HETEROGENEITY OF GENE EXPRESSION IN BOVINE SMALL FOLLICLES

N. Hatzirodos, H. F. Irving-Rodgers, R. J. Rodgers

Robinson Institute, Research Centre for Reproductive Health, Obstetrics and Gynaecology, University of Adelaide, Adelaide, SA, Australia

Small antral follicles <5 mm in bovine ovaries undergo one of two fates: further growth and selection to become the dominant follicle for ovulation, or atresia. Atresia can occur before, during or after selection. As follicle grow past >5 mm there is upregulation in expression of focimatrix genes and later upregulation of the LH receptor and steroidogenic enzymes, especially aromatase, in the granulosa cells. For follicles at sizes >5 mm entering atresia the granulosa cells are the first in the follicle to die. Thus expression of genes in granulosa cells is critical to the fate of the follicle. To examine granulosa cells of small follicles we collected bovine ovaries and dissected follicles, removed part of the follicle wall for subsequent classification of health or atresia, and harvested the remaining granulosa cells for RNA isolation. Follicles examined included small follicles (<5 mm), both healthy (n = 10) and atretic (n = 5), and healthy large follicles (>10 mm, n = 4). RNA was hybridized to Affymetrix GeneChip Bovine Genome Arrays and the results were analysed using Partek Genomics Suite software. The number of genes which were 2 fold differentially regulated between large and small follicles by Benjamini Hochberg post hoc test (False Discovery Rate, $P < 0.05$) was 2408 and between healthy and atretic small follicles was 4931. The coefficient of variation (CV; SD/mean × 100) for the expression level of each gene for each group was calculated. A gene frequency distribution indicated greater heterogeneity in expression levels in small follicles in comparison to large follicles. Furthermore, the greatest variability in genes in small follicles includes those that are either up or down regulated due to atresia or growth. We therefore conclude that variability in small follicles is a consequence of alternative fates that small follicle can undergo.

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PROGESTERONE PRODUCTION FROM GRANULOSA CELLS OF SOWS IS ENHANCED EQUALLY BY OMEGA-3 DERIVED PROSTAGLANDIN E3 AND OMEGA-6 DERIVED PROSTAGLANDIN E2

R. Smits[1], D. T. Armstrong[2], L. Ritter[2], M. Mitchell[2], M. B. Nottle[2]

[1]Research and Innovation, Rivalea Australia, Corowa, NSW, Australia

[2]Robinson Institute and School of Paediatricts and Reproductive Health, University of Adelaide, Adelaide, SA, Australia

Caughey et al (2005) reported that prostaglandins derived from omega 3 sources eicsapentaenoic acid (EPA C20:5) and docosahexaenoic acid (DHA C22:6) have different properties to those derived from the preferred substrate, arachidonic acid (ARA C20:4; n-6). Armstrong et al (2006) demonstrated that PGE2 increased progesterone when porcine granulosa cells were cultured in vitro with hCG. We hypothesized that PGE3 which is derived from EPA will produce a lower steroidogenic response as progesterone from isolated granulosa cells collected from pre-ovulatory sow ovaries.

Ovaries were collected from slaughtered sows and follicles between 3–8 mm were aspirated and through a series of wash steps in HTCM (Hepes TCM 199). Mid-sized granulosa cells were recovered in a solution of BTCM (bicarbonate TCM) containing IGF-1. 0.5×10^6 cells/mL were cultured in 250 μL of Control (BTCM only), PGE2 or PGE3 (320 ng/mL in BTCM, Cayman Chemical Co.) treatments with IGF1 at 25 ng/mL. Cultures were incubated for 22 h at 38°C. Cultures were centrifuged and the supernatant was analysed in duplicate for progesterone. Data was analysed by Univariate GLM ANOVA. There was no significant difference between PGE2 and PGE3 treatments, however the main effect of PGE significantly increased progesterone production relative to the control (P = 0.017). Granulosa cells cultured with omega 3 derived PGE3 did not produce significantly lower progesterone levels than those with PGE2. We conclude that both PGE2 and PGE3 promote a steroidogenic response in cultured porcine granulosa cells.

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Table 1.

[ab]Mean values with different superscripts are significant ($P < 0.05$).

	BTCM (0)	PGE2 (320 ng/mL)	PGE3 (320 ng/mL)	*P* value
Progesterone (ng/mL)	1.20 ± 0.36^a	3.04 ± 0.59^b	2.55 ± 0.25^b	0.017

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X-LINKED INHIBITOR OF APOPTOSIS PROTEIN (XIAP) EXPRESSION PATTERNS IN THE SHEEP OVARY

H. R. Douglas, I. C. Kokay, D. R. Grattan, P. R. Hurst

Anatomy and Structural Biology, University of Otago, Dunedin, New Zealand

At reproductive age, the ovary undergoes continual cyclicity of follicles due to multiple positive and negative signals that promote follicle growth and development, selection for ovulation, or atresia. The majority of follicles undergo atresia, a degenerative process involving granulosa cell apoptosis. This process is executed by caspases, which are cysteine proteases. Caspases are potently inhibited by XIAP providing a potential mechanism to control follicular atresia. The study objective was to test the hypothesis that XIAP will show elevated expression in healthy antral follicles compared to atretic antral follicles and will show an inverse relationship with active caspase-3 immunoreactivity in the same antral follicle during the sheep estrous cycle. Reproductively mature Romney ewes (n = 9) had estrous cycles synchronized with a prostaglandin F2α analogue, then tissue was collected on days 14, 15 and 16 of the subsequent natural cycle. Analysis involved determining the presence and localization of XIAP using in situ hybridization histochemistry and immunohistochemistry then comparing XIAP expression patterns with distribution of active caspase-3 protein. XIAP mRNA was not detected in primordial, primary and secondary follicles. In contrast, XIAP protein was present from the primary stage onwards. Antral follicles showed positive XIAP mRNA and protein expression in both granulosa and thecal cell layers and antral follicles on the same tissue section showed variable expression. All day 14 antral follicles were positive for XIAP mRNA expression irrespective of the level of active caspase-3 immunoreactivity, whereas an inverse relationship between active caspase-3 and XIAP was apparent in the majority of day 15 and 16 antral follicles. XIAP protein was widely expressed in active caspase-3 negative antral follicles and indicated a negative correlation with the onset of active caspase-3 expression in the majority of follicles. These results indicate that XIAP may regulate follicular atresia and act as an indicator of follicular health in the sheep ovary.

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EXPRESSION AND REGULATION OF TWO PROTO-ONCOGENES *BCL2* AND *FOS* IN THE PREIMPLANTATION MOUSE EMBRYO

X. L. Jin, C. O'Neill

Sydney Centre for Developmental and Regenerative Medicine, University of Sydney, Sydney, NSW, Australia

Development of the preimplantation embryo requires survival signalling by autocrine embryotrophins. Paf is the first defined embryotrophin and acts via a receptor- and phosphatidylinositol-3-kinase -dependent induction of intracellular calcium transients.[1,2] These calcium transients induce a calmodulin-dependent nuclear accumulation and phosphorylation of the Creb transcription factor at the time that definitive transcription from the embryonic gene is initiated in the 2-cell embryo.[3] A round 4084 loci possess CRE elements including Bcl2 and Fos.[4] In this study we examined the role of Paf in the transcription of the key proto-oncogenes, Bcl2 and Fos in the early mouse embryo. Quantitative RT-PCR detected Bcl2 and Fos in oocytes and some cohorts of zygotes, but not in 2-cell, 8-cell and blastocysts. Immunolocalization of Bcl2 and Fos showed little staining in oocytes and zygotes but increased staining in the 2-cell to blastocyst stages. Paf treatment of 2-cell embryos caused an α-amanitin-sensitive expression of Bcl2 and Fos for 20 min that subsided by 40 min. This transcription was blocked by inhibition of calcium (BAPTA-AM) or phosphatidylinositol-3-kinase signa l ling (LY 294002). Analysis of individual embryos showed most zygotes, but only ~12% of 2-cell embryos had detectable Bcl2 and Fos. A significantly smaller proportion of $Ptafr^{-/-}$ (Paf-receptor null) 2-cell embryos had detectable Bcl2 transcript and embryo culture (which diminishes Paf signalling) decreased transcription of Bcl2. This dichotomous pattern of transcript expression is consistent with the known periodic actions of Paf (with a periodicity of 90–120 min)[2] and the relatively short-half life of the resulting transcripts. Bcl2 antagonist (HA14- 1) caused a dose-dependent inhibition of cultured zygotes to develop to morphological blastocysts, which was partially reversed by exogenous Paf. The results show for the first time the autocrine embryotrophi n Paf induces periodic transient expression of key proto-oncogenes and provides evidence for a role of embryotrophins in the onset of gene expression in preimplantation embryo.

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DIETARY OMEGA-3 FATTY ACID SUPPLEMENTATION ALTERS EMBRYO DEVELOPMENT AND METABOLISM IN SOWS

M. Mitchell[1], R. Smits[2], N. O. Palmer[1], A. N. Filby[1], M. Lane[1]

[1]Robinson Institute, School of Paediatrics and Reproductive Health, University of Adelaide, Adelaide, SA, Australia
[2]Rivalea (Australia) Pty Ltd, Corowa, NSW, Australia

The long-chain omega-3 polyunsaturated fatty acids (LCPUFAs) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have diverse biological effects, including the improvement of cardiovascular health and asthma. The aim of this study was to examine how dietary LCPUFA supplementation of sows influenced follicular dynamics, embryo development and metabolism. A total of 30 sows per treatment were fed a control diet (CD: standard commercial ration) or an omega-3 supplemented diet (O-3: diet formulated with 3 g fish oil/kg) for 6 weeks. Ovaries were collected at slaughter and weighed 3–5 days post-weaning. Oocytes were aspirated and counted from 3 different follicle size groups (1–4mm, 4–8mm and 8+ mm) and matured in vitro for 44–46 h, prior to (a) fertilization using IVF and analysis of subsequent embryo development in culture, (b) assessment of embryo respiration rate (EmbryoScope technology, UniSense, Denmark) and glucose metabolism (radiolabelled glucose technique). Granulosa cells collected at aspiration were analysed for gene expression using q-PCR. Despite no effect of O-3 supplementation on ovary weight nor the proportion of oocytes that were fertilised and cleaved, there tended to be more medium and large follicles for the O-3 fed group. Significantly more embryos from sows fed O-3 diets developed to blastocyst stage ($P < 0.05$) and these tended to have an increased number of cells ($P = 0.06$). At the zygote stage, diet didn't influence embryo respiration rate but glucose utilisation by blastocysts was significantly lower for O-3 treatment compared to CD ($P < 0.006$). Gene expression of the ER2-α, FSH-receptor, or the prostaglandin receptors EP2 and EP4 did not differ, but there tended to be reduced expression of progesterone receptor (PR) and cycloxygenase-2 (COX-2) in granulosa cells from O-3 sows ($P < 0.07$). In summary, O-3 dietary supplementation changed follicular growth, embryo development and blastocyst metabolism. Further studies are ongoing to determine the effect of supplementation on embryo survival and litter size.

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METHYL BINDING DOMAIN PROTEIN (MBD1) IN THE NUCLEUS OF THE MOUSE ZYGOTE

Y. Li, X. L. Jin, C. O'Neill

Sydney Centre for Developmental and Regenerative Medicine, University of Sydney, Sydney, NSW, Australia

MBD1 is one of five proteins which bind methylated DNA and regulate gene transcription. The binding of these proteins, particularly MBD1, is commonly used as a proxy measurement of global CpG methylation. Since methylation is reported to be highly dynamic during the first cell-cycle, with reported asymmetric global demethylation of the paternal and maternal genomes by the time of syngamy, we were interested to assess the pattern of staining of the MBD1 during this stage of development. A specific antibody to MBD1 was shown by Western analysis to detect in zygotes a protein of predicted mass. Using immunolocalization, however, we found no staining in pronuclei. Brief acid treatment (10 min, 4 M HCl) followed by immunolabelling revealed strong pronuclear MBD1 staining throughout the maturation of the zygote and on metaphase chromosomes, indicative of epitope masking under normal staining conditions. Upon unmasking by acid treatment zygotes collected fresh from the oviduct did not show consistent differences in MBD1 staining between the maternal or paternal chromosomes or pronuclei, but for those embryos produced by IVF we found more MBD1 staining in the male paternal pronucleus. Brief treatment with trypsin caused a marked loss of MBD1 staining and this treatment increased the extent of staining of 5-methylcytosine. These results show that MBD1 antigen persists on DNA after treatments normally used for the detection of 5-methylcytosine. MBD1 at least partially masks methylcytosine from immunological detection and the results therefore raise the possibility that the reported changes in genome methylation in the zygote are a consequence of the binding of MBD1. If MBD1 binding is truly a proxy for methylation, the persistence of high levels of MBD1 throughout the first cell-cycle questions the current paradigm of global demethylation during zygote maturation.

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THE RELATIONSHIP BETWEEN DNA FRAGMENTATION IN MOUSE OOCYTES AND GENITAL TRACT MICROORGANISMS

J. E. Harris[1], E. S. Pelzer[1], J. A. Allan[2], E. Whiteside[1], L. De Boer[1], C. L. Knox[1]

[1]Cell and Molecular Biosciences, Institute of Health and Biomedical Innovation, Kelvin Grove, QLD, Australia
[2]The Wesley Hospital, Wesley Monash IVF, Brisbane, QLD, Australia

Colonising bacteria detected within the follicular fluid of women undergoing assisted reproductive technology cycles has been associated with decreased embryo transfer rates and decreased pregnancy rates. The bacteria isolated from the follicular fluid of these women, at the time of trans-vaginal oocyte retrieval include Gram-positive anaerobic rods, Gram-negative anaerobic rods, streptococci, staphylococci, and lactobacilli. Some of these bacteria are opportunistic pathogens in the female genital tract. The expression of virulence factors including hyaluronidase and DNase, could affect the structural integrity of the oocyte and induce DNA fragmentation and apoptosis. Hyaluronin is a major carbohydrate component of the cumulus oocyte complex that could be a target for bacterial hyaluronidase. Other virulence factors associated with bacteria detected in cases of genital tract infections include the hydrolytic enzymes sialidase, β-galactosidase and β-N-acetylhexosaminidase (Howe 1999). Production of reactive oxygen species as a result of the presence of bacteria and bacterial heat shock proteins have been suggested as mechanisms responsible for increased DNA fragmentation and apoptotic progression of male gametes in colonised semen. Previous studies have not confirmed a direct relationship between sperm morphology and the level of sperm DNA fragmentation. DNA fragmentation in oocytes in relation to the presence of bacteria remains to be investigated. Oocyte quality is only determined by the assessment of cumulus cell morphology prior to in-vitro fertilisation. In-vitro testing of oocyte DNA integrity under various conditions may further the understanding of oocyte quality. In this study, mouse oocytes were used to investigate the structural and DNA integrity of oocytes after in vitro exposure to *Lactobacillus* spp., *Streptococcus* spp., and *Staphylococcus* spp. and determine whether the morphological appearance of the oocyte was predictive of the level of DNA fragmentation and whether DNA fragmentation can be attributed to certain bacterial species found colonising follicular fluid.

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ANALYSIS OF PROTEINS SECRETED BY THE PREIMPLANTATION MOUSE EMBRYO

R. Gentles, C. O'Neill

Sydney Centre for Developmental and Regenerative Medicine, University of Sydney, Sydney, NSW, Australia

Many embryos generated by assisted reproductive technologies do not have the capacity for full development. A non-destructive test that allows the most viable embryos to be identified would allow development of a range of strategies to improve these technologies. Analysis of the proteinaceous secretome of the preimplantation mouse embryo by electrospray mass spectrometry of tryptic digests of embryo-conditioned culture media identified 20 peptides.[1] Lactate dehydrogenase beta (LDH) and protein disulfide isomerase (PDI) were consistently among the most abundant peptides identified. PDI has been found to be secreted and surface expressed in a number of other cell types suggesting a biologically significant role. LDH-beta is a marker of plasma membrane integrity and is widely used in medicine for assessment of cardiac health. The aim of this study was to assess the expression of these proteins in the early embryo to gain some understanding of the potential significance of their presence within the embryonic secretome. Zygotes (B6CBF2) were collected 20–22 h post hCG. They were cultured in groups of 10 embryos for 96 h in 10 mL of modHTF 30 μg BSA/mL media. Embryonic protein was extracted for western blot analysis or embryos subjected were to immunolocalization studies for PDI and LDH. Western blot analysis revealed presence of proteins of expected molecular mass. Staining for both proteins occurred at high levels at each stage of development throughout pre-implantation development. Staining was predominantly cytoplasmic and excluded from nuclei. Cultured blastocysts had less LDH than fresh but the level for PDI was similar for both. Staining of non-permeabilized embryos revealed extracellular staining of PDI. The results show high levels of cellular expression of two proteins reported to be released by the embryo in vitro.

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STUDY OF APHRODISIAC DRUG ACID PHOS ON MALE ALBINO RAT

C. K.I. Deshmukh

P.G.Department of Zoology, S.G.B Amravati University, Amravati, Maharashtra, India

The male albino rat, Rattus norvegicus maintained in the laboratory by supplying regularly food and water. Acid phos is a well known aphrodisiac drug from homeopathy medical system. Doses of 200 gm of 30 number globules made from sugar of milk and moistened by 15 ml of acid phos (H_3PO_3) of 6 potency. Group-I, II and III experimental rats were carried out for 15, 30, and 15 day recovery period respectively. Appreciable behavioral changes and changes in the body weights were noticed. In 15, 30 and in 15 days of recovery period, the acid phosphatase, SGOT and albumin were significant ($P < 0.05$) while alkaline phosphatase, SGPT, cholesterol, glucose, total proteins and globulin was found non-significant but A : G ratio was increased significantly in 30 days of treatment. The weight of liver, kidney, and testis has found linear increased relationship with the body weight but significant ($P < 0.050$) increased in the weight of seminal vesicleand body weight in the experimental rat. Various histo -architectural changes were observed in the tissues of liver, kidney, testis and seminal vesicle. Both liver and kidney showed degenerative changes after 15 and 30 days.Tetraploid stages of liver pernchymal cells were predominant in the experimental rats while in 15 days of recovery period, both attained the recovery. In 30 days, the diameter of seminiferous tubules is markedly reduced, with thin unfolded mucosa. In 15 days of administration of acid phos, the intertubular spaces between the seminiferous tubule were also reduced. The number of spermatids was increased in recovery period, the testis showed the recovery. In 15 days of administration, the secretion in the lumen of seminal vesicles increased related with the structure of the epithelium of seminal vesicles while in 15 days of recovery period, the seminal vesicles showed recovery of secretory activities with pseudostratified epithelium. All the results are discussed detailed in paper.