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ABSTRACTS

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001

ISOLATION AND CHARACTERISATION OF PORCINE ES CELLS

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Embryonic stem (ES) cells have the capacity for self renewal, can remain undifferentiated in long term culture and can contribute to all the cells in the body including the germ cells. ES cells have been isolated in mice and have also been described for humans. However despite considerable effort for more than two decades ES cells which can contribute to the germline are yet to be isolated for the pig or any domestic species for that matter. We have developed a new method for isolating porcine ES cells which uses whole embryos cultured in alpha MEM with 10% serum replacement plus additives under 5% O_2. Unlike methods employed previously this method results in homogenous outgrowths whose cells resemble ES cells and which express Oct 4 and Nanog and SSEA-1 [1]. These cells can be passaged and cryopreserved repeatedly resulting in the establishment of cell lines at similar efficiencies to that reported previously for 129Sv mice [2]. These cells can form embryoid bodies and can be differentiated to various cell types representative of all three germ layers [3]. Following their injection into blastocysts these cells localise /become incorporated in the inner cell mass and can be used to produce chimaeras when these embryos are transferred to recipient animals [2]. To date we have produced chimaeric pigs from one male ES cell line [2]. These are currently being mated to demonstrate germline transmission. Future studies will examine the applicability of our method to other species commencing with mice and cattle before extending these to humans.

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(3) Vassilieva S, Vassiliev I, Beebe LFS, Harrison SJ, McIlfatrick SM, Ashman RJ, Nottle MB. (2009). In vitro developmental potential of porcine embryonic stem cells. Proceedings 7th Annual Meeting. International Society for Stem Cell Research. 2009. Abstract 349. Barcelona, Spain.

002

REGULATION OF PLURIPOTENCY AND CELL CYCLE IN FETAL GERM CELLS

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The germ cell lineage is unique in that it must ensure that the genome retains the complete developmental potential (totipotency) that supports development in the following generation. This is achieved through a number of mechanisms that prevent the early germ cell lineage from somatic differentiation and promote the capacity for functional totipotency. Part of this process involves the retained germ line expression of key genes that regulate pluripotency in embryonic stem cells, embryonic germ cells and some embryonal carcinoma cells, the stem cells of testicular tumours. Despite this, germ cells are not intrinsically pluripotent and must differentiate along the male or female pathways, a process which requires commitment of the bi-potential primordial germ cells to the spermatogenic (male) pathway and their entry into mitotic arrest, or to the oogenic pathway (females) and entry into meiosis. This involves robust regulation of regulatory networks controlling pluripotency, cell cycle and sex specific differentiation. Our work aims to further understand the mechanisms controlling differentiation, pluripotency and cell cycle in early male and female germ cells. Our data shows that mitotic arrest of male germ cells involves strict regulation of the G1-S phase check-point through the retinoblastoma protein. In addition, suppression of pluripotency in differentiating male germ cells involves post-transcriptional regulation of OCT4, transcriptional regulation of *Sox2* and *Nanog* and methylation of the *Sox2* and *Nanog* promoters. Further understanding of these processes promises to lead to a greater understanding of the molecular mechanisms underlying control of pluripotency, cell cycle and differentiation in the germ line and the initiation of germ cell derived testis tumours.

003

ENDOMETRIUM: CINDERELLA TISSUE IN A STEM CELL WORLD

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Despite human endometrium undergoing more than 400 cycles of regeneration, differentiation and shedding during a woman's reproductive years, and that in non-menstruating species (eg rodents) there are cycles of endometrial growth and apoptosis, endometrial stem/progenitor cells have only recently been identified. Since there are no specific stem cell markers, initial studies using functional approaches identified candidate epithelial and stromal endometrial stem/progenitor cells as colony forming cells/units (CFU) (1). Further evaluation of key stem cell properties of individual CFU demonstrated that rare $EpCAM^+$ epithelial cells and $EpCAM^-$ stromal cells underwent self renewal by serial subcloning >3 times and underwent >30 population doublings in culture. Clonally-derived epithelial cells differentiated into $cytokeratin^+$ gland-like structures. Single stromal cells were multipotent as they differentiated into 4 mesodermal lineages; myogenic, adipogenic, osteoblastic and chondrogenic, suggesting that human endometrium contains a rare population of epithelial progenitor cells and mesenchymal stem cells (MSC) (2). Transplantation of freshly isolated human endometrial cells into immunocompromised mice reconstructed endometrial tissue that responded to estrogen and progesterone (3). Endometrial MSC can be prospectively isolated by co-expression of CD146 and PDGFRβ (4), but not Stro-1, a bone marrow MSC marker (5). Currently there are no known markers of endometrial epithelial progenitor cells. Endometrial cancer tissue harbours a small subpopulation of clonogenic, self-renewing, tumour-initiating cells, producing tumours that recapitulate parent tumours in histoarchitecture and differentiation markers (ERα, PR, cytokeratin, vimentin) when xenografted into mice, suggesting they are cancer stem cells. Candidate epithelial and stromal stem/progenitor cells have been identified in mouse endometrium as label retaining cells (LRC) in the luminal epithelium and perivascular cells at the endometrial-myometrial junction, respectively (6). It is likely that endometrial stem/progenitor cells play key roles in the development of gynecological diseases associated with abnormal endometrial proliferation such as endometriosis and endometrial cancer (7).

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(4) Schwab KE & Gargett CE Hum Reprod 2007; 22: 2903–11.
(5) Schwab KE et al Hum Reprod 2008; 23: 934–43.
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005

PREPARING FERTILE SOIL: THE IMPORTANCE OF ENDOMETRIAL RECEPTIVITY

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The human endometrium is receptive for implantation of a blastocyst, for only 4–5 days in each menstrual cycle. Failure of implantation is a major reason for infertility in women, and the inability to achieve endometrial receptivity is responsible for much of the failure of reproductive technologies. Endometrial receptivity requires alterations in the uterine luminal and glandular cells, particularly in terms of their secretory capacity and altered expression of adhesion molecules, along with decidualization of the endometrial stroma, which in women is initiated during the receptive phase, regardless of the presence of a blastocyst. Increased leukocyte numbers are also important. The microenvironments provided by the endometrium during the receptive phase and which support implantation are highly complex and constantly changing. The present review summarizes work from our laboratories and others, regarding these microenvironments, how they impact on receptivity and how they are disturbed in infertile women. Such microenvironments can also be manipulated to provide new contraceptive strategies for women.

007

INFLAMMATORY PATHWAYS IN ENDOMETRIAL CANCER

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Endometrial cancer is the most common gynaecological malignancy and accounts for 5% of cancers in women (http://info.cancerresearchuk.org/cancerstats/). The majority of endometrial cancers occur in post-menopausal women and 80% of patients are diagnosed when the tumour is confined to the uterus (stage 1 disease). Many of the risk factors for developing endometrial cancer are associated with excess exposure to oestrogen unopposed by progesterone. It is well established that local inflammatory pathways contribute to the initiation and progression of endometrial cancers via the release of local mediators to facilitate immune cell recruitment, angiogenesis and neoplastic cell proliferation and metastasis. Prostaglandins are one class of molecules that are important mediators of these processes. Prostaglandins are bioactive lipids produced from arachidonic acid by cyclooxygenase (COX) enzymes and specific terminal prostanoid synthase enzymes. Following biosynthesis, prostaglandins are rapidly transported outside the cell and exert an autocrine/paracrine function by coupling to specific prostanoid G protein-coupled receptors (GPCR). Expression of COX enzyme, synthesis of prostaglandins and expression of various prostaglandin receptors are elevated in pathologies of the endometrium including cancer. Using genome wide array analysis, we have identified target genes that are regulated by prostaglandins such as PGF2α via interaction with its GPCR - FP receptor. Gene ontology analysis have highlighted significant elevation in expression of genes involved in inflammatory and vascular processes which are central to endometrial function. Moreover, using an array of cellular and molecular techniques and in vivo models we have established an important role for PGF2α-FP interaction in regulating inflammatory and vascular functions. To-date, suppression of the activity of prostaglandins in pathology has focussed on using inhibitors of cyclooxygenase enzymes, which are key enzymes in the pathway to prostaglandin synthesis. However, this has been associated with serious cardiovascular side effects. Development of novel drugs that target specific receptors may provide better therapeutic alternatives.

009

KALLIKREIN-RELATED PROTEASES AS NOVEL THERAPEUTIC TARGETS IN PROSTATE AND OVARIAN CANCER

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The kallikrein-related (KLKs) peptidases are implicated in prostate and ovarian cancer invasion/metastasis via activation of growth factors, proteases and extracellular matrix degradation involved in. In our published work, we used cell biology approaches to show novel associations of KLK peptidases with processes indicative of metastasis and the potential of our novel sunflower trypsin inhibitor scaffold-engineered KLK4 inhibitor. Our current studies are directed towards discovering the precise KLK target proteins/substrates and the subsequent signalling pathways involved in these events in order to determine their therapeutic target potential. In this regard, we are using novel tissue engineered biomimetic 3D gel matrices to better mimic the *in vivo* micro-environment of prostate cancer cells especially in bone metastasis and peritoneal invasion in ovarian cancer. Pilot studies show that PC3 cells cultured on an osteoblast-derived bone matrix undergo an EMT-like change but remain dispersed on the cell surface. In contrast, LNCaP cells cluster aligning with the fibrillar structure as they invade into the bone matrix as typically seen *in vivo*. KLK4 proteolysis of the osteoblast-derived bone matrix has identified additional novel substrates. In addition, we are exploring the cell biology that underlies the reported high KLK4 or KLK7 levels associated with poorer outcome in women with epithelial ovarian cancer (EOC). Of note, KLK4 or KLK7 transfected SKOV3 EOC cells have increased chemoresistance to taxol and/or cisplastin suggesting a mechanism for this poor outcome. Furthermore, KLK7 transfected SKOV-3 cells form multicellular aggregates (MCA) in agarose suspension (a process indicative of peritoneal tumour cell spread seen in ascites fluid clinically) which can be reversed by a KLK7 blocking antibody indicating the critical role played by KLK7 in this event. These new paradigms are providing novel information on the role of KLK peptidases in prostate and ovarian cancer progression and their potential as novel therapeutic targets.

020

IN VITRO MATURATION OF FARM ANIMALS OOCYTES: A USEFUL TOOL FOR INVESTIGATING THE MECHANISMS LEADING TO FULL TERM DEVELOPMENT

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Due to logistical and economical reasons assisted reproduction of domestic animals has been based mostly on the use of oocytes isolated from ovaries collected at the slaughterhouse. In order to propagate valuable or rare genetic material, perform somatic cell nuclear transfer, generate genetically modified animals it was essential to obtain fully competent oocytes that would allow full term development of the in vitro produced embryos. Such demanding need has soon made clearly evident the crucial role played by oocyte quality, how easy it is to compromise its developmental potential and the fact that it is impossible to restore it once it has been lost. Almost three decades after the first bovine, sheep, goat, horse and pig in vitro generated offsprings were born, a large body of information has accumulated on the mechanisms regulating oocyte competence and on how the latter may be preserved during all the required manipulations. The amount of knowledge is far from being complete and many laboratories are actively working to further expand it. In this review we will highlight the aspects of the ongoing research in which we have been actively involved.

035

THE ROLE OF THE SERTOLI CELL IN REGULATING SPERMATOGENESIS, IMMUNE RESPONSES AND INFLAMMATORY DISEASE: MULTIPLE FUNCTIONS, COMMON MECHANISMS?

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There is increasing evidence that the Sertoli cell, in addition to modulating responses to direct antigenic challenges (eg. intratesticular allografts), is central to the response of the testis to inflammation and infection. Systemic inflammation exerts an inhibitory effect on spermatogenesis, which has been attributed to the effects of fever, vascular disturbances, or loss of androgenic support. However, recent studies point to more direct effects of inflammation on spermatogenesis. The discovery that Sertoli cells express Toll-like receptors (TLR), and react to TLR ligands by producing inflammatory cytokines and other mediators, provides a mechanism to account for this direct inhibition. Moreover, the pattern of cytokines produced by the Sertoli cell during inflammation is highly characteristic. For example, when stimulated with TLR ligands the Sertoli cell produces the pro-inflammatory cytokines, interleukin-1α (IL1α) and IL6, and the regulatory cytokine, activin A, but does not produce IL1β and tumour necrosis factor-α, which are major pro-inflammatory products of activated macrophages. The disruptive effects of inflammation on spermatogenesis may be attributed to the elevated production of these cytokines, all of which have stimulatory or inhibitory effects on germ cell mitosis, meiosis and apoptosis and Sertoli cell tight junction formation, In addition, activation of TLR/IL1 mediated inflammatory pathways in the Sertoli cell inhibits its ability to respond to its principal trophic hormone, follicle-stimulating hormone. Studies on the regulation of these interactions will further establish the role of the Sertoli cell in inflammation and infection. However, such studies also have important implications for normal Sertoli cell function, as TLRs can respond to endogenous ligands as well. Consequently, the Sertoli cell may be viewed as a sentinel cell, supporting and protecting spermatogenesis when conditions are optimal, but rapidly shutting down spermatogenesis in the presence of infection or illness. Intriguingly, these apparently disparate roles appear to involve common inflammation-related mechanisms.

036

SEX SPECIFIC FUNCTION OF THE HUMAN PLACENTA: IMPLICATIONS FOR FETAL GROWTH AND SURVIVAL

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The placenta plays a central role in the development of the fetus by modulating the supply of nutrients and oxygen throughout pregnancy. We have identified that the placenta adapts to the presence of a maternal pathophysiology in a sexually dimorphic manner which results in differences in fetal growth. We have reported that the female fetus reduces her growth in response to chronic maternal asthma which ensures her survival in the presence of an acute asthma exacerbation. Conversely the male fetus continues to grow normally in the presence of maternal asthma but this is associated with a poor outcome in the presence of an acute exacerbation. We propose that the sexually dimorphic response of the fetus is derived from differences in placental adaptation to a pathophysiological condition. In the presence of a female fetus and maternal asthma, we have observed global gene changes in the placenta accompanied by significant alterations in microRNA expression. Downstream of these alterations we have observed differences in protein expression especially in relation to placental cytokines and the glucocorticoid receptor. In the presence of a male fetus there are fewer changes in global placental gene and microRNA expression, and we have observed no alterations in expression of placental cytokines or the glucocorticoid receptor. These differential adaptations ensure increased survival of the female fetus and continued growth of the male fetus in adverse conditions.

037

IMMUNE-LIKE MECHANISMS ASSOCIATED WITH OVULATION

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Ovulation is the unique biological process by which a mature oocyte and surrounding somatic cells, the cumulus cell-oocyte complex (COC), are released from the surface of the ovary into the oviduct for transport and fertilization. Ovulation is similar to an inflammatory response: the follicles become hyperemic, produce prostaglandins and synthesize a hyaluronan-rich extracellular matrix. However, this view of ovulation may be too restrictive and need to be broadened to encompass the innate immune cell surveillance response system. This hypothesis is being proposed because ovarian granulosa cells and cumulus cells express and respond to innate immune cell related surveillance proteins (Toll-like receptors 2 and 4) and cytokines such as interleukin 6 (IL6) during ovulation. In addition, recent studies indicate that the ovulation process that is set in motion by the surge of luteinizing hormone (LH) is mediated, in large part, by the EGF-like factors (Amphiregulin, epiregulin and betacellulin) and their critical activation of RAS, most probably KRAS that is expressed at high levels in granulosa cells, and the mitogen activated protein kinases, MAP3/1 (ERK1/2). Mice in which granulosa cells are depleted of ERK1/2 fail to ovulate, oocyte meiosis does not resume, COC expansion is impaired and luteinization is blocked. Thus the global molecular reprogramming of granulosa cell gene expression patterns is completely derailed. Supported, in part by NIH-HD-16229, -16272 and -07495 (SCCPIR).

046

FUTURE PROOFING AUSTRALIA'S MAMMALIAN BIODIVERSITY USING GENOME RESOURCE BANKING AND ART: WHERE ARE WE UP TO?

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The establishment of a functional genome resource bank for the genetic management and future proofing of Australian native mammals sounds great in theory, but what is the reality of this idea. In order to understand the current rate of progress in this area, we will present an overview of the inherent structural and physiological limitations of non-eutherian mammalian reproduction in terms of gamete biology and ART. For the male, these include (1) an unique mode of spermatid condensation that imparts the need for major structural changes to sperm morphology during epididymal transit, (2) a lack of cysteine protamines and disulphide bonds in the sperm chromatin that predisposes the nucleus to post-thaw chromatin relaxation, (3) an extremely stable acrosome, which to date, has not been possible to experimentally react in vitro, (4) unusual lipid composition in the plasma membrane that potentially makes the sperm cell resistant to cold shock trauma and (5) the need, in some species, for extremely high concentrations of cryoprotectant, that paradoxically, appear to be cytotoxic to the spermatozoon. Female limitations include, (1) the production of a large yolky oocyte and resulting embryo, making it difficult to cryopreserve, (2) a small and technically challenging complex reproductive tract that makes gamete recovery and artificial insemination problematic and (3) a general lack of information on marsupial reproductive physiology and behaviour that has hindered the development of protocols for timed induction of oestrus and ovulation. We shall also identify, socio-political and ethical limitations holding back the application of assisted breeding technology in these species.

047

SPERM COMPETITION, INBREEDING AND THE EVOLUTION OF SUPERIOR EJACULATES

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The production of viable sperm is essential for male reproductive success. However, because females in many species mate with several males during a single reproductive episode, leading to sperm competition, a male's reproductive success also depends critically on the ability of his sperm to compete efficiently with those from rival males for fertilizations. Therefore, males who regularly encounter sperm competition are expected to produce high quality ejaculates. Here, I will provide an overview of how sperm morphology and performance are influenced by sperm competition, both within and between species, using recent empirical examples. Having established the importance of producing high quality ejaculates in males experiencing sperm competition, I will then examine the reproductive consequences of producing sub-optimal sperm. Given the well known role that inbreeding plays in reducing genetic quality and reproductive success, I will focus in particular on how inbreeding acts to reduce sperm quality. Finally, I will examine the consequences of inbreeding for male reproductive success in species where sperm competition is rampant. Together, these results highlight the evolutionary importance of sperm competition and inbreeding in shaping ejaculate traits.

048

FUNCTIONAL DIFFERENCES BETWEEN SEX-SORTED AND NON-SORTED SPERM

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The development and application of flow cytometric sorting for the pre-selection of sex has progressed at an increasing rate since the first report of live pre-sexed offspring of rabbits (2). The technique has been extended to production of pre-sexed offspring of numerous species and sorted bull semen is now widely available commercially around the world. Due to the stresses involved in the sex-sorting process, sex-sorted sperm may be functionally compromised in terms of reduced motility and viability, and their fertilising lifespan within the female reproductive tract may be reduced. Consequently, fertility *in vivo* may be compromised. However, improvements to the technology and a greater understanding of its biological impact on the sperm have facilitated recent developments in sheep, and we have demonstrated that sex-sorting is capable of selecting a functionally superior ram sperm population in terms of both *in vitro* and *in vivo* function. This has resulted in high fertility after intrauterine insemination of sex-sorted ram sperm (1). Unfortunately, to date, these results have not been matched in other species.

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059

POOR GROWTH BEFORE BIRTH IMPAIRS INSULIN SECRETION – WHAT WE HAVE LEARNT ABOUT THE MECHANISMS FROM THE PLACENTALLY-RESTRICTED SHEEP

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Diabetes occurs when insulin secretion fails to increase sufficiently to compensate for developing insulin resistance. This implies that the increased risk of diabetes in adults who were small at birth reflects impaired insulin secretion as well as their well-known insulin resistance. More recently, direct evidence has been obtained that adults and children who were growth-restricted before birth secrete less insulin than they should, given their level of insulin resistance. Our research group is using the placentally-restricted (PR) sheep to investigate the mechanisms underlying impaired insulin action (sensitivity and secretion) induced by poor growth before birth. Like the intra-uterine growth-restricted (IUGR) human, the PR sheep develops impaired insulin action by adulthood, but has enhanced insulin sensitivity in infancy, associated with neonatal catch-up growth[1,2]. Impaired insulin action begins to develop in early postnatal life, where although basal insulin action is high due to enhanced insulin sensitivity, maximal glucose-stimulated insulin action is already impaired in males[3]. Our cellular and molecular studies have identified impaired beta-cell function rather than mass as the likely cause of impaired insulin secretion, and we have reported a novel molecular defect in the calcium channels involved in the insulin secretion pathway in the pancreas of these lambs[3]. Upregulation of IGF-II and insulin receptor are implicated as key molecular regulators of beta-cell mass in the PR lamb[3]. By adulthood, both basal and maximal insulin action are profoundly impaired in the male lamb who was growth-restricted at birth[2]. These studies suggest therapies to prevent diabetes in the individual who grew poorly before birth should target beta-cell function, possibly in addition to further increasing beta-cell mass, to improve insulin secretion capacity, and its ability to increase in response to development of insulin resistance. We are now using the PR sheep to test potential therapies, since the timing of pancreatic development and hence exposure to a growth-restricting environment, is similar to that of the human.

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(3) KL Gatford, SNB Mohammad, ML Harland, MJ De Blasio, AL Fowden, JS Robinson, JA Owens (2008) Endocrinology 149: 5118–27.

060

OVER FEEDING EARLY IN LIFE AND RISK OF OBESITY: INSIGHT FROM THE RODENT

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While adult lifestyle factors undoubtedly contribute to the incidence of obesity and its attendant disorders, mounting evidence suggests that programming of obesity may occur following over-nutrition during development. As hypothalamic control of appetite and energy expenditure is set early in life and can be perturbed by certain exposures such as under-nutrition and altered metabolic and hormonal signals, in utero exposure to maternal obesity related changes may contribute to programming of obesity in offspring. Data from animal studies indicate both intrauterine and postnatal environments are critical determinants of the development of pathways regulating energy homeostasis. Experimental evidence in rat studies from our laboratory points to an additive detrimental impact of high fat diet consumption after weaning in animals born of obese mothers. Deleterious effects of high fat diet during pregnancy on metabolic profile, adiposity and cardiac hypertrophy were enhanced by postnatal over consumption. Even modest early postnatal overfeeding induced by litter size reduction leads to increased adiposity. Studies are needed to determine to what extent the effect of maternal and early nutritional changes persist. This presentation summarizes recent evidence of the impact of maternal obesity on subsequent obesity risk, paying particular attention to the hypothalamic regulation of appetite, and markers of metabolic control. There is an urgent need to investigate the mechanisms underlying the trans-generational effects of maternal obesity due to an extraordinary rise in the rates of maternal obesity.

061

PATERNAL FACTORS INFLUENCING GESTATIONAL OUTCOME IN OFFSPRING

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Fetal programming can often be attributed to sub-optimal, but potentially modifiable, maternal factors such as smoking and poor nutrition. Much of the literature in this field points to factors that cause intrauterine growth restriction (IUGR) and the long term consequences for offspring health. It is not greatly appreciated, however, that other complications that may occur with, or independently of, IUGR predispose offspring, and their mothers, to poor health. These include preeclampsia and gestational diabetes. Elevated maternal BMI increases the risk for most pregnancy complications. Our new data show that paternal obesity (BMI>30) and waist circumference >102cm are associated with IUGR. We have also identified polymorphisms in a number of genes that regulate how the placenta differentiates and invades the maternal decidua, and how the mother adapts to pregnancy, that are associated with adverse pregnancy outcomes. Excitingly, many of these are polymorphisms in the paternal genome. One might reasonably expect that these would be found in imprinted genes expressed only from the paternal allele. However, we have also found several non-imprinted genes in which paternal genotype has a significant influence on pregnancy outcome both on maternal and infant disease states, but also on fetal and placental growth parameters. Furthermore, these genes interact with the maternal environment including diet and smoking to profoundly affect maternal and infant health. Consequently we now propose a complicated model of the control of optimal placental and fetal growth and pregnancy outcome that includes important genetic contributions from both parents to placental genotype that regulate conceptus growth and function. Importantly, paternal genotype can influence placental gene expression and the myriad of placental hormones and growth factors secreted into the maternal circulation that modulate maternal adaptation to pregnancy and, in susceptible women, these interact with maternal genotype, BMI and lifestyle to cause poor maternal and infant health.

062

DEVELOPMENTAL ORIGINS OF TYPE 2 DIABETES: EPIGENETIC MECHANISMS IN BETA CELL FAILURE

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The abnormal intrauterine milieu of intrauterine growth retardation (IUGR) permanently alters gene expression and function of pancreatic b -cells leading to the development of diabetes in adulthood. Expression of the pancreatic homeobox transcription factor *Pdx1* is permanently reduced in IUGR and epigenetic modifications are responsible for this decrease. Exendin-4 (Ex-4), a long-acting glucagon-like peptide 1 (GLP-1) analog, given on days 1–6 of life increases *Pdx1* expression and prevents the development of diabetes in the IUGR rat. Here we show that Ex-4 increases USF-1 and PCAF association at the proximal promoter of *Pdx1*, thereby increasing histone acetyl transferase (HAT) activity leading to a permanent increase in histone H3 acetylation and H3K4 methylation. Normalization of these histone modifications precludes DNA methylation thereby preventing silencing of *Pdx1* in islets of IUGR animals. These studies demonstrate a novel mechanism whereby a short treatment course of Ex-4 in the newborn period prevents diabetes in adulthood by restoring *Pdx1* promoter chromatin structure thus preserving *Pdx1* transcription.

065

THE MAMMALIAN OOCYTE: FROM BENCH TO CLINIC

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The mature mammalian oocyte is the central link between generations. It is not only responsible for the transfer of the female genome between generations, but also largely determines embryo and early fetal developmental potential. For any female, oocytes are in limited supply and are easily damaged, such that the availability of high quality or developmentally competent oocytes is a fundamental rate-limiting factor in female fertility. This is particularly relevant in Australian society today with the steadily rising age to first conception which adversely affects oocyte quality and female fertility. Yet despite years of research and clinical IVF we still have a poor understanding of the molecular and cellular processes that control oocyte quality. It is clear that oocytes acquire developmental competence in the ovarian follicle. The acquisition of competence necessitates communication between the oocyte and maternal systems, a process which endows developmental potential as the oocyte grows and matures inside the follicle. At the cellular level this is achieved by bi-directional communication between oocytes and their companion somatic cells [1]. Over the past 10 years my laboratory has focused heavily on the nature of these oocyte-somatic communication axes and their impact on oocyte quality. Over this period, our work and that of others has shaped a new paradigm in ovarian biology, which is that the oocyte is not passive in the follicle, but rather that it actively directs the differentiation of its neighbouring somatic cells into cumulus cells through the secretion of GDF9 and BMP15 growth factors [2]. In doing so, oocytes dictate the function of their neighboring cumulus cells, directing them to perform functions needed for the appropriate growth and development of the oocyte. For example, cumulus cells supply oocytes with an array of nutrients, substrates and regulatory molecules such as cAMP, many directly through gap-junctions. These communication axes establish and maintain an elaborate and intricate local oocyte-cumulus auto regulatory loop that is required to enable post-fertilisation development. A clear clinical application of this new knowledge is in Artificial Reproductive Technologies, in particular oocyte in vitro maturation (IVM) [3]. IVM biotechnologies have the capacity to capture the vast supply of oocytes in the mammalian ovary and generate mature oocytes in vitro. Generating offspring using IVM is already a clinically and commercially viable biotechnology in livestock breeding programs, particularly in cattle. IVM is a particularly attractive technology for the treatment of human infertility, as it removes the need for expensive and potentially harmful ovarian hyperstimulation protocols used in clinical IVF. However, widespread application of IVM in humans requires an increase in efficiency and further examination of safety of the technology. Recent work from my laboratory has increased IVM success rates in animals by using GDF9 and BMP15 in IVM [2, 3] and by developing a new system of "Induced-IVM" that more closely resembles the mechanisms of oocyte maturation in vivo. Most recently, the latter approach has led to substantial increases in embryo yield and pregnancy outcomes to levels equivalent to hormone-stimulated IVF [4]. The next challenge is to adapt these new approaches to clinical/field conditions to provide new opportunities for infertile women and for the development of a wide range of reproductive biotechnologies.

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SRB ORALS

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IN VIVO DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS TO UTERINE TISSUE

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The endometrium undergoes cyclic regeneration. This regeneration has been attributed to adult stem progenitor cells and developmental mechanisms [1, 2]. A better understanding of human endometrial development may shed light on the mechanisms involved in endometrial regeneration and on early origins of adult endometrial disease. The lack of human fetal endometrial tissue has impeded research in early human endometrial development. We hypothesized that directed differentiation of human embryonic stem cells (hESC) to human endometrial tissue by neonatal mouse uterine mesenchyme represents a novel system to study early development of human endometrium. Recent studies have shown that the neonatal mouse uterine mesenchyme is extremely inductive and undergoes reciprocal signalling with human endometrial epithelial cells [3]. Our aim is to establish a xenograft tissue recombination protocol based on a model for human prostate tissue differentiation using hESC [4]. Our method involved formation of embryoid body (EB) with GFP labelled hESC (ENVY) [5] for recombination with 2x0.5mm pieces of epithelial-free uterine mesenchyme from postnatal day 1 mice. Upon fusion in culture, the recombinant tissue is grafted under the kidney capsule of NOD/SCID mice for 4–12 weeks and monitored by in-vivo imaging. Immunohistochemical analysis of recombinant grafts 4 weeks post transplantation (n=4) revealed immature CK8+CK18+Hoxa10+ human epithelial cells surrounded by mouse mesenchymal cells suggesting differentiation of hESC to epithelial cells possibly of endometrial lineage. The ER+PR+SMA+Hoxa10+ mouse mesenchymal cells surrounding human glands differentiated into SMA+ cells possibly via reciprocal signalling from human epithelial cells. At 8 weeks, we found several CK18+/Hoxa10+ human glands co-expressing CA125. These glands are supported by Hoxa10+ human stromal cells. Further experiments are underway to induce the expression of ER and PR in Hoxa10+ epithelial cells which will be crucial in revealing their endometrial lineage.

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ISOLATION OF PUTATIVE EMBRYONIC STEM CELLS FROM CLONED PIG EMBRYOS

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The isolation of embryonic stem cells from cloned embryos (NT-ESC) from domestic animals would have a number of biomedical and agricultural applications. Putative ESC lines from *in vivo* derived and *in vitro* produced pig embryos were recently established using a new isolation method[1]. The aim of the current study was to determine whether NT-ESC lines could be isolated from cloned pig embryos using this method. To do this we determined initially whether the treatment of embryos with Trichostatin A (TSA), a histone deacetylase inhibitor, could increase the number of cloned embryos that develop to the blastocyst stage because TSA has been shown to increase blastocyst development and NT-ESC isolation efficiencies in mice[2]. Cloned embryos were produced as described previously[3]. Briefly, in vitro matured sow oocytes were enucleated, fused with adult fibroblasts using an electrical pulse and activated about 1.5 hrs later with a second electrical pulse. Reconstructed embryos were then cultured in modified NCSU23 with or without 50nM TSA treatment for the initial 24 hours of culture. Embryo development was assessed on day 6. Treatment with TSA increased the number of cloned embryos that developed to the blastocyst stage (143/471; 30%) compared with control (54/353; 15%; $P<0.0001$). Blastocysts were then plated by mechanical depression onto mitotically inactivated mouse embryonic fibroblast feeder layers in a serum-free culture system on day 7. There was no significant difference in the efficiencies of establishment of homogeneous primary outgrowths between TSA treated (17/96; 18%) and control blastocysts (8/43; 19%). Thirteen homogenous outgrowths from the TSA treated group were vitrified at passage 2 or 3. Sublines are currently being characterised to determine their pluripotency.

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THE EFFECT OF INSULIN ON EMBRYONIC STEM CELL PROGENITOR CELLS IN THE MOUSE BLASTOCYST

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Human ESCs are produced from embryos donated at the mid-stage of pre-implantation development. This cryostorage reduced viability. However, it has been shown that this can be improved by the addition of growth factors to culture medium. The aim of the present study was to examine whether the addition of insulin to embryo culture medium from the 8-cell stage of development increases the number of ES cell progenitor cells in the epiblast in a mouse model. In vivo produced mouse zygotes (C57Bl6 strain) were cultured in G1 medium for 48h to the 8-cell stage, followed by culture in G2 supplemented with insulin (0, 0.17, 1.7 and 1700pM) for 68h, at 37^{o}C , in 5% O_2, 6%CO_2, 89% N_2. The number of cells in the inner cell mass (ICM) and epiblast was determined by immunohistochemical staining for Oct4 and Nanog. ICM cells express Oct4, epiblast cells express both Oct4 and Nanog. The addition of insulin at the concentrations examined did not increase the ICM. However, at 1.7pM insulin increased the number of epiblast cells (6.6±0.5 cells vs 4.1±0.5, P=0.001) in the ICM, which increased the proportion of the ICM that was epiblast (38.9±3.7% compared to 25.8±3.4% in the control P=0.01). This indicates that the increase in the epiblast is brought about by a shift in cell fate as opposed to an increase in cell division. The effect of insulin on the proportion of cells in the epiblast was investigated using inhibitors of phosphoinositide3-kinase (PI3K) (LY294002, 50µM); one of insulin's main second messengers, and p53 (pifithrin-α, 30µg/ml); a pro-apoptotic protein inactivated by PI3K. Inhibition of PI3K eliminated the increase caused by insulin (4.5±0.3 cells versus 2.2±0.3 cells, P<0.001), while inhibition of p53 increased the epiblast cell number compared to the control (7.1±0.8 and 4.1±0.7 respectively P=0.001). This study shows that insulin increases epiblast cell number through the activation of PI3K and the inhibition of p53, and may be a strategy for improving ESC isolation from human embryos.

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ROLE OF CANDIDATE STEM/PROGENITOR CELLS IN A MOUSE MODEL OF ENDOMETRIAL MENSTRUAL BREAKDOWN AND REPAIR

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Adult stem/progenitor cells (SPC) identified in human and mouse endometrium may be responsible for its remarkable regenerative capacity (1), however a functional role for SPC in menstruation is yet to be established. This study aimed to identify label retaining cells (LRC) as candidate epithelial SPC involved in the rapid re-epithelisation of the uterine surface in a mouse model; tissue SPCs are quiescent and will retain label (BrdU), while label is diluted out as transit amplifying cells proliferate. Mice were ovariectomised and endometrial breakdown and repair induced, mimicking menstruation in women (2). BrdU (50µg/g) was administered intraperitoneally 8.5 days before endometrial repair. Tissue was collected to assess initial labelling, and following four chase periods prior to and during endometrial repair (n=3–5 animals/group) and immunostained for BrdU. LRC were categorised as minimal (<50% nuclear label), partial (50–75%) and full (100%) and counted in the luminal (LE) and glandular (GE) epithelial compartments for each group. The majority of LE (91.4±1.9%; mean±SEM) and 35±3.8% of GE were initially labelled. During breakdown and repair the percentage of full LE LRC (38±13.1% vs 1.3±1.1%) and partial LE LRC (49.1±4.3% vs 1.8±0.7%) significantly decreased ($p \leq 0.01$) whilst minimal LRC significantly increased (12.9±3.5% vs 85.2±4.3%; $p \leq 0.001$) indicating dilution of BrdU label as cells proliferated. In contrast full, partial and minimal GE LRC did not significantly change throughout endometrial breakdown and repair. The rapid dilution of BrdU label in the LE suggests that the transit amplifying population are responsible for the rapid proliferation observed, whilst the lack of change of BrdU in the GE suggests that GE may be a source of SPC. Double immunofluorescence and confocal microscopy are currently underway to further characterise the LRC population in this model. This study provides some of the first insights into the contribution of candidate SPC to endometrial repair.

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PROLIFERATION ABILITY, TELOMERASE ACTIVITY AND MOLECULAR CHARACTERIZATION OF PLURIPOTENT CELL LINES FROM IVF AND PARTHENOGENTIC PIG EMBRYOS

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Porcine pluripotent ES cell lines are a promising tool for biotechnology, biomedical and developmental biology studies. However, no conclusive results have been obtained to derive genuine ES cells in the pig. Here we compare derivation efficiency of putative ES cells from IVF versus parthenogenetic pig embryos. We describe proliferation ability and doubling time, we study pluripotency markers and telomerase activity (TA) of the cell lines obtained. Pig oocytes were either fertilized in vitro or parthenogenetically activated. Blastocysts were subjected to immuno-surgery. Inner cell mass were plated and outgrowth expansion was monitored daily. Self renewal molecules were studied by RT-PCR and/or immunocytochemistry for up to 42 passages. TA was measured every five passages. The results obtained indicate that stable cell lines can be generated from IVF and parthenogenetic embryos. The latter appeared less resilient to immuno-surgery but demonstrated a higher ability to produce outgrowths. 77% of the parthenogenetic lines vs only 33% of the IVF ones expressed pluripotency markers and displayed high TA. Regardless to their origin, colonies showed a latency growth period in the 48 hours after plating, they grew exponentially between day 3 and 6 and then, proliferation rate was greatly reduced. Doubling time was estimated to be 31.5 hours. In both IVF and parthenogenetic cell lines, positivity for Oct-4, Nanog, Sox-2, Rex-1, SSEA-4, Alkaline phosphatase, TRA-1-81 and STAT3 was detected; no signal for LIF-Receptor beta and gp130 was shown. These results indicate that the main pluripotency network related molecules are expressed in the porcine species, while a classical LIF-Receptor beta- gp130-STAT3 activation pathway does not appear to be involved in the maintenance of self renewal. Finally, every cell lines expressed high TA, which was turned down once cells were induced to differentiate, indicating a physiologically normal control of TA in these cells.

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TRANSPLANTED GERM CELLS CAN COLONIZE THE GONADS OF SEXUALLY COMPETENT FISH AND PRODUCE FUNCTIONAL GAMETES

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Germ cell (GC) transplantation (GCT) has great potential for seed production and conservation of valuable germlines. Currently available approaches to GCT in fish rely on sophisticated equipment and skills for cell transplantation into the blastodisc of embryos and/or the peritoneal cavity of small, sometimes few millimeters long larvae. Moreover, transplanted individuals may take years to grow to maturity, adding to the cost of producing surrogate gametes. In this context, the use of intragonadal GCT into sexually competent recipients that have been experimentally depleted of endogenous GCs might overcome these constraints. Here we demonstrate the feasibility of xenogeneic GCT in sexually competent fish. Spermatogonial cells isolated from pejerrey (Odontesthes bonariensis, Atherinopsidae) donors were implanted surgically into the testes of congeneric Patagonian pejerrey (O. hatcheri) that were severely depleted of endogenous GCs by treatment with Busulfan (40 mg/kg) and elevated water temperature (25°C) (Fig. 1).

Donor cell behavior inside the recipient gonads was tracked using fluorescent cell linkers (CFDA-SE and PKH-26) and showed that transplanted spermatogonial cells were able to migrate towards, settle and multiply at the blind ends (cortical region) of the seminiferous lobules. The presence of donor-derived sperm was confirmed by PCR in 20% of the surrogate Patagonian pejerrey fathers at 6 months and fertilization of pejerrey eggs with surrogate sperm produced 1.2–13.3% pure pejerrey offspring (Fig. 2). These findings indicate that transplantation of spermatogonial cells into sexually competent fish can shorten considerably the production time of surrogate gametes and offspring. Ongoing studies are examining (low-tech) refinements in the proposed approach, such as non-surgical transplantation of GCs through genital papilla, and the suitability of GCT for generation of female gametes, for which cryopreservation techniques have not yet been developed. The results obtained so far have been encouraging and these developments will make GCT invaluable for the timely rescue of fish species facing imminent extinction.

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IL-6 INCREASES THE SHEDDING OF NECROTIC TROPHOBLASTS FROM PLACENTAL EXPLANTS

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Preeclampsia (PE) is characterised by elevated maternal blood pressure, preceded by endothelial cell dysfunction. Dead trophoblasts, shed from the placenta may be one of the factors that trigger PE. Women with PE frequently have elevated serum levels of inflammatory markers such as, IL-6 and TNF a but their functional significance is unclear. In this study we investigated whether these or other cytokines can alter trophoblast shedding from placental explants. Placental explants were treated with 9 different cytokines for 72 hours. Shed trophoblasts then were harvested using our published method[1]. The numbers of trophoblasts shed were quantified by automated cell counter. Expression of active of caspases 3&7 by the shed trophoblasts was determined using a FLICA kit. The trophoblasts shed from cytokine-treated or control explants were exposed to endothelial cell monolayers and endothelial activation determined by ELISA for cell surface ICAM-1. Treatment of explants with IL-6 caused a 50% increase ($p=0.001$), while TNF a and TGF b 1, caused smaller significant increases in the numbers of trophoblasts shed. Trophoblasts shed from explants treated with IL-6, TGF b 1 , or TGF b 3 expressed significantly less active caspases 3&7 than controls or trophoblasts shed from explants treated with other cytokines. Exposing trophoblasts shed from IL-6- or TGF b 1-treated explants to endothelial cells caused a significant ($P<0.001$) increase in endothelial activation. Normally trophoblasts shed from the placenta die by an apoptosis-like process and their phagocytosis by endothelial cells is silent but a shift to shedding of necrotic trophoblasts can lead to endothelial cell activation[2]. However, it remains unclear what might trigger a shift from apoptotic to necrotic trophoblast death. This study suggests that IL-6 and possibly other cytokines can alter both the number and the nature of shed trophoblasts such that the trophoblast are more necrotic and their phagocytosis by maternal endothelial cells could contribute to the pathogenesis of preeclampsia.

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INTERLEUKIN 11 AND LEUKEMIA INHIBITORY FACTOR REGULATE CYTOKINE NETWORKS IN HUMAN FIRST TRIMESTER PLACENTA

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Inadequate trophoblast invasion is thought to be involved in the pathogenesis of preeclampsia (PE). Shallow trophoblast invasion has been implicated to lead to placental hypoxia and a localised overproduction of pro-inflammatory cytokines. Interleukin (IL) 11 and leukemia inhibitory factor (LIF) are IL6-type cytokines produced at the maternal-fetal interphase and regulate human trophoblast migration/invasion, but their mechanisms of action are unknown. We aimed to determine the effect of hypoxia on human placental IL11/LIF secretion and the effect of IL11/LIF on placental cytokine secretion. As an in vitro model we cultured human first trimester placental villous explants (gestation weeks 7-9) in serum free conditions under either 2% (hypoxia) or 20% oxygen (normoxia) (N=8/gp) for 48h. Medium was assayed for IL11/LIF by ELISA. The effect of IL11/LIF (N=6/gp) on placental explant cytokine secretion (26 cytokines) was analysed using a quantitative multiplex immunoassay. Data was expressed as pg/mg wet weight and then as % change vs control (100%).

IL11 secretion from explants was decreased by 76±5% ($p<0.01$) under hypoxia vs normoxia while LIF secretion did not differ significantly. Under normoxia the most highly abundant cytokines were IL6, IL8 and MCP-1 while moderate levels of G-CSF, CX3CL1, IP-10, and PGDF were present in conditioned medium. IL11 increased IL10 secretion while it decreased G-CSF, TNFa, IL1receptor antagonist (Ra), IL6 and PDGF secretion ($p<0.05$) vs control. Similarly LIF decreased G-CSF, TNFa, IL1Ra and additionally IL8 secretion ($p<0.05$) vs control. IL6 and VEGF secretion were increased ($p<0.05$) while MCP-1 was reduced ($p<0.05$) by hypoxia vs normoxia.

This study identified for the first time that IL11 was reduced by hypoxia and identified a profile of cytokines secreted by placenta. IL11 and LIF regulated similar and unique anti-inflammatory cytokines in first trimester placenta. Whether placental IL11 or LIF is altered in women with PE remains to be elucidated however this study suggests mechanisms of action and confirms the importance of IL11/LIF in placentation.

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FUNCTIONAL ROLE OF HTRA3 IN TROPHOBLAST CELL INVASION DURING HUMAN PLACENTAL DEVELOPMENT

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Controlled invasion of extravillous trophoblast (EVT) through the maternal decidua is important for placental development and function. 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 and is down-regulated in invading EVTs, while up-regulation of decidual HtrA3 controls the process. The current study investigated HtrA3 expression in human endometrial stromal cells (HESC) during decidualization in vitro and whether HtrA3 inhibits EVT cell invasion. Stromal cells isolated from human endometrium were decidualized in vitro with estrogen, progesterone and cAMP. Quantitative RT-PCR and western showed HtrA3 mRNA and protein expression was significantly increased in decidualized HESC compared to controls. Indirect immunofluorescence showed homogeneous pattern and increase in intensity of HtrA3 staining in decidualized HESC compared to non-decidualized cells. HTR-8 cells derived from first trimester of pregnancy EVT showed higher levels of HtrA3 mRNA expression compared to other human choriocarcinoma cell lines (AC-1M88, AC-1M32, JEG-3 and BeWo). Both intracellular and extracellular HtrA3 staining was observed in HTR8 cells. Functional role of HtrA3 in cell invasion was determined in HTR-8 cells using an in vitro invasion assay. Exogenous addition of mutant HtrA3 (inhibitor) resulted in a significant increase in HTR-8 cells invading through matrigel coated membrane compared with controls. TGFβ-1 (as positive control) completely inhibited invasion of HTR-8 cells. HtrA3 is tightly regulated during decidualization of HESC in vitro. Inhibition of HtrA3 activity in trophoblastic HTR-8 cells increased invasiveness supporting its functional role during placental development.

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TROPHOBLAST DEPORTATION IS DEPENDENT UPON CASPASES 3, 8 AND ROCK

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Background: Trophoblast deportation is the process whereby multinucleated fragments of the syncytiotrophoblast are shed from the placenta into the maternal blood. It is estimated that 150,000 are shed from the placenta and deported daily in normal pregnancy and that more are shed during preeclampsia[1]. In normal pregnancy deported trophoblasts are thought to die by apoptosis, which is also increased in villous trophoblast in preeclampsia[2]. However, experimental confirmation that apoptosis leads to trophoblast shedding is required and it is not clear which components of the apoptotic pathway are involved in trophoblast shedding. Objectives: To determine the effect of inhibiting caspase 3 (executioner), caspases 8 and 9 (initiators), and Rho-associated kinase (ROCK; bleb formation) on the number of trophoblasts shed from first trimester human placentae. Methods: Using an in vitro placental explant model of trophoblast deportation, first trimester placentae were cultured for 72 hours in media containing specific inhibitors of ROCK, caspases 3, 8 or 9. Trophoblasts shed from quintuple explants/inhibitor from five placentae were depleted of contaminating leucocytes and erythrocytes, labelled with trypan blue and the sizes and numbers of shed trophoblasts quantified using a Nexcelom automated counter. Results: The number of trophoblasts that were shed from the explants was significantly increased ($p=0.04$) when caspase 3 (2.4 fold) and caspase 8 (2.7 fold) were inhibited. There was no significant change following caspase 9 inhibition. The number of shed trophoblasts was significantly decreased when ROCK was inhibited. None of the inhibitors significantly altered the size of the shed trophoblasts. Conclusion: Our data suggest that the apoptosis pathway is involved in trophoblast shedding in vitro from first trimester placentae. That caspase 8 but not caspase 9 affected shedding suggests trophoblasts from normal placentae are induced to die via the extrinsic apoptosis pathway. Aberrant regulation of the apoptosis pathway may contribute to pregnancy pathology.

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EFFECTS OF CAMP AND SERUM ON HUMAN TROPHOBLAST CELL VIABILITY AND DIFFERENTIATION

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The formation of syncytium is a pivotal event for trophoblast cells to interact with the placental bed. While cAMP is regarded as an inducer of syncytialisation, the affect of different culture conditions on this cAMP effect has not been explored. Therefore, the effects of cAMP on cell differentiation and viability in the presence or absence of serum were investigated in the human choriocarcinoma cell lines, BeWo and JEG-3. We observed that in the absence of cAMP, BeWo cells grew best in media containing 10% FCS, followed by media containing charcoal-stripped 10% FCS (10%CCS), and less well in serum-free media. In the presence of cAMP (0.25~1.5 mM), our observations suggest different cellular programmes may be in play. Treatment of BeWo cells with 0.75 mM cAMP for 24h and 48h, in the absence of serum, increased cell viability (MTT assay) by 25.1% and 46.1% respectively, compared to the control cells. Interestingly, this cAMP effect on cell viability was not observed in the JEG-3 cell line. In contrast, BeWo cell viability was decreased by 49.5% and 25.2%, and by 27.5% and 31.1% in JEG-3 cells, when the cAMP stimulated cells were cultured for 48h in 10% CCS and 10% FCS media, respectively. In addition, we observed a change in BeWo, but not JEG-3, cell morphology to a spindle-like shape with pseudopodia when cAMP stimulated cells were cultured in media containing 10% CCS or 10% FCS for greater than 24h. Since the process of syncytialisation may involve apoptotic events, we speculate that the different effects of cAMP on cell viability in trophoblast cells may be related to syncytialising factors contained in serum media. Further study will clarify whether serum promotes syncytium formation, while the lack of serum based factors could switch the cellular programme from one of syncytialisation toward a more proliferative type.

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LIF REGULATION OF EPITHELIAL CELL FUCOSYLTRANSFERASE EXPRESSION IN MOUSE ENDOMETRIUM DURING EARLY PREGNANCY

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Fucosyltransferase (FUT) enzymes are key regulators of glycosylated structures mediating embryo attachment to uterine epithelial cells at implantation. The identity of local regulatory signals is unknown. We have previously shown that macrophage co-culture significantly increases epithelial cell FUT2 and FUT4 mRNA expression *in vitro*, and the effect of co-culture is replicated with macrophage conditioned media. We aimed to define the identity of macrophage-secreted agents active in regulating FUT expression in mouse uterine epithelial cells, and to investigate the importance of macrophages for FUT expression *in vivo*. FUT1, FUT2, and FUT4 mRNAs were measured by qRT-PCR and data was normalised to β-actin mRNA in mouse uterine epithelial cells after culture with cytokines known to be secreted by macrophages. mRNA was also quantified in luminal epithelium laser-microdissected from mouse uterus on day 4 after mating with intact males or seminal vesicle deficient (SVX) males, to induce normal or depleted uterine macrophage populations respectively. Lectin staining on day 4 pc was quantified using ImageJ software in an alternate model of transient, systemic macrophage ablation following diphtheria toxin administration to CD11b-DTR transgenic mice. Epithelial FUT2 mRNA expression was specifically enhanced *in vitro* by addition of rLIF (2 ng/ml) (mean relative expression ± SEM, control 100 ± 5.6; rLIF 162.1 ± 11.5). Depletion of macrophages by mating with seminal vesicle deficient males reduced epithelial FUT2 mRNA expression on day 4 pc (intact 100 ± 9.1; SVX 73.5 ± 8.6). Depletion of macrophages in the CD11b-DTR mouse model caused a 30% reduction in the expression of the resulting glycoprotein epitope (α1,2 fucose) as observed by intensity of endometrial epithelial UEA-1 staining (control 100 ± 10; CD11b-DTR 72 ± 9) 24 hr post diphtheria toxin administration. In conclusion, these data demonstrate that endometrial epithelial FUT2 mRNA synthesis in preparation for embryo implantation is mediated via LIF and potentially other factors secreted from macrophages recruited during the inflammatory response to insemination. Uterine macrophage abundance and phenotype may thus be a determinant of receptivity to implantation.

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DIFFERENTIAL DEVELOPMENT OF SEX-RELATED CHARACTERS OF THE GSP AND PNP/DO CHICKENS AFTER LEFT-OVARIECTOMY

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To elucidate the strain difference in the sex reversal of genetic female to phenotypic male, GSP and PNP/DO females were left ovariectomized (ovx) between one day to three days after hatching and the degree of masculinization based on sex-related characters, histological analysis of the right gonad and hormone assay were assessed at one year of age. The GSP and PNP/DO inbred lines were both derived from the Fayoumi breed and are only differentiated based on red blood cell antigens type carried by each of the inbred line. Comb and wattles were found to be significantly bigger ($P<0.05$) in the GSP ovx compared to the PNP/DO ovx, although male plumage pattern were more pronounced in the PNP/DO ovx. Spurs were observed both in the GSP and PNP/DO ovx with no significant difference ($P > 0.05$) in length to the respective male controls and body weight were not significantly different ($P>0.05$) to the female controls. The size of the right gonad were significantly bigger ($P<0.05$) in the GSP ovx than the PNP/DO ovx. Positive correlations were found in the sex related characters as well as plasma testosterone level and the right gonad weight both in the GSP and PNP/DO ovx except for the spur length which resulted into a negative correlation in the PNP/DO ovx. Histological analysis revealed that the right gonad of PNP/DO ovx are morphologically developed compared to GSP ovx showing more advance stages of spermatogenesis. It could be inferred that PNP/DO females which exhibit hereditary persistent right oviduct, are more responsive to the masculinizing effect of ovariectomy compared to GSP females, suggesting that genetic background may have a possible contribution on the degree of masculinization and subsequent development of sex related characters.

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DEVELOPMENT OF A METHOD OF SEMINIFEROUS TUBULE TRANSFECTION *IN VITRO* TO DEFINE POSTMEIOTIC GENE REGULATION

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Postmeiotically expressed genes in the testis are essential for the proper progression of spermatogenesis, and yet, aside from the construction of individual transgenic mice using specific promoters to drive reporter plasmids, there are only very limited possibilities for relevant and quantitative analysis of gene promoters. This is due to the special nature of post-meiotic haploid cells, which to date are not represented in any appropriate cell-lines. Here we report the development of novel methodology using isolated and cultured rat seminiferous tubules in a multiwell format, into which promoter-reporter constructs can be introduced by a combination of microinjection and electroporation. Culture conditions were developed which allowed the continued incubation of isolated rat seminiferous tubules for up to 48h without obvious cell death and loss of post-meiotic cells. Transfection of intact seminiferous tubules by microinjection and electroporation was optimized to achieve high expression efficiencies of control plasmids, using either fluorescent protein or luciferase as reporters, thereby allowing both morphological as well as quantitative assessment. Successful transfection was achieved into all cell types except for mature spermatozoa. However, there appeared to be only limited cell-type specificity for the promoters used, even though these had appeared to be specific when used in transgenic animals. We have devised a methodology which allows relatively high throughput analysis of post-meiotic gene promoters into primary cells of intact seminiferous tubules. An apparent lack of cell-type specificity suggests that the gene fragments used do not contain sufficient targeting information, or that the transient episomal expression of the constructs does not encourage appropriate expression specificity. The results also highlight the doubtful interpretation of many studies using heterologous transfection systems to analyse post-meiotically expressed genes.

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HUMAN CUMULUS-OOCYTE COMPLEXES SECRETE CUMULUS EXPANSION ENABLING FACTOR(S)

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Interactions between the oocyte and its companion somatic cells are crucial to establish and maintain a highly specialized microenvironment required for oocyte viability. Specifically, cumulus cell expansion in the mouse is reliant on oocyte-secreted factors (OSF). Little is know about factors secreted by the human oocyte and how they may interact with cumulus cells. Therefore, the aim of this study was to establish whether human cumulus oocyte complexes (COC) produce OSF that induces cumulus expansion. COC of patients undergoing routine clinical IVF were cultured individually for 6h following oocyte retrieval. The human oocyte conditioned medium (HOCM) was collected. The bioactivity of OSF in the HOCM was assessed using an established assay of cumulus expansion of mouse oocytectomized complexes (OOX). Cumulus expansion was assessed blinded using the scoring system; 1 (no expansion) to 4 (maximally expanded) and gene expression was assessed by real time RT-PCR. Culture of OOX in control media with or without FSH did not induce expansion. Similarly, OOX cultured in HOCM without FSH did not expand. However, culture of OOX in HOCM with FSH significantly induced expansion (2.4±0.1 compared with control 1.1±0.04, $P<0.05$). Furthermore, this expansion was not different to OOX co-cultured with human (2.9±0.1) or mouse (2.6±0.1) denuded oocytes. Cumulus/OOX gene expression of hyaluronan synthase-2 and cyclooxygenase-2 was significantly up-regulated 4–5 fold when OOX were cultured in HOCM compared to control ($P<0.05$). Interestingly, different patients produced HOCM which resulted in different levels of expansion (range from 1.5–3.7). This study has established that human COC secrete paracrine factor(s) that enable cumulus expansion. This expansion was dependent on the presence of FSH. The identity of these factor(s) are currently unknown however it appears that COC from different patients produce differing levels of these cumulus expansion enabling factor(s).

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WHOLE BODY HEAT EXPOSURE INDUCES APOPTOSIS IN MOUSE CAUDAL EPIDIDYMAL SPERMATOZOA

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Introduction: The aim of the present study was to determine the immediate effects of whole body heating on sperm numbers, motility and apoptosis. Material and Methods: C57BL/6 mice (n=7) were exposed to 37–38^{o}C (8 hours/day), for three consecutive days while control mice (n=7) were kept at 23-24^{o}C. Caudal epididymal spermatozoa were collected from control and heat treated mice 16 hours after the last heat treatment to determine the sperm number and motility using a Neubauer haemocytometer and sperm apoptotic changes by dual color flow cytometry using Annexin V/PE (Annexin V conjugated with phycoerythrin) and 7AAD (7-amino-actinomycin D) stains. Results: There were no significant differences ($p>0.05$) in sperm numbers between heat treated and control mice, however heating did result in a significant reduction in sperm motility ($p<0.05$). Apoptosis staining identified four different subpopulations of spermatozoa: (a) live spermatozoa (Annexin $V^{-}/7AAD^{-}$), (b) early apoptotic spermatozoa with exteriorized phosphotidylserine (PS) receptor and intact plasmalemma (Annexin $V^{+}/7AAD^{-}$), (c) late apoptotic spermatozoa with PS receptor translocation and leaky plasmalemma (Annexin $V^{+}/7AAD^{+}$) and (d) dead spermatozoa with damaged plasmalemma with no detectable PS receptor (Annexin $V^{-}/7AAD^{+}$). Heating resulted in significant reduction in the percentage of live spermatozoa ($p<0.05$), an increase in early apoptotic ($p<0.05$), late apoptotic ($p<0.05$), and dead spermatozoa ($p<0.05$). Conclusion: This study shows that mice exposed to whole body heat exposure of 37–38^{o}C for 8 hours per day for three consecutive days exhibited early and late apoptotic changes to epididymal spermatozoa. These findings suggest possible adverse effects of exposure to high temperature on the viability of human spermatozoa in the epididymides. In addition, these findings reinforce the importance of temperature during sperm preparation procedures in infertility clinics, and research laboratories.

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EFFECTS OF SUPPLEMENTAL VITAMIN ON THE SEMEN CHARACTRISTICS OF MARKHOZ GOATS DURING NON-BREEDING SEASON

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The aim of the study was to investigate the effect of dietary supplementation of vitamin E on the semen characteristics of the Markhoz goats. Eighth bucks were randomly allocated into two groups, and received two different diets: unsupplemented diet (control) and supplemented diet with vitamin E (0.30 g/Kg DM). All experimental diets were formulated according to AFRC 1998. Semen was collected at 14-days intervals from June 17, 2006 to September 2, 2006 (non-breeding season) using artificial vagina. Semen characteristics were evaluated including semen volume, sperm concentration, live sperm percentage, percentage of motility and progressive motility. This characteristics were evaluated at two weeks intervals the trial. The total number of spermatozoa per ejaculation was calculated by multiplication of the semen volume with sperm concentration. Sperm motility was also analyzed by placing a sample on pre-warmed (37ºC) microscope slide covered with a cover slip, and examined under a high power microscope at a magnification × 200. Data was analyzed using proc MIXED in SAS program. Significant effect ($P \leq 0.05$) of the week (sampling time) was observed for all the parameters except for semen volume. Vitamin E supplementation significantly improved ($P \leq 0.05$) total number (Control: $263.7 \times 10^7 \pm 17.506$ vs. Vit E: $320.95 \times 10^7 \pm 17.506$) and sperm concentration (Control: $301.79 \times 10^7 \pm 13.657$ vs. Vit E:$386.57 \times 10^7 \pm 13.657$), motility (Control: 77.27% ±0.89 vs. Vit E:82.6% ±0.89) and progressive motility (Control:4.208 ±0.138 vs. Vit E:4.229 ±0.138), percentage of viability (Control:80.57% ±0.89 vs. Vit E: 85.9% ±0.89). The results suggested that the supplemental Vitamin E may improve the semen quality and fertility in the Markhoz goats.

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THE COMPARISON OF SPERM FREEZABILITY USING TWO EGG YOLK-FREE DILUENTS IN ZANDI RAM

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The aim of this study was to investigate the effect of two kinds of commercial egg yolk-free extenders: Bioxcell and AndroMed on in vitro sperm function of sheep. A total of 10 ejaculates were collected from 5 adult Iranian Zandi rams using artificial vagina. Semen samples were mixed and diluted with Bioxcell or AndroMed. Freezing was conducted using imv semi-automatic equipment. The percentage of motility and progressive motility of sperm were evaluated before freezing (at 37°C), after refrigeration (at 5°C) and thawing, and recovery rate was calculated. Data was analyzed using proc GLM of SAS. The effect of extender on sperm qualitative characteristics after thawing was significant. Mean percentage of motility, progressive motility and recovery rate were higher ($P \leq 0.01$) in AndroMed than Bioxcell. Results suggested that AndroMed in comparison with Bioxcell had more ability to preserve sperm quality during freezing process.

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CHARACTERISATION OF HEPARAN SULPHATE PROTEOGLYCANS IN THE MATURING CUMULUS OOCYTE COMPLEX

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Many growth factors including members of the transforming growth factor beta (TGFβ) superfamily and epidermal growth factor (Egf)-like ligands signal via interactions with heparan sulphate proteoglycans (HSPGs). Cell surface HSPGs can act by sequestering ligands at their site of action, by presenting a ligand to its signalling receptor, or by preventing ligand-receptor interaction. The oocyte secreted factors (OSF) growth differentiation factor 9 and bone morphogenetic protein 15 are members of the TGFβ superfamily that act selectively on cumulus cells. Conversely Egf-like ligands are secreted by mural granulosa cells and transmit LH-induced signals to cumulus cells. We investigated the possibility that HSPGs contribute to the spatially restricted responses these signals exert on cumulus cells. Syndecan-1 and Glypican-1 are cell surface HSPGs that are involved in numerous biological processes, including growth factor regulation, cell proliferation and differentiation. Microarray analysis showed Syndecan-1 and Glypican-1 mRNA expression induced 6-fold ($P=10^{-9}$) and 3-fold ($P=10^{-7}$) respectively in Egf+FSH stimulated cumulus oocyte complexes (COCs). Furthermore, Syndecan-1 and Glypican-1 mRNA were induced 27- and 16-fold respectively in COCs after hCG treatment of mice. Syndecan-1 and Glypican-1 protein was localised specifically to the COC through immunohistochemical analysis. In Vitro Maturation (IVM) of oocytes is a valuable alternative to gonadotropin mediated superovulation, but IVM COCs are less competent than those matured in vivo. Several components of the COC have been shown to be altered in IVM, including the chondroitin sulphate proteoglycan Versican. COCs from mice that underwent IVM in the presence of Egf+FSH and cilostamide for 16 hours had >16 fold reduced mRNA for Syndecan-1 when compared with In Vivo matured COCs. The lack of Syndecan-1 in IVM COCs could reduce signalling capacity of growth factors including OSFs. This may contribute to the reduced capacity of IVM oocytes to fertilise and produce a healthy embryo, and ultimately, a healthy offspring.

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THE EFFECT OF BMPR-IB IMMUNIZATION IN IMMATURE FEMALE MICE

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The female reproductive system is regulated by well known endocrine signaling components and the less well understood autocrine and paracrine signaling components by growth factors and their receptors. The type I Bone Morphogenetic Protein Receptor IB (BMPR-IB) is a serine/threonine kinase that facilitates the signaling of various TGF-β family members. BMPR-IB is believed to have a substantial role in reproduction as demonstrated by the hyperprolific effect on ovulation in Booroola ewes which harbor a natural BMPR-IB receptor mutation, as well as the sterility induced by the BMPR-IB knockout in female mice. Maturation of the ovary and enhancement of follicular growth are known to be dependent on gonadotrophin stimulation from the pituitary. This study aims to elucidate the role of BMPR-IB in the female reproductive system by passive immune neutralization. Immature female mice (21 days old) were passively immunized against BMPR-IB via subcutaneous injections of 100 µl PBS containing anti BMPR-IB Ig in the absence and presence of equine chorionic gonadotrophin (eCG). The preparations where administered on day one and three of a four day experiment. On day four mice were asphyxiated with CO_2 and the ovaries were removed and weighed. In immature mice immunization against BMPR-IB ovarian weights were not different from control, while eCG significantly increased ovarian weight compared to the controls. In combination immunization against BMPR-IB further augmented the gonadotrophin-stimulated increase in ovarian weight to a significant degree. The suppressive effect of BMPR-IB signaling on follicular development in response to gonadotrophin stimulation indicates a possible developmental role of this receptor in the initiation of follicle growth in response to increased circulatory gonadotrophins and prevention of precocious puberty.

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OXYGEN REGULATED GENE EXPRESSION IN MOUSE CUMULUS CELLS

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Hypoxia inducible factors (HIFs) are heterodimeric transcription factors that mediate the expression of a range of genes in response to low oxygen. Previously we showed that subsequent developmental outcomes were influenced by oxygen levels during in vitro maturation. The aim of the current study was to examine the effects of varying oxygen concentration during in vitro maturation of mouse COCs on expression of HIF target genes in the cumulus cells. I mmature COCs were collected from the ovaries of eCG-stimulated CBAB6F1 females (21 d) and cultured for 17–18 h under 2, 5 or 20% O_2. Hyaluronidase-treated and recovered cumulus cells were collected and mRNA extracted for analysis. A microarray approach (Affymetrix 430_2) was used to identify genes in cumulus cells that were differentially expressed under varying oxygen concentrations (2, 5, 10 and 20%). This revealed 218 differentially expressed probes, of which 34 were up-regulated with decreasing oxygen levels. The great majority of these were classified as HIF-regulated genes. Specific analysis from real time RT-PCR of HIF regulated target genes Slc2a1, Ldha, Pgk1, Eno1, Ndrg1, Bnip3 were all significantly up-regulated (by at least 5-fold) when cells were cultured at 2% or 5% oxygen, when compared to 20% oxygen. Hif-1a mRNA decreased when cumulus cells were cultured in 2%, compared to 20% oxygen. This study demonstrates that cumulus cell gene expression is influenced by oxygen concentration, and suggests that these effects are mediated by the HIF transcription factors.

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EXTRACELLULAR ROLES FOR PROREGIONS OF MOUSE BMP15 AND GDF9 IN VIVO

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Bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9) are essential for normal follicular development and ovulation in mammalian species. As TGF-beta family proteins, BMP15 and GDF9 are expressed as pre-pro-mature proteins, with a signal region prompting extracellular secretion, a proregion that is post-translationally cleaved, and a mature protein that is known to be biologically active as a non-covalently interacting dimer. Until recently, the proregion was thought primarily to facilitate correct folding and dimerization of the mature proteins within the oocyte prior to processing and cleavage. However, our in vitro work with recombinant mouse BMP15 and GDF9 has shown that each of the proregion, mature protein and the non-processed promature protein are secreted from transfected 293H cells. We showed non-covalent interactions existing between the proregion and mature protein of each of BMP15 and GDF9, and between the BMP15 proregion and GDF9 mature protein. Importantly, a mouse BMP15 proregion antibody was able to abolish cooperative BMP15 and GDF9 bioactivity measured using a granulosa cell thymidine incorporation bioassay, providing strong evidence for an extracellular role for the mouse BMP15 proregion. Currently, to find out whether BMP15 and GDF9 proregions have extracellular roles in vivo, our investigation has utilised knock-down of BMP15 and GDF9 proregion proteins by mouse immunisation. Ovaries of mice immunised with the GDF9 proregion had significantly increased numbers of corpora lutea ($p<0.005$), while ovaries from mice immunised with BMP15 proregion peptides had significantly fewer corpora lutea ($p<0.005$). These findings provide the first evidence that the proregions of mouse GDF9 and BMP15 have different physiological roles outside the oocyte. Our future aim is to elucidate the function of these proregions, how these may differ between BMP15 and GDF9, and whether these aspects differ between species with different ovulation quota.

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REGULATION OF GDF-9 AND GDF-9B BY FSH IN PREANTRAL FOLLICLE CULTURES

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GDF-9 and -9B (BMP-15) are oocyte-derived members of the TGF-β superfamily. In the mouse ovary, the absence of GDF-9 leads to an arrest of follicle development at the primary/preantral stage. As a result GDF9 deficient mice are infertile[a] because follicle development does not reach a stage where ovulation and oocyte release can occur. In contrast, GDF-9B knockout mice are subfertile[b]. GDF-9 was shown to act via TGFβRI and BMPRII and GDF-9B via BMPRIB and BMPRII. We have much to learn about what regulates the production of GDF-9, GDF-9B and the expression of its receptors. These studies investigated whether FSH, an important mediator of folliculogenesis, plays a role in this regulation. Preantral follicles (110–135 μm in diameter) were isolated from 18 day old C57BL/6 mice using fine needles. Follicles were cultured (30–35 per well) for 7 days with varying doses of FSH (0–100ng/ml). RNA was extracted, reverse transcribed and real time PCR was carried out with primer sets for GDF-9, GDF-9B, TGFβRI, BMPRII and GAPDH. Immunohistochemistry was conducted on sections of formalin-fixed 18 day old mouse ovary using antisera directed at TGFβRI and BMPRII. GDF-9 and GDF-9B mRNAs were downregulated by FSH treatment (compared to untreated control). There was no effect of FSH on the expression of either receptor. TGFβRI and BMPRII receptor proteins were localised to the cytoplasm of oocytes and granulosa cells in 18 day old mouse ovary. Both were mostly localised to secondary follicles, lighter TGFβRI staining was found in less mature follicles. Receptors for GDF9 signalling were both present consistent with direct effects of GDF9 on ovarian function. GDF9B might also have an effect although it remains to be seen if the type 1 receptor is localised to the mouse ovary. Further studies are required to investigate receptor regulation.

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ACTIVIN A AND OVARIAN FOLLICLE DEVELOPMENT

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A significant developmental stage in ovarian folliculogenesis is the acquisition of gonadotropin sensitivity by ovarian follicles. Activin has previously been suggested to be involved in the responsiveness of granulosa cells to FSH (1). Therefore, the role of activin was investigated using a 'physiological' culture system to determine if pathways exist to transduce activin signals within the postnatal rat ovary. Organ cultures with day 4 whole ovaries were employed in order to assess the potential impact of Activin A on follicle growth and transition from the primordial through to the primary and later preantral stages of development. Ovaries were isolated and cultured for 10 days with the addition of supplemented DMEM/Hams F-12 media (2) and either FSH (100ng/ml), Activin A (50ng/ml), or a combination of the two. Media and treatments were refreshed every alternate day. At the end of the culture period, ovaries were fixed and sectioned, or placed immediately into Ultraspec for RNA extraction for future real-time PCR. Sections were used for morphological assessment and ovarian follicle counting of primordial, primary and preantral follicles. An evaluation of atresia by the detection of apoptotic cells was undertaken using terminal deoxynucleotidyl transferase (TdT) mediated dUTP-biotin nick-end labeling (TUNEL). Primary follicle numbers increased significantly ($P<0.05$) in the combined treatment group whereas, preantral follicle numbers increased significantly ($P<0.0001$) when treated with Activin A alone. This is consistent with a morphological appraisal of atresia where a decrease in atresia was found in primordial and primary follicles, supporting the primary follicle development data and Activin A treatment alone resulted in more healthy primary and preantral follicles than atretic ones . Therefore, a stimulatory role for Activin A both in the presence of FSH (primary follicle development) or alone (preantral follicle development) has resulted in more follicles making the transition from the primordial to primary stages, as well as to the later preantral stages.

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DNA METHYLATION IN THE 2-CELL MOUSE EMBRYO IS THE RESULT OF DNMT ACTIVITY DURING DEVELOPMENT FROM THE ZYGOTE TO THE 2-CELL STAGE

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In an accompanying abstract we show for the first time that global demethylation of both paternally- and maternally-derived genomes occurs prior to syngamy. It is commonly considered that new methylation of the genome does not commence until late in the preimplantation stage. Yet embryos during cleavage stage are known to show DNA methylation. This creates a paradox, if global demethylation occurs by the time of syngamy yet remethylation does not occur until the blastocysts stage, how can cleavage stage embryos possess methylated DNA. We examined this paradox. We examined DNA methylation in 2-cell embryos by confocal microscopy of anti-methylcytosine immunofluorescence and propidium iodide co-staining of whole mounts. We confirmed that DNA in late zygotes was substantially demethylated in both the male and female pronuclei. By the 2-cell stage, embryos collected direct from the oviduct showed high levels of cytosine methylation. We assessed whether this accumulation of cytosine methylation during the early 2-cell stage was a consequence of DNA methyltransferase (DNMT) activity. This was achieved by treating late stage zygotes with the DNMT inhibitor RG108 (5 μM) for the period of development spanning pronuclear stage 5 to early 2-cell stage. The embryos that developed in the presence of the DNA methyltransferase inhibitor showed significantly less methylcytosine staining than the embryos in the untreated culture conditions ($P<0.001$). Treatment of embryos during this period with RG108 significantly reduced their capacity to develop to normal blastocysts, indicating that this early DNA re-methylation reaction was important for the normal development of the embryo. Our results show for the first time that de novo methylation of the genome occurs as early as the 2-cell stage of development and that this is mediated by a RG108-sensitive DNMT activity. The results substantially change our understanding of epigenetic reprogramming in the early embryo.

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REGULATION OF STRESS PROTEIN GENES DURING PRE-IMPLANTATION EMBRYO DEVELOPMENT IN MICE BY GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR (GM-CSF)

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In vitro culture has been shown to be detrimental for pre-implantation embryo development and this has been associated with culture stress and elevated expression of apoptotic genes. Granulocyte-macrophage colony-stimulating factor (GM-CSF) has been shown to promote development and survival of both human and mouse pre-implantation embryos. To investigate the mechanism of action of GM-CSF in mouse embryos, gene expression was examined in *in vitro* cultured blastocysts with and without recombinant mouse GM-CSF (rmGM-CSF) and *in vivo* blastocysts flushed from Csf2 null mutant and wild-type mice. Microarray analysis of the effect of GM-CSF on transcription profile implicated apoptosis and stress response gene pathways in blastocyst responses to rmGM-CSF *in vitro*. Groups of 30 blastocysts were collected from *in vitro* cultured and *in vivo* developed blastocyst were analysed using quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR analysis of *in vitro* blastocysts revealed that addition of rmGM-CSF causes differential expression of several genes associated with apoptosis and cellular stress pathway, including Cbl, Hspa5, Hsp90aa1, Hsp90ab1 and Gas5. Immunocytochemical analysis of common proteins of the apoptosis and cellular stress response pathways BAX, BCL2, TRP53 (p53) and HSPA1A/1B (Hsp70) in *in vitro* blastocysts revealed that HSPA1A/1B and BCL2 proteins were less abundant in embryos cultured in rmGM-CSF, but BAX and TRP53 were unchanged. In *in vivo* developed blastocysts, Csf2 null mutation resulted in elevated levels of only the heat shock protein Hsph1, suggesting that *in vivo*, other cytokines can compensate for GM-CSF deficiency as the absence of GM-CSF has a lesser effect on the stress response pathway. We conclude that GM-CSF is a regulator of the apoptosis and cellular stress response pathways influencing mouse pre-implantation embryo development to facilitate embryo growth and survival, and the effects of GM-CSF are particularly evident in *in vitro* culture media in the absence of other cytokines.

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IMPLICATIONS FOR FETAL AND PLACENTAL DEVELOPMENT FOLLOWING MITOCHONDRIAL PERTURBATION IN THE EMBRYO

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The environment an embryo is exposed to can profoundly influence peri- and post-natal development despite having some capacity to adapt. Whilst the mechanisms responsible remain largely unknown, mitochondria are a likely target. In this study we deliberately perturbed mitochondrial function in the mouse embryo, using a model we have established that shows step-wise changes in embryo metabolism and development. The aim of this study was to provide direct evidence implicating mitochondrial dysfunction in the embryo with perturbed fetal and placental development. Zygote stage embryos were recovered from superovulated female mice and cultured in control conditions to the 2-cell stage. Embryos were then allocated to one of three treatments; control media (0μM-AOA), 5μM or 50μM of the known mitochondrial inhibitor, Amino-Oxyacetate, in the absence of pyruvate (5μM-AOA, 50μM-AOA). Embryos were cultured to the blastocyst stage, then transferred to pseudopregnant recipients, with fetal and placental parameters measured on day 18 of pregnancy. Implantation rates and fetal survival for both 5μM-AOA and 50μM-AOA was comparable to control embryos. For 5μM-AOA there was a significant reduction in placental weight (P=0.02) but normal fetal weight, and a significant increase in fetal: placental weight ratio (P=0.002) relative to the control, suggesting increased placental efficiency. When mitochondria were further perturbed (50μM-AOA), the fetuses and placentas were both considerably compromised: that is, decreased fetal and placental weights (P=0.002), reduced placental diameter (P=0.03) and decreased fetal crown rump length (P=0.07). This study demonstrates that mitochondrial function in the embryo impacts on peri-natal development, providing compelling evidence for mitochondrial function involvement in the mechanisms underpinning "embryo programming". This data suggests a threshold effect, whereby embryos can only adapt up until a point after which development is compromised. Further elucidating these mechanisms is important for understanding how maternal environments and embryo culture systems determine development of future offspring.

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EPIGENETIC REPROGRAMMING IN ZYGOTES INVOLVES THE GLOBAL CYTOSINE DEMETHYLATION OF BOTH THE PATERNAL AND MATERNAL GENOMES

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Epigenetic reprogramming is essential for normal development and has been held to occur in a different manner for the paternally and maternally inherited genomes. The current paradigm implicates active global demethylation of the paternal pronucleus soon after fertlization, but passive demethylation of maternally-derived genome over many cell-cycles. This parent-of-origin difference has been difficult to reconcile with other biological processes prompting us to re-examine this evidence. DNA methylation levels were examined in mouse zygotes by immunolocalization with methylcytosine specific antibodies. Zygotes were isolated from the oviduct at times after hCG and staged for pronuclei maturity (PN1-5, least to most mature) or metaphase commencement. We found methylation levels to be high in PN1-2 stage pronuclei but then progressively declined. By PN5 stage methylcytosine staining was greatly diminished. Yet, contrary to the current paradigm, demethylation generally occurred in both the male and female pronucleus. We found no methylcytosine staining in any metaphase chromosomes. The contrast of our results with those widely cited prompted us to review the methodology previously used. In previous studies zygotes that had been collected after fertilization and then cultured in vitro, or produced by IVF and then cultured were used. When we prepared zygotes by these methods we found that many PN5-stage cultured zygotes displayed relatively more demethylation of the male pronucleus than the female. When zygotes were generated by IVF this asynchrony was further exacerbated. In contrast to the zygotes collected directly from the reproductive tract, metaphase chromosomes in cultured post-syngamal zygotes commonly showed extensive methylcytosine staining. Our results show that the normal process of epigenetic reprogramming in the mouse involves global demethylation of both the paternal and maternal genomes. This was variably perturbed (particularly in the female pronucleus) by IVF and zygote culture.

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A ROLE FOR SIRTUIN 3 IN THE DEVELOPING MAMMALIAN EMBRYO

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Pre-implantation embryo development relies critically on the balance between cytoplasmic and mitochondrial metabolism for the generation of metabolic intermediates such as NAD^+. SIRT3 is a mitochondrial sirtuin with NAD^+-dependant deacetylase activity that, targets glutamate dehydrogenase (GDH). In this study we characterised SIRT3 mRNA, protein and activity through pre-implantation development and determined whether modulation of SIRT3 activity influenced GDH activity. Embryos (zygotes, 2-cell, 8-cell and blastocyst stages) were recovered from female CBA/C57Bl6 mice following ovarian stimulation and mating with CBA/C57Bl6 males. Expression of SIRT3 mRNA was measured using real-time RTPCR, protein localisation examined using immunohistochemistry and SIRT3 activity measured using a Fluor-de-Lys SIRT3 fluorescent assay. Functional GDH activity was assessed in 2-cell embryos indirectly by measuring glutamine oxidation, following culture from zygote to 2-cell in the presence of nicotinamide, (a sirtuin inhibitor), G1.2 media, or simpleG1 media, compared to in vivo controls. SIRT3 mRNA was detected at all stages of development, with significantly greater levels expressed in the blastocyst. SIRT3 protein was localised predominantly around the nucleus of zygote and 2-cell embryos, and was mainly cytoplasmic in 8-cell embryos and blastocysts. SIRT3 activity remained constant throughout pre-implantation development, and tended to increase at the blastocyst stage. Glutamine oxidation was reduced for embryos cultured in G1.2 media relative to in vivo controls (0.14 pmol/e/hr vs 0.21pmol/e/hr), and this was further reduced by the addition of nicotinamide (0.07pmol/e/hr). Embryo culture in perturbing simpleG1 increased glutamine metabolism (0.33pmol/e/hr). In conclusion, SIRT3 mRNA, protein and activity was detected throughout pre-implantation development. Modulation of sirtuins by nicotinamide decreased glutamine metabolism, likely as a result of decreased deacetylation, thus decreased activity of GDH. SIRT3 can translocate to the mitochondria during cellular stress, thus the increased glutamine metabolism in simpleG1 conditions may be caused by translocation of SIRT3 to mitochondria, potentially increasing GDH deacetylation and enzymatic activity.

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REDUCED OOCYTE DEVELOPMENTAL COMPETENCE DURING THE PERIOD OF SEASONAL INFERTILITY IN PIGS

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Reduced farrowing rate due to early pregnancy loss is a manifestation of seasonal infertility in pigs. It has been hypothesised that the early disruption of pregnancy is due to poor oocyte developmental competence. The aim of this study was to determine if there are seasonal differences in oocyte developmental competence. Ovaries were collected from sows slaughtered 4 days after weaning. Cumulus-oocyte complexes (COCs) recovered from small (3–4mm) and large (5–8mm) antral follicles were morphologically graded and subjected to parthenogenetic activation following in vitro maturation (IVM) during the winter (n=1419) and summer (n=2803). Cumulus expansion was assessed subsequent to IVM. Data were analysed using a generalised linear mixed model in GenStat release 10. There was an effect of season on oocyte grade, with a larger proportion of oocytes collected in summer being graded suitable for IVM, compared with winter oocytes ($P<0.05$). A larger proportion of COCs had expanded cumulus during the winter than in the summer, which suggested that the preovulatory LH surge had already occurred. There was a season x follicle size interaction affecting cumulus expansion ($P<0.05$). There were no seasonal effects on the proportion of oocytes reaching metaphase II or cleaving after parthenogenetic activation. However, the proportion of oocytes from large follicles that developed to the blastocyst stage was higher in winter than in summer (55% vs 23%; $P<0.05$). There was no effect of season on the proportion of oocytes developing to the blastocyst stage from small follicles. However, during summer there was a reduction in the cell number of blastocysts derived from small follicles ($P<0.05$). Our results suggest that porcine oocytes are less able to reach their full developmental potential during the period of seasonal infertility, and that the associated pregnancy losses are due to reduced oocyte developmental competence.

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MICROARRAY ANALYSIS OF FOETAL MOUSE BRAIN FOLLOWING INDUCTION OF MITOCHONDRIAL DYSFUNCTION DURING PRE-IMPLANTATION EMBRYO DEVELOPMENT

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Environmental stress can disrupt mitochondrial function in the pre-implantation embryo, subsequently hindering embryo viability. Brain tissue is also sensitive to developmental perturbations, and we have previously discovered genes involved in neurological function and epigenetic modification are differentially expressed in blastocysts following mitochondrial dysfunction by amino-oxyacetate (AOA). In this study CBAxC57Bl6 2 cell stage mouse embryos were cultured in 5μM-AOA without pyruvate for 72h to induce mitochondrial dysfunction. Blastocyst stage embryos were then transferred to pseudopregnant recipients and the expression profile of day 18 foetal brains was interrogated using microarray. mRNA from mouse whole brain (4 per treatment) was extracted and analysed using an Affymetrix gene array. Ingenuity Pathway Analysis software identified persistent alterations in gene expression pathways in foetal brain after AOA treatment during embryo culture, that were subsequently confirmed by qPCR. Expression was significantly increased by both array and qPCR (>1.5 fold, $p<0.05$) for; 1) Eomes (1.9, 2.9 fold respectively), a T-box transcription factor involved in differentiation, cell death and development, 2) Nr4a3 (1.8, 2.2 fold respectively), a steroid hormone receptor and putative transcriptional activator and 3) Nola3 (1.7, 1.9 fold respectively), a small nucleolar ribonucleoprotein involved in rRNA processing. Neurological disease, behavioural disorders, carbohydrate metabolism, cellular growth and proliferation, cell death, DNA replication, recombination and repair pathways also showed altered gene expression (>1.25 fold). qPCR was performed on 28 genes exhibiting the greatest change in expression. 24/28 genes confirmed the array data, and of the 4 genes that did not; two had expression not detected by qPCR (Snhg1, Speer6-ps1), and two contradicted array results (Atp1b3 $p=0.05$, Stk38l $p=0.06$). This study links mitochondrial dysfunction during early embryo development and persistent molecular changes in the developing foetal brain. This indicates that insults incurred during early embryo development can cause permanent changes that we predict results from aberrant epigenetic modification.

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INDUCED OOCYTE IN VITRO MATURATION (IVM) SUBSTANTIALLY IMPROVES EMBRYO YIELD AND PREGNANCY OUTCOMES

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Oocyte maturation in vivo is a highly orchestrated, induced process, whereby 3'-5'-cyclic adenosine monophosphate (cAMP)-mediated meiotic arrest is overridden by the gonadotrophin surge prior to ovulation. However, in vitro matured (IVM) oocytes resume maturation spontaneously hence compromising developmental competence. Hence we hypothesized that establishing an induced system in vitro would synchronise oocyte-somatic cell communication leading to improved oocyte quality. Bovine or mouse oocytes were treated for the first 1–2 h in vitro with the adenylate cyclase activator forskolin and a non-specific phosphodiesterase (PDE) inhibitor, IBMX, which substantially increased cumulus-oocyte complex (COC) cAMP (180 vs. 2 fmol/COC , treated vs. control, $P<0.001$) to in vivo physiological levels. To maintain oocyte cAMP levels and prevent precocious oocyte maturation, oocytes were then cultured with an oocyte-specific (type 3) PDE inhibitor, cilostamide. The net effect of this system ("Induced IVM") was to increase oocyte-somatic cell gap-junctional communication (bovine: 1000±148 vs. 340±73 units; treated vs. control, $p<0.05$) and to slow meiotic progression through prophase I to metaphase II, extending the normal IVM interval (bovine: 30 vs. 24h, mouse: 22 vs. 18 h; treated vs. control). These effects on oocyte and somatic cell functions had profound consequences for oocyte developmental potential. In bovine, Induced IVM more than doubled embryo yield (69% vs. 27%; treated vs. control, $p<0.05$). In mouse, Induced IVM increased blastocyst rate (86% vs. 55%; treated vs. control, $p<0.05$), implantation rate (51 vs. 25%), fetal survival rate (29% vs. 5%) and fetal weight (0.9g vs. 0.5g, $p<0.01$). All these developmental outcomes in mice were restored, by using Induced IVM, to levels obtained from in vivo matured control oocytes (conventional IVF). In conclusion, Induced IVM mimics some of the characteristics of oocyte maturation in vivo and substantially improves oocyte developmental outcomes. This should lead to an increase in the use of this technique in reproductive biotechnologies.

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IMMUNE-DEVIATING CYTOKINES DETERMINE THE MATERNAL T CELL RESPONSE DURING PREGNANCY AND TOLERANCE OR REJECTION OF THE CONCEPUTS

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In healthy pregnancies the maternal immune system establishes paternal antigen-specific tolerance allowing survival of the semi-allogeneic conceptus. The cytokine environment is a key factor in determining the phenotype of antigen-specific lymphocytes, influencing the development of either cytotoxic or tolerogenic cells. We hypothesized that the cytokine environment at the time of priming to paternal antigens influences the phenotype of the maternal T cell response and pregnancy outcome. Transgenic Act-mOVA male mice expressing chicken ovalbumin (OVA) ubiquitously provided OVA as a model paternal antigen. OVA is present within the semen of Act-mOVA mice and is inherited and expressed by the conceptus tissue. OVA-reactive CD8+ OT-I T cells were activated with OVA in the presence of various immune-deviating cytokines in vitro, before transfer at 3.5 dpc to C57Bl/6 (B6) females gestating OVA-expressing fetuses. Pregnant mice received either naïve OT-I T cells, cytotoxic OT-I T cells stimulated in vitro in the presence of IL-2 or OT-I T cells stimulated in vitro in the presence of TGFβ1 and IL-10, two factors present in the uterus and associated with immune tolerance. Immunohistochemistry was utilized to demonstrate that OT-I T cells infiltrate into the implantation site. Cytotoxic OT-I T cells caused fetal loss, while OT-I T cells activated in vivo or in vitro with TGFβ1 and IL-10 did not cause fetal loss. Additionally, cytotoxic OT-I T cells did not affect B6 x B6 matings, demonstrating the antigen-specific nature of the T cell-mediated fetal loss. Collectively these experiments show that maternal antigen-reactive T cells activated in vivo in the cytokine environment of the mated uterus are tolerogenic, not cytotoxic, and implicate TGFβ1 and IL-10 as key elements of that environment. We conclude that the cytokine environment at the time of priming to paternal antigens influences the T cell phenotype and impacts upon maternal immune tolerance and fetal survival.

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SPATIAL REGULATION OF APC^{CDH1} INDUCED CYCLIN B1 DEGRADATION MAINTAINS GV ARREST IN MOUSE OOCYTES

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Within the mammalian ovary, oocytes remain prophase I arrested until a hormonal cue triggers meiotic resumption. The E3 ubiquitin ligase, Anaphase-Promoting Complex with its co-activator Cdh1 (APC^{Cdh1}) is known to be essential for this process by promoting cyclin B1 degradation via the 26S proteasome. Cyclin B1 is the regulatory subunit of Maturation-Promoting Factor, forming a heterodimer with CDK1, which is essential for nuclear envelope breakdown (NEB). Here we describe the intracellular partitioning that would explain how Cdh1 activity is fine-tuned, such that cyclin B1 is maintained at sufficient levels to allow oocytes to resume meiosis but not so high as to cause premature meiosis re-entry. Using RT-PCR we detected only one splice variant in mouse oocytes, Cdh1α, which possessed a nuclear localisation signal. By immunofluorescence we confirmed the nuclear location of Cdh1 and degradation machinery components including essential subunits of the APC and 26S proteasome. In all systems studied, cyclin B1 shuttles between the cytoplasm and nucleus, with nuclear localisation occurring just before NEB. We reasoned therefore that the nuclear localisation of the APC^{Cdh1} and 26S proteasome would aid in maintaining low nuclear levels of cyclin B1. Using two GFP-coupled cyclin B1 mutants, which differed in their intracellular location we found that nuclear accumulation of cyclin B1 was necessary in order for it to promote meiotic resumption because over-expression of nuclear-cyclin B1 accelerated entry into meiosis, whereas cytoplasmic-cyclin B1 did not. However, in milrinone-arrested GV oocytes rates of nuclear-cyclin B1 degradation were 5 fold higher than cytoplasmic-cyclin B1. Therefore we conclude that in oocytes, an increase in the nuclear-cytoplasmic ratio of cyclin B1 is an essential step in meiotic resumption, and that nuclear APC^{Cdh1} activity guards against early meiotic resumption, until the degradation machinery is overwhelmed by cyclin B1 translocation.

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EPIDERMAL GROWTH FACTOR RECEPTOR/MAPK3/1 PATHWAY CROSS-TALK ENABLES GROWTH DIFFERENTIATION FACTOR 9 TO SIGNAL THROUGH SMAD2/3 IN MOUSE GRANULOSA CELLS

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Oocyte-secreted growth differentiation factor 9 (GDF9) plays a critical role throughout folliculogenesis. It has been shown to control many functions of granulosa cells, including gene expression, steroidogenesis and proliferation. This study investigates the cellular requirements that allow GDF9 to act on granulosa cells. Our results showed that GDF9 (20 ng/ml)-stimulated mouse granulosa cells ^{3}H-thymidine incorporation was inhibited by a type 1 receptor Alk4/5/7 inhibitor (SB431542, 5 μM), by an epidermal growth factor (EGF) receptor inhibitor (AG1478, 5μM) and a MEK1 inhibitor (U0126, 10 μM). Interestingly, activin A- and TGFβ-stimulated ^{3}H-thymidine incorporation shared similar inhibitor sensitivity. Moreover, when denuded oocytes were used as the mitogenic agent, SB431542, AG1478 and U0126 all prevented the increase in ^{3}H-thymidine incorporation. Oocyte-stimulated ^{3}H-thymidine incorporation in secondary follicles and cumulus-oocyte complexes were also sensitive to Alk4/5/7, EGF receptor and MEK1 inhibition. Basal and EGF-stimulated levels of phopho-MAPK3/1 were inhibited by using the EGF receptor inhibitor, but were not affected by inhibition of Alk4/5/7 or by adding GDF9 in granulosa cells. Using granulosa cells transfected with a SMAD3-luciferase reporter construct, GDF9-stimulated SMAD3 response could be inhibited by Alk4/5/7, EGFR and MEK1 inhibitors. Genes involved in cumulus cells expansion (Ptx3 and Has2) were upregulated in granulosa cells by co-culturing with denuded oocytes and that upregulation was inhibited by Alk4/5/7 as well as by EGF receptor inhibition. These results suggest that TGFβ superfamily members signalling through Smad2/3 share a common requirement of EGF receptor-dependant phospho-MAPK3/1 throughout folliculogenesis. These results strongly suggest that, apart from its role in the transmission of the ovulatory LH signal within the ovarian follicle, EGF receptor pathway might serve as modulators of GDF9 action on granulosa cells. Hence the interaction between endocrine and oocyte signalling may be mediated at the level of MAPK and Smad2/3 cross-talk in granulosa cells.

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FATTY ACID OXIDATION IS ESSENTIAL FOR OOCYTE DEVELOPMENTAL COMPETENCE

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Oocyte lipid composition and developmental competence are influenced by dietary fat yet whether lipids are metabolised by the oocyte or essential for subsequent embryo development is largely unexplored. Fatty acid oxidation (FAO) is largely overlooked as an energy source for the oocyte, despite generating several-fold more energy than glucose oxidation. FAO requires the rate-limiting enzyme carnitine palmitoyltransferase-1 (Cpt1) and the metabolite Carnitine to shuttle fatty acids into mitochondria for energy production. Analysis of Cpt1 mRNA during oocyte maturation showed that Cpt1 expression was hormonally induced ($p<0.05$) in the cumulus oocyte complex (COC), peaking at 10h following ovulatory hCG treatment. In contrast, Cpt1 was not hormonally regulated in granulosa cells ($p>0.05$). To investigate the role of Cpt1-mediated FAO during oocyte maturation we measured FAO in oocytes in the presence and absence of Carnitine and inhibited FAO to determine its importance for oocyte developmental competence. Levels of FAO in COCs were assessed as metabolism of the fatty acid ^{3}H-palmitate. During oocyte maturation there was a 2.1-fold increase ($p<0.0001$) in FAO compared to immature COCs. Carnitine supplementation led to a further 3.7-fold increase ($p<0.001$), while inhibition of Cpt1 with Etomoxir resulted in a 6.5-fold decrease ($p<0.0002$) in FAO during oocyte maturation. FAO inhibition had no effect on cumulus expansion. However inhibition of FAO during oocyte maturation followed by IVF and embryo culture in the absence of inhibitor, resulted in significantly decreased numbers of embryos developing ‘on time' ($p<0.002$). This is the first identification of hormonal induction of Cpt1 and Cpt1 mediated FAO in the COC during oocyte maturation. Further, the results demonstrate that oxidation of fatty acids by the oocyte is essential for oocyte developmental competence and can be modulated by Carnitine. These findings provide a potential mechanism by which dietary fat, obesity or metabolic disorders including CPT deficiency lead to infertility.

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SPECIFICITY STUDIES ON THE IMMUNOSUPPRESSIVE AND CYTOTOXIC ACTIVITIES OF LYSOPHOSPHATIDYLCHOLINES (LPCS) OF GONADAL ORIGIN

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The immunosuppressive activity of ovarian follicular fluid and testicular interstitial fluid is due to the presence of several LPCs, but the specificity of this inhibition is a potential source of controversy, as these molecules also possess lytic activity. In the following study, we compared the immunosuppressive and cytotoxic activities of the two most abundant gonadal LPCs, 1-palmitoyl-sn-glycero-3-phosphocholine (16:0aLPC) and 1-oleoyl-sn-glycero-3-phosphocholine (18:1aLPC), together with a number of related lysophospholipids (LPs), using a T-cell activation inhibition assay and an ovarian granulosa cell viability assay. Both the immunosuppressive and cytotoxic activities of the LPCs were blocked by serum (>5%) and serum albumin (>5mg/ml) *in vitro*. In the absence of serum proteins, the most immunosuppressive LPCs were 16:0aLPC, 18:0aLPC, 18:1aLPC and platelet activating factor (PAF; 1-O-palmitoyl-2-O-acetyl-sn-glycero-3-phosphocholine) with IC_{50} values of 1.2-4.3 μM. Curiously, PAF was the LPC most cytotoxic to granulosa cells (IC_{50} 10 μM). The other immunosuppressive LPCs exhibited cytotoxicity within the range of 40-50 μM, *i.e.* at doses 10-50-fold higher than their immunosuppressive concentrations. Comparison of LPs of different structures indicated that optimal immunosuppression is related to a phosphocholine, but not serine, ethanolamine or phosphate group, at sn-3, and an ester- or ether-linked fatty acid of chain length C16-C18 at sn-1. Acetylation of sn-2, as in PAF, had only minor effects on immunosuppressive activity, but greatly increased cytotoxicity. These data establish that inhibition of activated T-cells is not a direct consequence of the cytotoxicity of these molecules, although some structural features that contribute to lytic activity, such as fatty acid chain length, overlap with the ability to confer immunosuppression. On the basis of these data, we propose that the effects of LPCs on T-cell proliferation may not be mediated by a specific lock-and-key receptor, but rather by a direct interaction with the cell membrane at concentrations significantly below their lytic concentrations.

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ELUCIDATION OF THE MOLECULAR MECHANISMS THAT UNDERPIN CAPACITATION-ASSOCIATED SPERM SURFACE REMODELLING

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Recent research from our laboratory has provided evidence that sperm-egg interaction is mediated by a multimeric sperm receptor complex. Furthermore, we have demonstrated that this complex is assembled on the sperm surface during the final phase of their maturation, a process known as capacitation. The mechanisms underpinning this capacitation-associated surface remodelling remain poorly understood and are the subject of our current investigation. Specifically we have focused on whether this process is driven by vesicle mediated, intracellular trafficking of proteins. For this purpose we have examined the presence and physiological significance of a key part of the molecular machinery necessary for this form of trafficking, namely the enzyme dynamin. Our studies revealed that sperm are endowed with at least two isoforms of dynamin (1 and 2) both of which reside within the peri-acrosomal region of the sperm head, a location compatible with a role in sperm membrane remodelling. Consistent with this putative role, it was demonstrated that dynamin 1 was phosphorylated during capacitation, a post-translational modification that has been causally linked to both its activation and to the capacitation-associated surface remodelling of mouse spermatozoa. Furthermore, it was demonstrated that pharmacological inhibition of dynamin activity led to a concomitant reduction in the ability of spermatozoa to bind to the zona pellucida of homologous oocytes. This suppression was also correlated with reduced surface expression of a number of proteins including a subset of putative sperm-zona pellucida receptors. Collectively these data support the novel hypothesis that dynamin does participate indirectly in sperm membrane remodelling events by virtue of its ability to mediate intracellular trafficking.

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REGION- AND TIME-DEPENDENT CHANGES IN STRUCTURE AND CELLULAR TURNOVER IN ANDROGEN DEPRIVED MOUSE EPIDIDYMIS

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Epididymal maturation of spermatozoa including acquisition of motility and fertilizing ability depends on androgens both directly from testis and indirectly via the circulation. Androgen action via androgen receptor (AR) can cause both proliferative and anti-proliferative effects (1,2) so we have analysed changes in mouse epididymis following androgen deprivation either by orchidectomy or in prostate epithelial AR knockout (PEARKO) males with reduced androgen action also in epididymis (3). Structural changes (stereology), proliferation (PCNA) and apoptosis (TUNEL) were compared between mature intact males, orchidectomized males 3 day (3d) or 3 weeks (3w) after castration and PEARKO males (3) in the caput and cauda epididymis regions. In caput, epithelial volume decreased while stroma increased in castrates but not in PEARKO whereas lumen volume decreased only after 3 weeks of castration. Proliferating cells (per 100 tubules) were significantly increased 2.8-fold in PEARKO and 6.6-fold in 3d castrate group whereas in the 3wk castrate group proliferation was significantly decreased compared with intact controls. Apoptosis significantly increased by 3.3, 42 and 5.7-folds in PEARKO, 3d and 3wk castrate groups, respectively, compared with intact controls. In the cauda epididymis, castration significantly decreased the volume of lumen and increased stromal volume relative to intact controls. Epithelial proliferation was increased by 20-fold in 3d castrates compared with intact controls. Castration significantly increased apoptosis by 19 and 4.3-folds in 3d and 3wk castrates, respectively, compared with intact controls. We show that the androgen deprivation triggers changes in epididymis structure and cellular turnover in a region-specific and time-dependent manner. The results also reveal a role of testicular factors (presumably androgens) in suppression of epithelial proliferation in the epididymis.

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THE REGULATION OF TRP53 IN SPERM

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TRP53 is a tumour suppressor protein that is a universal sensor of cell stress. Upon ejaculation, sperm undergo a process of capacitation which allows them to become fertile. We have previously shown that mouse and human sperm possess TRP53. In this study we analyzed the regulation TRP53 presence in sperm. Mouse sperm were collected from the epididymides and incubated for various times in fertilisation medium. TRP53 was detected by both Western blot analysis and immunolocalization. We found that sperm collected directly from the epididymis generally had little or no detectable TRP53. The level increased with time in culture over a period of 120 min. Most of the TRP53 was detected in the midpiece of sperm although it was also detected in the head of a small proportion of sperm. The increase in TRP53 with time accompanied the increase in the proportion of sperm undergoing the acrosome reaction. Yet, Trp53-null sperm still underwent the acrosome reaction at a normal rate. By contrast, sperm that were prevented from undergoing capacitation and the acrosome reaction (by the removal of albumin or calcium from media) showed a marked reduction in the amount of TRP53 detected. This shows that TRP53 may be dependent upon capacitation, but the reverse was not the case. Inhibition of protein synthesis by puromycin did not block the time-dependent increase in TRP53 in sperm. Canonically, the TRP53 level is controlled by its rate of degradation by MDM2-ubiquitin mediated proteolysis. We found that MDM2 was present in sperm and inhibition of MDM2 (by Nutlin-3) caused an acute increase in TRP53 detected in sperm. This study shows that TRP53 levels are acutely regulated in sperm during the time that sperm acquire the capacity to fertilise, yet sperm lacking TRP53 are capable of fertility. Identification of the role for this TRP53 in sperm is under investigation.

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THE DEVELOPMENT OF THE PHALLUS IN A MARSUPIAL

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The phallus, the limbs and the tail are all considered appendages in the developing mammal. In mice, several key genes including *FGF8*, *BMP4*, *SHH*, *DLX5* and *DLX6* are known to control the precise pattering of the limb and phallus in the fetus (reviewed in Yamada et al. 2006). The signalling cascade in both appendages begins with SHH interacting with FGF8. In humans, disruptions to these gene pathways result in malformations of both limbs and phallus because these appendages share conserved elements in patterning and development (Yamada et al. 2003). However, this is a poorly researched area so additional models are needed to provide a greater perspective into mammalian embryonic patterning especially of the external genitalia. In marsupials, since most sexual differentiation occurs after birth, the developing phallus and limbs are accessible at stages that occur in utero in humans and other eutherian mammals. We have used the tammar as an alternative model to understand the differentiation of the phallus and limb. FGF8, SHH and megalin mRNA and protein are all expressed in the limb and phallus from the early embryo until post-natal stages. SHH and megalin were co-localised in the urethral epithelium of the tammar phallus. This is the first study to detect megalin in the developing mammalian phallus. SHH is a critical gene in patterning the appendages, and megalin is a transport protein that ferries steroids into the cell where they can bind to their cognate receptor. It has a high affinity for the potent androgen androstanediol, an androgen critical for virilisation of the tammar phallus (Leihy et al. 2004). Further examination is underway to confirm whether the expression of the genes patterning the phallus in the mouse are conserved in the marsupial and whether megalin plays any role in the patterning of the phallus, possibly mediated by SHH.

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DEVELOPMENT OF A MODEL SYSTEM TO ASSESS THE REGULATION OF LEYDIG STEM CELL DIFFERENTIATION

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The Leydig cells of the testis are responsible for the production of ca. 95% of all circulating androgens in the adult male. There is substantial evidence now that loss of Leydig cell (LC) functionality may account in part for declining androgen levels in the ageing male. Some of this loss of functionality appears to be due to disruption of early LC differentiation processes caused by exposure to environmental endocrine disrupting substances. We have made use of the ability of the alkylating agent ethane dimethane sulfonate (EDS) to destroy specifically all immature and adult LC to develop a model system which allows us to quantitatively assess the differentiation of a new population of LC from resident stem cells in the testis. Following a single bolus injection of EDS into adult Sprague Dawley rats, unless additional testosterone is present to suppress the HPG axis, new LC redifferentiate from the resident stem cells in the testis. These are small spindle-shaped mesenchymal cells which are located in the peritubular region adjacent to the seminiferous tubules. In the absence of mature LC these mesenchymal cells differentiate first into LC progenitor cells, then immature LC, and finally into adult LC, following a precisely defined kinetic which can be mapped using quantitative RT-PCR for LC specific genes in whole testis samples. We are using this model to examine the effect of in vivo application of known environmental endocrine disrupting agents on LC differentiation, and hence their potential to cause long-term loss of LC functionality.

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THE FIRST EVIDENCE OF HIGH SUSCEPTIBILITY TO COLD SHOCK BY THE SPERMATOZOA OF A MARSUPIAL, THE FAT TAILED DUNNART (*SMINTHOPSIS CRASSICAUDATA*)

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Carnivorous marsupials are native Australian predators including the highly threatened northern quoll (Dasyurus hallucatus) and Tasmanian devil (Sarcophilus harrisii). These species are currently actively managed in captive populations but assisted reproductive techniques such as gamete banking may also contribute to their conservation. Previous studies on a model dasyurid, the fat tailed dunnart (Sminthopsis crassicaudata), have found that spermatozoa do not survive freezing and thawing using a variety of freezing protocols and cryoprotectants. We have re-examined cold shock to investigate problems with sperm cryopreservation in S. crassicaudata. Epididymal spermatozoa were rapidly cooled to 0.5ºC in a pre-cooled tube held in an iced water slurry and upon re-warming the spermatozoa were non-motile (n=6). The addition of up to 20% egg yolk, which is considered protective to the spermatozoa of cold shock sensitive eutherians, did not improve the outcome (n=6). Similarly when S. crassicaudata spermatozoa were rapidly cooled to 4ºC, just 2% remained motile upon re-warming (n=10). However when spermatozoa were combined with at least 10% egg yolk and rapidly cooled to 4ºC only small reductions in motility were observed upon rewarming (n≥8). In order to achieve motile spermatozoa at 0ºC, controlled rate cooling at 0.5ºC/minute was examined. In the absence of egg yolk there was a decline in the percentage of motile spermatozoa below 4ºC (n=6). However if spermatozoa were combined with at least 10% egg yolk there was no significant loss of motility at temperatures as low as 0ºC (n=6). This study has revealed that at least one species of marsupial is highly susceptible to cold shock. These paradigm shifting findings give direction to future experiments aiming to develop a robust technique for sperm preservation in endangered dasyurids.

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ROLE OF RNA-BINDING PROTEIN, MUSASHI-1 (MSI-1), IN MURINE FOLLICULOGENESIS AND OOCYTE DEVELOPMENT

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Follicular development and oocyte maturation in mammals requires the temporal and spatial control of protein production. Consequently, it is hypothesised that the preovulatory follicle represses mRNA translation until specific proteins are required during oocyte maturation. Increasingly RNA-binding proteins are being recognised as important contributors to germ cell development, particularly during oocyte transcriptional quiescence. We have identified the presence of RNA-binding protein musashi-1 (Msi-1) mRNA within the mouse ovary and mature mouse oocyte, where the protein is believed to act as a translational repressor by binding to specific sequences within the 3' UTR of target mRNA molecules. Recent studies in various mammalian systems have identified p21 ^{WAF1}, cdkn2a, notch and m-numb as potential targets of Msi-1. We have also identified morf4l1 as a potential target through preliminary pulldown and microarray analysis using a GST tagged Msi-1 recombinant protein. To further study these potential targets, a transgenic Msi-1 mouse was produced to overexpress the RNA-binding protein in the developing oocyte. Real time PCR, performed on intact ovaries of WT and Tg mice, has so far demonstrated a 1.5-fold increase in Msi-1 expression in tgMsi-1/+ ovaries, above WT ovary expression. Real time PCR analysis of Msi-1 target mRNA expression has also shown an overall increase in expression in the tgMsi-1/+ ovaries of p21 ^{WAF1} (~2.5-fold), cdkn2a (~2-fold), and notch (~3-fold). However m-numb and morf4l1 do not appear to be targets of Msi-1 in the oocyte, with no significant difference in expression between the WT and tgMsi-1/+ ovaries analysed. Functional quantification of oocyte development reveals a significantly less oocytes produced from superovulated juvenile mice compared with wild type litter mates. Therefore, preliminary analysis suggests that Msi-1 may play a role in binding the transcripts of genes necessary for cell cycle regulation and chromatin remodelling, characteristic of meiotic progression and oocyte development.

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TRP53 REGULATES THE FORMATION OF A PLURIPOTENT INNER CELL MASS IN THE EARLY EMBRYO

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The developmental viability of the early embryo requires the formation of the inner cell mass (ICM) at the blastocyst stage. The ICM contributes to all cell lineages within the developing embryo in vivo and the embryonic stem cell (ESC) lineage in vitro. Commitment of cells to the ICM lineage and its pluripotency requires the expression of core transcription factors, including Nanog and Pou5f1 (Oct4). Embryos subjected to culture in vitro commonly display a reduced developmental potential. Much of this loss of viability is due to the up-regulation of TRP53 in affected embryos. This study investigated whether increased TRP53 disrupts the expression of the pluripotency proteins and the normal formation of the ICM lineage. Mouse C57BL6 morulae and blastocysts cultured from zygotes (modHTF media) possessed fewer ($p < 0.001$) NANOG-positive cells than equivalent stage embryos collected fresh from the uterus. Blocking TRP53 actions by either genetic deletion ($Trp53^{-/-}$) or pharmacological inhibition (Pifithrin-α) reversed this loss of NANOG expression during culture. Zygote culture also resulted in a TRP53-dependent loss of POU5F1-positive cells from resulting blastocysts. Drug-induced expression of TRP53 (by Nutlin-3) also caused a reduction in formation of pluripotent ICM. The loss of NANOG- and POU5F1-positive cells caused a marked reduction in the capacity of blastocysts to form proliferating ICM after outgrowth, and a consequent reduced ability to form ESC lines. These poor outcomes were ameliorated by the absence of TRP53, resulting in transmission distortion in favour of $Trp53^{-/-}$ zygotes ($p < 0.001$). This study shows that stresses induced by culture caused TRP53-dependent loss of pluripotent cells from the early embryo. This is a cause of the relative loss of viability and developmental potential of cultured embryos. The preferential survival of $Trp53^{-/-}$ embryos after culture due to their improved formation of pluripotent cells creates a genetic danger associated with these technologies.

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MACROPHAGES ARE ESSENTIAL FOR MAINTENANCE OF EARLY PREGNANCY THROUGH REGULATION OF CORPUS LUTEUM FUNCTION

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Macrophages are abundant within the ovary, and have been identified in the corpus luteum (CL) of most species studied, including in rodents and human. Through their secretory products, macrophages are thought to be involved in ovarian tissue remodelling, including luteinization, and in regulating steroidogenesis [1, 2]. Macrophages co-cultured with granulosa or luteal cells act to stimulate progesterone secretion [3]. To determine the impact of macrophage ablation during early pregnancy, we utilised the macrophage specific CD11b-DTR diphtheria toxin receptor (DTR) transgenic mouse to cause transient systemic ablation of macrophages via the administration of DT (25 ng/g). The effects of macrophage ablation during the pre-implantation phase of pregnancy were evaluated and implantation sites were counted. Ablation of macrophages on day 1 pc (n=13 wild-type; n=9 CD11b-DTR) or day 4 pc (n=6 CD11b-DTR; n=9 wild-type) caused complete pregnancy loss in all DT-treated CD11b-DTR mice, while wild-type mice maintained viable pregnancies [mean ± SEM implantation sites = 5.0 ± 1.4 (day 1 treated), and 6.0 ± 1.9 (day 4 treated)]. Serum progesterone was analysed 24 h following macrophage ablation. A single DT injection on day 3 pc significantly reduced serum progesterone (P_4) levels [n=7 wild-type P_4 (ng/ml)= 29.6 ± 3.3; n=8 CD11b-DTR = 11.1 ± 2.1]. The administration of exogenous P_4 (2 mg) on each of day 4-7 pc prevented fetal loss in DT-treated CD11b-DTR mice (n=6; implantation sites = 7.8 ± 1.6), while no pregnancies remained viable in DT-treated mice administered vehicle only (n=9). In conclusion, luteal insufficiency appears to be the cause of pregnancy failure following macrophage ablation. These data indicate a critical role for macrophages in corpus luteum function in early pregnancy.

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PATERNAL OBESITY IMPAIRS SPERM FUNCTION AND SUBSEQUENT EMBRYO AND PREGNANCY OUTCOMES

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Despite the increased prevalence of obesity in males of reproductive age, the effects of male obesity on conception and pregnancy have been largely ignored. Hence, the aim of this study was to elucidate the effects of paternal Diet Induced Obesity (DIO) on sperm function, embryo development and pregnancy. Six week old C57BL/6 male mice (n=36) were allocated to either standard chow or a high fat diet (HFD). After eight weeks, mice were either sacrificed and spermatozoa assessed, for motility, reactive oxygen species (ROS) and DNA damage or mated and zygotes collected and cultured to the blastocyst stage. Blastocyst development, cell number and apoptosis were assessed, and fetal outcomes analyzed following embryo transfer. Differences between treatments were assessed using GLM. The percentage of motile spermatozoa was decreased (36% vs. 44%, $p<0.05$) in the HFD group compared to controls. Intracellular ROS were elevated (692units vs. 409units, $p<0.01$) in the HFD group compared to controls. Overall levels of sperm DNA damage were also increased (1.64% vs. 0.17%, $p<0.05$) in the HFD group. Blastocyst development was reduced when males were fed a HFD (64% vs. 84%, $p<0.05$). Similarly, blastocyst cell number (37.9±2.8 vs. 46.6±2.5, $p<0.05$), inner cell mass number (11.4±0.9 vs. 15.3±0.9, $p<0.05$) were reduced and apoptosis (12.8±1.9 vs. 6.6±0.6, $p<0.05$) increased in embryos sired by a male fed a HFD. Implantation (86.7% vs. 72.5%, $p<0.05$) and fetal development (38.7% vs. 22.5% $p<0.05$) were also significantly reduced when blastocysts came from a male fed a HFD. This is the first report providing comprehensive evidence that paternal DIO significantly impairs embryo quality and pregnancy rates. These effects may be related to the observed increase in oxidative stress and sperm DNA damage. These data provide compelling evidence that male obesity impacts on male fertility, embryos as well as pregnancy and therefore studies in human are warranted.

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HORMONAL REGULATION OF MIRNA IN THE TESTIS

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Acute suppression of circulating reproductive hormones (FSH and testosterone) inhibits sperm release (spermiation) (1), although the molecular mechanisms of spermiation failure are poorly understood. Micro-RNAs (miRNAs) are small non-coding RNAs that regulate protein expression, and are essential for normal spermatogenesis. Recent studies suggest that miRNAs are exquisitely sensitive to hormonal control by FSH, LH and testosterone (2-4). This suggests that hormonal regulation of miRNAs in the testis following acute hormonal suppression may contribute to spermiation failure. Therefore, we hypothesised that gonadotrophin regulated miRNAs control spermiation outcome. We used array analysis to show that miRNA expression is hormonally regulated by FSH and testosterone in our rat in vivo model of spermiation failure and also in primary rat Sertoli cells by. qPCR validation revealed that miR-7b, -23a, -30c, -125b, -148b, -197, -483, -592, and -690 are all hormonally sensitive testicular miRNAs. Bioinformatic analyses of potential gene targets of these miRNAs predicted numerous protein components localised in the testicular tubulobulbar complex (TBC). The TBC is a podosome-like structure found between Sertoli cells and adjacent germ cells in the testis, and is thought to internalise intact inter-cellular structures and regulate spermatid head shape prior to spermiation. WASP, a TBC protein that regulates actin filament dynamics, contained a conserved binding site for miR-690 within its 3'UTR. Increased miR-690 expression following hormone suppression corresponded to a decrease in WASP protein expression in vivo and in vitro. In addition, transfection of miR-690 into HEK293T cells down-regulated WASP protein. Our results suggest that following hormone suppression, miR-690 is stimulated in the Sertoli cell, thereby inhibiting WASP protein expression. We conclude that miRNA-mediated disruption of TBC integrity potentially regulates spermatid disengagement. This study describes new molecular mechanisms in the testis that may control spermiation outcome of potential significance in male hormonal contraception.

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SMAD3 DOSAGE INFLUENCES TESTICULAR MATURATION

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Activin A, a TGF-beta superfamily ligand which signals via Smad2 and Smad3, is critical for normal mouse testis development and quantitatively normal sperm production. Whereas activin enhances immature Sertoli cell proliferation (1), excessive activin production causes Sertoli cell tumours (2); this is alleviated when mice lack Smad3 (3). Sertoli cells exhibit developmentally regulated Smad utilization in activin signalling. Immature Sertoli cells signal via Smad3 while the onset of Smad2-mediated signal transduction correlates with Sertoli cell maturation (4). This change coincides with decreased testicular Smad3 production at puberty and a shift in follicle stimulating hormone (FSH)-induced Smad transcription, from Smad3 in 6 dpp (days post partum) Sertoli cells to Smad2 in 15 dpp cells. These findings suggest that Smad3 is more important for testis development than adult spermatogenesis. To test this hypothesis, we examined testis development in $Smad3^{+/-}$ and $Smad3^{-/-}$ mice. At 7 dpp, testis weight and cord diameter were reduced in $Smad3^{-/-}$ mice, indicating impaired Sertoli cell proliferation. Levels of FSH, a potent Sertoli cell mitogen, were unaltered. Histological analysis revealed advanced spermatogenesis in heterozygous mice, with round spermatids already present at 16 dpp. Quantitative PCR also identified advanced Sertoli and germ cell maturation in $Smad3^{+/-}$ mice, while Leydig cell maturation appeared unaltered. Adult $Smad3^{+/-}$ and $Smad3^{-/-}$ mice were fertile, but had smaller testes. This is the first study relating Smad3 levels to puberty onset and identifies the $Smad3^{+/-}$ mouse as a model of peripheral precocious puberty with otherwise normal physiological status, i.e. no gonadal tumours and normal FSH levels. These results demonstrate that FSH influences testis growth and maturation by regulating Smad3 expression and highlights the importance of testing whether environmental factors, toxicants and endocrine disruptors affect Smad3 expression, thereby leading to altered testis development.

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THE CAPACITATION INDUCED FORMATION OF A MULTIMERIC SPERM-ZONA PELLUCIDA RECEPTOR COMPLEX

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The ability of mammalian spermatozoa to fertilize the oocyte is dependent on a complex cascade of biophysical and biochemical changes collectively known as capacitation. This final phase of sperm maturation is characterised by a dramatic remodelling of the sperm surface architecture to render the cell competent to recognise and bind to the zona pellucida. Although the current paradigm suggests this interaction is mediated by a single receptor-ligand interaction, recent evidence emerging from our laboratory suggests that this event is more complex and involves the capacitation-dependent formation of a multimeric zona pellucida receptor complex. Through the novel application of Blue Native PAGE and Far Western blotting, we have recently identified the first such complex that displays the anticipated affinity for the zona pellucida. LCMS analysis revealed that this high molecular weight complex is comprised of a family of 8 chaperonin subunits that form an active t-complex polypeptide-1 (TCP-1) complex. In addition, this complex was also shown to contain an N-acetylglucosaminyltransferase, GCnT2, an enzyme with affinity for N-acetylglucosamine. Importantly, this sugar forms part of the zona pellucida glyco-matrix and has previously been implicated as a ligand for sperm interaction. Consistent with this notion, incubation of sperm with anti-GCnT2 antibodies or N-acetylglucosamine competitively inhibited sperm-zona pellucida interactions in a dose dependent manner. Collectively, this data raises the intriguing possibility that chaperonin proteins participate in the assembly and/or presentation of key zona adhesion molecules during capacitation. Future work is aimed at identifying additional zona receptors that may reside within this complex.

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LIPOTOXICITY MEDIATED ENDOPLASMIC RETICULUM STRESS, MITOCHONDRIAL DYSFUNCTION AND APOPTOSIS CONTRIBUTE IMPAIRED OOCYTE QUALITY IN RESPONSE TO OBESITY

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In obesity, accumulation of lipid in non-adipose tissues, a process termed lipotoxicity, is associated with endoplasmic reticulum (ER) stress, mitochondrial dysfunction and ultimately apoptosis. We have previously shown that diet-induced obesity in mice causes impaired oocyte developmental competence, but whether this is due to activation of lipotoxicity pathways in the ovary is not known. The present study examined the hypothesis that diet-induced lipid accumulation in the cumulus oocyte complex (COC) disrupts ER homeostasis and mitochondrial membrane potential which leads to apoptosis. COCs and mural granulosa cells were collected from ovaries of adult mice fed a high fat (HFD) or control diet for 4 weeks. ER homeostasis was assessed by measuring expression of known ER stress marker genes, GRP78, ATF4 and CHOP. COCs from mice fed HFD showed significantly increased expression of GRP78 and ATF4. There was a similar trend towards increased expression in granulosa cells. Mitochondrial function was assessed by measuring membrane potential using the dual emission probe JC-1. In COCs from mice fed HFD there were reduced numbers of active mitochondria but instead large aggregated clusters of inactive mitochondria. Apoptosis in granulosa cells was determined by DNA laddering assay which showed significantly increased DNA fragmentation in cells from mice fed HFD. Apoptosis was also assessed by TUNEL staining of paraffin embedded ovaries from identical treatment groups. Ovaries from HFD mice appeared to have increased TUNEL positivity in both granulosa and cumulus cells. Our results demonstrate that the ER stress, mitochondrial dysfunction and apoptosis are markedly increased in granulosa cells and COCs from mice fed HFD, suggesting that lipotoxicity contributes to the impaired oocyte quality and reduced fertility observed in response to obesity.

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INVOLVEMENT OF TRP53 IN SIRT1 FUNCTION DURING EMBRYO DEVELOPMENT

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Sirtuin 1 (SIRT1) is an NAD^+-dependant deacetylase with significant functions in cell survival and metabolism, including glucose homeostasis and mitochondrial physiology. TRP53 is a universal effector of cellular stress responses and is an important target of SIRT1. Transcriptional activity of TRP53 in the pre-implantation embryo is associated with retarded development, however examination of SIRT1 function and how it relates to TRP53 activity remains to be elucidated. We therefore assessed whether SIRT1 is involved in pre-implantation embryo development and determined whether TRP53 interacts with SIRT1 function. Zygotes were collected from superovulated female mice and cultured to the blastocyst stage in optimised conditions (F1 mouse strain, G1/G2 series sequential media, 5%CO_2, 5%O_2, 90%N_2, group culture) or compromised conditions (C57Bl/6 strain, mHTF static media, 5%CO_2 in air, individual culture). Embryo development and blastocyst cell number was assessed following exposure to a SIRT1 inhibitor (0, 1, 10, 100 or 1000μM sirtinol). In subsequent experiments, embryos were cultured in a 2x2 factorial design (±1μM sirtinol and ±30μM pifithrin-α (TRP53 inhibitor)) and embryo development and cell number determined. Sirtinol caused a dose-dependent reduction in total cell number in blastocysts during culture in both optimised and compromised conditions ($p<0.05$), while the rate of development of zygotes was reduced for embryos in compromised but not optimised conditions ($p<0.05$). When SIRT1 was inhibited, in the presence or absence of TRP53 inhibition, blastocyst development and cell number for embryos in optimal conditions was unchanged. However, blastocyst development (83% vs 55%, $p<0.05$), and cell number (39 vs 54 cells, $p<0.05$) was reduced when SIRT1 was inhibited in compromised embryos, and in the absence of TRP53, development was resolved to control levels. These results show that SIRT1 is important for embryo development, particularly under compromised conditions, and that TRP53 is a likely target for SIRT1 deacetylase activity in the mammalian embryo.

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XENOBIOTICS; INFLUENCE ON OVARIAN FOLLICULAR DEVELOPMENT

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The mammalian female reproductive lifespan is largely defined by a finite pool of ovarian follicles established around the time of birth. It is now understood that certain synthetic chemical compounds, known as xenobiotics, can cause premature ovarian senescence through the destruction of small ovarian follicles. Although the ovotoxic effects of these chemicals are well documented, the exact molecular mechanisms behind their action are only just becoming understood. Recent evidence suggests that bioactivation of xenobiotics by Phase I detoxifying enzymes may lead to the generation of free oxygen radicals (ROS), which we suspect may perturb intracellular signalling pathways in primordial follicles. In this study we attempted to identify ovarian follicle signalling pathways activated by xenobiotic exposure using ovotoxic agents which target immature follicles. Neonatal ovaries obtained from 3/4-day old Swiss mice were exposed to either 4-Vinylcyclohexene (25μM), Methoxychlor (25μM) or Menadione (5μM) for 96hrs using our in vitro culture system. Total RNA was then collected and analysed using Affymetrix Mouse Genome 430 2.0 Arrays. Bioinformatic analysis identified between ~500–1000 genes with a two-fold significant difference in gene expression ($p<0.05$) for each xenobiotic compared to the control. Differentially expressed genes were analysed for pathways and molecular functions using Ingenuity Pathways Analysis (Ingenuity Systems). In agreement with the current literature, many of the genes belonged to toxic response pathways, such as; Xenobiotic metabolism (10); p53 (15) and Apoptosis (11) signalling. However, the vast majority of the differentially expressed genes belonged to canonical pathways implicated in follicular development, such as PI3K/AKT (18), Wnt/ b -catenin (21), and JAK/Stat (8) signalling. Further qPCR analysis has confirmed a substantial increase in the transcription factor Sox4 and cell cycle inhibitor Cdkn2a in 4-Vinylcyclohexene and Menadione treated ovaries respectively. These results suggest that xenobiotics which target primordial follicles may exert part of their ovotoxic effects by perturbing signalling pathways involved in follicular activation and development.

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EFFECT OF KOREAN RED GINSENG EXTRACT IN STEROID-INDUCED POLYCYSTIC OVARY MURINE MODEL

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Experimental induction of polycystic ovary (PCO) resembling some aspects of human PCO syndrome was produced using the long-acting compound estradiol valerate (EV). Our previous study on the role of Korean red ginseng total saponins in a steroid-induced PCO rat model demonstrated that electro-acupuncture modulates nerve growth factor (NGF) concentration in the ovaries. In fact, the involvement of a neurogenic component in the pathology of PCO-related ovarian dysfunction is preceded by an increase in sympathetic outflow to the ovaries. In the present study, we tested the hypothesis that Korean red ginseng extract (KRGE) administration modulates sympathetic nerve activity in rats with PCO. This was done by analysing NGF protein and NGF mRNA expression involved in the pathophysiological process underlying steroid-induced PCO. EV injection resulted in significantly higher ovarian NGF protein and NGF mRNA expression in PCO rats compared to control rats, and PCO ovaries were counteracted by KRGE administration with significantly lower expression of NGF protein and NGF mRNA compared to EV treated ovaries. These results indicate that EV modulates the neurotrophic state of the ovaries, which may be a component of the pathological process by which EV induces cyst formation and anovulation in rodents.

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CD44 SIGNALLING IN THE CUMULUS OOCYTE COMPLEX DURING OVULATION

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Oocytes develop within ovarian follicles that nurture and regulate oocyte maturation. The LH surge induces a cascade of gene expression leading to formation of the hyaluronan (HA) rich cumulus matrix around the oocyte. This cumulus oocyte complex (COC) is composed of high concentrations of HA cross-linked by several HA-binding proteins. Null mutation of several COC matrix genes results in ovulation defects demonstrating the importance of the composition and structure of the COC; but the mechanisms by which the matrix promotes ovulation are unknown. We hypothesised that HA, via activation of its receptor CD44 on cumulus cells, regulates cytoskeletal function, cell adhesion and migration, and that acquired cumulus cell motility facilitates ovulation. We investigated cellular signaling and cellular phenotypes occurring in response to the formation of the HA-rich COC matrix. Expression of CD44 was upregulated 5 to 6-fold in cumulus cells following 6 or 12h hCG (LH analog) stimulation. Signal transducers of CD44 action; Tiam1, a guanine exchange factor, and Rac1, an actin cytoskeleton remodelling Rho-family GTPase, were present in cumulus cells but not regulated by hCG. Induction of migratory and invasive capacity of cumulus cells by hCG was demonstrated using transwell migration and ECM invasion assays. Cumulus cell migration increased 8-fold 10h after hCG compared with cumulus cells from untreated mice. These cumulus cells also showed the capacity to invade through a matrigel barrier. Inhibitors of the CD44-assembled cell migration complex demonstrated the importance of this pathway in the migratory and invasive phenotype of cumulus cells. These results demonstrate that CD44 is a key factor in the assembly of a macromolecular complex facillitating cell motility in cumulus cells at the time of ovulation, and suggest that cumulus cells in the expanded COC undergo epithelial-mesenchymal transition to become invasive motile cells which may play a key role mediating ovulation.

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EFFECTS OF ALBENDAZOLE ON OVARIAN OESTROGEN SYNTHESIS IN THE RAT

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Albendazole is a drug commonly used for treatment of helminth infestation in human and livestock populations. Recent studies show that albendazole reduces ovarian follicular fluid oestrogen levels in sheep[1], but the mechanism involved is unknown. The aims of this study were to determine whether albendazole exerts similar effects on ovarian oestrogen levels in the rat, and to assess the effects of albendazole on expression of key steroidogenic genes in the rat ovary. Oestrus cycles were continuously monitored in Wistar rats by vaginal smears. Commencing at proestrus, albendazole was administered for 12 days in drinking water (approximate dose 15 mg/kg/day). Plasma and whole ovaries were collected on the fourth proestrus (1500–1600h). A second group of rats were treated similarly except that pseudopregnancy (PSP) was induced by mating with a vasectomised male at the second proestrus. Plasma and the non-luteal ovary were collected on day 8 of PSP. Oestradiol was extracted from plasma and ovaries with ethyl acetate and concentrations measured by a chemiluminescent assay. Expression of steroidogenic acute regulatory protein (StAR), P450 side chain cleavage (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), aromatase and 20α-hydroxysteroid dehydrogenase (20α-HSD) mRNAs were measured by RT-PCR. Oestrus cyclicity, ovarian weight and mating behaviour were all unaffected by albendazole in cycling and PSP rats, although as expected levels of oestradiol were lower in PSP. In ovaries of cycling rats albendazole did not affect oestradiol concentrations but reduced ovarian P450scc mRNA expression (by 65%; P=0.024) and there was a trend for an increase in 3β-HSD (P=0.09) and aromatase expression (P=0.12). Expression of the other steroidogenic genes was unaffected and no changes in gene expression were observed in PSP rats. In conclusion, albendazole treatment reduced ovarian P450scc in cycling rats but did not inhibit ovarian oestradiol synthesis or reproductive function.

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EXPRESSION PATTERNS OF EXTRACELLULAR MATRIX IN MICE OVARIES

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Extracellular matrix (ECM) has been shown to have distinct expression patterns during bovine follicle and corpus luteum (CL) formation and regression. To date little is known about ECM patterns during follicle and CL formation in the mouse ovary. Twenty nine day old mice were treated with PMSG on experimental day 0 and 1 to induce follicle development, and subsequently with hCG on day 2 to induce ovulation. Ovaries were collected for immunohistochemistry on days 0, 2 and 5 (n = 10 per group). Another group was similarly treated but was additionally mated, and ovaries examined in the pregnant mice on experimental day 11 (n = 7). The follicular basal lamina (BM) of all developmental stages contained collagen IV α1 and α2, laminin α1, β1 and γ1 chains, nidogen 1 and 2, and perlecan. Collagen XVIII was only found in BMs of primordial, primary and some preantral follicles, whereas laminin α2 was only present in some preantral and antral follicles. BMs of atretic follicles showed similar composition to healthy follicles. A specialized matrix of the membrana granulosa (focimatrix) was detected in both healthy and atretic follicles. The focimatrix contained collagen IV α1 and α2, laminin α1, β1 and γ1 chains, nidogen 1 and 2, perlecan and collagen XVIII, but not laminin α2. The immunostaining in CL was restricted to capillary sub-endothelial basal laminas and contained collagen IV α1 and α2, laminin α1, β1 and γ1 chains, nidogen 1 and 2, perlecan and collagen XVIII. No parenchymal matrix, as observed in cow and human, was detected. In addition, laminin α4 and α5 were not immunolocalised to any structure in the mouse ovary. The composition of the BM of follicles in the mouse ovary is similar to cow and rat, but the appearance and composition of the focimatrix differs from bovine ovaries.

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EFFECT OF A59V LOCUS IN THE LEPTIN GENE ON LENGTH OF PREGNANCY IN IRANIAN HOLSTEIN COWS

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We investigated effect of A59V polymorphism in the leptin gene on length of pregnancy. Blood was collected from 255 Holstein cattles belonging to four different herd managements in Isfahan province. Genomic DNA extracted from whole blood. Genotypes of A59V locus were identified with PCR-RFLP technique. Amplified region is located in exon three of leptin gene. The genomic bovine leptin sequences, which consist of three exons, were obtained from Gene Bank (Accession number U50365). The polymerase chain reaction was used to amplify the 331 bp DNA fragments from genomic DNA. The PCR reaction contained 100 ng of genomic DNA, 0.3 µM of each primer, 1.5 mM $MgCl_2$, 200 µM dNTP, 10mM Tris HCl, 50 mM KCl and 1 U Taq-polymerase in total volume of 20 µL. Sequences of primers that were used in PCR were reported previously by Haegeman et al. (2000). Conditions for PCR were 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 30 s. Followed by final extension for at for 15 min 72°C. Digestion of PCR product of 331 bp with 5 U of HphI (Fermentas) in 20 µL of reaction volume at 37°c for 8 h and analyzed on 8% no denature polyacrylamyde gel. Allele A in the A59V locus was the allele not digested by restriction enzyme, allele B was the restriction enzyme-digested PCR product. Digestion revealed 3 genotypes, AA (331 bp), AB (331, 311, and 20 bp), and BB (311 and 20 bp). Significances of the genotype effects were estimated by GLM procedure of SAS. This study showed that genotype effect on length of pregnancy were significant ($P<0.01$). Animals homozygous for allele A had higher length of pregnancy (($P<0.01$, AA=279.17±0.47, AB=276.96±0.57, BB=274.8±2.2).

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MATERNAL NUTRITION AND GESTATIONAL AGE AFFECT PLACENTAL MICRORNA EXPRESSION IN THE GUINEA PIG

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Maternal undernutrition restricts placental growth and nutrient supply to the fetus, but induces compensatory alterations in structure and function of the placenta. Maternal undernutrition in guinea pigs also restricts placental growth and alters structure, and changes expression of Igf1, Igf2, Slc2a1, Slc38a2 mRNA in mid and late gestation, consistent with nutritionally induced changes in nutrient transport across the placenta. MicroRNAs are non-coding RNAs that regulate expression of target genes by translational inhibition and mRNA degradation and are present in the mammalian placenta. Effects of maternal undernutrition on their expression are unknown. We hypothesised that altered expression of key functional genes in the placenta in maternal undernutrition are in part due to altered expression of regulatory microRNAs. The effect of maternal food restriction on the expression of microRNAs in the guinea pig placenta was examined at D30 and D60 of gestation (term = D70). Guinea pigs were fed either ad libitum (AL) or restricted (R). MicroRNA expression was determined by Exiqon microarray v.8.1. In AL placentas, 119 microRNAs were upregulated ($p<0.05$), whilst 40 were down-regulated ($p<0.05$) at late compared to early gestation. In R placentas, 163 microRNAs were upregulated ($p<0.05$), whilst 123 were down-regulated ($p<0.05$) at late compared to early gestation. Of the 20 most abundant up-regulated microRNAs miR-Plus (ID 17871) and hsa-miR-411 were altered only in AL and hsa-miR-376a and -376b were altered only in R placenta. Of the 20 most abundant down-regulated microRNAs, 13 were altered only in AL and 14 only in R placentas. Placental expression of microRNAs changed with gestation, and maternal undernutrition modified this pattern and altered expression of many additional microRNAs in the guinea pig placenta. This suggests that miRNAs and factors that influence their expression may play a role in the structural and/or functional development of the placenta and hence fetal growth.

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THE POTENTIAL ROLE OF MICRORNAS IN THE DEVELOPMENT OF THE HUMAN PLACENTA IN EARLY PREGNANCY

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Placental functional development is characterised by dynamic and co-ordinated changes in expression of genes that drive invasion, differentiation and growth. These changes may arise in part from altered expression of microRNAs (miRNAs) via their regulatory networks. MiRNAs are short, single-stranded, non-coding RNAs involved in the post-transcriptional repression of gene expression. MiRNAs bind to complementary sites in the 3'UTR of target mRNAs to repress or silence translation. MiRNAs have been detected in the mammalian placenta, but their patterns of expression throughout pregnancy have not been systematically characterized. Using microarrays, miRNA gene expression was compared at two stages (6-8 weeks, 10–12 weeks) in early gestation, in chorionic villi of human placentas (term ~40 weeks). Putative and validated targets of differentially expressed miRNAs were extracted from freely accessible databases, miRBase [1], PicTar [2], TargetScan [3] and miRecords [4]. 15 miRNAs were differentially expressed between these gestational ages ($p<0.05$). 11 of these miRNAs were upregulated in 10–12 week villi and 4 were downregulated. Many of the differentially expressed miRNAs are members of the same polycistronic clusters, suggesting that these miRNAs may be co-expressed. Shared targets of differentially expressed miRNAs from the same clusters were assessed using Ingenuity Pathways Analysis, to search for significantly represented molecular networks. All downregulated miRNAs at 10-12 weeks shared 35 putative targets and fell into 1 of 2 clusters, on chromosome 13 or X. Previously validated targets include PTEN [5], Notch1 [6], VEGFA [7], CDKN2A [8] and DHFR [9]. Six of the upregulated miRNAs at 10–12 weeks are members of 3 clusters on chromosome 19, 9 and X. Networks targeted by these cluster members include PTEN, HIF1α and IL-12 signalling. Together all of these processes are active and important in early placentation and their predicted targeting by differentially expressed miRNAs is consistent with an important role in placental development.

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PATERNAL AND FETAL SINGLE NUCLEOTIDE POLYMORPHISMS IN KDR GENE ASSOCIATE WITH PREECLAMPSIA AND INTRAUTERINE GROWTH RESTRICTION

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Introduction: Preeclampsia (PE) and intrauterine growth restriction (IUGR) together contribute to maternal and neonatal morbidity and mortality. Abnormal placental angiogenesis is implicated in these pregnancy complications. KDR is the main receptor for vascular endothelial growth factor, a potent angiogenic factor which regulates placental angiogenesis. Derangements in KDR expression are known to result in abnormal angiogenesis. We aimed to determine whether polymorphisms in KDR gene (KDR T604C and KDR C1192C) are associated with PE and IUGR. Methods: 1169 nulliparous pregnant women and their partners were recruited prospectively at the Lyell McEwin Hospital and women monitored throughout pregnancy. PE and IUGR were classified using strict guidelines. Uncomplicated pregnancies were deemed controls. Peripheral blood was collected from couples and cord blood collected at delivery. DNA extraction from buffy coats and genotyping were performed at the Australian Genome Research Facility using the Sequenom MassARRAY system . Genotypes for PE (n=63) and IUGR (n=94) were compared with controls (n=373) and analysed using ANOVA and Chi Square. Odds Ratios (OR) were calculated. Results: Paternal and neonatal KDR T604C were associated with PE (p=0.028, OR=1.9, 95%CI=1.08–3.34 and p=0.008, OR=2.5, 95%CI=1.3–4.78). Paternal and neonatal KDR T604C were associated with IUGR (p=0.005, OR=2.01, 95%CI=1.24–3.25 and p=0.01, OR=1.15, 95% CI=1.22–3.79). Neonates with KDR T604C CC genotype were 144.5g lighter than those with the TT genotype (p=0.05). Mean customised birth weight centile was 8.7 lower for fathers with KDR T604C CC genotype compared to CT (p=0.041). KDR C1192T SNP was not associated with outcome. Conclusion: Our results suggest that KDR T604C polymorphism is associated with both PE and IUGR. We are the first to demonstrate an association between paternal KDR polymorphisms and pregnancy complications. Paternal genes acting via the placenta appear to contribute to the risk of PE and IUGR. Ongoing research will determine the role of these polymorphisms in placental angiogenesis.

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THE EFFECT OF MATERNAL FOLIC ACID SUPPLEMENTATION THROUGHOUT PREGNANCY ON NEURODEVELOPMENT, MOTOR FUNCTION AND BEHAVIOUR OF PROGENY IN THE RAT

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Background: Maternal folic acid supplementation (mFAS) during early pregnancy is recommended to reduce the incidence of neural tube defects and has recently been associated with improved neurodevelopment in children. However, the effect on neurodevelopment of mFAS from before conception and throughout pregnancy is unknown. We examined the effect of mFAS throughout the gestational period on postnatal growth, neurodevelopment and early adult motor function and behaviour in rat offspring. Methods: Female Wistar Rats were fed either a control (folic acid 2mg/kg, n=6) or moderate mFAS diet (folic acid 6mg/kg, n=6) from two weeks before mating with Lewis males, until birth of progeny. Male and female progeny (Control=36, mFAS=36) were weighed on postnatal day (PD) 3, 7, 14, 21, 40 and 90, and underwent various tests between PD4 and 14: righting reflex, palm-grasp reflex, negative geotaxis, forelimb hanging, ascent test and eye opening. Locomotor/exploratory behaviour, motor coordination and anxiety were assessed using an open field test (PD52), rotarod (PD55) and elevated plus maze (PD58) (Control=24, mFAS=24). Results: mFAS did not alter maternal weight gain, litter-size at birth or progeny growth between PD3-90. mFAS tended to increase righting reflex time (p=0.057) and impair ascent ability (p=0.085). Negative geotaxis time was reduced at PD7 but not later (Diet x Age p=0.051). mFAS increased the proportion of progeny with eyes open at PD14 (p=0.008) and tended to increase forelimb hanging time(p=0.097). mFAS did not alter motor learning/function (rotarod), but increased ambulatory and exploratory behaviour (open field test; p=0.027). Conclusions: mFAS delays some early aspects of neurodevelopment including neonatal postural reflex maturation and proprioceptive/vestibular function, but accelerates others such as eye opening. However, the open field test indicated that mFAS improved the offspring's locomotion and exploratory behaviours in adulthood. Further studies will differentiate the neurodevelopmental effects of mFAS around conception from gestation-long mFAS.

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MATERNAL FOLIC ACID SUPPLEMENTATION INDUCED ALTERATIONS IN METABOLIC HEALTH OF PROGENY: ROLE OF MICRORNA REGULATORY NETWORKS

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Background: Nutrition in early life can influence metabolic functionality in later life, in part via heritable epigenetic changes, which modify gene expression without altering DNA sequence. Folate supplies methyl groups for the methylation of DNA and histones, both major epigenetic marks that change dynamically in utero. We have recently shown that maternal folic acid supplementation (MFAS) in the pregnant rat increases insulin sensitivity in adult male progeny, while decreasing that of females. The molecular basis of this is unknown but microRNAs may play a role. MicroRNAs are epigenetically regulated non-coding RNAs that downregulate post-transcriptional expression of their targets. MFAS may modulate epigenetics and expression of microRNAs and their targets in adult progeny to alter insulin sensitivity. Aims/Hypotheses: The effect of MFAS before and throughout pregnancy on microRNA expression in liver and skeletal muscle of adult progeny was determined. Methods: Female Wistar rats were fed Control (n=11) or Folic Acid Supplemented (n=9) diets containing either 2 or 6 mg folic acid/kg respectively, from two weeks before mating and throughout pregnancy. One male and female progeny per litter were sacrificed on postnatal day 90 and microRNA expression was determined by Exiqon microRNA microarray v.8.1. Results: MFAS altered hepatic microRNA expression in adult male progeny, but did not alter that in females. Sixteen hepatic microRNAs were differentially expressed, with five predicted in silico (rno-miR: 23a, 23b, 212, 298 and 325-5p) to target several key insulin signalling molecules (p85α, p110β, Akt2, and Prkcz). miR-122a, which promotes cholesterol and lipid synthesis in vivo, was also downregulated. MFAS did not alter microRNA expression in skeletal muscle of adult male or female progeny. Conclusions: MFAS alters hepatic microRNA expression in adult male progeny. Changes in their expression together with their targets in insulin signalling pathway may initiate increased insulin sensitivity in adult male progeny.

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THE IMPRINT STATUS AND EXPRESSION OF *INS* IN THE TAMMAR WALLABY, *MACROPUS EUGENII*

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Genomic imprinting is an epigenetic mechanism that differentially regulates the expression of certain genes, resulting in expression from only one parental allele. It is presumed to have first evolved after the divergence of therian mammals from the monotremes. One imprinted gene, INS is maternally imprinted (paternally expressed) in the eutherian and marsupial yolk sac[1,2]. INS encodes the precursor to the hormone insulin, which regulates carbohydrate metabolism and has a role in cell growth and, by regulating amino acid and fatty acid transporters, protein synthesis. In rats, mice and several other mammals insulin, in addition to cortisol and prolactin, is an absolute requirement for the onset of lactation and the synthesis of milk[3]. As imprinting plays an important role in regulating nutrition and growth the role of imprinted genes in the placenta has been the focus for imprinting research. Since the mammary gland provides a critical source of nutrition for the neonate in all mammals it is possible that genomic imprinting may have developed and been maintained in this organ. Given that marsupials deliver tiny, altricial young, it is in the relatively long and complex lactation phase where the mother has most control of the young's growth. Therefore, there may be greater selection for genomic imprinting in the marsupial mammary gland than in the eutherian mammary gland. This study examined the expression and the imprint status of INS in the mammary gland and neonatal tissues of the tammar wallaby, Macropus eugenii. INS expression was detected using PCR and direct sequencing provides evidence of INS imprinting in the mammary gland. This is the first study to identify imprinting in the mammary gland of a marsupial and the first to identify INS imprinting outside of the yolk sac.

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PLASTICITY IN IMPRINTING OF BOVINE *IGF2R* CORRELATES WITH TOTAL EXPRESSION LEVELS IN FETAL TISSUES

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The multifunctional insulin-like growth factor 2 receptor (IGF2R) facilitates endocytosis and subsequent clearance or activation of a variety of ligands involved in cell growth and motility. Thus, the IGF2R gene has a major role in embryonic development and fetal growth. Murine Igf2r is subject to genomic imprinting and maternally expressed in peripheral fetal tissues. However, data on imprinting of IGF2R in human is still controversial with biallelic expression, partial imprinting, and monoallelic expression reported for fetal tissues [1, 2, 3]. Data from additional species may help to understand fetal IGF2R expression. The bovine is similar to human in that it is outbred and monotocous. We have analysed bovine Day 153 fetuses (55% to term, n=40) with Bos primigenius indicus and B. p. taurus genetics to determine the imprinting status of IGF2R in fetal brain, liver and skeletal muscle. Sequencing of PCR amplicons from IGF2R exon 48 revealed a polymorphic microsatellite and 14 SNPs. These were used to identify 15 heterozygous fetuses informative for imprinting analysis. We found biallelic expression of IGF2R in fetal brain and predominantly maternal expression in fetal liver and skeletal muscle. However, we observed considerable plasticity in imprinting in liver and skeletal muscle with paternal expression levels of 7%–21% and 4%–21%, respectively. Fetal liver samples with B. p. indicus maternal genetics showed significantly higher mean paternal expression levels than those with B. p. taurus maternal genetics ($P<0.05$). Real-time qPCR showed a significant relationship between imprinting and total IGF2R expression level within both tissues ($P<0.05$). Our data indicate plasticity in imprinting of IGF2R that could fine tune expression levels in fetal tissues.

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MATERNAL OBESITY IS ASSOCIATED WITH AN INCREASED INCIDENCE OF PROSTATE ABNORMALITIES IN ADULT RAT OFFSPRING

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The World Health Organization has stated that 75% of adults worldwide are overweight, and in Australia nearly 25% of men are obese. Obesity is associated with an increased risk of cardiovascular disease, type 2 diabetes and cancer, with 30 to 40% of the latter possibly preventable by maintaining a healthy weight (The International Association for the Study of Obesity). Prostate cancer is the most commonly diagnosed cancer in men and there is increasing evidence that obesity increases the risk of prostate cancer mortality. High birth weight, an indication of excess nutrition during foetal development, has been associated with an increased risk of childhood and adult obesity, and for cancer. Using an animal model, we investigated whether obese mothers are more likely to have obese sons who are at an increased risk of developing prostate abnormalities and thus prostate cancer, in adulthood. Female rats were fed with either a control diet (4g fat/kg) or high fat diet (100g fat/kg) from before mating and throughout pregnancy. Prostate tissues were collected from the male offspring at 90 days (post-puberty) and 180 days (young adult). Histological analysis of the day 90 prostates identified hyperplasia in 100% of the ventral lobes (VL) and 64% of the dorsolateral lobes (DLP) in offspring of the maternal high fat group compared to 0% in each respectively, in those of the maternal control diet group. The VL is the most hormone sensitive prostate lobe of the rat, while the DLP is considered the equivalent of the human peripheral zone, the region from which the majority of human prostate cancers arise. These results suggest for the first time that maternal high fat diet may induce prostate abnormalities in male offspring that may in turn, predispose to an increased risk of prostate cancer in later life.

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MUTANT STEROIDOGENIC FACTOR-1 FROM PATIENTS WITH DISORDERS OF SEX DEVELOPMENT SHOW REDUCED ACTIVATION OF THE TESTIS-SPECIFIC ENHANCER OF *SOX9*

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The orphan nuclear hormone receptor Steroidogenic Factor 1 (SF1; NR5A1) is expressed throughout hypothalamic, pituitary, gonadal and adrenal tissues. Naturally occurring human mutations combined with mouse knockout models have revealed a critical role for SF1 as a transcription factor at multiple stages during gonadal development and during development of the adrenal. Missense mutation or truncation to SF1 in XY humans cause Disorders of Sex Development (DSD) with variable phenotypes. The precise mechanisms of SF1 action that fail in human DSD are not fully determined. This work aimed to utilise naturally occurring DSD-causing mutations in SF1 to increase our understanding of the sex determining function of SF1 in the developing male gonad. Recent work by others (1) identified SOX9 as a key target gene of SF1 during testis determination. SF1 activates Sox9 through a testis-specific enhancer element, termed TES. We tested the abilities of eleven clinical SF1 mutations to activate TES in reporter assays in HEK293T cells. Eight of the eleven SF1 mutants showed considerably reduced activation of TES compared to WT SF1. Furthermore, all mutations causing moderate to severe DSD phenotypes correlated with a more severe impairment of TES activation. In addition, all eleven of the mutants showed reduced synergistic activation of TES in co-transfection with the testis-determining co-factor SRY. Overall, this biochemical analysis of the function of mutant SF1 from DSD patients suggests that a failure of SOX9 up regulation, due to reduced activation of TES during testis development, could be the primary cause of the DSD in some patients with SF1 mutations.

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IDENTIFICATION AND CHARACTERISATION OF SURFACE PROTEIN COMPLEXES IN HUMAN SPERMATOZOA

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Upon leaving the testis mammalian spermatozoa are functionally incompetent and are thus unable to fertilize an oocyte. As the spermatozoa ascend the female reproductive tract, functional maturity is achieved through a complex cascade of biophysical and biochemical changes known as capacitation. An important aspect of this final maturation phase is the remodelling of the sperm surface architecture to enable it to interact with the zona pellucida, a glycoprotein matrix that surrounds the oocyte, and initiate fertilisation. While originally thought to be underpinned by a simple lock and key mechanism, emerging evidence has suggested that this interaction may instead be mediated by a multimeric recognition complex that is formed on the sperm surface during capacitation. However, to date the presence and composition of such a complex has yet to be described. Through the application of Blue Native Polyacrylamide Gel Electrophoresis (BN-PAGE), we have provided evidence that human spermatozoa express a number of high molecular weight protein complexes on their surface. Furthermore, the affinity of these surface expressed complexes for the zona pellucida was assessed utilising solubilised human zona pellucida and the technique of Far Western Blotting. Among the complexes that showed affinity for the zona pellucida we identified one comprising 14 subunits of the 20S proteasome. Interestingly, the 20S proteasome has previously been implicated in various aspects of mammalian fertilisation, including zona pellucida penetration and the acrosome reaction, although its precise role in these events has yet to be elucidated. Collectively, these results demonstrate the presence of multimeric protein complexes on the surface of human spermatozoa, and support their putative role in the initial interaction between the sperm and the zona pellucida. Our current research is focused on elucidation of the role of the 20S proteasome in human sperm-zona binding and further investigation of surface expressed protein complexes.

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SERTOLI CELL-SPECIFIC DISRUPTION OF THE ANDROGEN RECEPTOR DNA-BINDING DOMAIN REVEALS DIFFERENTIAL TEMPORAL CONTROL OF DISTINCT ANDROGEN-REGULATED GENES

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Androgen receptor (AR) actions are vital for spermatogenesis. However, in postnatal development male germ cells do not express AR, highlighting its key role in testicular somatic cells. We recently used a Cre-loxP strategy to determine the in vivo requirement of AR DNA-binding in Sertoli cell (SC) function. Transgenic (Tg) mice with Cre expression targeted by SC-specific AMH or Abp promoters were crossed with floxed-Ar (Ar^{flox}) mice for Cre-loxP inframe deletion of Ar exon 3, which encodes a zinc finger essential for the DNA-binding domain (DBD). SC-specific mutated $AR^{\Delta ex3}$($SCAR^{\Delta ex3}$) produced infertile *AMH*.$SCAR^{\Delta ex3}$ and Abp.$SCAR^{\Delta ex3}$ males. Testes from adult homozygous $TgCre^{(+/+)}$ AMH.$SCAR^{\Delta ex3}$ or Abp.$SCAR^{\Delta ex3}$ males were 30% of normal size and exhibited meiotic arrest, whereas testes from hemizygous $TgCre^{(+/-)}$ Abp.$SCAR^{\Delta ex3}$ males were larger (47% normal) with more postmeiotic germ cell development. Despite marked Leydig cell hypertrophy, testicular expression of the adult Leydig marker Hsd3b6 (RT-PCR) and normal intratesticular testosterone levels (LC-MS/MS) in $SCAR^{\Delta ex3}$ males indicated the presence of morphologically distinct but functional adult Leydig cells. SC-specific mutated $AR^{\Delta ex3}$ was predicted to disrupt classical AR-regulated pathways via loss of direct DNA interaction. Androgen-repressed testicular Ngfr expression (known to be via non-classical AR pathways) was not upregulated in $SCAR^{\Delta ex3}$ testes, suggesting maintenance of a non-classical mechanism independent of AR-DBD. In contrast, SC-specific Rhox5 and Eppin transcription, regulated by divergent or classical androgen-response elements respectively, were both decreased in postnatal $SCAR^{\Delta ex3}$ vs. control testes, demonstrating SC-specific AR function as early as postnatal day 5. However, Rhox5 expression declined dose-dependently, whereas Eppin expression increased, in adult $TgCre^{(+/-)}$ and $TgCre^{(+/+)}$ $SCAR^{\Delta ex3}$ testes, revealing differential temporal control for distinct AR-regulated transcripts. Thus, our $SCAR^{\Delta ex3}$ paradigm displayed dose-dependent TgCre-disruption of meiotic competence and post-meiotic development as well as gene expression, and represents a unique model to selectively differentiate AR-regulated genes.

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TGFB SIGNALING IN AN IN VITRO SEMINOMA MODEL

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Testicular cancer, the second most common malignancy in young men, has a 95% cure rate but can result in infertility or subfertility. Its incidence has increased significantly in recent decades (1). This cancer is thought to arise during embryogenesis, based on the persistence of embryonic germ cell markers such as Blimp1 (2), Oct3/4(3) and Nanog (3) in adult seminoma cells. TCam2 cells are a recently characterised in vitro seminoma model (4). We show by Q-PCR and immunofluorescence that they also express these early germ cell markers. TGFβ signaling plays a key role during germ cell development, and is implicated in the development of testicular cancers (5,6). To investigate this further, we first determined whether the pathway is active in TCam2 cells. By Q-PCR we demonstrate expression of the TGFβ downstream transcription factors Smad 2, 3 and 4, and Activin type I and II receptors. Importantly, ActRIIA, which is undetectable in adult testicular germ cells, but readily detected in human foetal germ cells (7) and clinical seminoma samples (6), is readily detectable at both the mRNA and protein level in TCam2 cells. Furthermore, 24 hour treatment with Activin (5 and 50ng/ml) or BMP4 (5 and 50ng/ml) induces a 3–4 fold increase in ActRIIA mRNA levels, but not ActRIA, ActRIB or ActRIIB. Strikingly, in TCam2 cells BMP4 and to a lesser extent retinoic acid, but not activin, support survival and proliferation of TCam2 cells in the absence of serum. This is consistent with known roles of BMP4 and retinoic acid in enhancing murine foetal germ cell proliferation/self-renewal and survival (8,9), and activin inhibition of foetal murine germ cell proliferation (10). This study is the first to demonstrate a functional response in seminoma cells consistent with their foetal germ cell-like identity and forms the basis for future mechanistic analyses of the role of TGFβ signaling in human testicular cancer.

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IDENTIFICATION OF DECIDUALISATION- INDUCED PROTEIN CHANGES IN HUMAN ENDOMETRIAL STROMAL CELLS BY PROTEOMICS

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Decidualisation of human endometrial stromal cells (HESC) is pivotal for successful implantation and can be induced by cAMP analogues such as 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) and ligands to enhance cellular cAMP levels. The resulting decidualisation HESC is recognised by morphological changes and cellular products such as prolactin or insulin-like growth factors. The regulation of proteins during decidualisation HESC has yet to be clearly identified. The aim of this study was to identify proteins that are altered during decidualisation. HESC were isolated and decidualised with 500μM of 8-Br-cAMP for 72 hours. Decidualisation success was determined by prolactin assay. 2D differential in-gel electrophoresis (DIGE) was used to examine differentially expressed proteins between control and 8-Br-cAMP treated HESC. The proteins of interest were validated by Western blot and/or immunohistochemistry. A total of 88 differentially expressed proteins were identified by matrix assisted laser desorption/ ionisation (MALDI) and/ or liquid chromatography mass spectrometry (LC-MS). The proteins that were up regulated during decidualisation include insulin growth factor binding protein as well as caldesmon, tropomycin, actin, tubulin, SRC substrate cortactin and calponin-1 which contribute to the re-organisation of cell cytoskeletal restructuring and remodelling. Proteins associated with cellular growth and transformation such as annexin, nuclear migration factor protein and elongation factor 1B were also up regulated. These results suggest that the process of decidualisation is complicated requiring synergy and cross-talk between structural and cell growth proteins and pathways.

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DEVELOPMENT OF A NON-HORMONAL CONTRACEPTIVE: VAGINAL DELIVERY OF A LIF INHIBITOR: ITS TISSUE DISTRIBUTION AND EFFECT ON IMPLANTATION IN MICE

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Uterine leukaemia inhibitory factor (LIF) is obligatory for fertility in mice and associated with infertility in women. Intraperitoneal injection (IPI) of a long-acting LIF inhibitor conjugated to polyethylene glycol (PEGLA) blocks endometrial LIF action preventing implantation[1]. Thus, PEGLA is a promising non-hormonal contraceptive. In women, vaginally applied (VA) compounds preferentially localize to the uterus suggesting a desirable delivery method for contraceptive purposes; however this has not been examined in mice. We aimed to compare the effects of VA and IPI PEGLA on tissue distribution and implantation in mice. Non-pregnant or mated female mice (Pregnant day [D] 2 [D0: day of plug], n=3/group) were given ^{125}I-PEGLA by IPI ($3x10^6$cpm) or VA ($7x10^5$cpm). ^{125}I-PEGLA was measured in blood and tissue at various time points. To block implantation, mated mice (n=4/group) were given PEGLA or control by IPI (500μg/injection) or VA (300μg/injection) at 1200h and 2200h on D2 and 1000h on D3 and the uterus examined for implantation sites on D6. ^{125} I-PEGLA accumulated in blood and uterus following IPI more rapidly (10min cf 30min), reached a higher concentration (10min, 6h and [blood] 24h; $p<0.05$) and remained longer (24h cf 6h) compared to VA. The percentage of protein-bound ^{125}I in blood was higher following IPI (79.4+1.9%) than VA (47.5+6.7%) at 6h ($p<0.05$). Following IPI, ^{125}I-PEGLA accumulated in the liver, gall bladder and stomach (2h) and spleen (24h) compared to control while no specific tissue accumulation was observed following VA. PEGLA prevented implantation following IPI ($p<0.05$) and reduced the size of implantation sites and decidualization in VA compared to controls. This study demonstrated differences in tissue distribution between VA and IPI PEGLA. It showed that VA PEGLA acted on the uterus in mice albeit to a lesser extent than IPI. It suggests that VA PEGLA is a potential route of delivery for contraceptive purposes.

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ENDOMETRIAL REPAIR - ENTER THE MATRIX

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Menstruation and endometrial repair are two opposing functions which 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 have utilised two models of endometrial repair, a mouse menstruation model[3] , and an endometrial luminal epithelial cell-line (ECC-1)[4] with the aim of defining the adhesion and ECM molecules important for endometrial repair. Uterine horns (repairing and non-repairing control from each of 4 mice) were subjected to laser-capture microdissection of the repairing luminal epithelium (LE) and immediate sub-luminal stroma. RNA was extracted and processed for pathway-focused array. ECC-1 cells were grown to over-confluence, wounded and allowed to repair. RNA was extracted on each day during repair and used for oligo-microarray analysis. Menstruating/repairing human endometrium was investigated for expression of ECM molecules by immunohistochemistry. A host of ECM molecules (ADAMTS's, MMP's, cadherins, integrins and matrix proteins) were shown to be altered in the repairing murine endometrium. In agreement with published data[2], fibronectin and integrins α5 and β1 were elevated at the time of repair. However, in our models, fibronectin was regulated mainly in the stromal compartment, demonstrated by its absence in the ECC-1 model and concentrated stromal immunolocalization in human endometrium. Both integrins displayed regulated expression in the ECC-1 model and localised to repairing LE of human endometrium. Other noteworthy ECM molecules regulated solely within the murine model include integrins (αM, αL, β2) and selectins expressed on lymphocytes, which may reflect the importance of these cells in endometrial repair[5]. In conclusion, these data suggest the ECM contributes to the dynamic changes observed during endometrial repair and these models may enable further insight into the roles of each cellular compartment.

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THE EPIDERMAL GROWTH FACTOR (EGF) FAMILY IN THE ENDOMETRIUM AND BLASTOCYST OF THE TAMMAR WALLABY, *MACROPUS EUGENII* DURING EMBRYONIC DIAPAUSE

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Embryonic diapause, a suspension of cell division and growth at the blastocyst stage, is widespread amongst mammals, but is especially common in the kangaroos and wallabies. In the tammar, *Macropus eugenii*, the sequence of endocrine events leading to embryonic diapause and reactivation are well defined (1). The blastocyst can remain in diapause for up to 11 months without cell division, measurable metabolism or apoptosis occurring (2). The ovarian hormones, especially progesterone, exert their effects on the blastocyst by alterations in the endometrial secretions (3), but the molecular cross-talk between the endometrium and blastocyst is unknown. There is increasing evidence for the involvement of leukaemia inhibitory factor (LIF) but the epidermal growth factor (EGF) family of growth factors are also likely to be involved. This study examined the expression of EGF and HB-EGF as well as their receptors, ERBB1 and ERBB4, in the tammar endometrium and blastocyst at entry into, and reactivation from, diapause. The genes for these factors were highly conserved in the tammar with orthologues in human and mouse. Quantitative RT-PCR of all four factors in the endometrium showed that expression changed with stage. Although expression levels of both receptors did not change between diapause and reactivation, both HB-EGF and EGF levels increased at reactivation from diapause and levels of HB-EGF decreased at entry into diapause. All factors were immunopositive in the endometrium. Studies underway will determine whether the cellular location and quantity of these factors change with entry into or exit from diapause, and define the molecular interactions occurring between the blastocyst and endometrium. These results are consistent with a role for the EGF family of growth factors in the control of embryonic diapause in tammars.

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THE ROLE OF PROPROTEIN CONVERTASE 6 DURING DECIDUALIZATION: REGULATION OF BONE MORPHOGENETIC PROTEIN 2 ACTIVATION

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Proprotein convertase 5/6 (PC6), a member of the proprotein convertase (PC) family, is a critical endometrial factor for implantation. PC6 is up-regulated in the endometrium specifically at implantation in association with epithelial differentiation (in human and monkey) and stromal cell decidualization (in the mouse, human and monkey). PC6 is the only PC member that was significantly up-regulated during decidualization. Knockdown of PC6 inhibits decidualization. PCs function by converting a range of important precursor proteins into their bioactive forms. One group of such proteins is the transforming growth factor beta (TGF-beta) superfamily proteins. They are first synthesized as larger biologically inactive precursors, and then are processed by PCs into their active forms. Bone morphogenetic protein 2 (BMP2) is a TGF-beta superfamily member and demonstrated to be essential for decidualization. We hypothesized that BMP2 is one of the proteins that PC6 activates during decidualization. Freshly isolated stromal cells from human endometrium were decidualized in culture with and without inhibition of PC6 activity. The full-length (precursor, non-active) and processed (activated) forms of BMP2 were determined in cellular lysates and media. The precursor form of BMP2 was reduced whereas the active form was increased during decidualization. Inhibition of PC6 activity inhibited decidualization, and this inhibition was accompanied by a total inhibition of the production of active BMP2. To further confirm the role of PC6 in activating BMP2 in decidualization, active BMP2 was added into cells and the decidualization arrest caused by PC6 inhibition was partially rescued. This study demonstrated that PC6 regulates decidualization by activating molecules such as BMP2 that are essential for decidualization.

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GDF-9, BMP-15 AND ACTIVIN A CONTRIBUTE TO SEMINAL FLUID SIGNALLING IN HUMAN CERVICAL EPITHELIAL CELLS

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We have previously shown that in women, as in animal species, introduction of seminal fluid at coitus induces cytokine expression and a local inflammatory-like response in the female reproductive tract. The response is characterised by induction of mRNAs encoding several pro-inflammatory cytokines including GM-CSF, IL-1α and IL-6, as well as chemokines IL-8, MIP-3α and MCP-1. Recent studies in our laboratory have focused on identifying active signalling agents present in human seminal plasma. To date we have shown that all three mammalian isoforms of TGFβ (TGFβ1, TGFβ2 and TGFβ3) and 19-hydroxy PGE are key regulators of this response. The current study aimed to determine whether other members of the TGFβ superfamily including GDF-9, BMP-15 and Activin A also contribute. To investigate this we utilised immortalised Ect1 ectocervical epithelial cells, which mimic the response of primary ectocervical epithelial cells. Ect1 cells were incubated with increasing doses (0.5, 5.0, 50 or 500 ng/ml) of rGDF-9, rBMP-15 or rActivin A and production of pro-inflammatory cytokines and chemokines was assessed in 24 hour post-treatment supernatants using Luminex microbead technology. BMP-15 was found to stimulate GM-CSF production (2-fold), while GDF-9 and Activin A both stimulated IL-6 production (2.4-fold and 80% increases respectively), all acting in a dose-dependent manner. In contrast, all three factors inhibited IL-8 production by Ect1 cells. These data demonstrate that GDF-9, BMP-15 and Activin A are new seminal fluid signalling agents capable of targeting female reproductive tract epithelial cells and inducing different response profiles compared with TGFβ and 19-hydroxy PGE. The relative bioavailability of these factors in seminal fluid would therefore influence the profile of inflammatory response in the female partner, regulating immune responses to male seminal antigens as well as sexually transmitted pathogens.

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A MECHANISM UNDERLYING DISORDERS OF SEX DEVELOPMENT CAUSED BY *DAX1* DUPLICATION

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The DAX1 protein is an orphan nuclear hormone receptor expressed in developing and adult hypothalamic, pituitary, adrenal and gonadal tissues. In humans, duplication of the DAX1 gene at locus Xp21 causes Disorders of Sex Development (DSD), whereby XY individuals develop as females, due to the failure of testicular development. DAX1 acts as a co-factor for nuclear receptor-mediated transcription of steroidogenic genes. In mice, overexpression of a Dax1 transgene causes delayed testis cord formation, a milder phenotype than that seen in human (1). Exactly how DAX1 duplication interferes with typical testicular development is unclear but a 'window' of DAX1 activity was proposed (2). In order to identify the mechanism of DAX1 action when overexpressed in the developing XY gonad, we have used both in vivo and in vitro approaches. We hypothesised that, when present in excess, DAX1 must repress the action of early testis-forming genes. We investigated the effect of Dax1 over expression, using the Dax1 transgenic mouse line, Dax1812 (1), on expression of Sox9, a critical testis-forming gene. Immunostaining of Dax1812 gonads revealed reduced Sox9 expression, suggesting excess Dax1 antagonises Sox9 upregulation during the early stages of sex determination. To determine whether antagonism of Sox9 was occurring at the transcriptional level we assessed the effect of excess Dax1 on the activity of the Testis-Specific Enhancer of Sox9 (TES), which drives Sox9 transcription in the developing XY gonad (3). In combination, the in vivo and in vitro evidence strongly suggests that Dax1, when present in excess, can repress Sox9 expression through TES and that this repression occurs through inhibition of Steroidogenic Factor-1 activity. With this work we have identified a potential mechanism for disruption of the male-specific sex determination pathway caused by DAX1 duplication and leading to DSD in XY individuals.

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RAT TESTICULAR MACROPHAGES EXHIBIT AN "ALTERNATIVELY ACTIVATED" RESPONSE TO LIPOPOLYSACCHARIDE (LPS), INTERFERON-Γ (IFNΓ) AND INTERLEUKIN-4 (IL-4), CONSISTENT WITH IMMUNE PRIVILEGE

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Testicular macrophages (TMs) are implicated both in the response of the testis to invading pathogens and supporting the immunosuppressive environment that protects developing germ cells (immunoprivilege). Macrophages are classified into two general phenotypes: "classically activated" (M1), which undergo inflammatory responses to LPS and IFNg, and "alternatively activated" (M2), defined by anti-inflammatory activity and regulated by IL-4. Our aim was to establish whether TMs have an M2 phenotype, consistent with immunoprivilege. Rat TMs and bone marrow-derived macrophages (BMMs) were isolated from adult rats, and cultured with LPS, IFNg and/or IL-4 for 2-3h. mRNA expression was measured by real-time RT-PCR and protein production was measured by ELISAs. Compared with BMMs, TMs stimulated with LPS and IFNg, either individually or in combination, expressed low levels of pro-inflammatory cytokines, such as IL-1β, IL-1α and tumour necrosis factor-α, intermediate levels of IL-6, but much greater levels (8-fold) of the anti-inflammatory cytokine, IL-10. TMs also displayed elevated constitutive expression of IL-10 and responded to IL-4, unlike BMMs. However, TMs expressed relatively low levels of another immunoregulatory cytokine, transforming growth factor-β1. After FACS-sorting of TMs using an antibody to CD163, a surface marker associated with M1-M2 progression, $CD163^+$ TMs produced high levels of IL-10 constitutively and after stimulation, whereas $CD163^-$ cells produced little or no IL-10. Unexpectedly, both $CD163^-$ and $CD163^+$ TMs expressed similar levels of most pro-inflammatory genes. These data indicate polarisation of TMs towards the M2 phenotype, characterised by production of IL-10 and responsiveness to IL-4, although the polarised TMs continue to express pro-inflammatory cytokines, albeit at significantly lower levels than other macrophages. The M2 phenotype is consistent with a role in testicular immunosuppression, but may also contribute to fibrosis, which is associated with testicular responses to vasectomy, cryptorchisism and infertility.

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CHARACTERISATION OF THE *IN VITRO* FUNCTION OF ACTIVIN AC

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Activins are members of the TGF-β superfamily that signal via type II and type I receptor subunits and intracellular Smads[1]. Activin A stimulates FSH release from the pituitary and is also a potent growth and differentiation factor in many physiological systems[2]. Over-expression of the activin-β_C subunit in vitro leads to a reduction in activin A and an increase in activin AC[3]. Transgenic mice over-expressing activin-β_C show decreased circulating activin A, implying that activin AC may also be formed in vivo [4]. Recently recombinant activin AC has become available, therefore this study examines the in vitro function and mechanism of action of activin AC. Activin AC stimulates FSH release in LβT2 cells and is a negative growth regulator in LNCaP cells, however the potency of activin AC is 8-10 fold less than activin A. Incubation of LNCaP cells with activin receptor antibodies (ALK4, ActRIIA, ActRIIB) abolishes the growth inhibitory effects of activin AC. Activin AC binds to ActRIIB, however a 20–30 fold decrease in both the potency and affinity of activin AC is evident compared to activin A. In addition, activin AC increases Smad-2 phosphorylation. These results indicate activin AC utilises the same receptors and intracellular signalling molecules as activin A. The activin A antagonists, follistatin and activin C[4], also antagonise the growth inhibitory effects of activin AC and reduce Smad-2 phosphorylation and Smad-4 expression. This study shows for the first time that the in vitro function of activin AC is similar to activin A, albeit at a lower potency and provides the impetus to determine the physiological role of activin AC in vivo.

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CHARACTERIZATION AND FUNCTION OF THE BULL SPERM PROTEIN SPAM1: TWO DISTINCT ISOFORMS WITH DISTINCT ROLES

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We identified an 80 kDa bull sperm protein (p80) that possesses homology with the Sperm adhesion molecule 1 (Spam1), a GPI-anchored glycoprotein conserved amongst mammals that is required for fertilization. Since bovine Spam1 had not been identified, the aim of this project was to determine if p80 is the bovine Spam1 and to test the hypothesis that it plays a role in gamete interaction during bovine fertilization. Amino acid sequence deduced from 3`/5`Race confirmed that homology between p80 and Spam1 in various species ranged from 47 to 61%. It also revealed specific differences including the absence of a GPI-anchor, the presence of a transmembrane domain, and N- and O- glycosylation sites. By generating and characterising antibodies against p80 N- and C-terminal domains, the protein orientation in the sperm membrane was evaluated. We identified two populations of p80 on the sperm head: one internalised in the anterior region and the second localised on the post-acrosomal region with its hyaluronidase domain exposed to the extracellular environment. Proteomic and immunologic analyses revealed that the p80 post-acrosomal population is a shorter isoform originating from the epididymis while the full length p80 located on the anterior region originates from the testis. Finally, the potential function of p80 during the sperm/zp interaction was evaluated by sperm/zona pellucida (zp) binding assay. The C-terminal extremity of p80 was implicated in sperm binding to the zp by antibody and native protein competition. Furthermore, glycosylation was not required during this interaction since deglycosylated p80 in the incubation medium had the same inhibitory effect on zona binding as the native p80. Collectively, the results demonstrated that p80 is the bovine Spam1, and that two isoforms are present on bull sperm. The hyaluronidase activity of the post-acrosomal isoform is required for cumulus penetration, while the other one participates in sperm/zp binding during fertilization.

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HEPATOCYTE-NUCLEAR FACTOR 3-ALPHA (HNF-3A) EXPRESSION IN THE DEVELOPING PROSTATE OF THE TAMMAR WALLABY: A MARKER OF PROSTATE DIFFERENTIATION

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The prostate is the source of about 30% of the seminal fluid. The prostate develops from the urogenital sinus involving epithelial-mesenchymal interactions, and is dependent on androgen secretion for its differentiation. Most studies on prostatic development have focused on the mouse but the tammar may be an alternative model for some aspects of prostatic development due to its structural similarity to the human. The tammar prostate is a cone-shaped secretory gland which consists of a central zone, transition zone and peripheral zone, as in humans. In contrast, the mouse prostate is a multi-lobular organ. Although much is known of the hormonal control of prostatic development in the tammar [1,2,3], little is known about the specific markers of its differentiation. HNF-3α is a transcription factor that is expressed in the urogenital epithelium of the prostate in the mouse and human. This study characterised HNF-3α in the developing prostate of the tammar. The full length sequence of the gene was obtained in silico and confirmed by cloning. Tammar HNF-3α is highly conserved, sharing 82% nucleotide identity with the human and 80% nucleotide identity with mouse. We used RT-PCR to examine the temporal expression of HNF-3α, and found that it is expressed throughout prostate development in the male from immediately after birth to day 70, the latest stage examined. It is also expressed in the developing female urogenital sinus during a similar time period. HNF-3α protein has a spatial expression similar to that of the mouse, and it is localised to the nucleus of the urogenital epithelial cells. These results indicate that prostatic differentiation is conserved between the tammar (marsupial) and mouse (eutherian). Characterisation of HNF-3α in the tammar will also provide a useful tool in assessing prostatic differentiation in our ongoing prostatic organ culture experiments.

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NOVEL SIGNALLING IN MOUSE EMBRYONIC STEM CELLS GENERATES PRIMITIVE ECTODERM-LIKE CELLS

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The phenotypic status of embryonic stem (ES) cells is controlled in part by signalling pathways which translate inputs mediated by extracellular molecules. An important extracellular protagonist in mouse ES cells is LIF (leukaemia inhibitory factor) which interacts with the gp130–LIFR receptor complex to activate a number of downstream signalling pathways, including the STAT3, MEK/ERK and PI3K/Akt. These pathways, together with others, interact in complex and sometimes competing ways to generate the well-known characteristics of mouse ES cells of self-renewal, high rates of proliferation, and pluripotence. The addition of a second molecule, L-proline, to the extracellular environment alters the pluripotent status of mouse ES cells, converting them to a second pluripotent population equivalent to the primitive ectoderm of the pre-gastrulating embryo. This conversion, from ES cells to primitive ectoderm-like cells, primes the latter for directed differentiation to specific cell types (1). Here we show, using inhibitor studies and kinome array analysis, that this small molecule appears to work by (i) changing the balance in activity of signalling pathways already stimulated by LIF and (ii) activating additional signalling pathways. Specifically, L-proline rapidly further activates the LIF-stimulated MEK/ERK pathway, tipping the balance in favour of primitive-ectoderm formation and away from ES-cell self-renewal sustained by LIF-mediated activation of the STAT3 pathway. In addition, L-proline rapidly stimulates other pathways including p38, mTOR and PI3K/Akt each of which contributes, to a greater or lesser extent, to the conversion to primitive ectoderm-like cells. These results indicate that (i) L-proline acts in novel ways to stimulate embryo-like developmental progression in ES cells and (ii) through the addition of small, nontoxic activators and inhibitors of signalling pathways, the differentiation of pluripotent ES cells might be controlled sufficiently well for the homogeneous production of specific cell types suitable for use in animal models of human disease.

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SRB POSTERS

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THE MANIPULATION OF THE EPIGENETIC MARK HISTONE 3 LYSINE 9 TRIMETHYLATION IN DONOR CELLS AND ITS EFFECTS ON THE DEVELOPMENT OF CLONED MOUSE EMBRYOS

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To produce live cloned mammals from adult somatic cells the nuclei of these cells must be first reprogrammed from a very restricted, cell lineage-specific gene expression profile to an embryo-like expression pattern, compatible with embryonic development. Although this has been achieved in a number of species the efficiency of cloning remains very low. Inadequate reprogramming of epigenetic marks in the donor cells correlated with aberrant embryonic gene expression profiles has been identified as a key cause of this inefficiency. Some of the most common epigenetic marks are chemical modifications of histones, the main structural proteins of chromatin. A range of different histone modifications, including acetylation and methylation, exists and can be attributed to either repression or activation of genes. One epigenetic mark which is known to be very stable and difficult to remove during reprogramming is the trimethylation of lysine 9 in histone H3 (H3K9Me3). To test the hypothesis that H3K9Me3 marks are a major stumbling block for successful cloning we are attempting to remove these marks by overexpression of the H3K9Me3 specific histone demethylase, jmjd2b, in donor cells, prior to their use for nuclear transfer. We have engineered mouse embryonic stem (ES) cells for the tet inducible expression of a fusion protein with a functional jmjd2b or non-functional mutant jmjd2b histone demethylase. Approximately 94% and 88% of the cells can be induced for the expression of functional and mutant jmjd2b-EGFP in the respective ES cell lines. Immunofluorescence analyses have shown that induction of functional jmjd2b-EGFP results in an approximately 50% reduction of H3K9Me3 levels compared to non-induced cells and induced mutant jmjd2b-EGFP cells. The comparison of the in-vitro embryo development following nuclear transfer with induced and non-induced donor cells show significantly better overall development to blastocysts and morulae from induced donor cells with reduced H3K9Me3 levels.

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BACTERIOSPERMIA AND ITS CONTROL IN BUBALINE SEMEN

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The present study was designed to investigate the bacterial species incriminated in bubaline semen and to find out the effectiveness of antibiotics (GTLS; gentamycin, tylosin and linco-spectin or SP; streptomycin and penicillin) in cryodiluent on bacterial control and quality of buffalo bull spermatozoa. For this purpose four experiments were conducted. In experiment 1, a total of 11 bacterial species were identified from buffalo ejaculates. The predominant bacteria were Staphylococcus aureus, Escherichia coli and Pseudomonas aeruginosa in the ejaculates. In experiment 2, total aerobic bacterial counts in post-thaw samples were lower ($P<0.05$) in GTLS than in SP or NC (negative control). Fewer bacterial genera were identified in semen samples having GTLS than SP. Majority of the bacterial isolates from ejaculates showed more sensitivity towards GTLS than SP. In experiment 3, motilities (visual and computer-assisted), velocities (straight-line, average path and curvilinear), amplitude of lateral head displacement and plasma membrane integrity in post-thaw semen samples did not differ ($P>0.05$) due to antibiotics. Spermatozoal abnormalities (acrosome, head, mid-piece and tail) were lower ($P<0.05$) in GTLS and SP than in NC. In experiment 4, the fertility rates for SP-based vs. GTLS-containing frozen semen of buffalo bull were 42.8 and 55.2%, respectively. The results for GTLS were significantly higher than SP. The fertility rates also differed significantly in the first and second batch of inseminations performed with SP or GTLS-based cryopreserved semen of buffalo bull. In conclusion, a number of bacterial species are isolated from bubaline semen. Bacterial and seminal quality measured by standard laboratory tests and field fertility trials indicate that GTLS is more suitable in extender for cryopreservation of buffalo bull spermatozoa.

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COLD SHOCK DURING RAPID COOLING OF SPERM FROM A TROPICAL ANURAN, THE CANE TOAD, *BUFO MARINUS*

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Amphibian sperm are not considered to be susceptible to cold shock injury during cooling and cryopreservation. In this study we investigated the susceptibility of the tropical bufonid, the Cane Toad (Bufo marinus) to sperm cold shock. Sperm from testes macerated in 2x Simplified Amphibian Ringer (inactivated state) were diluted 1:6 in various cryodiluents containing 10% sucrose and 10–20% glycerol or DMSO, or were used directly as undiluted controls. Samples were cooled at three cooling rates ($1^oC\ min^{-1}$, $5^oC\ min^{-1}$ or placed directly on ice – rapid cooling) to 0^oC and then either warmed to room temperature and their motility and viability assessed after activation by dilution, or cryopreserved. Cryopreserved samples were stored in liquid nitrogen for two days and thawed at room temperature before assessment of motility and sperm viability. Cooling rapidly to 0^oC by directly placing samples on ice or cooling at $5^oC\ min^{-1}$ before warming to room temperature resulted in a significant decline in motility (all means less than 40% of control motility after 30 min at room temperature) in comparison to samples cooled slowly at $1^oC\ min^{-1}$ (all means greater than 80% motility; $p < 0.05$ to 0.01). Samples cryopreserved after cold shock (rapid cooling to 2^oC by immediate exposure of straws to 2^oC ambient temperature) versus samples cooled slowly ($1^oC\ min^{-1}$) to 2^oC prior to cryopreservation had significantly lower mean post-thaw motilities ($p<0.05$; in the range of 40–60% motile versus 80–95% for non-cold shocked). These data together indicate that the sperm of B. marinus undergo cold shock injury prior to freezing, and that post-thaw recovery after cryopreservation of cold-shocked sperm is substantially reduced.

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SUCCESSFUL IN VITRO FERTILIZATION (IVF) AFTER PRIOR FAILED IVF UTILIZING A PROPRIETARY BLEND OF SUPPLEMENTS

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Background: To date, little is known about the effect of supplements on the outcome of in vitro fertilization (IVF). The data on this matter is limited to measuring the overall pregnancy rate on a population of women who took a specific supplement, and not on IVF patients. Objectives: To demonstrate the positive role of an investigated supplement in the outcome of patients undergoing IVF. Method: 18 women undergoing IVF treatment were placed on a proprietary combination of vitamins and antioxidants designed to encourage blood flow and improve egg quality. The women were selected for this protocol mostly due to prior poor egg quality and/or large amount of embryo fragmentation .The women took supplementation twice daily for 4–12 weeks prior to transfer. The charts of the patients who used the supplements were used to obtain data. Previous failed IVF was defined as negative pregnancy. Successful IVF outcome was determined by positive chemical pregnancy and clinical pregnancy after one attempt. Range and mean was calculated for patient's age and number of failed previous IVF attempts. The previous IVF attempts were performed in different centers without using this supplement in all patients. Results: Eighteen patients used the supplement before and during their IVF cycles. Patient's age ranged from 28 to 44 with mean of 36.4 years. They had on average, 2 prior failed IVF attempts. Seventeen of 18 patients had successful IVF outcome. The failed patient required frozen testicular extraction of sperm (TESE), prior to IVF. Summary: Seventeen of 18 patients who used our supplements had successful IVF. These patients failed an average of 2 previous IVF attempts without using our supplements. Conclusion: Usage of our supplements is associated with improved rates of success in patients undergoing IVF with a history of prior failed IVF attempts. Larger studies need to be conducted.

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THE ROLE OF ADAMTS1 IN BREAST CANCER PROGRESSION AND METASTASIS

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The extracellular protease Adamts1 is a vital enzyme involved in normal development and has been associated with various processes of extracellular matrix (ECM) remodelling including ovulation and pathologies exhibiting disrupted ECM. In the PyMT-MMTV mouse mammary cancer model we have demonstrated reduced tumour growth and pulmonary metastasis incidence in Adamts1$^{-/-}$ mice. Furthermore, benign DCIS lesions of the mammary gland were significantly more common in Adamts1$^{-/-}$ mice. To better understand the mechanisms surrounding Adamts1 mediated progression from DCIS to invasive cancer we next investigated proliferation, apoptosis, blood vessel density and/or extracellular remodelling. Morphometric comparisons were performed using immunohistochemical markers of proliferation (Ki67) and apoptosis (active caspase3) as well as vasculature (CD34), and known Adamts1 proteolytic target, versican in wildtype and Adamts1$^{-/-}$PyMT tumours. Our analysis revealed that no significant difference in proliferation between the two genotypes, but Adamts1$^{-/-}$ had increased apoptosis compared with Adamts1$^{+/+}$ tumours. Contrary to previous reports suggesting Adamts1 is antiangiogenic, no significant difference was found in blood vessel density between Adamts1$^{-/-}$ compared with Adamts1$^{+/+}$ tumours. Interestingly, versican abundance in peritumoural stroma was lower in Adamts1$^{-/-}$ tumours. This latter finding may provide an explanation for the reduced metastasis incidence found after Adamts1gene inactivation, as many previous studies have associated peritumoral versican abundance to metastasic progression. Our findings increase the understanding of mechanisms whereby Adamts1 promotes breast cancer metastasis by showing its role in increasing cell survival, as well as modulating the stromal ECM environment through which it may promote invasion and metastasis. The involvement of Adamts1 in breast cancer progression presents this protease as a potential novel target for reducing metastasis incidence.

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MENSTRUAL CYCLE VARIATIONS IN PLASMA MICRORNA EXPRESSION PROFILES

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microRNAs are short, single-stranded RNAs that regulate gene expression at the post-transcriptional level. Plasma and serum microRNAs correlate closely with microRNA profiles of diseased tissue and have been explored as blood-based biomarkers for human diseases, including steroid-driven malignancies. However, reproductive steroid signalling regulates the expression of specific microRNAs and this could impact the utility of microRNA biomarkers in reproductive aged women. We hypothesised that microRNA expression profiles are altered by steroid hormone fluctuations associated with the menstrual cycle. To test this hypothesis, plasma microRNA expression was measured in healthy women at 3 stages of a 28 day menstrual cycle; ie menstrual (day 3–5), follicular (day 9–13) and implantation window/secretory phase (day 18–22). Total RNA was extracted from plasma, multiplex reverse transcription was performed, and the cDNA pre-amplified prior to expression analysis of 667 microRNAs on Taqman low density PCR arrays (n=6 women). Preliminary data shows that up to 200 microRNAs may be detected with this methodology, and that at least 14 of these are differentially expressed (fold change $\geq \pm 1.5$) at follicular and secretory phase, as compared to menstrual phase. We plan to confirm these findings with standard Taqman microRNA assays (n=10 women). Our findings suggest that plasma miRNA expression profiles change over the menstrual cycle, and that this could confound microRNA-based diagnostic tests. We hope to demonstrate the most appropriate cycle phase for blood-based miRNA profiling, facilitating the development of plasma microRNA-based diagnostic tests and providing valuable information to researchers studying circulating microRNA profiles in reproductive aged women.

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TGFB REGULATES ENDOMETRIOSIS- LIKE LESION DEVELOPMENT IN MICE INDEPENDENTLY OF THE RUNX-2/OPN PATHWAY

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TGFβ is likely to significantly influence endometriotic lesion development, as TGFβ KO/SCID mice with no host-derived TGFβ activity have smaller human ectopic endometrial lesions than control mice. TGFβ potentially acts via RUNX2, a transcription factor that directly upregulates OPN transcription in osteoblasts, as we have identified RUNX-2 and OPN gene expression at high levels in nude mouse endometriosis-like lesions, in human endometrial stomal cell cultures and in the stroma of endometriotic tissues. We hypothesised that inhibition of RUNX-2 would suppress OPN production and result in reduction of endometriotic lesion formation and size. As P38/MAPK inhibitors suppress TGF-β mediated RUNX-2 transcription, we utilised the nude mouse model to test whether the P38/MAPK inhibitor FR167653 would suppress OPN production in endometriotic tissues resulting in smaller lesions. FR167653 (30 mg/kg twice a day) or placebo was administered for either 10 or 14 days to nude mice (16 in each group) implanted with human endometrial tissue xenografts from 4 different women. The size and weight of the lesions were measured and immunohistochemical analysis of OPN, αSMA (myofibroblasts) and F4/80 (macrophages) was carried out. There was no difference in size or weight of the lesions, and there was no overt difference in any of the staining parameters explored. The inhibition of p38/MAPK did not alter the size of the nude mouse lesions nor OPN staining within these lesions despite being administered at a maximal therapeutic dose. This suggests that TGFβ regulation of endometriotic lesion development is mediated by an alternate molecular pathway.

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REGULATING MURINE FOLLICLE DEVELOPMENT BY STIMULATION OF THE JAK2/STAT3 SIGNAL TRANSDUCTION PATHWAY

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Follicular development requires the recruitment of primordial follicles into the growing follicle pool following initiation of multiple cytokine signalling pathways. Suppression of follicular development is thought to be key to maintaining the population of primordial follicles and allowing for controlled release of these follicles throughout the reproductive lifespan of the female. However, little is known of the processes and signalling molecules that suppress primordial follicle activation and early follicle growth. Our group has identified significant upregulation of the Janus Kinase 2 (JAK2)/ Signal Transducer and Activator of Transcription 3 (STAT3) signalling pathway inhibitor the Suppressor of Cytokine Signalling 4 (SOCS4) that coincides with the initial wave of follicular activation in the neonatal mouse ovary. Further studies by our group have localised the SOCS4 protein to the granulosa cells of activating and growing follicles, suggesting SOCS4 expression may be linked to follicular activation. We have focused on examining protein localisation and gene expression patterns of the eight SOCS family members CIS and SOCS1-7. We have recently demonstrated that co-culture of neonatal ovaries with Kit Ligand (KL) for 2 days increases the mRNA levels of all SOCS genes. We also demonstrated the co-localisation of SOCS2 proteins with the KL receptor c-kit in the mural granulosa cells of antral, and large pre-antral follicles suggesting a significant role for SOCS2 in the later stages of follicular development. We have also shown that culturing ovaries with the potent JAK2 inhibitor AG490 substantially reduces mRNA levels of all SOCS and STAT genes that we have so far measured. We hypothesise a significant role for JAK2/STAT3 signalling in promoting the activation and early growth of ovarian follicles. Our investigations have identified significant roles for JAK2/STAT3 and the SOCS family in the regulation of ovarian follicle development.

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TRANSFORMING GROWTH FACTOR INDUCED PROTEIN TGFBI PROMOTES OVARIAN CANCER CELL MOTILITY AND ADHESION TO PERITONEAL CELLS

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Ovarian cancer is characterized by metastases to the peritoneal surface lining the abdominal cavity. It remains unclear which factors promote the implantation of ovarian cancer cells onto the peritoneal lining. We have recently investigated interactions between ovarian cancer cells (OVCAR-5, OVCAR-3, and SKOV-3) and mesothelial cells isolated from omental tissues (LP-9). We conducted a proteomic screen of the conditioned medium of co-cultures of ovarian cancer and mesothelial cells. One of the molecules identified to be modulated is the extracellular matrix adhesion protein, transforming growth factor-beta-induced protein (TGFβI, also known as b ig-H3 or keratoepithelin) which is induced by transforming growth factor-beta in many cell types which has been shown to promote adhesion and migration of hepatoma and astrocytoma cells and enhance colon cancer cell extravasation. In this study we investigated the expression of TGFβI in ovarian cancer tissues and the effects of recombinant TGFβI on ovarian cancer motility and adhesion to peritoneal mesothelial cells. In functional assays, treatment with recombinant TGFβI significantly increased adhesion of all three ovarian cancer cell lines to LP-9 mesothelial cells by up to 25% ($P<0.01$) and increased motility in OVCAR-5 cells by 62% ($P<0.001$). Furthermore, addition of a neutralising TGFβI antibody reduced OVCAR-5 adhesion to LP-9 to 79% of control level ($P<0.001$). TGFβI produced by LP-9 cells was processed to smaller forms when co-cultured with ovarian cancer cell lines by western blotting. MALDI-TOF/TOF mass spectrometry identified TGFβI processing at both the N and C terminal domains. The addition of broad spectrum protease inhibitors blocked the TGFβI processing and reduced OVCAR-5 adhesion to LP-9 cells to 60% of control level ($P<0.001$). We conclude that although some ovarian cancer cells produce low levels of TGFβI, TGFβI abundantly expressed by peritoneal mesothelial cells can promote ovarian cancer cell adhesion and motility.

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THE EFFECTS OF HIGH FAT DIET ON LIPID LOCALISATION IN THE PERI-OVULATORY CUMULUS OOCYTE COMPLEX

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Intracellular neutral lipids are stored in discrete droplets that are surrounded by lipid associated proteins, such as adipophilin and perilipin, which control cellular lipid metabolism by regulating the access of lipases. The role of lipids in oocyte maturation is unclear, although they have a potential role as an energy source for the oocyte and early embryo. To elucidate potential mechanisms controlling lipid utilisation in the peri-ovulatory cumulus-oocyte-complex (COC) we 1) localised lipid droplets by immunohistochemistry for adipophilin and perilipin and direct staining of neutral lipids with BODIPY and 2) investigated whether a high fat diet can alter oocyte lipid quantity or localisation. Ovaries were isolated from 21day old mice before and 10h after the ovulation stimulus hCG. Adipophilin and perilipin were both detected by immunohistochemistry in peri-ovulatory follicles with similar localisation before and after hCG. In separate experiments, adult mice were fed a high fat or control diet for 4 weeks and COCs were isolated from preovulatory follicles prior to hCG or from the oviduct 13h after hCG stimulation followed by BODIPY staining and quantification with confocal microscopy. BODIPY staining showed that COCs possess low levels of lipids evenly distributed in the oocyte before hCG but increased lipid assembled as droplets in the oocyte after ovulation. In mice fed a high fat diet, intracellular lipids were markedly increased in both the cumulus cells and oocytes from preovulatory and ovulated COCs. The ubiquitous expression of lipid droplet proteins in the peri-ovulatory follicle together with the changes in neutral lipid storage concurrent with ovulation suggests that lipid metabolism play an important role in oocyte release, transport and/or developmental competence. Furthermore, the dramatic effect of dietary fat on COC lipid content may contribute to the impaired oocyte quality we have observed in obese mice as well as reduced fertility in obese women

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COMMERCIAL EQUINE CHORIONIC GONADOTROPHIN ISOFORM COMPOSITION, IMMUNOACTIVITY, AND BIOACTIVITY IN A MURINE MODEL USING OVARIAN AUGMENTATION AND TESTICULAR INTERSTITIAL CELL BIOASSAYS

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Equine chorionic gonadotrophin (eCG) is a placental glycoprotein hormone that is harvested from the plasma of pregnant mares for the formulation of commercial products which are used in a variety of assisted reproductive procedures in livestock. It is well documented that the bioactivity of gonadotrophin products is highly variable. The aim of this study was therefore to determine how different eCG products affected target tissues and cells. Isoforms of eCG were separated with iso-electric focusing and immunoactivity measured with RIA. For in vivo bioassay, dose-response eCG treatments were administered subcutaneously to immature female mice with follow-up treatment on day 2. On day 3 the mice were asphyxiated and ovaries dissected and weighed relative to standard. For in vitro bioassay, adult male mice were asphyxiated, testes removed, decapsulated, dispersed, strained and washed with DMEM:F12 0.1% BSA. The cell stock was counted and diluted for culture at 20,000 cells/well. Cell viability was determined using trypan blue. Five doses were tested for each eCG product. The cells were incubated at 32ºC for 3 hours in 5.0% CO_2 in humidified conditions. Media was collected after 3 hours and immediately assayed for testosterone with RIA. Product eCG with ~90% isoforms with pH 3.0–3.7 and ~10% with pH 3.8–4.4, showed 29% greater biologically activity in the ovarian augmentation assay and 44.8% more immunoactivity then stated product bioactivity. Product eCG with ~70% isoforms with pH 3.0–3.7 and ~30% with pH 3.8–7.4 was 3.0% less effective in vivo and had 7.9 % less immunoactivity then stated bioactivity, however, in vitro testosterone production was more effectively stimulated then with the previous more acidic eCG product. Our study shows a selective difference in commercial eCG biological activity that appears to depend on subtle isoform heterogeneity. Future studies aim to determine the specific actions of eCG isoforms in target tissues.

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LOSS OF BETAGLYCAN DISRUPTS SOMATIC CELL DIFFERENTIATION IN THE FETAL MOUSE OVARY

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Betaglycan (TGFBR3) is an accessory receptor that modulates the activities of members of the TGF-beta superfamily of growth factors which regulate many aspects of reproductive biology. We reported the presence of betaglycan on somatic and germ cells in fetal murine ovary from 12.5 dpc (1). However, the role of this receptor in early ovarian development is poorly understood. We hypothesised that, given its expression pattern, betaglycan could serve as a TGFb accessory receptor on both oogonia and somatic cells during early ovarian development. In the current study, we examined the Tgfbr3 (betaglycan) null and wildtype ovary from 11.5-14.5 dpc. During this window of development, no overt morphological phenotype was detected, and cell proliferation (PCNA immunostaining) and apoptosis (active caspase 3 immunostaining) were unchanged in the null ovary. To determine whether somatic and/or germ cell markers were altered in the absence of betaglycan, quantitative real time PCR analysis was conducted using total RNA derived from 11.5-14.5 dpc betaglycan wildtype, heterozygous, and null ovaries (n=3 at each age/genotype). From 12.5 dpc, genes associated with the differentiation of the female somatic lineages Wnt4, Fst, Bmp2 were significantly decreased in the null ovary by 30%-50% ($P<0.05$) while germ cell markers (Oct4, Mvh) showed no significant changes compared to wildtype ovary. Genes associated with the testis-differentiation pathway (e.g. Sox9, Cyp11a1, Cyp17a1, Hsd3b, Insl3, Dhh) were not aberrantly expressed in betaglycan null or heterozygous ovary, with expression levels very low to undetectable. Collectively, these data suggest that while betaglycan does not appear to play a key role in establishing the structure of the ovary, loss of this receptor results in a disruption to the differentiation of ovarian somatic cells.

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THE REPRODUCTIVE PHENOTYPE OF THE IKKB CONDITIONAL KNOCKOUT MOUSE

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Nuclear factor-κB (NF-κB) designates a family of transcription factors that have been shown to modulate antiviral, inflammatory and immune responses. Activation of NF-κB is dependent on IKKβ a component of the IκB kinase (IKK) complex which promotes degradation of IκB inhibitory proteins and allows nuclear translocation of NF-κB. Our studies in ovarian granulosa cell tumour cell lines (COV434 and KGN) indicate that NF-κB signalling is constitutively activated. FSH has been reported to increase XIAP expression through NFκB activity in granulosa cells, but beyond that the role of NFκB signalling in folliculogenesis has not been elucidated. To establish the significance of NF-κB signalling in the ovary (and testis), we have generated a gonadal specific IKKbeta conditional knockout mouse. A transgenic mouse line containing floxed IKKβ alleles (gift of M Karin, UCSD) was crossed with a cre mouse line (gift of M Matzuk, BCM) expressing the recombinase in anti-Müllerian hormone receptor expressing cells (granulosa cells or Sertoli cells). The resulting mice will not express IKKβ in granulosa cells or Sertoli cells and thus cannot activate the classical NFκB signalling pathway. On histological assessment, the ovaries and testes from flox x cre (heterogenous) mice appear normal with follicles of all developmental stages and corpora lutea. Preliminary data suggests that breeding with the heterogenous females resulted in increased litter sizes. The histology of the testes is also unremarkable. The mice homozygous for the deletion of IKKβ in the granulosa cells appear healthy and a preliminary assessment does not reveal gross morphological abnormalities of the ovaries. The results of detailed histological and overall assessment of these mice will be presented. These IKK conditional knockout mice should provide insights into the role of NFκB signalling in gonadal function.

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EFFECTS OF INHIBITION OF HEPERANASE BY SULPHATED MALTOHEXAOSE ON OVULATION, CORPORA LUTEA FORMATION AND PREGNANCY

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Heparan sulphate proteoglycans such as perlecan and collagen XVIII, are present in the follicular basal lamina, focimatrix and sub-endothelial basal laminas in follicles, but following the LH surge the follicular basal lamina and focimatrix are degraded. Heparanase, which degrades the glycosaminoglycan heparan sulphate, is upregulated in granulosa cells during ovulation. We examined the effects of heparanase inhibition by maltohexaose sulphate treatment on ovulation, formation and vascularisation of corpora lutea, and the establishment of pregnancy. Female twenty-five days old mice were treated with 0.3 mg (>25 mg/kg) maltohexaose sulphate or saline daily for four days from time 0 h and subjected to induction of ovulation by treatment with PMSG (5 IU at 0 h) and hCG (5 IU at 48 h) and mated with a fertile male. Mice were sacrificed at time 0 h, 48 h, 120 h and day 11 (relative to the first injection of PMSG) (n = 20 per group), and ovaries were collected and pregnancy status examined. One ovary was stained with haematoxylin and eosin to measure numerical densities of antral follicles and corpora lutea in the ovary, as well as volume density of luteal tissue. Immunohistochemistry for CD34 (endothelial cell marker) and perlecan (localized to sub-endothelial basal lamina) was conducted to quantify the degree of vascularisation of the corpora lutea. In control mice there was a reduction in the number of antral follicles with increasing age, but no effect of treatment with sulphated maltohexaose was observed on antral follicle development. Nor was there any effect of sulphated maltohexaose on corpora lutea formation, or embryo implantation. In addition, this treatment did not have any effect on the vascularisation of the corpora lutea. These findings suggest that heparanase activity may not be critical during the later stages of follicle growth, ovulation and formation of corpora lutea.

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THE EFFECTS OF ECG INJECTION ON SUPEROVULATORY RESPONSES BEFORE VERSUS AFTER CIDR REMOVAL IN IRANIAN SHALL EWES

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The aim of this study was to investigate the effects of eCG injection on ovarian responses 2 days before or immediately after CIDR removal in a superovulation program during non-breeding season in Iranian Shall ewes. 12 ewes were kept inside for the duration of the experiment and were fed a live weight maintenance ration. The ewes were synchronized by CIDR. The day of CIDR insertion was considered as the starting point of the experiment (day 0) . The ewes received 1500 IU eCG on day 12. Ewes were randomly assigned to 1 of 2 groups, and CIDR removed on days 12 and 14 in groups 1 and 2, respectively. Ovarian follicular activity was monitored by transrectal ultrasonography daily from the initiation day of superovulatory stimulation (day 12) until the day of estrus (day 14), and once 7 days after estrus (day 21) to monitor the number of corpus lutium. During examination the animals were held in a standing position. Ultrasonographic scanning of both ovaries was recorded using a MP4 player . The number of medium and large follicles (≥3 mm) were recognized, measured and mapped to their location using printed images of both ovaries. Data were analyzed using GLM procedure of the SAS. The results presented in table 1 showed that the ovarian responses in terms of the number of ≥3 mm follicles at estrus and CL 7 days later were greater ($P<0.05$) in group 1 ewes. The results indicated that the superior time for eCG injection is on the day of CIDR removal.

Table 1. The mean number of medium and large follicles and the number of CL

	Number of ≥3 mm follicles			Number of CL 7 days after estrus
Experimental days	12	13	14	21
Group 1 (n=6)	0.66±0.2	4.50±0.2[a]	3.60±0.8[a]	3.50±0.2[a]
Group 2 (n=6)	0.83±0.2	2.50±0.6[b]	2.33±0.6[b]	2.16±0.6[b]

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RELATIONSHIPS OF LUTEAL PHASE VARIABLES (PRIOR TO AI) WITH FOLLICULAR WAVES IN DAIRY COWS

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This study was done to consider relationships of luteal phase variables (prior to AI) with follicular waves. The estrous cycles of 10 cows were synchronized with 2 im injections of prostaglandin F2α given 11 d apart. The cows were randomly assigned to 1 of 2 treatments. Cows in the control treatment received no treatment, whereas GnRH6 cows received a GnRH injection on d 6 of the estrous cycle (estrus = d 0). Daily, from estrus d 0 to the next estrus d 23, cows had their ovaries scanned by ultrasound. Blood samples were collected by tail following each ultrasound examination from estrus until next estrus (estrus = d 0). Concentrations of plasma progesterone were determined by radioimmunoassay kit. The limit of detection of the assay was 0.125 ng/mL and the intra- and interassay coefficients of variation were 8.4% and 10.2%, respectively. Data that were analyzed by using PROC GLM of SAS. For comparisons between groups, the 2-sample t-test was used for continuous traits, such as size of cl or hormone concentrations. Prospective comparisons of indices of progesterone indicated that the length of luteal lifespan was longer in three-wave than in two-wave cows ($P<0.01$). Plasma progesterone concentrations were similar at peak and measured as area under the curve on day 5 through 17 preceding insemination in two-wave (6.70±0.30 ng/ml) and three-wave cows (7.30±0.50 ng/ml). Length of the luteal phase (defined as from the day of estrus until the last day on which plasma progesterone remained greater than 2 ng/ml) was <2 days shorter in two-wave cows than in three-wave cows (16.7±0.30 vs. 18.40±0.50 d; $P<0.05$). In addition, the day of peak progesterone occurred earlier in two-wave cows (13.50± 0.40 vs. 16.30±0.70 d; $P<0.05$).

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RELATIONSHIP BETWEEN THE OVARIAN STATUS ON THE INITIATION DAY OF SUPEROVULATION AND SUPEROVULATORY RESPONSES IN CATTLE

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The purpose of this experiment was to determine the influence of the ovarian status on the initiation day of superovulation (d 0) on superovulatory responses in terms of the number of large follicles (≥7mm) at estrus (d 4) and CL 7 days after estrus (d 11). Ultrasonography was performed on d 0, 4 and 11of the experiment. Animals conventionally superovulated (400 mg Folltropin-V) between d 8 to 12 of the estrous cycles and 102 superovulation cycles were considered in the present experiment. Data were analyzed using GLM procedure of the SAS. In these cases, the number of follicles was determined in each of the 2 studied classes (SF, 4 to 6 mm; and LF, ≥7mm) on d 0. Subsequently, each of these classes was divided into two subgroups (Low, High) based on the number of follicles observed on d 0 (Table 1). The number of LF at estrus in each of the 2 studied classes (Low vs. High) were (13.02±1.02 vs. 19.7±1.69, $p<0.01$; and 16.23±1.59 vs. 14.75±1.48, $p>0.1$) and the number of CL on d 11 were (9.75±0.57 vs. 12.75±0.98, $p<0.01$; 10.91±0.8 vs. 10.85±0.65, $p>0.1$). Therefore, the ovarian status on d 0 in terms of the number of SF affected the number of LF (d 4) and CL (d 11). In addition, the number of superovulatory cycles with low number of SF was 2 times more than the ones with a high number of SF on d 0; also 49 out of 102 superovulatory cycles had at least 2 LF on the initiation day of superovulation (Table 1). These results suggested that follicular puncture 1–2 days before a conventional superovulation treatment may increase the superovulatory responses.

Table1. Number of follicles and superovulation cycles in each subgroup

Follicular classes at estrus	Number of follicles		Superovulation cycles	
	Low	High	Low	High
4 to 6 mm	≤5	≥6	68	34
≥ 7 mm	≤1	≥2	53	49

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INFLUENCE OF THE DURATION OF CIDR INSERTION ON OVARIAN RESPONSES IN AN ECG PROGRAM DURING NON-BREEDING SEASON IN EWES

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The aim of this study was to investigate the effect of 14 versus 7 days CIDR insertion before eCG treatment on superovulatory responses during non-breeding season in ewes. 10 Iranian Shall ewes, between 2 and 3 years old were kept inside during the experiment period and were fed a live weight maintenance ration. Ewes were randomly assigned to 1 of 2 groups . CIDR was inserted to the ewes of group 1 for 14 days and for 7 days to group 2. Each ewe received 1500 IU eCG on the day of CIDR removal . Ovarian follicular activity was monitored by transrectal ultrasonography on the days of eCG treatment, estrus, and 7 days after estrus to monitor the number of corpus lutium. During examination the animals were held in a standing position. Scanning of both ovaries was recorded using a MP4 player . The number of medium and large follicles (≥3 mm in diameter) were recognized, measured and mapped to their location using printed images of both ovaries. Data were analyzed using GLM procedure of the SAS. The results presented in table 1 shows that the ovarian responses in terms of the number of ≥3 mm follicles at estrus is greater ($P<0.05$) in group 1 than group 2 ewes. The mean number of CL after eCG injection in group 1 and 2 were also significantly different (3.6±0.24 vs 2.4±0.51; $P<0.05$) . The results showed that the 14 days synchronization before eCG treatment during non-breading seasons in ewes had the beneficial effects on ovarian responses.

Table 1. The mean number of medium and large follicles and the number of CL

	Number of ≥3 mm follicles		Number of CL
Days	eCG treatment	Estrus	7 days after estrus
Group 1 (n=5)	1.60±0.24	5.00±0.31[a]	3.60±0.24[a]
Group 2 (n=5)	1.40±0.24	3.60±0.24[b]	2.40±0.51[b]

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RELATIONSHIP BETWEEN THE OVARIAN STATUS ON THE INITIATION DAY OF SUPEROVULATION AND THE OVULATORY FOLLICLES AT ESTRUS IN SHALL IRANIAN EWES

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The objective of this experiment was to determine the influence of the ovarian status on the initiation day of superovulation (day 14) on superovulatory response in terms of the number of ovulatory follicles (F, ≥4mm) at estrus (day 16). Estrous cycles of 50 Shall Iranian ewes were synchronized by CIDR. The day of CIDR insertion was considered as the starting point of the experiment (day 0). At the time of CIDR removal (day 14), 650 IU of eCG was administered to ewes. Ultrasonography was performed on days 14 and 16. Data were analyzed by means of the GLM procedure of the SAS. In these cases, the number of follicles were determined in each of the three studied classes (<2, 2 to 4, and ≥4 mm) on day 14. Subsequently, each of these classes was divided into two subgroups (Low, High) based on the number of follicles observed on day 14 (Table 1). The number of large follicles at estrus in each of the three studied classes were (3.96±0.28 vs. 4.78±0.26, $p<0.05$; 4.31±0.35 vs. 4.41±0.24, $p>0.05$; and 4.50±0.23 vs. 4.07±0.37, $p>0.05$) respectively. The number of medium and large follicles on day 14 did not affect the superovulatory response. However, the high number of small follicles on the initiation day of superovulation increased the number of ovulatory follicles at estrus. Since, 23 out of 50 ewes had a low number of small follicles on day 14 (Table 1), which may suggest that an injection of FSH 1–2 days before initiation of eCG treatment enhance the superovulatory response.

Table1. Number of follicles and superovulation cycles in each subgroup

Follicular classes	Number of follicles		Number of ewes	
	Low	High	Low	High
<2 mm	≤6	≥7	23	27
2 to 4 mm	≤2	≥3	16	34
≥4 mm	≤1	≥2	14	36

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EFFECT OF KISSPEPTIN ON REPRODUCTIVE FUNCTION IN SEASONALLY ACYCLIC RED DEER HINDS

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A preliminary study was conducted to examine whether the marked inhibition of ovulation in seasonally acyclic red deer could be overcome by use of kisspeptin to stimulate gonadotrophin release in these animals. After 14 days progesterone priming with intravaginal CIDR-G devices, non-lactating and lactating hinds (n = 8 in both cases) received 0.50 μmol murine kisspeptin-10 in saline solution delivered intravenously in 30 hours, either continuously or as 4-hourly boluses, and lactating hinds (n = 8) received saline solution intravenously as controls. Kisspeptin treatment caused an elevation ($P < 0.05$) of the mean plasma LH concentration at 2 h (0.36 + 0.237 versus 0.11 + 0.006 ng/ml, treated versus controls, respectively). Two weeks later there was a tendency (Chi-square = 9.286, 4 d.f., P = 0.054) for an increase in occurrence of medium and larger sized ovarian follicles in treated hinds. However, no ovulations resulted from the infusions and there was no effect on timing of the onset of ovulatory activity during the subsequent breeding season, as determined from circulating progesterone concentrations. It is concluded that kisspeptin stimulates the reproductive axis in seasonally acyclic red deer hinds, however the dose or mode of administration required to produce ovulations may be higher than what is effective for sheep in an equivalent reproductive state.

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THE ASSOCIATION OF FOLATE PATHWAY ENZYME POLYMORPHISMS AND PREGNANCY OUTCOME

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The folate, vitamin B12 and vitamin B6 (one-carbon) metabolic pathway is essential for the synthesis of precursors used in DNA synthesis, repair, and methylation. We hypothesise that single nucleotide polymorphisms in genes encoding enzymes in this pathway can disrupt these processes leading to adverse pregnancy outcomes. We investigated associations of six candidate polymorphisms in five genes related to one-carbon metabolism with risk for adverse pregnancy outcome in 586 nulliparous Caucasian couples with normal fertility. Chi-square analysis was used to compare genotype frequencies with pregnancy outcomes. Pregnancies were classified as healthy (n=261), preeclampsia (PE, n=38), gestational hypertension (GHT, n=32), small-for-gestational-age (SGA, n=60) and PE+SGA (n=22). Associations between maternal, paternal and neonatal genotypes with customised birthweight centiles and placental weight were determined using ANOVA with SIDAK post-hoc analyses. The maternal MTR 2756 G allele was associated with decreased placental weight (–87g, P=0.040). Both paternal and neonatal MTR 2756 G alleles were associated with lower birthweight (–12%, P=0.028 and –10%, P=0.039) while the latter was also associated with PE+SGA (P <0.000). Neonatal MTRR GG genotype was associated with GHT and PE with SGA (P=0.033, P=0.011). Neonatal MTHFD1 GG genotype was twice as frequent in PE and GHT (P=0.037; P=0.019) while neonatal TCN2 GG genotype doubled in SGA (P=0.042) compared with healthy pregnancies. Our findings indicate that genetic variation in multiple one-carbon metabolism genes may influence risk of adverse pregnancy outcome. MTR with cofactor vitamin-B12, catalyses the methylation of homocysteine to methionine. Formation of methionine through this pathway is important for synthesis of phospholipids, proteins, myelin, DNA, RNA and S-adenosyl methionine. TCN2 encodes the vitamin-B12 transport protein and MTHFD1 catalyses the conversion of one-carbon derivatives of tetrahydrofolate, which are substrates for methionine, thymine and purine synthesis and are important for healthy placental and fetal development. Larger studies are needed to further examine gene/gene and gene/diet interactions in this pathway.

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PLACENTAL ANTIOXIDANT ENZYMES IN RAT PREGNANCY SHOW ZONE- AND STAGE-DEPENDENT VARIATION

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Placental oxidative stress plays a key role in the pathophysiology of placenta-related disorders including preeclampsia. Protection from oxidative stress is provided by antioxidant enzymes which inactivate reactive oxygen species (ROS). The rat placenta consists of two major zones, the junctional (JZ) and labyrinth (LZ), and because only the LZ grows in late gestation we hypothesized it generates more ROS and thus requires greater antioxidant protection. Our previous studies on expression of the antioxidants superoxide dismutase (SOD)-1, SOD-2 and catalase support this hypothesis. Here, we extend these observations to include mRNA expression of SOD-3 and thioredoxin reductases (Txnrd-1, -2, -3) and activities of SOD, hydrogen peroxide (H_2O_2) scavenging and xanthine oxidase (XO). Placental oxidative damage was assessed by measurement of F_2-isoprostanes and TBARS concentrations. We also measured the effects of maternal dexamethasone treatment, since glucocorticoid excess is known to induce oxidative damage in other tissues. Placentas were collected from untreated mothers on days 16 and 22 (term=day 23) and on day 22 after dexamethasone treatment from day 13 (1 μg/ml drinking water). SOD-3, Txnrd-1, -2, and -3 mRNAs were measured in JZ and LZ by qRT-PCR. F_2-isoprostanes were measured by GC-MS and kit assays were used to measure TBARS and the activities of SOD, H_2O_2 scavenging and XO. In both placental zones, expression of SOD-3 and Txnrd-1 mRNAs and H_2O_2 scavenging activity decreased from day 16 to 22, whereas XO activity increased. Dexamethasone treatment increased H_2O_2 scavenging in both zones, but had no effect on SOD or XO activities or antioxidant mRNA expression. Despite predicted increases in placental ROS generation in late pregnancy and after dexamethasone, neither F_2-isoprostanes nor TBARS were increased. These and our previous data suggest that endogenous protection against oxidative stress is abundant in the rat placenta and provides protection against potential oxidative insults including glucocorticoid excess.

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SPECIES DIFFERENCES IN THE REQUIREMENT OF SMAD2/3 AND MAPK SIGNALLING FOR CUMULUS CELL EXPANSION

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Cumulus cell expansion is necessary for female fertility. In the mouse, cumulus expansion is dependent on activation of two cumulus signalling pathways: 1) mitogen-activated protein kinase (MAPK3/1) by FSH/EGF and 2) Smad2/3 signalling by oocyte-secreted factors. This study was conducted to determine the involvement of these two signalling pathways in pig cumulus expansion.

Abattoir-collected pig COC and eCG-primed mouse COC were cultured for either 24 or 20 hours respectively in the presence of FSH and treated with increasing doses of either a MAPK 3/1 inhibitor (U0126), it's inactive analogue (U0124), or kinase inhibitors SB431542 and dorsomorphin that prevent phosphorylation of Smad2/3 and Smad1/5/8, respectively, or a vehicle control. Morphological cumulus expansion was measured using an established subjective scoring system. At a dose of 10μM, U0126 completely abolished mouse expansion (0±0, $p<0.05$) and caused severe impairment of cumulus expansion in porcine COC (1.23±0.09, $p<0.05$). U0124 and dorsomorphin were unable to inhibit cumulus expansion in either species. SB431542 was also ineffective at blocking pig cumulus expansion (2.6±0.16 $p<0.05$) despite using a dose 4 times higher (8μM) than that required to ablate mouse COC expansion. To determine if pig oocytes activate cumulus cell Smad2/3 or 1/5/8, a Smad-luciferase reporter assay was undertaken. In contrast to mouse oocytes which only activated the Smad2/3 pathway, pig oocytes activated both pathways. This suggests that the failure of SB431542 to prevent pig cumulus expansion is not due to inadequate oocyte activation of Smad2/3. These results demonstrate that while activation of both MAPK3/1 and Smad2/3 pathways are required for mouse cumulus cell expansion, pig COC expansion only requires the MAPK3/1 pathway. Neither species require Smad1/5/8 pathway activation. This study provides new insight into the differing signalling mechanisms required for mammalian cumulus cell expansion.

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ROLES FOR HUMAN CHORIONIC GONADOTROPHIN IN EMBRYO-ENDOMETRIAL CROSS-TALK DURING BLASTOCYST IMPLANTATION

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Emerging evidence suggests an important role for the early embryo product human chorionic gonadotrophin (hCG) in embryo-endometrial interactions critical for successful embryo implantation[1]. The human endometrium is also a source of hCG, with maximal expression of hCG and its receptor, hCG /LHR, in endometrial epithelial cells during the window of implantation in vivo[2,3], and in primary endometrial epithelial cells (EECs)[3]. Implantation is tightly regulated by growth and regulatory factors produced within the embryo-endometrial microenvironment. We hypothesise that embryo/endometrial-derived hCG mediates the molecular cross talk vital for successful implantation. The main objective of this study was to investigate the effect of hCG on the production of a selected cohort of 42 cytokines and growth factors by EECs. These included those with both known and previously unidentified roles during implantation. The secretory profile of cytokines/growth factors produced by EECs was also analysed. EECs (n=8 cultures) were isolated from biopsies collected from fertile cycling women. Cells were treated without or with recombinant hCG for 48 hr and conditioned media collected for quantitative analysis using Luminex™ multiplex technology. For the first time, a secretory profile of 42 cytokines and growth factors produced by EECs was established, as was the identification of fibroblast growth factor-2 (FGF-2) secretion by human endometrial epithelium. hCG (2 IU/ml) significantly increased the production of a number factors including those with known roles during trophoblast migration and adhesion (CX3CL1; 71±31%, CXCL10; 67±24%, CCL4; 87±12%), in trophoblast differentiation (IL-1α; 68±31%) and with unidentified roles during implantation (CCL22; 78±40%, GM-CSF; 45±16%, FGF-2; 50±25%; all $p<0.05$). Upregulation of the known hCG regulated proteins, VEGF and LIF, validated this study. These findings clearly support roles for the embryo /endometrium via hCG in actively contributing to the molecular cross-talk during the early stages of implantation.

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(2) Zimmerman et al. (2009) Biol Reprod 80, 1053–1065.
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GENETIC POLYMORPHISMS IN INTERLEUKIN-1 FAMILY MEMBERS AND PREGNANCY OUTCOME

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Poor placental development is associated with a variety of common, potentially life-threatening pregnancy complications, including preeclampsia (PE) and small-for-gestational-age (SGA) babies. The placenta and fetus express a combination of maternal and paternal genes. Interleukin-1 alpha (IL-1α) and -1 beta (IL-1β) promote trophoblast invasion by upregulating expression of matrix metalloproteinases, while interleukin-1 receptor antagonist (IL-1RN) acts as a competitive inhibitor of IL-1 a and IL-1β by binding to the IL-1 receptor. We hypothesis that polymorphisms which decrease IL-1 activity, within IL-1α rs1800587 (C-allele) and rs17561 (T-allele), IL-1β rs16944 (C-allele) and IL-1RN rs454078 (A-allele) affect placental development and thus pregnancy outcome. To determine the effect of the IL-1 SNPs on pregnancy outcome, blood was collected prospectively from pregnant women at 15 weeks gestation and from their partners and babies at birth. DNA was extracted and genotyped by Sequenom MassArray. Pregnancies were fully characterised and classified into control or PE, PE+SGA, SGA after delivery by an experienced obstetrician. The IL-1α rs1800587 fetal genotype was associated with PE+SGA and smaller head circumference at birth. IL-1α rs17561 fetal genotype was associated with PE+SGA, and a smaller head circumference and body length at birth. The frequency of IL-1β C-allele was associated with PE in neonates, and in fathers the CC homozygote genotype was associated with decreased placental weight at birth. The IL-1RN rs454078 maternal genotype was associated with PE and maternal waist circumference while fetal AA genotype was associated with SGA and decreased placental weight at birth. Genotypes which decrease the production of IL-1α and IL-1β while increasing IL-1RN will reduce proinflammatory cytokines and thereby affect the invasive potential of placental trophoblasts. We have shown that these polymorphisms in both parents may be associated with poor pregnancy outcome. Suggesting that both partners contribute to placental differentiation and function.

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EXTENSIVE LYMPHATIC REMODELLING OCCURS IN HUMAN PLACENTAL BED DURING THE FIRST 20 WEEKS OF PREGNANCY

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The aim of this study was to describe the distribution of lymphatics in human placental bed from 6–20 weeks of pregnancy, with emphasis on the lymphatics surrounding the spiral arterioles as they undergo remodelling associated with trophoblast invasion. Placental bed biopsies were obtained following elective termination (n=41, 6–18 weeks gestation) and at term (n=5). Samples were routinely processed for serial sectioning at 3um to allow structural relationships to be compared in consecutive sections. Double immunohistochemistry protocols were used to identify lymphatic vessels in conjunction with one of: blood vessels, smooth muscle, epithelium and trophoblast, or proliferating cells. Immunostaining demonstrated that lymphatic vessels were present in abundance in the hypersecretory endometrium throughout all stages of gestation, and were prominent encircling spiral arterioles and adjacent to the endometrial glands. In contrast, the decidua was nearly always devoid of lymphatics, and in particular they were absent from the vicinity of spiral arterioles that were surrounded by decidual cells. In hypersecretory endometrium lymphatic vessel profiles varied greatly in architecture and size, and included focal accumulations of endothelial cells, through to fully formed lymphatic vessels. There was evidence of lymphatic vessel endothelial cell proliferation in all the different vessel types. The trophoblast appeared to have no overt influence on the structure or appearance of the lymphatic endothelium, with no evidence of incorporation into, or distortion of, the lymphatic vessel walls. Obvious increases were noted in the size of the lymphatic vessel profiles in hypersecretory endometrium as gestation progressed. The study provides the first detailed description of the placental bed lymphatics and their relationship to other endometrial structures during human pregnancy. We have shown that lymphatic vessels are absent from the decidua, apparently regressing as surrounding stromal cells decidualise. In contrast, the underlying hypersecretory endometrium contains abundant lymphatics which increase in size as pregnancy progresses.

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THE INTERNALIZATION OF ANTIPHOSPHOLIPID ANTIBODIES INTO TROPHOBLASTS CORRELATES WITH THE EXPRESSION OF MEGALIN

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Antiphospholipid antibodies (aPL) are autoantibodies that increase the risk of preeclampsia nine fold. We have recently shown aPL increase in the number of syncytial knots shed from placental explants and also change the trophoblast death process towards necrosis. Shedding of necrotic syncytial knots is thought to contribute to the pathogenesis of preeclampsia. Antiphospholipid antibodies but not control antibodies, are internalised into the syncytiotrophoblast suggesting a specific mechanism for internalisation of the aPL. Megalin is known to be an endocytic receptor for the antigen of aPL. We believe that the internalisation of aPL into the syncytiotrophoblast is required to for aPL to affect trophoblast shedding and in this study began to investigate the hypothesis that megalin mediates aPL-internalisation. Monoclonal aPL, IIC5 or ID2, were incubated with monolayers of the trophoblast cell lines, Jar, Jeg 3 or BeWo, or first trimester placental explants for 24 hours. Internalisation of aPL into trophoblasts was determined by fluorescent immuno-staining as was the expression of megalin using an antimegalin antibody (Sigma). Experiments were repeated at least three times. Confocal microscopy demonstrated that the syncytiotrophoblast of explants and BeWo cells, but not Jar or Jeg3 cells, internalised the aPL. Despite treating explants and BeWos with the same amount of aPL the level of aPL internalised by the syncytiotrophoblast of explants was greater than the level internalised by BeWos. Megalin was expressed strongly by the syncytiotrophoblast and weakly by BeWos but was not expressed by Jars or Jeg3 cells. The internalization of aPL into syncytiotrophoblasts may play an important role in regulating trophoblast death leading to aberrant shedding of syncytial knots. This study provides preliminary evidence that megalin expression correlates with the ability of trophoblasts to internalize aPL suggesting it may be the receptor that mediates aPL internalization into trophoblasts making this pathway a potential therapeutic target.

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PROPROTEIN CONVERTASE 6 PLAYS A CRITICAL ROLE IN MODULATING THE HUMAN ENDOMETRIAL EPITHELIUM FOR RECEPTIVITY AND IMPLANTATION

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Successful embryo implantation is an important step in establishing pregnancy, requiring a healthy embryo and a receptive endometrium. Establishment of endometrial receptivity involves morphological and physiological changes initially in the endometrial epithelium, but 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 present study aimed to determine whether PC6 is a critical regulator in the endometrial epithelium for receptivity and implantation. We examined whether endometrial epithelial PC6 dys-regulation is associated with implantation failure in women and whether knockdown of PC6 by siRNA in human endometrial epithelial cells affects embryo adhesion in a cell culture model. Endometrial PC6 expression was assessed by immunohistochemistry in the mid-secretory phase of the menstrual cycle (receptive phase) in two unique clinical cohorts comprising women of known fertility and infertility (with no obvious gynecological disorders, and with fertile males). Endometrial epithelial PC6 levels were significantly lower in infertile vs fertile women in both cohorts. To further establish that PC6 is important for receptivity, a cell model relevant to human implantation was used involving co-culture of uterine epithelial cells with mouse embryos. The epithelial cells were stably transfected with PC6 siRNA and PC6 knock down was confirmed at the levels of mRNA, protein, and activity by real-time RT-PCR, Western blotting and activity assay respectively. Embryos readily adhered to normal epithelial cells, but the adhesion was significantly reduced in the PC6 knockdown epithelial cells. We are currently using proteomics technology to identify the pathways affected by PC6 knockdown. These results strongly suggest that PC6 plays a critical role in modulating the human endometrial epithelium for receptivity and implantation.

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LYVE-1 AND VEGFR-3 POSITIVE LYMPHATIC VESSELS ARE LARGELY RESTRICTED TO THE MYOMETRIUM IN THE MOUSE UTERUS

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The human endometrium contains a distinct population of lymphatic vessels, with reduced numbers of vessel profiles in the functionalis relative to the basalis and underlying myometrium. The mechanisms restricting lymphatic vessel development within the functionalis and the consequences of this distribution for endometrial function have not been investigated. To determine how the distribution of endometrial lymphatics is regulated, an *in vivo* animal model is required for mechanistic studies. As an initial step, we described the distribution of lymphatic vessels and the expression of Vegf-C and Vegf-D within the mouse uterus using immunohistochemistry. Uterine tissues were collected from mice during the oestrus cycle (diestrus, proestrus and oestrus, n=7–9 per group), during early pregnancy (days 1–4, n = 4–5 per group), and from ovariectomised mice treated with vehicle, estradiol-17β or progesterone treatment (n=7–8 per group). Uterine sections were immunostained with antibodies against Lyve-1, Vegfr3, Vegf-C and Vegf-D. Lyve-1 positive lymphatic vessels were almost exclusively observed in the connective tissue between the longitudinal and circular muscle layers of the myometrium. Lymphatic vessel profiles were rare within the endometrium and were only observed on 24% of the sections examined. The Vegfr3 immunostaining was less robust than Lyve-1 with considerable variation in non-lymphatic staining among the different sections (which did not relate to a particular reproductive state or treatment group). Despite the variability, the pattern of lymphatic vessels was as seen with the Lyve-1 antibody. Vegf-C and Vegf-D immunostaining was present in all uterine compartments (epithelium, stroma, myometrium), however, only minimal changes were noted in expression across the oestrus cycle, during early pregnancy, or in hormone treated mice. In conclusion, despite the presence of the key lymphangiogenic growth factors Vegf-C and Vegf-D, there are minimal lymphatic vessels present within the mouse endometrium. This implicates other factors in the mechanism restricting endometrial lymphatic vessel growth.

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SOLUBLE MEDIATORS IN THE HUMAN UTERINE CAVITY: POTENTIAL ROLES IN EMBRYO IMPLANTATION

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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 likely contain important mediators that modulate the endometrium and support the conceptus during implantation. Previously we identified that several chemokines were maximally produced during the MS phase by endometrial glandular epithelium (GE) (1, 2) and the presence of chemokine receptors on GE and human trophoblast (3). Furthermore recombinant human chemokines and endometrial epithelial cell-conditioned media stimulated trophoblast migration; this was attenuated by neutralizing specific chemokines (3). Chemokines also regulate a variety of adhesion and ECM molecules on trophoblast (4). Thus chemokines have important roles during embryo implantation. We hypothesized that chemokines are secreted into the uterine cavity and may act on the implanting blastocyst and the endometrium. This study aimed to identify chemokines in uterine fluid (collected by flushing the uterine cavity with 5mls of saline) from fertile women during the proliferative (non-receptive; n=4) and MS (receptive; n=4) phases of the cycle, and from women with unexplained infertility during the MS phase (n=4). Uterine fluid was analyzed using quantitative Milliplex^{TM} Luminex® 42-plex cytokine/chemokine assays revealing the presence of IL-8, CCL2, CCL4, CCL7, CCL11, CCL22 and CX3CL1 in uterine fluid from all women. Importantly chemokine profiles were altered with both cycle phase and fertility; for example CCL4 and CCL22 levels were lower in the infertile cohort, where as CCL2 levels were higher in uterine fluid collected during the proliferative phase. Identifying the soluble mediators in human 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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MODULATION OF THE MATERNAL IMMUNE SYSTEM DURING EARLY BOVINE PREGANCY

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Over the past three decades, there has been a significant decline in dairy cattle fertility. A large proportion of pregnancy losses are believed to occur during the pre-implantation period, when the developing embryo is elongating rapidly and signalling its presence to the maternal system. The molecular mechanisms that prevent progression of the estrous cycle and allow the allogenic embryo to survive within the maternal environment are not well understood. To gain a more complete picture of these molecular events, global transcriptional profiling was performed using endometrial tissue from reproductive day 17 pregnant and non-pregnant (cycling) Holstein-Friesian dairy cattle. Microarray analysis revealed 357 differentially expressed transcripts (with ≥2 fold change in expression; P-value < 0.05) between pregnant and cycling animals in both caruncular and intercaruncular tissue. Additionally, 122 and 26 differentially expressed transcripts were identified only in either caruncular or intercaruncular endometrium, respectively. Pathway analysis of differentially expressed genes revealed enrichment for genes involved in interferon signalling and modulation of the immune response in pregnant animals. Many of these genes have already been described in relation to bovine reproduction. However, this analysis also revealed a number of genes not previously identified in this context, providing further insight into the mechanisms by which the embryo regulates the maternal immune response.

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CO-ORDINATED GENE EXPRESSION IN BOVINE GRANULOSA CELLS PRECEDES FOLLICULAR DOMINANCE

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During growth of bovine follicles, one emerges as the largest and dominant follicle. What regulates dominance remains unknown, but candidates include oestradiol, TGFB1, and recently CYP11A1 and focal intra-epithelial matrix (focimatrix). The two to four largest follicles were dissected from pairs of bovine ovaries and follicular fluid collected. A portion of the follicle wall was histologically classified for follicle health or atretia, and granulosa cells harvested for quantitative RT-PCR. Messenger RNA levels of focimatrix (COL4A1, LAMB2, HSPG2), steroidogenic enzymes (CYP11A1, CYP19A1) and TGFB1 genes were measured. Follicular fluid progesterone and oestradiol concentrations were measured by RIA. Follicles were identified as pre-deviated (before size-deviation) if the largest two or more healthy follicles were of equal size (6.7±0.1 mm, n = 14 animals, 35 follicles), and as post-deviated (after size-deviation) if they differed in size by 0.5–1.0 mm (7.2±0.2 mm; n = 11 animals, 26 follicles). For analyses, pre-deviated follicles were grouped into either the highest (oestradiol, CYP11A1) or lowest (TGFB1) expression (n = 14) and compared to the remaining follicles (n = 21). Deviated follicles were classified into dominant (n = 12) and subordinate (n = 14) based on diameter. Dominant follicles did not differ from subordinate follicles in any parameters measured, but were significantly larger than subordinate or pre-deviated follicles (P<0.01). For pre-deviated follicles grouped on oestradiol no parameters differed significantly, and when grouped on TGFB1, LAMB2 (P<0.05), HSPG (P<0.05), CYP19A1 (P<0.05) and TGFB1 (P<0.01) differed but levels were lower, not higher as expected. When grouped on CYP11A1, COL4A1 (P<0.05), LAMB2 (P<0.01), HSPG2 (P<0.01) and CYP19A1 (P<0.001) were significantly elevated in the high CYP11A1 group. This suggests that CYP11A1 and focimatrix might be important in follicle dominance.

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THE EFFECT OF ALTERING GLUCOSE LEVELS DURING COLLECTION AND MATURATION OF MOUSE OOCYTES ON SUBSEQUENT DEVELOPMENTAL COMPETENCE

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The preconception environment is known to influence oocyte developmental competence. In particular, hyperglycaemic conditions during cumulus-oocyte complex (COC) maturation result in decreased oocyte quality. This is, in part, due to perturbations in O-linked glycosylation in the cumulus cells. In embryos, even a brief exposure to glucose during early cleavage can have significant impact on O-linked glycosylation and further development. The aim of this study was to determine the effect of altering glucose concentrations during the collection and maturation phases of COCs on oocyte developmental competence. COCs were collected and matured for 17h at 37°C in 6% CO_2 with 0 or 10mM glucose in a 2 x 2 factorial design. A fifth group used standard concentrations of 0.5mM and 5.55mM glucose in the collection and maturation media respectively. Following maturation, oocytes were inseminated and cultured to the blastocyst stage. The average time for collection was 1 h. COCs exposed to 0mM glucose during collection and 10mM glucose during maturation had the greatest cumulus expansion despite no change in the proportion of COCs completing nuclear maturation. However, this same treatment group resulted in significantly lower blastocyst production than the control group (8.4% vs. 25.0%, $P<0.05$). These results show that glucose concentration in collection medium has a significant influence on maturation indices and oocyte developmental competence, as determined by blastocyst development rates. Our data further supports the concept that the conditions used for the collection of oocytes can have profound effects on subsequent development. We intend to investigate if these effects are related to perturbations in cumulus cell O-linked glycosylation.

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EXPRESSION AND LOCALISATION OF SIRTUIN 1 (SIRT1) IN CELLS OF THE MURINE OVARY

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Sirtuin proteins regulate cellular metabolism, survival and function via their deacetylase activity on target histones and proteins. SIRT1 is important in translating the beneficial effects of calorie restriction and in increasing longevity in lower organisms. Many proteins targeted by SIRT1 are important to fertility, including androgen and oestrogen receptors, and transcription factors such as FOXO. We examined the presence of SIRT1 in the ovary, and whether its localisation and expression levels change throughout the reproductive cycle and with female age. 21 day old female C57BL/6 mice were treated with gonadotrophins and ovaries collected from four mice each at 0h (unstimulated), 44h post-PMSG, 8h post-hCG, 11.5h post-hCG and 14h post-hCG. One ovary was fixed for immunohistochemistry, and from the other ovary, cumulus (CC) and granulosa (GC) cells were isolated for real-time RTPCR analysis. To examine the effect of maternal age, 21 day old (young) and 38 week old (old) female Swiss mice were stimulated and ovaries collected 16h later for immunohistochemistry. SIRT1 mRNA and protein was detected in GC and CC at all stages examined; expression was higher in GC compared to CC, and no difference was detected within cell type at different stages of follicle growth. Localisation of SIRT1 protein within the cell, and staining intensity, changed during antrum formation; there were more intensely stained GC towards the antrum, and staining was concentrated around the nucleus. Weak positive staining was detected in mural GC however, in CC, GC on the apical side of the follicle and cells of the ovarian surface epithelium strong staining was observed. We did not observe changes in protein localisation or staining intensity as a consequence of maternal age. SIRT1 mRNA and protein is present in cells of the ovary and studies to determine the potential role it plays in these cells is on going.