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ABSTRACTS

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1. EPIGENETIC PHENOMENA IN REPRODUCTIVE BIOLOGY

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At many levels from monozygotic twins, to standard laboratory animals, to cultures of differentiated cells, it is clear that genotype and phenotype are not synonymous. This is in part because of epigenetic changes. These are heritable changes in gene function involving modification in chromatin or in gene function but not structure. Epigenetic changes are seen at many points in development including development of the mammalian germ line. These modifications include demethylation and selective remethylation of the genome, chromatin remodelling, erasure of allele specific methylation of imprinted loci and re-activation of the silent X-chromosome. Imprinting is the epigenetic modification that marks a gamete's haploid genome as maternal or paternal. Imprinted genes have roles in prenatal growth, development of particular lineages, in behavior and in some diseases. Imprints are initiated during gametogenesis, inherited by mature gametes and transmitted to embryos. Whilst we do not as yet completely understand all the aspects of the linkages between methylation, chromatin remodelling and imprinting it is clear these epigenetic phenomena function in concert if development is to proceed normally. One example of where this may go awry is in cloning by nuclear transfer. Genes, which are silenced in somatic cell nuclei used as nuclear donors in cloning, must be reactivated for clones to develop normally. This requires reprogramming in an entirely different context than that applying during gametogenesis and within a much shorter time span. It is therefore not surprising that partial or total failure of reprogramming occurs. This will result in a range of phenotypes in clones with only a small percentage showing complete reprogramming. This correlates with the low percentage of healthy clones. Clearly more complete understanding of epigenetic processes would contribute substantially to our understanding of normal development and improve our of efficiency cloning. This is of particular importance if healthy cloned animals are to be produced for commercial purposes.

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2. EPIGENETIC INHERITANCE IN MAMMALS

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It is well recognised that there is a surprising degree of phenotypic variation among genetically identical individuals even when the environmental influences, in the strict sense of the word, are identical. Genetic textbooks acknowledge this fact and use different terms such as "intangible variation" or "developmental noise" to describe it. We believe that this intangible variation results from the stochastic establishment of epigenetic modifications to the DNA nucleotide sequence. These modifications, which may involve cytosine methylation and chromatin remodelling, result in alterations in gene expression that, in turn, affect the phenotype of the organism. Recent evidence, from our work and that of others in mice, suggests that these epigenetic modifications, which in the past were thought to be cleared and reset on passage through the germline, may sometimes be inherited to the next generation. This is termed epigenetic inheritance, and while this process has been well recognised in plants, the recent findings in mice force us to consider the implications of this type of inheritance in mammals. At this stage we do not know how extensive this phenomenon is in humans but it may well turn out to be the explanation for some diseases which appear to be sporadic or show only weak genetic linkage.

Morgan H., Sutherland H., Martin D.I.K. and **Whitelaw E.** (1999) Epigenetic inheritance at the agouti locus in the mouse. *Nature Genetics*, 23, 314-317.

Whitelaw E and Martin D.I.K (2001) Retrotransposons as epigenetic mediators of phenotypic variation in mammals. *Nature Genetics*, 27, 361-365.

Rakyan V K, Blewitt M, Druker R, Preis J, and **Whitelaw E** (2002) Metastable epialleles in mammals. *Trends in Genetics* (Vol 18, in press).

3. SUPRAMOLECULAR SCIENCE TO NANOTECHNOLOGY

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Many processes in biological and medicinal science are characterised by supramolecular chemistry. This chemistry is defined as non-molecular with non-covalent interactions or associations between biological molecules. Examples include gene regulation and transcription, protein-protein, protein-antibody interactions. Furthermore, the synthesis of proteins or cofactors is often achieved by use of chaperone proteins assisting the “intermediates” to exist in otherwise “reactive” environments prior to delivery or assembly of the mature biomolecule.

The most recent development in scientific technology has been the decrease in size from micro-scale to nano-scale. This has drawn into sharp focus the possible use of biological molecules as components in nano-scale devices. The types of technology envisaged includes photonic devices, such as Rhodopsin, molecular motors such as, ATPase, memory chips for quantum computers based on DNA, single molecule sensitive biosensors and targeted drug delivery and gene therapies. One of the major scientific developments that has led to this new nanotechnology is the availability of new materials such as microporous coatings, high tensile carbon tubes, and nanocomposite ceramics that can be used in bioelectronics. In addition, the development of Scanning Probe Microscopy has resulted in routine imaging at the molecular and atomic scale. This talk aims to demystify some of these components of nano-science and provide an awareness of possible applications in the bio-medical sciences.

Proteins and enzymes are particularly well designed to perform specific reactions with high selectivity and therefore they are ideal biological elements for biosensors. Of particular focus in this talk is the use of supramolecular strategies together with immobilised techniques for metalloproteins and enzymes. These methods enable the direct, real time monitoring of redox behaviour, which can be related to enzyme activity or function. One aim in this research is to use the molecular properties of proteins and enzymes with high specificity and selectivity to underpin the developments of a new generation of biosensors. The attachment of metalloproteins to an electrode surface also allows one to probe the thermodynamic and kinetic controls of their function. The methods used for enzyme immobilisation will be presented as well as examples of the possible scope for application to other areas of biological and medicinal science. The potential for new bio-medical diagnostics will be discussed.

4. GRAPES AND WINE FLAVOUR

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A brief overview of research into wine and grape flavour highlighting selected developments in the field over the last three decades. The talk will be concerned with factors that shaped the direction and evolution of wine flavour research rather than with details of the techniques that have been employed. For example, the focus away from wine and onto the grape for an understanding of varietal flavours, the recognition of a multiplicity of flavour active components, and the absence of single flavour impact compounds, and a better understanding of the origins of flavour components, are just some of the features that define our present knowledge of wine flavour composition. Some applications of the knowledge gained from the research and the potential of this to further enhance Australia's position as a premium wine producer will be discussed.

5. IRON IS HIGHLY GENOTOXIC TOWARD HUMAN SPERMATOZOA

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Human spermatozoa have been reported to be susceptible to the DNA damaging effects of pro-oxidants such as hydrogen peroxide (H₂O₂). However, DNA damage in individual genes has not been investigated in these cells. Thus, we have applied a quantitative PCR assay (QPCR) to measure gene-specific DNA damage in human spermatozoa treated with the pro-oxidants H₂O₂ and iron. **Methods:** Human spermatozoa were treated in vitro with hydrogen peroxide (0-5 mM) or Fe(II)SO₄ (0-400 μM) for one hour at room temperature. After DNA purification, gene-specific DNA damage was measured by QPCR. DNA damage (e.g., strand breaks, bulky adducts) blocks the progression of the PCR polymerase, resulting in decreased amplification of the target genes. The presence of single-strand DNA breaks and alkali-labile sites was determined by alkaline gel electrophoresis. **Results:** Millimolar concentrations of H₂O₂ were required to induce DNA damage in human spermatozoa as assessed by QPCR. After 5 mM H₂O₂, amplification was reduced by 40-50%, depending on the particular gene. The nature of this DNA damage was revealed by alkaline gel electrophoresis to be single-strand breaks and alkali-labile sites. In contrast, iron induced a significant amount of DNA damage after treatment with as little as 100 μM (25-40% decreased amplification), while 400 μM iron reduced amplification of target genes by greater than 90%. Interestingly, the DNA damage induced by iron was not due to strand breaks or alkali-labile sites as assessed by alkaline gel electrophoresis. **Discussion:** These results indicate that nuclear DNA of mature spermatozoa is relatively protected from the damaging effects of hydrogen peroxide; however, iron appears to be highly genotoxic to nuclear DNA of human spermatozoa. The two genotoxins also appear to induce distinctly different lesions. H₂O₂ induces strand breaks and/or alkali-labile sites, while iron does not induce these lesions. We hypothesize that iron is stimulating the release of genotoxic lipid peroxides, which then form covalent adducts with DNA bases. These results are the first to show that DNA damage can be measured in specific DNA sequences in male germ cells, and provide new insight into the susceptibility of human spermatozoa to genotoxic agents.

6. MORPHOLOGICAL MANIFESTATION OF Y CHROMOSOMAL MICRODELETIONS IN THE HUMAN TESTIS

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Infertility affects 10-15% of couples seeking offspring [1]. Between 2 - 20% of the severe cases carry Y-chromosomal microdeletions in the azoospermia factor (AZF) region [2]. These chromosomal microdeletions are associated with morphological disorders of spermatogenesis. Although the missing genes are described, no principle causing germ cell depletion is known. The aim of this study is to characterise biopsies of AZF patients more intensively to find similarities among the pathologies of such patients. Testicular biopsy specimens from 17 patients with AZFc deletions were analysed. In a randomised study the diameter of the tubules, the lumina, the lamina propria and the epithelia were measured and compared with those of patients with idiopathic Sertoli-cell-only (SCO) syndrome (n=11), mixed atrophy (n=10) and complete spermatogenesis (n=11). The biopsies were also stained for Sertoli cell-specific antigens. Additionally the distal and proximal regions of the microdeletion were analysed with new primers in order to characterise the microdeletion position and length [3]. By comparing the testis parameters of the AZFc deletion group with the control patients, an intermediate state between total SCO and complete spermatogenesis emerged. The diameters of the average tubule of patients with an AZFc deletion and patients with idiopathic SCO were significantly smaller than those of patients with complete spermatogenesis. The lumen diameter was significantly smaller compared to the control group with complete spermatogenesis and larger than in idiopathic SCO. Analysis of the chromosomal microdeletions demonstrated that all patients with a deletion solely in the AZFc-region have identical breakpoints. The immunohistochemical examination of the Sertoli cell proteins did not reveal any specific expression differences among the patient groups. AZFc microdeletions lead to alterations in testis morphology different from those in patients with mixed atrophy, although the germ cell status of both are identical. Patients with Y chromosome microdeletions show an intermediate status between idiopathic SCO and complete spermatogenesis. Sertoli cell function seems not to be altered [1] Nieschlag, E. (2000) Scope and goals of andrology. In: Nieschlag E. and Behre H. M. (eds.) Andrology: male reproductive health and dysfunction. 2nd ed. Springer, Heidelberg; 4-5.[2] Vogt et al. (1996). Human Molecular Genetics, 5, 933-943.[3] Kuroda-Kawaguchi et al. (2001) Nature Genetics, 29, 279-286.

7. LEPTIN INHIBITS BASAL BUT NOT STIMULATED TESTOSTERONE PRODUCTION BY THE IMMATURE MOUSE TESTIS

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Leptin is secreted into the blood by adipose tissue and was originally identified as a regulator of appetite and energy expenditure. It has subsequently been shown to be involved in many other physiological systems including reproduction. In humans and mice, plasma leptin levels are lower in males than in females even when corrected for body fat. Some authors have attributed this difference to a suppressive effect of testosterone on leptin production. However, we have recently shown that plasma leptin levels in both intact and castrate male sheep are lower than that found in females, (3) suggesting that the lower levels in males are not induced by testosterone. In contrast it has also been suggested that elevated leptin may suppress testosterone production, since obese men tend to have reduced fertility and reduced plasma testosterone. Recently it has been reported that leptin suppresses testosterone production in hCG stimulated adult rat testis slices (1) and purified Leydig cells (2). In contrast, we have reported that leptin has no effect on adult mouse Leydig cells so the aim of this study was to investigate the effect of leptin on testicular slices from immature (14 days old) and adult (60 days old) Swiss mouse testes. A biphasic response was observed with leptin suppressing testosterone production by up to 60% in the unstimulated immature testis at a dose of 6.25 ng/ml. In the hCG stimulated testis leptin had no effect on testosterone production. In the adult testis leptin had no significant effect on either stimulated or unstimulated testosterone production. In contrast purified Leydig cells from both immature and adult testes were not affected by leptin. These data suggest that physiological doses of leptin do not directly effect Leydig cell steroidogenesis but acts via paracrine signaling from other cell types within the testis.

(1) Tena-Sempere *et al.*, 1999 *Journal of Endocrinology* **161**: 211-218

(2) Caprio *et al.*, 1999 *Endocrinology* **140**: 4939-4947

(3) Kauter *et al.* 2000. *Journal of Endocrinology* **166**: 127-135

8. THE ROLE OF FGFR SIGNALLING IN SPERM TAIL DEVELOPMENT

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Unusual sperm tail morphology is present in a high percentage of infertile males, and because motile sperm are essential for unassisted fertilisation, research into sperm tail proteins will have an important impact on the way in which infertility is treated. A major focus of our group is to characterise the function of the outer dense fibres (ODF) of the sperm tail. ODF provide rigidity and directionality to tail movement, but the recent isolation of ODF proteins with enzymatic or regulatory characteristics, suggests they have a more active role in motility. Screening of a rodent testis library using an ODF specific antisera and 3'RACE identified SNT-2 (Suc-1 associated associated Neurotrophic Factor Target 2) as a potential ODF component. The SNTs are known FGF (Fibroblast Growth Factor) signalling adaptors and key components in the activation of the MAP kinase and PI3-kinase pathways via the FGF receptor. Subsequently Northern blot analysis revealed that SNT-2 is highly expressed in the testis and up-regulated concordant with the appearance of spermatids and sperm tail development. Further, Western blot analysis has demonstrated that rat sperm tails are immunopositive for FGFR-1, and there are large concentrations of FGFs within the epididymis. Collectively this data suggests that FGF signalling through SNT-2 is involved in sperm tail development/function. In order to test this hypothesis we have created transgenic mice that express a dominant-negative variant of the FGFR-1 in haploid germ cells. The transgene is a fusion of cDNAs encoding a truncated FGFR-1 (extracellular and transmembrane domains) and a flag-tag, driven by the mouse protamine promoter. Mice have been generated by microinjection and are currently undergoing genotyping prior to assessing spermatogenesis and fertility.

9. REDOX REGULATION OF SPERM FUNCTION

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Mammalian sperm function is redox regulated. Using a variety of chemiluminescent and colorimetric probes we have discovered multiple sources of redox activity in human spermatozoa. Thus NADH- and NADPH-dependent oxidoreductases have been detected in human sperm that generate profound chemiluminescent responses when lucigenin is used as the probe, but not luminol, isoluminol or MCLA. The activities observed when NADH or NADPH is used as substrate are highly correlated, but biochemically distinct. For example, the NADPH-dependent activity can be inhibited with diphenylene iodonium, while the NADH response cannot. On the other hand, both oxidoreductases can be suppressed with the thiol reactive agent, pCMBS and both enzyme systems are competent to reduce tetrazolium salt derivatives such as NBT and WST-1. The NADH-dependent oxidoreductase has been isolated by FPLC and a protein band correlating with enzyme activity was analysed by MALDI TOF. The isolated protein was identified as cytochrome b₅ reductase (R.2). Transient transfection of COS7 cells with this enzyme reveal an increase in NADH-dependent lucigenin chemiluminescence, consistent with a redox role for cytochrome b₅ in human spermatozoa. The NAD(P)H lucigenin reductase activity in human spermatozoa is significantly correlated with the induction of lucigenin-dependent chemiluminescent responses with 12-myristate, 13-acetate phorbol ester (PMA). Moreover, both the PMA and NAD(P)H responses are significantly elevated in populations of defective human spermatozoa recovered from the low density region of Percoll gradients. In contrast, the calcium-dependent chemiluminescent responses typical of NOX5 are similar in both Percoll fractions. These observations shed light on the biochemical nature of the lesions present in male infertility and will help resolve the aetiology of this condition.

10. DOES CALCIUM HAVE A ROLE IN SPERM HEAD ROTATION TO T-SHAPE ORIENTATION DURING CAPACITATION? A TALE OF TWO MARSUPIALS SPECIES, THE BRUSHTAIL POSSUM (*TRICHOSURUS VULPECULA*) AND THE TAMMAR WALLABY (*MACROPUS EUGENII*)

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Australian marsupial sperm attain T-shape orientation in the oviduct (1,2) as also during *in vitro* oviduct co-culture systems (3-5). The T-shape sperm in the brushtail possum were able to bind, penetrate and fuse with eggs (6,7). Here we propose a hypothesis explaining the mechanism by which sperm turn T-shape during capacitation. **MATERIALS AND METHODS** The preparation of oviduct-conditioned media (CM) and induction of T-shape orientation in sperm were as described previously (2-6). Ionic calcium in CM was estimated using iSTAT chip. The effects of tyrosine phosphorylation and various stimulators of cAMP pathways on sperm were according to Sidhu et al. (unpublished). **RESULTS AND DISCUSSION** The calcium levels in the possum oviduct is maintained at ~1mM as also in the oviductal CM produced *in vitro*. Estimating calcium in batches of CM indicated that the low levels of calcium are essential for optimal fertilization in the possum. Ejaculated wallaby semen contains 1.45-mM of calcium and sperm are streamlined, unable to fertilize eggs. Resuspending the wallaby ejaculated sperm in calcium-free buffer i.e. TALP and the possum epididymal sperm in low calcium buffer i.e. (pSOF media, calcium ~0.7 mM) turned them to T-shape within 2 h and that was reversible with 1.45 mM calcium. Increasing cAMP or tyrosine phosphorylation in sperm also turned them to T-shape orientation within 1 h. In the possum, >80% sperm turn T-shape in pSOF media and these sperm were unable to penetrate zona. Thus T-shape in sperm is not the sole criterion for capacitation. These results demonstrate that low levels of calcium are essential for obtaining T-shape orientation in sperm and the latter involves cAMP and tyrosine phosphorylation pathways and are essential for fertilization. **ACKNOWLEDGEMENT** This study was supported by the Marsupial CRC Australia and MAF Policy New Zealand. **REFERENCES** ¹Bedford, J.M. & Breed, W.G. (1994). *Biol. Reprod.* 50, 845-854. ²Molinia, et al. (1998). *J.Reprod. Fertil.* 112, 9-17. ³Sidhu, et al. (1998). *J.Reprod. Fertil.* 114, 55-61. ⁴Sidhu, et al. (1999a). *Biol. Reprod.* 61, 1356-1361. ⁵Sidhu, et al. (1999b). *Reprod. Fertil. Dev.* 11, 329-336. ⁶Mate et al. (2000). *Zygote* 8, 189- 196. ⁷Sidhu et al. (2001). *Proc. Aust. Soc. Reprod. Biol.* 32, 22. ksidhu@possum.bio.mq.edu.au

11. PROLACTIN IS A PREIMPLANTATION GROWTH FACTOR IN MICE

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Many hormones, cytokines and growth factors support optimal development of the preimplantation embryo. The close physiological relationship (1) between growth hormone, which has already been identified as a preimplantation growth factor (2) and prolactin, and evidence from gene knock-out models, implicated prolactin (3) and its receptor (4) in preimplantation development. The effects of prolactin on growth of mouse embryos *in vitro* were therefore investigated. Two-cell embryos were collected from superovulated Quackenbush mice 48 h post-hCG and incubated with ovine prolactin and/or antibodies against prolactin or the prolactin receptor for approximately 2 days. Gross morphological development and the number of cells in blastocysts and their component tissues were assessed by spreading 96 h post hCG blastocysts and nuclear staining. Confocal laser scanning immunohistochemistry (CLSIH) as described (2) demonstrated expression and localisation of prolactin and the prolactin receptor. Prolactin increased the total number of cells in the blastocyst. Concentration studies showed prolactin to be extremely potent in this role with $EC_{50} < 1$ pg/ml and concentrations above 10 pg/ml to be less effective. Addition of either of the blocking antibodies reduced cell number in blastocysts developing in the absence of exogenous prolactin and blocked the effects of added prolactin. CLSIH showed prolactin to be present in blastocysts that had been cultured 24 h. The receptors were present in oocytes and embryos throughout the preimplantation developmental stages, coincident with mRNA expression. This suggests that embryos express prolactin receptors that are responsive to prolactin secreted in an embryonic autocrine or paracrine regulation of cell number during preimplantation development. The predominantly trophectodermal location of prolactin receptors in the late blastocyst suggests that these receptors may be associated with regulation of implantation a major function of these cells.

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2. Pantaleon M, et al, (1997) Proc Natl Acad Sci USA. 94(10):5125-30.
3. Horseman ND, et al, (1997) EMBO J. 16(23):6926-35.
4. Ormandy CJ, et al, 1997 Genes Dev. 11(2):167-78.

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12. REAL-TIME PCR EXPRESSION OF HYPOXIA-INDUCIBLE FACTORS DURING BOVINE PREIMPLANTATION EMBRYO DEVELOPMENT

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Hypoxia-inducible factors (HIFs) are oxygen-sensitive bHLH-PAS transcription factors involved in the regulation of genes such as glycolytic enzymes, glucose transporters and vascular growth factors, necessary for adaptation to hypoxic conditions (1). Oxygen concentration significantly affects embryo development *in vitro*, whereby lower (5-7%) concentrations facilitate development. Furthermore, post-compaction bovine embryo development was further enhanced when O₂ levels were reduced from 7% to 2% (2), replicating the hypoxic conditions that occur in utero during early development (3). The aim of this study was to determine the level of expression of the two subunits HIF1 α and HIF1 β throughout preimplantation bovine embryo development. Bovine embryos were generated using standard protocols (2). Control RNA, for standard curves, was obtained from liver tissue. Oocytes/embryos were placed in TriReagent, snap frozen in liquid nitrogen and stored at -80°C. Total RNA was isolated and reverse transcribed to cDNA using random primers. Real-time PCR reactions were undertaken using SYBR green master mix (Applied Biosystems) on an ABI PRISM 5700 Sequence detection system using primers designed with Primer Express[®] (GenBank: HIF1 α AB018398, HIF1 β AB053954). All PCR products were sequenced to confirm identity. Real-time PCR demonstrated a temporal expression pattern of bovine HIF1 α and 1 β from immature and mature oocytes, presumptive zygotes, 2-8 cell and 9-16 cell embryos, compact morulae and blastocysts. HIF1 α progressively decreased during early cleavage, but increased at the blastocyst stage. HIF1 β expression also increased at the blastocyst stage, however was almost undetectable in immature and mature oocytes. This contrasts with all other cell types, as HIF1 β expression is ubiquitous. Moreover, cumulus cells express considerable levels of HIF1 β , suggesting that oxygen detection in oocytes may be mediated through cumulus cells. The presence of (and increase in) transcripts for both subunits of the HIF-1 complex suggests that hypoxia is an environmental stimulus that the post-compaction bovine embryo can detect and respond to at the molecular level, further supporting our hypothesis that oxygen is a regulator of early embryo development. Supported by NHMRC Project Grant 157941.

- [1]Wang, G. L. *et al.* (1995). *Proc. Nat. Acad. Sci. USA* 92:5510-4. [2]Thompson, J. G. *et al.* (2000). *J. Reprod. Fert.* 118:47-55. [3]Leese, H. J. (1995). *Hum. Reprod. Update* 1: 63-72.

13. EFFECT OF IN VITRO OXYGEN CONCENTRATION ON GLUCOSE TRANSPORTER-1 EXPRESSION IN THE MOUSE BLASTOCYST

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Preimplantation embryos develop *in vivo* under conditions of low oxygen. Oviductal and uterine oxygen concentrations are less than half that of atmospheric levels, with uterine O₂ concentrations of 3-5% reported around the time of blastocyst formation in several species (1). Decreasing the concentration of oxygen during *in vitro* culture, to more closely resemble *in vivo* conditions, facilitates embryo development (2) and these effects may be mediated through altered gene expression. Mouse preimplantation embryos express mRNA for hypoxia-inducible factor-1 α (3), an oxygen-regulated transcription factor which activates the expression of genes involved in the adaptation to low oxygen, including glucose transporter-1 (GLUT1). The aim of this study was to determine whether oxygen concentration regulates expression of GLUT1 in the mouse embryo. One-cell embryos were collected from CBAxC57Bl6 F1 mice 22 hours post hCG injection. Embryos were cultured in KSOM media under 7% O₂, 6% CO₂, 87% N₂. After 48 hours, compact morulae were transferred to 2%, 7% or 20% O₂ and cultured for a further 40 hours to the blastocyst stage. Embryos were snap frozen for RNA extraction. *In vivo* developed blastocysts were collected 88 hours post hCG. Extracted RNA was reverse transcribed and the abundance of GLUT1 mRNA and 18S rRNA was analysed by real-time RT-PCR using SyBr Green Master Mix in a GeneAmp 5700 Sequence Detection System (Applied Biosystems). GLUT1 results were normalised to 18S rRNA abundance. Oxygen concentration influenced GLUT1 gene expression (P<0.04). Levels of GLUT1 mRNA in embryos cultured under 2% O₂ were 118% of those observed in *in vivo* derived embryos. Embryos cultured under 7% or 20% O₂ expressed 59% and 84% as much GLUT1 as *in vivo* derived embryos, respectively. GLUT1 mRNA was increased in embryos cultured under 2% O₂, compared to those exposed to 7% O₂. These results suggest that the preimplantation embryo has the capacity to detect and respond to decreases in oxygen availability with changes in expression of hypoxia-regulated genes.

Supported by NHMRC Project Grant 157941 [1] Fischer B, Bavister BD. (1993) *J Reprod Fert* 99, 673-679 [2] Thompson JG et al (2000) *J Reprod Fert* 188, 47-55 [3] Harvey AJ et al. (2002) *Theriogenology* 57 (1), 496.

14. A COMPARISON OF STAINING METHODS USED TO DETERMINE NUMBERS OF CELLS IN PIG BLASTOCYSTS

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Total blastocyst cell numbers can be readily determined by staining nuclei with the fluorescent dye, bisbenzimidazole (Hoechst 33258). Differential labelling of trophoctoderm (TE) and inner cell mass (ICM) nuclei involves a number of treatments, including immunosurgery, which make the stained blastocysts extremely fragile. The aim of the study was to determine whether pig blastocysts subjected to differential labelling remain intact, enabling an accurate total cell count to be made. Day 7 parthenogenetic pig blastocysts were produced *in vitro* as described previously [1]. The activation treatment consisted of sequential incubations with 5 μ M ionomycin for 5 min and 2 mM 6-dimethylaminopurine for 3 h. Blastocysts were randomly allocated and stained. Bisbenzimidazole staining involved removal of the zonae and incubation with 50 μ M bisbenzimidazole for 20 min. Differential labelling involved removal of the zonae and sequential incubations with 10 mM trinitrobenzenesulphonic acid for 30 min, 0.2 mg/ml anti-dinitrophenol BSA for 30 min, a 1:5 dilution of guinea pig complement serum and 0.01 mg/ml propidium iodide for 30 min and 50 μ M bisbenzimidazole for 20 min. Stained blastocysts were mounted onto microscope slides and nuclei were visualized by epifluorescence microscopy. The experiment was replicated 3 times. The data was analysed by using the Student's *t*-test. There was no difference between the two staining methods in the total numbers of cells counted (Table 1), indicating that blastocysts subjected to differential labelling remained intact. In conclusion, the study demonstrates that the differential labelling procedure developed for pig blastocysts accurately determines the total cell number.

Table 1. Numbers of cells (mean \pm SEM) in day 7 parthenogenetic pig blastocysts.

Staining method	n	TE	ICM	Total
Differential labelling	78	41.9 \pm 1.9	9.9 \pm 0.9	51.8 \pm 2.4
Bisbenzimidazole	44	–	–	48.1 \pm 3.5

1. Grupen CG et al (1999) *Reprod Fertil Dev*, 11:457-462.

15. PARACRINE PERTURBATIONS ASSOCIATED WITH BOVINE NUCLEAR TRANSFER PREGNANCIES

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The regulation of fetal and placental growth and differentiation involve a variety of endocrine, autocrine/and or paracrine signals, including the insulin-like growth factor family (IGFs) and leptin. Increasing evidence suggests that paracrine signalling plays a fundamental role in placental growth and development. Alterations in the paracrine system in the endometrium may underlie pathological processes such as infertility, preeclampsia or endometrial neoplasia⁽¹⁾. Abnormal placental development and function and abnormal fetuses have been associated with *in vitro* derived embryos and especially embryos generated by nuclear transfer (NT)⁽²⁾. The aim of the research was to explore the hypothesis that paracrine factors involved in the regulation of normal placental growth, development and function are dysregulated in cattle pregnant with embryos generated by NT. Reproductive tracts from pregnant Friesian cows were obtained at days 50, 100 and 150 of gestation following embryo transfer with NT or *in vitro* produced (IVP) embryos or following artificial insemination (AI). Placental localisation of insulin like growth factor binding proteins 2 and 3 (IGFBP-2, -3) and leptin was performed by immunohistochemistry. Spatial and temporal differences in the localisation of IGFBP-2, -3 and leptin were observed in placental tissues from NT compared with AI/IVP pregnancies. At day 50 and 100 of gestation, increased immunostaining for IGFBP-2 was seen in the stromal tissue below the placentomes in NT pregnancies. Increased staining for IGFBP-3 was observed in uterine stromal tissue in NT pregnancies at all time points examined. Leptin immunostaining in the fetal villi in NT pregnancies was markedly increased at day 50 and 150 of gestation. In summary, the spatial and temporal patterns of expression of a number of growth factors involved in placental and fetal growth is altered in placental tissues from NT pregnancies. It is likely that placental dysfunction in NT pregnancies is associated with paracrine perturbations via a mechanism of action as yet unknown. (1) Reis FM, Cobellis L, Luisi S, Driul L, Florio P, Faletti A, Petraglia F. (2000) Paracrine/autocrine control of female reproduction. *Gynecol Endocrinol* 2000 Dec;14(6):464-75 (2) Wells, D. N., Misica, P. M., and Tervit, H. R. (1999) Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biology of Reproduction* 60 (4), 996-1005.

16. SURVIVAL OF FRESH AND VITRIFIED SHEEP IVP AND CLONED EMBRYOS AFTER EMBRYO TRANSFER

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The aim of this study was to examine embryo survival rates following the transfer of *in vitro* produced (IVP) and cloned sheep embryos cryopreserved using the Open Pulled Straw (OPS) vitrification method (1). Initially, vitrified Day 6 IVP blastocysts were transferred to recipients (two embryos per recipient) either within 1-2 h (=DELAYED; 34 embryos) or 2 min (=DIRECT; 30 embryos) of warming. No significant differences in pregnancy or fetal survival rates (76.5 and 66.7%; 52.9 and 46.7%, respectively) were observed between the methods. Both procedures were used in cloning experiments involving either serum-starved or actively growing adult granulosa and fibroblast donor cells and both *in vivo* and *in vitro* derived oocytes. Recipient pregnancy rates and fetuses going to term for *in vivo*-fresh (178 embryos), *in vivo*-OPS-DELAYED (15 embryos), *in vitro*-fresh (103 embryos), *in vitro*-OPS-DELAYED (113 embryos) and *in vitro*-OPS-DIRECT (28 embryos) groups were 53.7, 83.3, 43.1, 35.4 and 14.3% and 12.9, 33.3, 15.5, 5.8 and 0%, respectively. With *in vivo* derived embryos, fresh embryos yielded surprisingly significantly poorer results than vitrified embryos, although there were very small numbers in the latter group. Unlike the initial observations on IVP-embryos, the pregnancy rates achieved with *in vitro*-derived cloned embryos were significantly lower with OPS-DIRECT method as compared with OPS-DELAYED or fresh transfers, and fetal survival rates were significantly lower with both OPS-methods compared with fresh transfers. These results suggest that *in vivo*-derived cloned embryos endure vitrification better than *in vitro*-derived clones, and that the delayed transfer after OPS-vitrification is the preferred option for somatic cell cloned sheep embryos. (1) Vajta G, Kuwayama M, Holm P, Booth P, Jacobsen H, Greve T, Callesen H. A new way to avoid cryoinjuries of mammalian ova and embryos: the OPS-vitrification. *Mol Reprod Dev* 1998; 51: 53-58

17. ABNORMAL TROPHOBLAST MHC-I EXPRESSION AND ASSOCIATED ENDOMETRIAL LYMPHOCYTIC RESPONSE IN BOVINE NUCLEAR TRANSFER PREGNANCIES

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Somatic cell nuclear transfer (NT) has produced healthy “cloned” offspring in 7 mammalian species. Few NT embryos however, ever reach this stage as less than 2 out of every 100 NT embryos transferred into recipient females result in viable offspring (compared to 60/100 naturally fertilized embryos). In cattle, many of these losses occur around the time of placental attachment from the fourth week of gestation. Expression of major histocompatibility complex class I (MHC-I) by trophoblast cells and distribution of endometrial T-lymphocyte numbers were investigated as part of an assessment of the immunological status of NT pregnancies. Six 5 week old NT pregnancies were generated, all derived from the same fetal cell line. All 6 NT placentas displayed trophoblast MHC-I expression. None of 8 controls (4-7 weeks old) showed any MHC-I expression. Numbers of T lymphocytes (CD3 positive) were significantly higher in the endometrium of the majority of NT pregnancies compared with controls. Trophoblast MHC-I expression is normally suppressed during early gestation, and the observed MHC-I expression in the cloned pregnancies is likely to have induced a maternal lymphocytic response that would be detrimental to maintaining viability of the NT pregnancy.

18. CONCENTRATION OF PROGESTERONE AND SUPEROVULATORY RESPONSE WITH DIFFERENT DOSAGES OF FSH

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Seventeen Nelore (*Bos indicus indicus*) heifers were superovulated from Day 9 to 12 of the estrous cycle using two different dosages of FSH (250 or 500 UI). The objective was to measure the plasma concentration of progesterone during superovulation and embryo collection, and link the values to follicular dynamics and superovulatory response that were monitored daily by ultra-sound until the first insemination and embryo collection. Concentration of progesterone was analyzed every three days from Day 0 (estrous) until Day 12, at first artificial insemination and at embryo collection. Plasma was only analyzed in heifers that responded to superovulation. Heifers that displayed a low concentration of progesterone, suggesting the absence of a functional corpus luteum in the beginning of the superovulatory protocol, didn't have satisfactory results to FSH administration. However, more viable embryos/collection were not associated with higher plasma levels of progesterone on the day of first administration of FSH, as would be expected, but were related to individual heifer response. The plasma concentration of progesterone on the day of insemination was similar between heifers superovulated with 250 or 500 UI of FSH (Tab.1). However, on the day of embryo collection (d=7), the levels were inversely correlated to FSH dosage and directly related to the number of corpora lutea following superovulation (11.25 x 8.25 CL/donor). We conclude that superovulation with 250 UI of FSH was more efficient for stimulating more responsiveness follicles and the appearance of more corpora lutea; as a result, the secretion of progesterone was higher. Associated with this, all animals superovulated with 250 UI of FSH responded to the superovulatory protocol.

Table 1 – Plasma concentration of progesterone in Nelore heifers on the day of artificial insemination and the day of embryo collection.

Cycle day	Progesterone (ng/mL)	
	250 UI FSH	500 UI FSH
d=0 (Artificial Insemination)	0.51 ^{a,A}	0.35 ^{a,A}
d=7 (Embryos collection)	41.29 ^{a,B}	17.40 ^{b,B}

Averages followed by different lower case letter in same row are different (p<0,05)

Averages followed by different upper case letter in same collum are different (p<0,05)

19. EFFECT OF SEMINAL PLASMA ON FROZEN-THAWED BOAR SPERM

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Under optimal conditions, fertility after insemination with frozen boar sperm can be close to that achieved with chilled semen but, in the field, the levels are often substantially lower. We investigated the addition of seminal plasma (SP), reported to have beneficial effects in other species, before and after freeze-thawing, with the aim of improving post-thaw quality of sperm. The sperm-rich fraction of 3 ejaculates from each of 4 boars were collected and frozen, packaged in 0.5ml straws, according to the method of Bwanga et al. (1). The experimental groups had cooling extender supplemented with 100, 50 or 0% SP (standard protocol). After thawing, the samples were diluted in BTS containing 0, 5, 10, 20, 40, 80 or 100% SP. Post-thaw addition of SP in the range of 5-40% had a beneficial effect on initial motility and after 3h incubation at 37°C ($p < 0.05$). In particular, adding 10% SP to the post-thaw extender had a beneficial effect on motility at 0h (from $47 \pm 1.2\%$ with 0% SP to $58 \pm 0.9\%$ with 10% SP, $p < 0.05$). A concentration of 20% SP added post thaw gave optimal motility after 3h incubation at 37°C (from $29 \pm 1.8\%$ with 0% SP to $39 \pm 2.6\%$ with 20% SP, $p < 0.05$). Addition of 100% SP post-thaw had a significantly detrimental effect on motility at 0h (from $47 \pm 1.2\%$ with 0% SP to $24 \pm 5.6\%$ with 100% SP, $p < 0.05$) as well as a adverse, though not significant, effect on the number of intact acrosomes. There was no difference between 0 and 50% SP in the cooling extender on post-thaw sperm motility, but cryosurvival was lower with 100% SP ($p < 0.001$). Using CTC staining, there were more acrosome intact cells after thawing in samples without SP in the freezing medium compared to those frozen with SP. Work is in progress to establish the effect of adding SP in the range of 0-50% to the cooling extender prior to freezing.

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20. THE INFLUENCE OF ENVIRONMENTAL TEMPERATURE ON ELITE A.I. BULLS HOUSED IN A COOLING SHED OR FIELD

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Heat stress in cattle is associated with increased ambient temperature and/or humidity. In dairy cows this stress may be reduced with a cooling shed (using misted water and fans) (1). This type of shed may also prevent bulls suffering heat stress. Increasing scrotal temperature (with scrotal insulation) mimics increases in environmental temperature and reduces semen quality (2). Such changes in scrotal temperature can be measured using a non-contact infrared thermometer (IRT) (3). The aim of this study was to measure temperature differences between the cooling shed and the field, and between both individual bulls and groups of bulls, to identify those susceptible to heat stress. Scrotal and deep body temperatures were measured in 19 bulls housed at Genetics Australia, for the summers of 2001-2002, over 11 hot days ($\geq 30^\circ\text{C}$). Five bulls were housed in a cooling shed and 14 in the field. The fans and mist in the cooling shed were activated and remained on when ambient temperature was $\geq 27^\circ\text{C}$. Proximal, mid and distal scrotal temperatures were determined with an IRT (IRtec500+, $\pm 0.5^\circ\text{C}$, Eurotron). Core body temperatures were measured with a custom built rectal probe (Industrial Pyrometers Australia (IPA) accurate to $\pm 0.2^\circ\text{C}$). Environmental conditions were measured with data loggers ($\pm 0.1^\circ\text{C}$) (IPA) located in the cooling shed and field. Ambient field temperatures were hotter than the cooling shed (31.1°C vs. 30.2°C , $p < 0.05$) on days $\geq 27^\circ\text{C}$, but not on mild ($\leq 27^\circ\text{C}$) days (18.3°C vs. 18.9°C , $p > 0.05$). When ambient temperatures rose from $30-37^\circ\text{C}$, the respiratory rates, proximal, mid and distal scrotal temperatures of bulls in the field rose higher than bulls in the cooling shed. On hot days, the respiratory rates ($p < 0.05$) and rectal temperatures ($p < 0.05$) were lower in bulls in the cooling shed, while distal scrotal temperatures also appeared numerically cooler (not significant). The proximal and mid distal scrotal temperatures were not significantly cooler. Large individual differences existed in proximal, mid and distal scrotal and rectal temperatures ($p < 0.05$) between bulls in response to increasing environmental temperatures. Therefore although each bull reacts differently, bulls in the cooling shed appear to be able to thermoregulate more efficiently on hot days. (1) Armstrong DV (1994) *J Dairy Sci* 77:2044-2050, (2) Vogler CJ *et al* (1991) *J Dairy Sci* 74:3827-3835, (3) Brockway A (2001) *Proc Soc Repro Biol*, Abstract 19

21. FLOW CYTOMETRIC SORTING OF FROZEN-THAWED RAM SPERMATOZOA

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Sex pre-determination using sperm sorting and assisted reproductive technology (ART) is under development for wildlife species (1). Application is limited in situations where the sperm sorter is located a distance from the male(s). In this study, the quality of frozen-thawed, sorted ram spermatozoa was compared after three post-thaw processing methods. Semen from three rams (n=3 ejaculates per ram) was frozen using standard methods. An aliquot of each ejaculate was processed as a fresh control (FRESH; 2) in parallel with frozen samples. Frozen spermatozoa were thawed, prepared for sorting by no further processing (NEAT), washing (WASH) or gradient centrifugation (GRADIENT) and evaluated for motility at 1 h post-staining and motility and acrosome status at 0 and 4 h post-sorting. Samples were analysed using a high-speed sorter (SX MoFlo®, Cytomation Inc. CO, USA) after incubation with H33342 and food dye (2). Reanalysis of sorted spermatozoa revealed high levels of purity for X- and Y-enriched samples for all treatments (range: 84.7–91.1%). Percentage of motile spermatozoa prior to sorting was lower ($P<0.05$) for frozen-thawed samples (GRADIENT: $73.9\pm 3.7\%$; WASH: $32.2\pm 3.3\%$; NEAT: $32.7\pm 2.5\%$) compared to FRESH ($83.3\pm 1.2\%$). Post-sorting, percentage of motile spermatozoa for NEAT (0h: $60.0\pm 5.1\%$; 4h: $27.2\pm 6.1\%$) was lower ($P<0.05$) than that for FRESH (0h: $87.8\pm 0.9\%$; 4h: $83.3\pm 1.2\%$), WASH (0h: $80.0\pm 2.4\%$; 4h: $71.7\pm 3.6\%$) and GRADIENT (0h: $84.4\pm 1.3\%$; 4h: $77.2\pm 1.7\%$). There was a male effect ($P<0.05$) on the percentage of acrosome-intact spermatozoa but no effect ($P>0.05$) of time or treatment (range: 81.3–89.2%). Using a modified sperm migration test (3), sample aliquots were assessed for ability to penetrate an artificial cervical mucus at 0 h post-sorting. Vanguard sperm migration distance was lower ($P<0.05$) for NEAT (17.7 ± 1.7 mm) compared to WASH (29.1 ± 3.8 mm) and GRADIENT (28.4 ± 2.0 mm) and similar ($P>0.05$) to FRESH (23.7 ± 1.8 mm). In summary, high purity sorting of frozen-thawed ram spermatozoa was possible after processing to remove cryodiluent and samples showed only slight reductions in quality after sorting. The model has potential for use in sorting wildlife spermatozoa. (1) O'Brien *et al.* (2001) *Biol. Reprod. (Suppl. 1)* 64:158. (2) Hollinshead *et al.* (2001) *Proc. SRB* 32: 20. (3) Mortimer *et al.* (1990) *Hum. Reprod.* 5:835-841. Supported by XY, Inc., Zoological Parks Board of NSW and ARC.

22. EFFECT OF TIME OF INSEMINATION AND DOSE OF SORTED, CRYOPRESERVED RAM SPERM ON FERTILITY IN EWES

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Lambs have been produced after artificial insemination with cryopreserved sex-sorted sperm in sheep (1). This study aimed to determine the optimum time to inseminate ewes and the minimum effective dose of sorted frozen-thawed sperm to obtain commercially acceptable fertility. Semen samples from 3 rams were prepared for sorting, stained, incubated and analysed using a high speed cell sorter (SX MoFlo®; 1). Sperm were processed through the sperm sorter at 15,000-18,000/sec without sex-sorting. After each run 1.3×10^6 sperm were sorted for sex and analysed to determine purity (2). The sperm were frozen using standard techniques (3). A control sample from each ram's ejaculate was frozen without machine processing. Oestrus was controlled in 360 Merino ewes using progestagen-impregnated intra-vaginal pessaries (FGA, Vetrepharm) inserted for 12 days and an injection of 400 IU PMSG (Pregnecol, Vetrepharm) at sponge removal (SR). The time of ovulation was controlled by injection of $40\mu\text{g}$ GnRH (Fertagyl®, Intervet) 36h after SR. Ewes were inseminated with 1, 4 or 16×10^6 frozen-thawed processed sperm or 100×10^6 control frozen-thawed sperm into the uterus by laparoscopy 54, 58 or 62h after SR. Fifteen ewes not given GnRH were inseminated with control sperm at 54-58h. Pregnancy was diagnosed by ultrasound on d 57 and the data analysed by logistic regression. Motility of sperm after thawing was $42\pm 0.4\%$ (control) and $43\pm 0.6\%$ (machine processed). The numbers of ewes pregnant/inseminated (%) were 15/89 (16.9%), 22/90 (24.4%) and 28/90 (31.1%) for 1, 4 and 16×10^6 sperm doses, respectively ($p<0.001$), but pregnancy was not affected by time of insemination ($p=0.347$) or ram ($p=0.783$). There were no interactions between treatments. The results suggest that doses in excess of 16×10^6 sorted frozen-thawed sperm, deposited close to the anticipated ovulation time (58h; 12/30; 40.0% pregnant), are required to obtain fertility similar to the control (6/15, 40.0% pregnant). (1) Hollinshead *et al.* (2002). *Reprod. Fertil. Dev.*, submitted. (2) Johnson and Welch (1999). *Therio.* 52: 1323-1341. (3) Evans and Maxwell (1987). Salamon's Artificial Insemination of Sheep and Goats. Research supported by XY, Inc. and Australian Research Council.

23. THE EFFECT OF NUTRITION DURING THE CYCLE OF MATING ON OVIDUCTAL FLUID AMMONIA AND UREA LEVELS IN THE EWES

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This study examined the effect of changes in nutrition during the cycle of mating in the ewe on the concentrations of ammonia and urea in oviductal fluid. Twelve mature ewes (4-5 years, 58-67 kg) of comparable body condition were fed rations that provided either 1.5 x daily energy needs for maintenance (H) or 0.5 x maintenance needs (L). Nutritional treatments were imposed from 18 d before until 6 d after the expected time of ovulation. Oviducts were catheterised for four days before the start of the experiment and oviduct fluid was collected from day 0 until day 6 after ovulation. Half of the ewes were treated to superovulate (S) (using progestagen, FSH, GnRH treatment) while the remainder (NS) ovulated spontaneously following progestagen treatment. The data were analysed by ANOVA as a 2x2 factorial design. Body weight was significantly ($P < 0.001$) reduced for those sheep fed the L ration compared with H ration (L: -4.5 ± 0.43 kg, H: 1.17 ± 0.48 kg). The number of corpora lutea was not influenced by the dietary treatment. Differences in oviductal fluid ammonia concentrations approached significance ($P = 0.055$) for nutritional treatment (H: 2369 ± 160.3 $\mu\text{mol/L}$; vs. L: 1606 ± 143.3 $\mu\text{mol/L}$) but were not significantly different for ovulatory status (S vs. NS) or day of collection. Oviductal urea concentrations were significantly ($P = 0.005$) affected by treatment (H: 10.95 ± 0.59 mmol/L ; vs. L: 7.26 ± 0.29 mmol/L) and day of collection ($P = 0.012$, highest on day 5) but were not affected by ovulatory status. Both ammonia and urea have been associated in various ways with aberrant embryo development. The findings of this study indicate that short-term changes in nutrition can induce changes within the oviductal milieu that could provide a link between nutrition and embryo development and survival.

24. PITUITARY LH β - AND FSH β -SUBUNIT mRNA AND LH AND FSH CONTENT IN HEIFERS DURING AND AFTER TREATMENT WITH GnRH AGONIST

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Treatment with GnRH agonist is associated with delay in return to normal LH and FSH secretion and the aim was to determine whether this is a result of disruption of transcription or post-transcription regulation of LH and FSH (1,2). Beef heifers (2-y-old) were randomly assigned on live weight to 3 groups: Group 1, control (n=6), synchronised to day 11 of oestrous cycle on Day 31; Group 2 (n=6), GnRH agonist (deslorelin) treatment for 31 days; Group 3 (n=6), deslorelin treatment for 28 days. Animals were slaughtered on Day 31. Contents of anterior pituitary LH and FSH were determined by RIA, and LH β - and FSH β -subunit mRNA by Northern Blotting. Data were analysed by ANOVA. For Group 2, pituitary LH β -subunit mRNA was suppressed 60% and LH 95% while FSH β -subunit mRNA was suppressed 25% and FSH 90%, compared with controls. Three days after discontinuation of deslorelin, LH β -subunit mRNA and LH remained suppressed (50% and 95%, respectively). At this time FSH β -subunit mRNA was similar to controls while FSH remained 80% reduced.

Table 1. Anterior pituitary contents of LH, FSH (ng/mg tissue) and their β - and α -subunit mRNAs (relative units) for control heifers (Group 1), deslorelin-treated heifers (Group 2) and heifers 3 days after withdrawal of deslorelin (Group 3). Results are means \pm SEM.

	LH	LH β mRNA	FSH	FSH β mRNA	α -sub. mRNA
Group 1	466 \pm 81 ^a	1.22 \pm 0.14 ^a	44.13 \pm 4.28 ^a	1.65 \pm 0.31 ^a	1.46 \pm 0.39 ^a
Group 2	23 \pm 3 ^b	0.49 \pm 0.06 ^b	3.87 \pm 0.49 ^b	1.27 \pm 0.14 ^a	7.75 \pm 0.76 ^b
Group 3	26 \pm 4 ^b	0.60 \pm 0.11 ^b	7.32 \pm 1.39 ^c	1.66 \pm 0.18 ^a	6.34 \pm 1.04 ^b

^{a,b,c} Column means without common superscripts differ ($P < 0.05$)

Deslorelin suppressed LH β -subunit mRNA expression without significant effect on FSH β -subunit mRNA. As LH and FSH contents were more suppressed than their respective β -subunit mRNAs, it would appear that treatment with deslorelin can influence gonadotrophin synthesis by altering post-transcriptional mechanisms. [1] D'Occhio MJ et al. (2000) *Ann Rev Biomed Sci* 2: 91-112[2] Staton JM et al. (2000) *J Mol Endocrinol* 25: 17-34.

25. EXPRESSION OF GERM CELL AND SOMATIC CELL MARKERS DURING GONADAL DEVELOPMENT IN A MARSUPIAL

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In this study several germ cell and somatic cell markers that have been studied in the mouse and other species were investigated during gonadal development in the tammar wallaby (*Macropus eugenii*) to determine their potential for monitoring germ cell migration, germ-cell somatic cell, and to define their expression patterns during development. We studied the expression of anti-EMA-1, anti-SSEA-1, anti-C-kit, GCNA-1 and MIS by immunohistochemistry during embryonic, fetal and pouch young life and in the adult testis and ovary. EMA-1 and SSEA-1 were first detected in the germ cells of day 20 fetuses and were expressed up to day 19 post partum, but were absent from adult ovary and testis. C-kit protein was not detected in the germ cells of embryo and fetus but it was present at least from day 1 to 30 post partum in both male and female pouch young germ cells. In adult ovary, c-kit protein was detected in oocytes at all stage of development and also in the granulosa and theca cells. In adult testis this protein was detected in spermatocytes, spermatids and spermatozoa. C-kit protein was also detected in the neural tube, blood cells, the mesonephros of late fetuses and the mesonephros and skin of pouch young. GCNA-1 was not detected at any stage of germ cell development. MIS protein was expressed in the Sertoli cells at least from day 1 to day 26 of male pouch young, but decreased to become undetectable on day 90. MIS was not detected in pouch young ovary but it was detected in the granulosa cells of adult ovary. Thus this study confirms that EMA-1 and SSEA-1 can be used to identify germ cells during their migratory and proliferation phase in the Tammar, while c-kit protein can be used as germ cell marker during proliferation stage.

26. LEPTIN RECEPTOR mRNA EXPRESSION DURING FOLLICULAR DEVELOPMENT AND OVULATION IN THE IMMATURE PRIMED RAT OVARY¹

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Leptin is a hormone essential for normal reproductive function, produced primarily by adipose tissue but also by the ovary, with functional leptin receptors observed in ovarian tissue. Leptin administration increases oocyte maturation rates in mouse pre-ovulatory follicle-enclosed oocytes (1), and decreases both *in vivo* and *in vitro* rat ovulation rates (2). We aimed to determine whether expression of leptin receptor mRNA fluctuated during follicular development and rupture in the immature primed rat, as it is hypothesised that the biological activity of leptin is regulated via receptor availability. Immature rats were stimulated with eCG and 48h later with hCG, sacrificed and ovaries collected at various time points. RNA was isolated, reverse transcribed and cDNA subjected to real-time PCR amplification using SYBR Green, and specific primers for the long, functional or short, non-functional forms of the leptin receptor (Ob-Rb and Ob-Ra respectively) or luteinizing hormone/chorionic gonadotrophin receptor (LHCGR). Data were normalised relative to β -actin expression, and expressed relative to immature levels (Table 1).

	Immature	Hours post eCG			Hours post hCG		
		4h	26h	47h	h	h	4h
Ob-Rb	1.0	1.4 ± 0.4	0.8 ± 0.3	0.9 ± 0.4	.2 ± 0.2	.7 ± 1.4*	.1 ± 0.9
Ob-Ra	1.0	0.9 ± 0.2	1.0 ± 0.2	0.7 ± 0.1	.6 ± 0.5	.7 ± 1.5*	.7 ± 1.3
LHCGR	1.0	1.3 ± 0.2	2.0 ± 0.4	3.7 ± 1.5 ^a	.5 ± 0.9 ^a	.2 ± 0.01 ^b	.6 ± 0.1 ^c

Table 1. *Significantly different to all pre-ovulatory time points. Significantly different to; ^aimmature, ^ball other time points, ^c47h post eCG and 2h post hCG. ($P \leq 0.05$) (4-5 animals/time point).

Expression of mRNA encoding both forms of leptin receptor increased dramatically immediately prior to ovulation (h post hCG). LHCGR levels increased in response to eCG and decreased following hCG. These results demonstrate that there is cyclic regulation of leptin receptor and LHCGR expression, indicating they are directly or indirectly regulated by gonadotrophins. The ovarian cellular site of this increase is unknown, however the thecal compartment is a likely candidate, as high levels of leptin receptor protein have been demonstrated in these cells (1). [1] Ryan NK *et al* (2002) *Biol Reprod* 66(5) 1548-54. [2] Duggal PD *et al* (2000) *Endocrinol* 141(6) 1971-6. ¹Funded by NHMRC.

27. ANDROGEN RECEPTOR EXPRESSION IN OVARIAN EPITHELIUM IN MICE OF DIFFERING AGES AND TOTAL OVULATION NUMBER.

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One hypothesis of ovarian surface epithelial (OSE) carcinogenesis is that high levels of progesterone are protective, whereas high levels of androgen increase the risk of ovarian cancer (1). Epithelial-lined inclusion cysts are common in all women and are thought to be precursors of ovarian cancer (2). This study aimed to investigate spatial differences in expression of androgen receptor (AR) in the OSE and inclusion cysts in mice of varying ages and total lifetime ovulation number (total OV#).

Ovaries from Swiss Webster mice (total OV# median: range [n mice]) were collected from 3 month virgins (3 m; 113: 11-235 [55]), from 12 month old breeders (12 m; 217: 97-386 [21]) and from 8 month virgin mice, housed in split cages alongside a male, to induce continuous oestrous cycles (8 m; 629: 456-908 [16]). Ovaries were also collected from prepubertal mice (4 w; 0[9]) (3). AR immunohistochemistry was performed on 4 µm sections, cut from paraformaldehyde-fixed, paraffin-embedded tissue. Invaginations were defined as where OSE descended into the ovarian stroma, but remained connected to the OSE, while inclusion cysts were enclosed structures within the ovary, lined with epithelium. Inclusion cysts were observed only in 8 m and 12 m animals.

AR expression, was low in OSE from 3 m and 12 m animals, with very few squamous cells staining. However in 4 w and 8 m animals there were very few positive cuboidal cells also. In contrast the majority of cells lining inclusion cysts, with the exception of attenuated cells, were stained for AR. Low levels of AR expression were seen in invaginated OSE in 12 m animals. No differences were seen between invaginated epithelium and OSE in 3 m or 8 m animals. The strong upregulation of AR expression in cyst epithelial cells suggests that androgen responsiveness may play a role in the aetiology of ovarian cancer.

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28. LOCALISATION OF EPITOPES ON BRUSHTAIL POSSUM (TRICHOSURUS VULPECULA) ZONA PELLUCIDA 2 PROTEIN

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Immunisation of female possums (*Trichosurus vulpecula*) with recombinant possum zona pellucida 2 constructs (rZP2) led to 72-75% reduction in fertility, presumably through interference in secondary sperm-egg binding. In order to define those regions of the protein crucial to sperm-egg binding, antigenic regions on possum ZP2 protein were mapped. The amino acid sequence of full-length possum ZP2 protein was used to synthesise a complete set of overlapped 15-mer peptides. The peptides were used in a modified enzyme-linked immunosorbent assay (ELISA) to identify continuous epitopes recognized by antibodies in the sera of possums immunised with rZP2 constructs. Thirteen epitopes were located on possum ZP2 protein. Comparisons of the ELISA binding patterns of peptides to antibodies in the individual sera with the fertility status of the immunised possums revealed three significant peptide epitopes. One of the three epitopes bound to fixed possum spermatozoa from the caudal epididymis. Another corresponded to a similar region that has been shown to be integral to fertilisation in the mouse. The significance of these three individual epitopes in relation to fertilisation in the possum is currently under investigation.

29. EOSINOPHILS IN DEVELOPMENT AND FUNCTION OF REPRODUCTIVE TISSUES

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Eosinophils are a distinctive feature of some mucosal tissues, where they may be important in defence against infectious agents, but may also have a role in normal physiology by contributing to development and maintenance of epithelial integrity. Several recent studies have indicated that deficiencies in eosinophils or defects in eosinophil recruitment are associated with delayed postnatal mammary gland morphogenesis [1] and perturbations in the estrous cycle [2], [3]. These reports led us to examine the affect of an over-abundance of eosinophils on these processes. As interleukin 5 (IL-5) is central to the regulation of eosinophil growth and differentiation, eosinophilic IL-5 transgenic (IL-5 Tg) mice and normal wild type (WT) mice were compared to determine the impact of eosinophils on mammary gland development and reproductive function. Eosinophils were increased 13-fold and 4-fold in the uterus and mammary gland, respectively in IL-5 Tg mice compared to WT littermates. High numbers of eosinophils were also noted in the regressing corpora lutea of ovaries in IL-5 Tg mice whereas eosinophils were rarely seen in WT ovaries. Mammary gland development was transiently delayed in IL-5 Tg mice, with significantly less ductal extension and branching at 7 weeks of age. IL-5 Tg mice had significantly shorter estrous cycles than WT mice (4.7 ± 0.4 days versus 6.4 ± 0.5 days, respectively, $p = 0.03$). At day 18 of pregnancy, fetal resorptions are increased by 75% in IL-5 Tg heterozygote matings, and a decrease in fetal: placental ratio was observed (mean + SEM = 7.54 ± 0.09 in IL-5 Tg matings versus 8.26 ± 0.10 for WT, $p < 0.001$). Parturition was not compromised in IL-5 Tg mice, as judged by the gestation length and pregnancy outcomes. The birth weights and growth trajectories of pups were not significantly influenced by maternal IL-5 transgene expression. Our studies suggest that IL-5 transgenesis and/or eosinophilia may alter the estrous cycle and at least transiently delay ductal morphogenesis in the murine mammary gland. Eosinophils recruited into the ovary of IL-5 Tg mice may impact indirectly on both of these parameters. Despite these observations, the outcomes of pregnancy, parturition and lactation were not compromised by maternal over-expression of IL-5. *References:* [1] *Development*, 2000. **127**(11): p. 2269-82. [2] *J Reprod Fertil*, 2000. **120**(2): p. 423-32. [3] *Endocrinology*, 2001. **142**(10): p. 4515-21.

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30. EMBRYO DEVELOPMENT AND BIOTECHNOLOGY

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Mammalian preimplantation embryos are the only stage of development that may be grown in vitro independent of the maternal environment. The grown oocyte formed in the maturing follicle can be extracted from the ovary and either matured in vitro, or recovered after maturation in vivo, to produce viable embryos with a high developmental competence. This has enabled the in vitro production of mammalian embryos that may be fertilized and grown to the blastocyst stage in culture. While control of the germ cell, primary oocyte and growing oocyte in the preantral follicle is less well understood, it is possible to manipulate ovarian follicular dynamics by ovarian transplantation and may be controlled in the future by interference in the normal selection mechanisms of primordial follicle recruitment. Data is being generated from expression libraries in the human and other species to identify the key regulatory molecules for follicular and oocyte recruitment. This may be used to alter the reproductive life-span for the female.

The production of oocytes and embryos provides the basis for reproductive biotechnologies in domestic animal species and the human. Cattle reproductive technologies are well developed for production and human IVF is the foundation for the treatment of human infertility. The cattle industry accepts embryo production, embryo cryopreservation and embryo transfer as an integral part of the breeding strategies used. More recently, the cloning of animals, particularly cattle, has been explored with variable outcomes. The reprogramming of somatic cells by ooplasm doesn't completely reset the epigenetic regulators of gene expression, so that failure to erase the somatic epigenetic signature can result in abnormalities of development, including both placental and fetal compartments. This results in high rates of implantation failure, fetal growth and both anatomical and functional abnormalities which are often lethal. However, around 50% of offspring are viable and healthy despite the altered epigenetics. In the second generation the epigenetic irregularities are corrected during gametogenesis.

The ability to clone animals enables the production of transgenic animals in one generation. Other methods of transgenesis in animals include sperm-mediated transfection, which involves intracytoplasmic sperm injection (ICSI). Other new and interesting methods are being designed that involve the manipulation of gametogenesis.

Human IVF has evolved into a successful treatment for female and male infertility. ICSI may be used for testicular sperm and elongated spermatids. However, success with round spermatids or their precursors has not been clinically useful. The new and rapidly growing area of genetic diagnosis of point mutations and trinucleotide repeat disease in preimplantation embryos is an important new biotechnology in human medicine. These new molecular techniques are also being applied for aneuploid screening of IVF embryos, reducing the need for multiple embryo transfer for maintaining high pregnancy success rates. It is now possible to fingerprint sibling embryos to test new culture systems etc. With these capabilities, it is possible to identify genes that correlate with breast cancer and HLA type embryos for compatible transplants. Expression screening and apoptosis markers may also be used for determining embryo viability.

Preimplantation embryos are also a source of pluripotential embryonic stem (ES) cells. These may be derived from fertilized oocytes, parthenogenetic embryos and nuclear transfer embryos. It is of interest that embryos that have little or no developmental competence can produce ES cell lines with apparently complete ability to produce all types of terminal tissue types and to integrate in vivo into all tissues of interest. This may be similar to the ability of some adult cell types to transdifferentiate under experimental conditions in vitro and in vivo. These extraordinary cells may enable a new medical biotechnology of cell therapy for tissue repair and regeneration. These cells may also allow for gene therapy to be applied safely for the treatment of a wide range of genetic diseases.

These have been exciting times for biological sciences and promise a wonderful future research opportunity for young scientists. I thank all the scientists who have worked with me over the decades for the joy it has brought me for the contributions they have all made. I also acknowledge the funding of our creativity by numerous agencies and commercial bodies over the decades.

31. THE IMMUNOLOGICAL PARADOX OF PREGNANCY – SURVIVAL OF THE ULTIMATE ALLOGRAFT

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A successful pregnancy requires that the mother accepts a foreign body. This involves paradoxes for the immune system. The extensive genetic diversity between mother and offspring provides protein differences that, given exposure to the immune system, range from weakly to very strongly immunogenic. Thus the placenta and foetus represent “the ultimate allograft”. The highly active metabolic exchange across the placenta and dynamic developmental changes provide an opportunity for strong maternal immunity to develop. Establishment and maintenance of pregnancy involves immune tolerance, immunosuppression and immunomodulation. A state of selective maternal-anti-foetal immunosuppression exists which would be the dream of organ transplanters to mimic. Global immunosuppression obviously would be detrimental to the mother and a strong local anti-microbial immunity must be maintained.

The mammalian immune system has evolved to cohabit with microbes and parasites. It is perceived to consist of different but overlapping compartments, described as the Innate, Humoral and Cellular. These provide, respectively, extremely rapid containment and destruction of infecting organisms, an induced mechanism to recognise and remove particulate matter, and an induced mechanism to recognise and destroy infected cells in the body. These are achieved through quite distinct “pattern recognition” processes. It is apparent that selective control of each of these is crucial to successful pregnancy. A central feature of the immune system is the need to distinguish “self” from “foreign” and the provision of a variety of mechanisms to achieve this at very subtle levels. Its critical function to screen out mutating, potentially cancerous cells, is also relevant to our consideration of pregnancy and immunity. So how is the specific suppression induced?

There are several mechanisms thought to be crucial to the maternal / foetal immune state – perhaps more to be uncovered. Blocking the function of indoleamine 2,3-dioxygenase (IDO), a widely expressed enzyme responsible for tryptophan metabolism, results in maternal-anti-foetal immune responses. IDO is currently the most fashionable candidate to explain maternal immunosuppression. Secondly, control of the complement cascade, central to the innate immune system, is also essential to healthy pregnancy.

This presentation will introduce the immune mechanisms enabling the ultimate allograft, a foetus, to survive and thrive.

32. PRESENT AND FUTURE OPTIONS FOR PRESERVATION OF TESTIS TISSUE AND FUNCTION

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The testis releases two major products: male gametes and male sex steroids. While absent or insufficient secretion of steroids can easily be treated by administration of exogenous hormones, no cure has been established for a defect in germ cell development. The introduction of ICSI, however, has drastically changed the prospects for treatment, as generation of even a single postmeiotic male germ cell offers a chance of achieving a pregnancy. Therefore, new approaches based on male germ cell transplantation and testicular tissue grafting might be optimised to work for the generation of progeny. Interspecies transfer of germ cells showed that the recolonisation by stem cells is robust, as spermatogonial stem cells from phylogenetically distant species can recolonise the mouse testis. In contrast to the fact that spermatogonial stem cells are able to recolonise the seminiferous tubules, complete spermatogenesis is only observed when germ cells are transplanted between phylogenetically closely related species. We have developed an approach to infuse germ cells into monkey and human testes and tested whether germ cell transplantation can be applied as a tool for gonadal protection in non-human primates. However, due to the risk of tumour cell contamination germ cell transplantation will not eliminate the cryopreservation of sperm as a widely used tool and well accepted option for gonadal protection in postpubertal oncological patients. It appears likely that optimised techniques will instead become available to generate male gametes from preserved testicular material. One of the promising new approaches, and an alternative to germ cell transplantation, is grafting of testicular tissue. We have shown that testicular tissue from various newborn species grafted ectopically into the backskin of immunodeficient mice is capable of developing qualitatively complete spermatogenesis. Sperm obtained from these grafts have been used for the generation of progeny. Like the more advanced status of ovarian tissue grafting as a tool to protect fertility and substitute hormones in female patients, grafting of testicular tissue might become an important tool for fertility preservation and hormonal replacement therapy in the male.

33. DOES THE IMMUNE SYSTEM PLAY A ROLE IN REPRODUCTION? A CLINICIANS PERSPECTIVE

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Human reproduction is an enigma from an immunological perspective since the immune system can be responsible for infertility and pregnancy complications, while facilitating a successful reproductive outcome in the majority. In this presentation, a brief overview of the immune systems role in each stage of human reproduction will be presented, along with an example of the clinical consequences when things go wrong.

Cytokines are involved in the control of all aspects of ovarian function. including follicle growth, steroid production and ovulation. Around the time of ovulation a surge in local production of TNF- α , IL-1 β and GM-CSF results in the recruitment of macrophages into the follicle, which inturn release proteases and prostaglandins producing rupture of the dominant follicle and ovulation. Inhibition of these processes with non-steroidal anti-inflammatory drugs has been shown to inhibit ovulation and potentially result in infertility.

The presence of allogenic sperm in the female reproductive tract provides an immunological challenge to the female. In most cases, repeated exposure of a woman to sperm antigens will tollerise her towards her partners antigens, which inturn will enhance placental invasion and decrease her chances of developing pre-eclampsia. Alternatively, adverse maternal immune responses to sperm can lead to infertility. Examples include the ability of anti-sperm antibodies to block fertilisation or destruction of sperm by peritoneal macrophages activated by cytokines liberated from endometriosis.

The interaction between the embryo, endometrium and immune system has been extensively studied in rodents. Cytokines such as Il-1, IL-11, LIF, GM-CSF and CSF seem to benefit embryo development and implantation, while IFN γ and TNF α have a negative influence. While these cytokines may mediate their action directly on the embryo itself, many mediate their influence indirectly by modifying uterine leukocyte numbers and activity. Women experiencing recurrent miscarriage of immune aetiology have been identified as having abnormal endometrial cytokine expression and Natural Killer cell activity.

The immune system continues to play a role in human reproductive processes long after conception and early embryonic development. Inappropriate activation of the maternal immune system can lead to pre-term labour, fetal growth restriction and pre-eclampsia. Evidence will also be presented linking adverse maternal immune responses and cerebral palsy.

34. FINDING SOLUTIONS: SEMEN AND MATERNAL IMMUNE TOLERANCE

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Successful pregnancy requires a state of maternal immune 'tolerance' to accommodate antigens expressed by the conceptus. Implantation failure and placental pathologies such as recurrent miscarriage and pre-eclampsia largely reflect insufficiencies in maternal immune adaptation, but progress in devising therapeutic strategies to treat these conditions is stalled because the mechanisms underlying induction and maintenance of maternal tolerance are unknown. Increasingly, clinical and experimental data support the proposal that insemination has consequences for the reproductive process beyond delivery of male gametes. An emerging hypothesis is that insemination comprises the 'inductive phase' of the maternal immune response to pregnancy, since it is in the context of semen that the female tract is first and most frequently exposed to paternal transplantation antigens shared by the conceptus. Our studies in mice indicate (1) that at insemination, the maternal tract is better poised to process antigens than at any other stage of the cycle or pregnancy; (2) that semen contains high concentrations of 'immune-deviating' cytokines, including transforming growth factor (TGF) β and prostaglandin E, known to induce immune tolerance in other mucosae; (3) that exposure to semen is sufficient to induce a transient state of functional immune tolerance to paternal transplantation antigens, and (4) that insemination is causally linked to the activation and expansion of populations of lymphocytes recruited into the implantation site. Recent studies in women show that comparable events occur in the human cervix after intercourse. Together, these studies indicate that exposure to semen may promote the success of pregnancy through orchestrating a maternal immune response conducive to optimal placental invasion and function. Since it is the inductive phase that principally determines the strength and quality of any immune response and is most amenable to therapeutic manipulation, we expect that unravelling the cellular and molecular processes linking insemination with implantation will facilitate development of new clinical interventions for treatment of immune-based fertility disorders.

35. ESTRADIOL-MEDIATED CYCLIN D2 EXPRESSION: A ROLE FOR ER α ?

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Cyclin D2, a cell cycle regulator, plays a key role in the proliferation of ovarian granulosa cells (1) and thus follicle development. Follicle stimulating hormone (FSH) and estradiol (E2) have been shown to enhance cyclin D2 mRNA expression *in vivo* (2). It is unclear however, which estrogen receptor (ER), ER α or ER β , mediates this response. We addressed this question using cultured granulosa cells (3), which exclusively express cyclin D2 (no other cyclins). Granulosa cells were isolated from the ovaries of diethylstilboestrol (DES)-treated, 21 day old Sprague-Dawley rats. Cells in triplicate wells, were plated overnight (day 1), the media was changed (day 2) and treatments were added on day 3 for 2 h. FSH (100ng/ml) and E2 (1 and 10nM) were added separately and in combination. RNA purified from freshly isolated and cultured granulosa cells, was reverse transcribed and subjected to real time PCR analysis of cyclin D2, ER α , ER β and GAPDH (data normalisation) mRNAs. Experiments were repeated three times. Cyclin D2 mRNA expression by freshly isolated granulosa cells was elevated 4-fold in comparison to control cells plated for 2 days. In response to FSH, cyclin D2 mRNA expression was stimulated 2-fold (compared to control cells). In contrast, E2 at 1 and 10nM, failed to influence cyclin D2 expression. The combined FSH and E2 treatment gave results consistent with the stimulation of cyclin D2 mRNA by FSH alone. ER β mRNA expression by granulosa cells did not change with time in culture or in response to treatment. ER α mRNA expression by granulosa cells, down-regulated by *in vivo* DES treatment (3), was not restored with time in culture. We hypothesise that ER α not ER β is involved in the regulation of cyclin D2 expression by E2. The down-regulation of ER α mRNA, induced by the *in vivo* exposure to DES and its continued decline *in vitro*, may account for the inability of E2 to enhance cyclin D2 mRNA expression. These findings are consistent with our belief that ERs play different roles in the ovary: ER β mediates differentiated functions and ER α cellular proliferation. [1] Sicinski et al., (1996) *Nature* 384: 470-474. [2] Robker et al., (1998) *Molec. Endocrinol.* 12:924-939. [3] Drummond et al., *Molec. Cell. Endocrinol.* 149: 153-161. Supported by the NH&MRC of Australia (Regkey 983212).

36. PURIFICATION OF HUMAN GRANULOSA CELLS FROM FOLLICULAR FLUID SAMPLES FOR USE IN SERIAL ANALYSIS OF GENE EXPRESSION (SAGE)

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Follicular fluid collected at the time of IVF is a prime source of human granulosa cells. However, follicular fluid often contains red and white blood cells. This contamination must be removed for meaningful *in vitro* or molecular studies. Previous studies have purified granulosa cells by a combination of density gradients (1), the use of magnetic anti-CD45 beads (2) and also by flow cytometry (3). We propose a purification methodology which exploits clumps of mural granulosa cells present in follicular fluid. Follicular fluid samples were collected from female patients undergoing IVF for male factor infertility at the Otago Fertility Center, Healthcare Otago. Centrifugation through a 50% Percoll gradient (600g) was used to remove red blood cells. Cells were collected from the Percoll interface and viewed under a dissecting microscope. Clumps of granulosa cells were mechanically removed with a glass pipette and washed in Hams F10 media. To determine white blood cell content cells were stained with antibodies (CD3, CD14, CD16, CD19) and quantified by flow cytometry. White blood cell contamination was found to be between 2 and 4%. An FSH antibody confirmed purified cells were positive for FSH. In addition, cell clumps were examined by histology and electron microscopy and found to contain homogenous cells, which were identified as granulosa cells. This method provides a relatively simple and fast method to purify human granulosa cells, which is applicable to molecular studies.

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37. REMODELLING OF EXTRACELLULAR MATRIX IN BOVINE FOLLICLES AT OVULATION

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Within the membrana granulosa we have identified a unique extracellular matrix (fociemix) consisting of basal lamina like material that develops as follicles enlarge (1). To elucidate its role we examined its fate following ovulation, by immunohistochemical analysis of the composition of fociemix and the follicular basal lamina. Ovulation in *Bos indicus* cattle (426-562 kg) was synchronized as follows: Day 1; intra-vaginal progesterone releasing device (CIDR) plus 2 mg 17 β -oestradiol (Cidriol); Day 9; prostaglandin (Estrumate); Day 10; removal of CIDR. Animals were randomly allocated to 3 groups: Group 1, Control (n = 6), no further treatment; Group 2 (n = 8), injection of 25mg LH (Lutropin) i.m. on Day 11; Group 3 (n = 8), 25 mg i.m. LH on Day 10. Ovaries were collected on Day 12, such that Group 2 ovaries were collected 12 – 14 h and Group 3 38 - 40.5 h after LH. Pre- or recently-ovulatory follicles were dissected from the ovary, in an apical-basal orientation, and frozen in OCT compound or fixed in 2.5% glutaraldehyde. Immunohistochemistry was performed on OCT embedded tissue and the following basal lamina components localised: collagen type IV (α 1), laminin chains β 1, β 2, γ 1, nidogen, perlecan and the extracellular matrix proteoglycan versican. Group 1 follicles were 13-20 mm in diameter and Group 2 follicles 6-18 mm. Pre-ovulatory follicles in Group 3 measured 10, 11 and 18 mm, and other follicles had ovulated. Following ovulation the follicular basal lamina remained immunopositive for laminin chains β 2, γ 1 and nidogen. Laminin β 1 was not detected. Staining for the α 1 chain of collagen type IV was reduced compared with basal lamina staining of smaller antral follicles (2), and discontinuous in recently ovulated follicles, indicating degradation of the basal lamina. Perlecan was absent from the membrana granulosa and follicular basal lamina of most Group 3 pre- and post-ovulatory follicles as was versican. However, granulosa cells (especially Group 2 follicles) showed intense cytoplasmic staining for versican, suggesting it was up regulated. The findings indicate that matrix in the basal lamina and within the membrana granulosa of follicles actively changes at the time of ovulation. (1)Biol Reprod 2001: 64, Suppl 1, Abstract 101. (2)Biol Reprod 1998: 59, 1334-1341.

38. PROTEOGLYCANS: OSMOTICALLY ACTIVE MOLECULES IN FOLLICULAR FLUID

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During follicle development an antrum filled with follicular fluid develops. Follicular fluid contains proteins derived from plasma, at progressively lower concentrations than plasma for increasing sizes above 100 kDa. Follicular fluid also contains molecules, like proteoglycans (PGs), produced by the granulosa cells. PGs consist of glycosaminoglycans (GAGs) covalently attached to a protein core. GAGs have osmotic activity. We investigated the possibility that PGs create an osmotic gradient, driving the recruitment of fluid from the vascularised theca layer into the follicular antrum. Ovaries were collected at a local abattoir from non-pregnant cycling heifers. Antral follicles >11 mm were dissected and the follicular fluid aspirated. The remainder of each follicle was fixed in Bouin's solution for subsequent histological assessment of follicular health and atresia. Four individual pools of follicular fluids from healthy and atretic follicles were analysed. Pools were dialysed with membranes of differing molecular weight cut offs (10, 100, 300, or 500 kDa) for 24 h at 4C. The colloid osmotic pressure of the fluid was measured (Gonotec 050 colloid osmometer) with 10 kDa membrane. A reduction in osmotic pressure of 60 \pm SEM 6% and 80 \pm 13% in fluid from healthy and atretic follicles, resulted from the removal of molecules >500 kDa, indicating that large molecular weight molecules contribute significantly to the overall osmotic potential of follicular fluid. Pools were then treated with enzymes (DNAse, proteinase K, hyaluronidase, chondroitinase ABC, keratanase, collagenase, or heparinase) and then dialysed at 100 kDa cut off at 37C in 2M NaCl to prevent aggregation of macromolecules, followed by dialysis at 37 $^{\circ}$ C against H₂O. Osmotic pressure was reduced 42 \pm 2% and 33 \pm 3% by digestion of hyaluronic acid (0.5u/20 μ l hyaluronidase from *Streptomyces hyalurolyticus*, 4 h, 37 $^{\circ}$ C, pH 6) in healthy and atretic follicles, respectively. Digestion of GAG chains (0.02u/20 μ l, chondroitinase ABC from *Proteus vulgaris*, 4 h, 37 $^{\circ}$ C, pH 8) resulted in a reduction in OP of 54 \pm 14% and 20 \pm 3.6% in fluid from healthy and atretic follicles. This suggests that PGs in follicular fluid, too large to readily cross the follicular wall, can exert osmotic pressure. These PGs may therefore contribute to the accumulation of follicular fluid.

39. THE INFLUENCE OF THE OOCYTE DURING *IN VITRO* MATURATION ON THE METABOLISM OF BOVINE CUMULUS-OOCYTE COMPLEXES

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Interactions between the oocyte and surrounding cumulus cells are bi-directional and oocyte-secreted factors have profound effects on cell growth and differentiation. The aims of this study were to determine the influence of the oocyte on bovine cumulus cell metabolism during in vitro maturation. Cumulus-oocyte complexes (COC) were aspirated from antral follicles from abattoir-derived ovaries. Complexes were oocyctomised (OOX), leaving the cumulus and zona pellucida intact. Intact COC, OOX and OOX plus denuded oocytes (OOX+DO) were cultured individually in 10µl drops of IVM media (TCM 199 + pyruvate + BSA + LH + FSH) at 39°C in 5% CO₂ in air. Oxygen consumption measurements were taken at 1-4h, 11-14h and 21-24h of culture using a non-invasive microfluorescence method [1]. Spent media was collected and analysed for glucose and pyruvate consumption and L-lactate production [2]. The DNA content of individual complexes was quantified using PicoGreen fluorescence dye to enable metabolic measurements to be expressed per ng of DNA. There were no significant differences between COC, OOX and OOX+DO in any of the metabolic parameters measured, providing further evidence that oocyte-specific factors, while regulating cell proliferation, have few measurable effects on bovine cumulus mass expansion [3]. However, regardless of treatment, metabolic profiles changed over the 24h maturation period. There were significant increases (P<0.05) in the uptake of oxygen (4h: 50.2±5.64 vs. 24h: 94.0±14.9 pl/ngDNA/h), glucose (4h: 25.5±4.07 vs. 24h: 39.9±6.23 pmol/ngDNA/h) and pyruvate (4h: 2.15±0.22 vs. 24h: 4.69±0.85 pmol/ngDNA/h). L-lactate production remained low and constant over the 24h, with less than 3% of the consumed glucose being converted to L-lactate. Oxidation of glucose via oxidative phosphorylation accounted for less than 2% of that consumed. Little glucose is therefore utilised for ATP production, suggesting an alternative fate, such as synthesis of extracellular matrix during cumulus expansion. 1. Houghton, FD, et al (1996) *Mol Reprod Dev*, 44: 476-85. 2. Thompson, JG, et al (1996) *J Reprod Fertil*, 106: 299-306. 3. Ralph, JH, et al (1995) *Mol Reprod Dev*, 42: 248-53. Supported by ARC C00107702 and Cook Australia.

40. EXPRESSION WITHOUT SECRETION OF TGF-β1 AND TGF-β2 BY BOVINE AND MURINE OOCYTES

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It is now well established that oocytes are potent regulators of cumulus cell function and thereby their own microenvironment. This activity is commonly attributed to members of the TGF-β superfamily because of the capacity of these growth factors to mimic oocyte-secreted factors (1). The aims of this study were to examine in bovine and murine oocytes (1) the expression pattern of TGF-β1 and β2 mRNA throughout meiotic maturation, and (2) whether oocytes secrete TGF-β1 and β2. Follicular cells and oocytes were collected from abattoir derived bovine ovaries and from eCG-primed immature mice. RNA was extracted from follicular cells and from oocytes at defined stages of meiotic maturation; in mouse from both in vivo and in vitro matured oocytes at 0, 3, 6, 9 and 24h, and in cows from immature and in vitro matured oocytes. RNA was reverse transcribed and cDNA was amplified with specific primers targeted against TGF-β1 and β2. Both bovine and murine denuded oocytes (DO) expressed TGF-β1 and β2 mRNA at all stages of meiotic maturation. All somatic cells examined expressed mRNA for both TGF-β transcripts. The possibility of CC contamination of DO samples was excluded by screening for FSH receptor mRNA expression by PCR. In order to determine whether DO secrete TGF-β proteins, mural granulosa cells (MGC) from both species were cultured in the presence of either DO or TGF-β +/- a TGF-β pan specific neutralising antibody. MGC were assessed for [³H]-thymidine incorporation as a marker of DNA synthesis. [³H] counts were increased significantly (P<0.05) by TGF-β (3 or 14 fold above controls) and by DO (3 or 8 fold) in bovine and murine MGC respectively. The TGF-β antibody was able to completely neutralise the proliferative effects of exogenous TGF-β but not of DO. These results suggest that although oocytes from both species express TGF-β1 and β2 mRNA transcripts, these may be stored as maternal mRNA and not translated, indicating that TGF-β does not account for the potent mitogenic effects of oocytes on granulosa cells. (1) Salustri, A, et al (1990) *J. Biol. Chem.* 265:19517-23 Supported by NHMRC.

41. DEVELOPMENT AND ANTRUM FORMATION IN MARSUPIAL PREANTRAL FOLLICLES CULTURED IN A SERUM-FREE MEDIUM

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Culture of isolated preantral follicles is potentially an important source of mature eggs for research and assisted reproductive techniques, where an understanding of both the prepubertal and adult system is important. In the only published report of follicle culture in a marsupial species [1], the culture system supported follicular growth but no antral development was observed. The aim of this study was to compare the growth and development of preantral follicles from prepubertal and adult tammar wallabies, *Macropus eugenii*, in a serum-free culture system. Preantral follicles were dissected from ovaries of 6 prepubertal and 5 adult tammars and cultured individually in drops of medium (Dulbecco's modified essential medium with various additives) at 37°C in a humidified gas environment of 6% CO₂ in air for 4 days. Follicles were measured and observed each day. Growth rates were expressed in terms of proportional increase in volume over the culture period and the follicles were assessed for signs of antrum development. Almost all follicles increased in volume (Prepubertal, 53/54; Adult, 54/55) and there was no significant difference in the average volume increase between groups (Prepubertal, 48%; Adult, 59%; $p > 0.05$). There was, however, a significant difference between groups in the incidence of antrum formation (Prepubertal, 10/54; Adult, 0/55; $p < 0.05$). While both groups of follicles had a similar growth rate, only follicles from prepubertal animals showed signs of antrum formation. This may reflect the different prior exposure of the follicles to endogenous hormones. In the adult system, follicles are exposed to an indeterminate hormonal environment and consequently have more variability and a lower developmental potential than follicles from prepubertal animals. Modifications to the culture system may provide more appropriate conditions for follicle growth and development, in particular for antrum formation. This is the first report of antrum development in preantral follicles *in vitro* in a marsupial species. The culture system did not contain serum and indicates that antrum formation in the tammar may be independent of a number of growth factors that are usually present in serum.

1. Butcher L, Ullman SL. (1996). *Reproduction, Fertility and Development*. **8**: 535-539

42. INCORPORATION OF BRDU INTO THE OVARIAN SURFACE EPITHELIUM IN MATURE MOUSE OVARY SLICES MAINTAINED IN CULTURE

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The original aim of this work was to study the growth of small follicles *in vitro* using slices of mature mouse ovary in culture. An unexpected finding was that some ovarian surface epithelial cells (OSE cells) were found to incorporate bromodeoxyuridine (BrdU) suggesting the possibility of OSE proliferation. Two further cultures were undertaken in which 8 ovaries at each time were removed from 6 to 8 week old animals, dissected into thirds or halves and pooled before being cultured for 24, 48 and 72 hrs in Waymouth's medium containing 10µM BrdU. In addition adult mice were injected with 1mg BrdU every 8 hrs for 3 days to assess ovary BrdU labelling *in vivo*. Intact ovaries and ovary slices were fixed and sections stained immunohistochemically to localise BrdU. Healthy follicles with labelled granulosa were observed in ovaries from both the injected animals (51/57 labelled) and the cultured slices (24/32 and 35/43 labelled follicles after 72 hr in culture). As shown previously (Davies, B.R. et al, *Gynecol. Endocrinol.* **13**: 75-81, 1999.) less than 0.4% of >1000 OSE cells were found to be BrdU positive after injections into the animals. In culture, however, the OSE showed increasing BrdU incorporation with 5%, 20% and over 50% labelled cells at 24, 48 and 72 hr. respectively. No other tissues within the ovary were found to have significant numbers of labelled cells. In a few examples the mesothelial lining of the ligament suspending either the ovary or oviduct also had labelled cells. These results indicate the considerable potential for proliferation of OSE cells *in vitro*. BrdU incorporation may be induced by an OSE-specific proliferative factor, or factors present in the culture medium. Alternatively the removal of the ovary from the mouse may eliminate an inhibitory influence or be a wound-response to the act of dissection of the ovary. Whatever the reason, it remains to be determined if the cells of the OSE can proceed *in vitro* to enter and complete mitotic division.

43. SEX DIFFERENCES IN THE NEURONAL INPUTS FROM THE HYPOTHALAMUS AND BRAINSTEM TO THE REGION OF THE GONADOTROPHIN-RELEASING HORMONE (GnRH) NEURONES IN THE MEDIAL PREOPTIC AREA

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In sheep, the majority of GnRH neurones are located in the rostral preoptic area and are regulated by a complex network of neurones. The location of these neurones in the ram remains unknown. Retrograde tracing studies in the ewe have mapped the location of cells that project to the rostral preoptic area (1), but similar data are not available for the ram. The regulation of GnRH secretion in the sheep is sexually dimorphic and GnRH neurones in ram lambs receive about half the number of synaptic inputs as do ewe lambs (2). Thus, it cannot be assumed that the inputs to the GnRH neurones are the same in males and females. The retrograde tracer, FluoroGold, was used to trace the neuronal inputs from the hypothalamus and brainstem to the rostral preoptic area of the ram and to compare this with the ewe. Retrogradely labelled cells were observed in the same hypothalamic and brainstem regions as previously reported for the ewe (1) but the ram had a significantly ($P < 0.05$) greater number of retrogradely labelled cells/section in the dorsomedial nucleus than the ewe and fewer ($P < 0.05$) retrogradely labelled cells in the ventromedial nucleus. These nuclei have been implicated in the regulation of GnRH secretion (3) and our results may partially explain the sex differences in how GnRH secretion is regulated. Fluorescence immunohistochemistry was used to determine the neurochemical identity of some of these cells in the ram. Very few tyrosine hydroxylase-containing neurones in the A14 group ($< 1\%$), adrenocorticotrophic hormone ($< 1\%$) and neuropeptide Y-containing neurones (1-5%) in the arcuate nucleus contained FluoroGold. Within the brainstem, virtually all FluoroGold-containing cells in the A1 region and about half in the A2 region co-stained for dopamine β -hydroxylase. No other retrogradely labelled cells in the brainstem were noradrenergic. Although dopamine, β -endorphin, and neuropeptide Y have been implicated in the regulation of GnRH secretion in males, it is unlikely that these neurotransmitters regulate GnRH secretion via direct inputs to GnRH neurones. (1) Tillet et al. 1993. *J Comp Neurol* 330:195-220. (2) Kim et al. 1999. *Biol Reprod* 61:599-605. (3) Scott et al. 2000. *Anim Reprod Sci* 60/61:313-326.

44. *IN VITRO* REGULATION OF ACTIVIN / INHIBIN SUBUNITS, FOLLISTATIN AND BAMBI IN THE 3 DAY OLD RAT TESTIS BY ACTIVIN, FOLLISTATIN AND FSH

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Members of the transforming growth factor (TGF) β superfamily and their antagonists are produced by several cell testicular types during early postnatal development. We have observed a discrete switch from activin to follistatin expression within gonocytes as they transform into spermatogonia, and we have illustrated their functional effect on somatic and germ cells (Meehan *et al* 2000. *Dev Biol* 220:225). This led to the hypothesis that these proteins may regulate their own expression and thereby modulate this stage of testicular differentiation. Using 24 hour cultures of 3 day old rat testis fragments and real-time PCR analysis, expression levels of activin and inhibin subunits (α , β A and β B), follistatin and BAMBI mRNAs were measured in response to activin A, follistatin and FSH relative to media alone. BAMBI is a pseudoreceptor that antagonises signalling by activin, TGF β and some bone morphogenetic proteins, while both follistatin and inhibin can inhibit activin signalling. The inhibin α subunit mRNA was increased 3-fold by FSH (to 280% of control, $p < 0.001$), while FSH had no significant effect on the other target mRNAs. Addition of follistatin to these cultures reduced follistatin mRNA to 38% ($P < 0.001$) of the control group value. Activin A and the combination of FSH and follistatin also partially reduced levels of follistatin mRNA. In contrast, activin A and follistatin treatments each increased activin β B mRNA (180% $p < 0.001$ and 160% $p < 0.05$, respectively). BAMBI mRNA was significantly reduced by the addition of activin A (67% $p < 0.05$), but neither follistatin nor FSH alone or in combination affected BAMBI expression. This study provides evidence that activin and follistatin may regulate their own actions during postnatal testis development. The data indicate the presence of a negative auto-feedback loop involving follistatin. In contrast, the effect of activin A to inhibit expression of BAMBI and to stimulate activin β B subunit mRNA levels would reinforce the maintenance of activin bioactivity. This finding correlates with the upregulation of BAMBI mRNA observed in germ cells using *in situ* hybridisation as gonocytes transform into spermatogonia and activin A protein is lost. This approach has identified the potential for interactions between activin and its regulatory molecules at the onset of spermatogenesis.

45. REGULATION OF SERTOLI CELL ACTIVIN AND INHIBIN SECRETION BY HORMONES, CYTOKINES AND THE SEMINIFEROUS EPITHELIAL CYCLE

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Activin A and the inflammatory cytokines, interleukin-1 α (IL-1 α) and IL-6, are produced by the Sertoli cell and are implicated in control of spermatogonial and spermatocyte development. Activin A also inhibits IL-1 and IL-6 production and action in several other systems, and therefore is a potential negative regulator of testicular inflammation. However, there have been few studies describing the production and regulation of activin A in the testis. Sertoli cells from immature (20 day-old) or adult rats were cultured for 48h in the presence of IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1ra), IL-6, the inflammatory mediator lipopolysaccharide (LPS from *E. coli*), testosterone and/or ovine FSH or dibutyryl cAMP (cAMP). Adult rat seminiferous tubules were stage-dissected and incubated with test substances for 72h. Dimeric activin A and inhibin B in the culture media were measured by specific ELISAs. In immature and adult Sertoli cells, activin A secretion was stimulated by IL-1, and inhibited by FSH/cAMP. Conversely, inhibin B was stimulated by FSH/cAMP and inhibited by IL-1. LPS induced a large increase in activin A, which was partially inhibited by IL-1ra, and a decrease in inhibin B secretion by the Sertoli cells. In cultured seminiferous tubules, activin A secretion occurred across all stages, with a distinct peak of secretion at stage VIII. This secretion was almost completely blocked by IL-1ra. In contrast to isolated Sertoli cells, activin A secretion by cultured seminiferous tubules at stages VII-VIII was stimulated by cAMP. Inhibin B secretion was stimulated in all stages by cAMP, but not by IL-1. These data confirm that Sertoli cell activin A production is positively regulated by endogenous and exogenous IL-1 via a cAMP-independent pathway, although other locally-produced cytokines also may be involved. Activin A is negatively regulated by FSH/cAMP, but this arm of the regulation is modulated by specific germ cell associations. Inhibin B, on the other hand, is positively regulated by FSH/cAMP, and negatively regulated by IL-1. There is reciprocal control of activin A and inhibin B throughout the cycle of the seminiferous epithelium by IL-1 and FSH, which has important implications for control of spermatogonial development and the effects of inflammation on the pituitary-testicular axis.

46. CHARACTERISATION OF PLASMA INHIBIN FORMS IN FERTILE AND INFERTILE MEN

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Inhibin B is the major feedback regulator of FSH in the male. In vitro data suggest that precursor high mol wt forms of inhibin have reduced bioactivity. The aim of this study was to determine the levels of high mol wt forms of inhibin B and inhibin α -subunit in plasma in fertile men and men with various forms of infertility. Plasma from control fertile men (n=11), and men classified by testicular histology as hypospermatogenesis (HypoSG, n=4), Sertoli Cell Only (n=4), germ cell arrest (n=2) and Klinefelter's Syndrome (n=3), and following chemotherapy (n=4) were fractionated using a combined immunoaffinity chromatography, preparative SDS-PAGE and electroelution procedure. Inhibin forms were determined by ELISAs for inhibin B, total inhibin (all forms of inhibin containing the α C fragment) and pro- α C. In fertile men and men with HypoSG, inhibin B was identified as mature (26-30k) and precursor (60k) forms with similar proportions (29.1% vs 25.2%, respectively) of the 60k form in both groups. Inhibin B levels were too low to be assessed in the other infertile groups. Precursor forms of pro- α C (46k, pro- α N- α C) also showed no differences between control (7.4%) and all infertile groups (7.4-11%).

To establish if the Pro- α C ELISA was detecting the precursor forms of inhibin B containing the pro- fragment, normal male plasma was repeatedly immunoabsorbed with antiserum (INPRO) to the pro- region and the remaining immunoabsorbed plasma then immunoabsorbed with antiserum to the α C subunit. No inhibin B was detected in the INPRO-absorbed sample and the profile of immunoactivity as determined by Pro- α C and total inhibin ELISAs were similar. Similarly, the profiles of inhibin B and total inhibin in the α subunit-absorbed sample were identical. These data indicate that the pro- α C ELISA is detecting all forms of the monomeric α C subunit but not the precursor inhibin B forms and that the inhibin B ELISA is detecting all dimeric inhibin B. The two ELISAs are thus separately detecting all the free α subunit and inhibin B in male plasma.

It is concluded that a) the Pro- α C and inhibin B ELISAs provide an unambiguous assessment of the levels of these inhibin forms in the circulation and b) the proportion of precursor inhibin B forms in plasma (25-30%) is unchanged in men with sub-fertility.

47. CONTRACEPTIVE EFFICACY OF A DEPOT ANDROGEN AND PROGESTIN COMBINATION IN MEN

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Hormonal male contraception was theoretically feasible for decades with formal proof of concept demonstrated in landmark WHO male contraceptive efficacy studies in early 1990s. Combined testosterone plus progestin treatment is the most promising strategy, but no contraceptive efficacy studies are reported. The superior efficacy plus lesser demands on compliance suggest depot regimens are ideal for hormonal male contraception. We completed the first study estimating the contraceptive efficacy of a depot hormonal regimen, an androgen/progestin combination, in men. Fifty-five men in stable relationships seeking a change in contraceptive method were administered testosterone (four 200 mg implants, 4 or 6 monthly) and a progestin (300 mg DMPA, 3 months). Once sperm output was suppressed (<1M/mL, two consecutive months), men entered a 12 month efficacy period when all other contraception was ceased. Sperm output was monitored monthly. There were no pregnancies in 426 person-months (35.5 person-years) of efficacy exposure (upper 95% CL for contraceptive failure rate, 8% per annum). Mean sperm density fell rapidly (by 88% @1 month and 98% @2 months) allowing nearly all men to enter efficacy within 3 months (50% @ 1 month, 83% @ 2 months, 94% @ 3 months). Only 2/55 (3.6%) men were unable to enter efficacy due to insufficient suppression of sperm output. A few men treated with T implants at 6 month intervals demonstrated androgen deficiency symptoms &/or escape of spermatogenic suppression (predicted by suboptimal gonadotropin suppression) between months 5-6; men receiving T implants at 4 months intervals had no androgen deficiency nor loss of gonadotropin and sperm output suppression. Recovery was slower than anticipated (median time 6-9 months to sperm density 20M/mL) presumably due to DMPA kinetics. Discontinuations were for protocol (12), personal (10) and medical (5) reasons but there were no serious adverse effects related to drug exposure. We conclude that the first prototype depot androgen/progestin combination regimen provides high contraceptive efficacy with satisfactory short-term safety but slow recovery of spermatogenesis. Larger studies with purpose-developed depot combinations are required to clarify the overall safety and efficacy of the most promising approach to hormonal male contraception. This study was supported by CONRAD.

48. PRESENCE OF IMMUNE MODULATING MOLECULES IN BOAR SEMINAL PLASMA

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Factors in seminal plasma are recognised as potential modulators of reproductive success in the pig. Experiments in rodents and more recently in pigs indicate roles for specific proteins in seminal plasma in inducing cellular and molecular changes in the female reproductive tract. An active constituent of murine seminal plasma has been identified as the potent immune modulating cytokine transforming growth factor beta (TGF β)¹. In vitro experiments suggest the response to TGF β is further influenced by interferon (IFN) γ and lipopolysaccharide (LPS). To investigate whether immune modulating molecules are present in pig semen, the content of TGF β ₁, TGF β ₂, IFN γ and LPS in boar seminal plasma were measured. Semen was collected from 42 Large White boars of similar age and of known fertility, and seminal plasma was prepared by centrifugation. Commercial ELISA assays were used to measure TGF β ₁, TGF β ₂ (both Promega) and IFN γ (Endogen) and LPS was measured by Limulus Amebocyte Lysate assay (Bio Whittaker). TGF β ₁ and TGF β ₂ were detected in all samples [median (range) = 185 (91-423) and 50.1 (26-101) ng/ml respectively], IFN γ was detectable in only 2 samples (39 & 40 pg/ml) and endotoxin was present in 30 samples [12.5 (11-193) EU/ml]. Variation over time and effect of frequency of collection on TGF β content was also evaluated. Content of both isoforms varied <18 % within individual boars over 4 months (n = 42 boars, 4-8 collections). When semen was collected 3 times within a week (n = 3 boars), TGF β content was diminished by up to 60% by the third collection. This data demonstrates that cytokines, in particular TGF β are present in large quantities in boar seminal plasma and that the level of this cytokine varies between boars. Cytokine content remains relatively constant within an animal over time but decreases in response to frequent collection of semen. These findings show that immune modulating moieties, with the potential to cause beneficial type 2 skewing of the maternal immune response in early pregnancy, might be the active fertility modulating constituents of seminal plasma. This information might provide the basis for development of prognostic assays for the evaluation of fertility in pigs. *Supported in part by the Australian Pork Limited.* [1]Tremellen KP, Seamark RF and Robertson SA: *Biol. of Reprod.* **58** (5) 1217-1225 (1998).

49. INFLUENCE OF OXYTOCIN ON OXYTOCIN RECEPTOR AND 5 α -REDUCTASE TRANSCRIPTION IN THE RAT PROSTATE

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Dihydrotestosterone (DHT) is essential for normal function and growth of the prostate. DHT is produced by the reduction of testosterone, catalysed by 5 α -reductase. Two isoforms of the 5 α -reductase enzyme exist. In the rat prostate type I is expressed in the epithelium and type II in stromal cells. Oxytocin increases the activity of this enzyme but it is unclear how. Neither expression nor distribution of oxytocin receptor have previously been described in the rat prostate. This study aimed to determine the distribution of oxytocin receptor, and to investigate if oxytocin influences expression of its receptor and the isoforms of 5 α -reductase. Adult male Wistar rats were treated daily with either 5 μ g/Kg oxytocin, 2 μ g/Kg of a specific oxytocin antagonist (OTA), both oxytocin and OTA, or saline (control) respectively for 3 days. On day 4 rats were euthanased by CO₂ inhalation and the ventral prostate removed. Western blot and immunohistochemistry, employing a polyclonal antibody to the 3rd intracellular loop of the oxytocin receptor, identified a specific peptide of ~60 kDa and localised oxytocin receptor to both the stromal tissue and epithelium. Total RNA was used in a semi-quantitative RT-PCR approach to estimate relative levels of expression of 5 α -reductase type I and II, and oxytocin receptor with levels normalised against a β -actin amplicon as internal control. Oxytocin treatment significantly decreased ($P < 0.05$) oxytocin receptor mRNA to 50% that of control. In contrast, oxytocin treatment significantly increased ($P < 0.001$) 5 α -reductase II expression by 44%. Both changes were inhibited by OTA, indicating a specific action. No significant difference was found in levels of 5 α -reductase I expression between treatment groups. In conclusion, the localisation of the oxytocin receptor would potentially allow oxytocin to affect both isoforms of 5 α -reductase. The increase in only type 2 expression suggests that activity of both enzymes are regulated differently by oxytocin. Furthermore, downregulation of oxytocin receptor by its ligand provides another site of control.

50. INTROMISSION FAILURE LEADS TO INFERTILITY IN TGF β 1 NULL MICE

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Transforming growth factor β 1 (TGF β 1) is a multifunctional cytokine suggested to play several important roles in male reproductive function including regulation of spermatogenesis, steroidogenesis and modulation of development of the seminal vesicle, penis and prostate. The TGF β 1 null mutant mouse (TGF β 1^{-/-}) offers the unique opportunity to study the role of TGF β 1 in vivo. We have previously found that TGF β 1^{-/-} male mice are infertile with none of eight null mutants producing mating plugs or pregnancies when housed with females for several weeks. The reproductive organs of TGF β 1^{-/-} males are of normal weight and histology, and spermatogenesis proceeds normally. We have extended investigations into this phenotype by analysis of mating behaviour of TGF β 1^{-/-} males. Adult TGF β 1^{+/-} and TGF β 1^{-/-} males were housed singly and a receptive superovulated B10 female introduced, 2 hours of interaction between each male and female was recorded at 2200-2400 hours using a digital video camera under red light. Data on the latency, number and duration of occurrences of male behaviours including (1) anogenital investigation; (2) mounting; (3) intromission and (4) ejaculation was recorded. Both TGF β 1^{+/-} and TGF β 1^{-/-} males displayed initial sexual interest in the female by way of anogenital investigation. Four of six TGF β 1^{-/-} males mounted the female, while all TGF β 1^{+/-} males mounted females. Two of six TGF β 1^{-/-} males also showed intromission behaviour however these displays were shorter in duration than intromission behaviour displayed by controls and may be false intromissions. All TGF β 1^{+/-} males ejaculated during the test period, and did not display further sexual behaviour. In contrast, no TGF β 1^{-/-} males ejaculated during the test period and these mice continued to display mounting behaviour leading to significantly more total mounts over the 2 hour period [average(range), 11(10-27) versus 48(0-71) total mounts, TGF β 1^{+/-} and TGF β 1^{-/-} respectively]. However as TGF β 1^{+/-} males displayed the behaviour for a shorter period, the overall rate of mounting behaviour was comparable [0.39(0.19-0.59) versus 0.47(0.39-0.75) total mounts per minute, TGF β 1^{+/-} and TGF β 1^{-/-} respectively]. These data demonstrate that TGF β 1^{-/-} males show normal sexual interest and behaviour but fail to intromit successfully and therefore cannot inseminate females. We conclude that intromission failure is the primary cause of infertility in TGF β 1 null mutant male mice.

51. IMMUNOREGULATORY CYTOKINES IN SEMINAL PLASMA OF MEN WITH INFLAMMATION OR SPERM AUTOIMMUNITY

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Inhibition of T-lymphocyte function *in vitro* by seminal plasma is due to several factors, including prostasomes, oxidised polyamines, prostaglandins of the E series, and immunoregulatory cytokines. We have previously shown that, following removal of the prostasomes and polyamine activity, there is an inverse relationship between T-lymphocyte inhibitory activity in seminal plasma and autoimmune infertility associated with sperm antibodies (1). This observation suggested reduced immunosuppression by seminal plasma could play a role in the development of male autoimmune infertility. Suppression of immune responses normally involves immunoregulatory cytokines, and the contribution of specific cytokines was investigated in the following study. Seminal plasma samples were collected with informed consent from the following groups of patients attending Monash IVF: normospermic men, men with clinically significant sperm autoantibodies (>50% IgA and/or IgG), and men with evidence of ongoing inflammatory reactions as indicated by the presence of leukocytes (0.78-3.2 million/ml) in the seminal plasma. Prostaglandins and other small MW factors were removed by dialysis. Immunosuppressive activity was measured by an *in vitro* bioassay employing dose-dependent inhibition of rat T-lymphocyte proliferation. The immunoregulatory cytokines activin A, interleukin-10 (IL-10) and transforming growth factor- β 1 (TGF- β 1), were measured by ELISA. Total immunosuppressive activity was significantly increased in men with sperm antibodies, and reduced in the inflammation group. However, both activin A and IL-10 levels were significantly elevated in men with leukocytes. The number of leukocytes in seminal plasma was positively correlated with activin A and IL-10 levels, and showed a significant negative correlation with active TGF- β 1 levels. There was no relationship between any cytokine and antibody status. The data suggest that the reduction in immunosuppressive activity during inflammation was not due to a reduction in seminal plasma levels of activin A, IL-10 or TGF- β 1, and was most likely due to an increase in pro-inflammatory cytokines. The reduced immunosuppressive activity previously observed in seminal plasma of men with sperm autoimmunity appears to be the result of a reduction in small MW factors, presumably prostaglandins. Clinically, reduction in immunosuppressive activity of the seminal plasma, due to alterations in inflammatory and regulatory cytokine levels, may negatively impact upon spontaneous pregnancy rates in couples where the male partner has leukocytospermia. (1) Imade *et al.*, 1997 *Human Reproduction* **12**: 256-262

52. SEASONAL CHANGES IN THE ABUNDANCE OF 14 kDa SEMINAL PLASMA PROTEINS SHOWN TO BIND TO RAM SPERMATOZOA

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Spermatozoa mature and attain their function as they pass through the epididymis and then the male reproductive tract on ejaculation. During this time, they are exposed to secretions from the epididymis and the accessory sex glands and acquire several proteins. Changes in the protein composition in these secretions may affect the biological function of spermatozoa. The abundance of a few seminal plasma proteins in bull semen has been correlated with the fertility of the bulls (1). In seasonal breeders like the ram, semen collected outside the breeding season is of lower fertility than semen collected from the same animals during the breeding season. These differences are not fully accounted by changes in sperm numbers alone and changes in seminal plasma total protein concentration and composition associated with the seasons has also been observed (2, 3). The interaction of these seminal plasma proteins with sperm may influence functions, such as fertilizing ability and the protection of sperm membrane from cold shock damage (4). Semen was collected from rams in the breeding and non-breeding season using an artificial vagina and the seminal plasma separated by centrifugation. EDTA and benzamide was added as protease inhibitors to 1 mM final concentration. The sperm cell pellet was washed three times with phosphate-buffered saline (PBS) and sperm membrane proteins extracted in Tris-buffered Triton X-100. Epididymal sperm were also subjected to the same treatment. All protein samples were stored frozen as aliquots at -20°C prior to analysis. 300 μ g of protein from each were subjected to 2-dimensional gel electrophoresis in 20 x 20 cm gels. Four protein spots of approximately 14 kDa were found to be less abundant in seminal plasma samples collected in the non-breeding season. These proteins were also bound to membranes of ejaculated sperm even after extensive washing with PBS and were extracted along with the sperm membrane proteins with detergents. However, the same protein spots were absent from membrane proteins extracted from epididymal sperm. We are currently attempting to identify these proteins and elucidate their function.[1] Killian *et al.* (1993) *Biol. Reprod.* **49**: 1202-1207 [2] Smith *et al.* (1999a) *Proc. NZ Soc. Anim. Prod.* **59**: 223-225 [3] Smith *et al.* (1999b) *Proc. Aust. Soc. Reprod. Biol.* **30**: 40.[4] Barrios *et al.* (2000) *Biol. Reprod.* **63**: 1531-1537.

53. IDENTIFICATION AND LOCALISATION OF A MESOTOCIN RECEPTOR IN THE PROSTATE OF THE BRUSHTAIL POSSUM

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Oxytocin is present in the prostate of the eutherian mammals and has been implicated in the regulation of prostate contractility and growth [1]. The marsupial produces mesotocin, rather than oxytocin, and a mesotocin receptor has been identified in the Tammar wallaby prostate [2]. The aim of this study was to identify a mesotocin receptor within the possum prostate and investigate the localisation of the receptor protein in the adult prostate in and out of the breeding season. Groups of possums (n=6-10), trapped alive in the wild, were killed at two monthly intervals during the year and prostate tissue collected. The prostates were divided into cranial and caudal portions and either frozen or fixed in 10% formalin. Immunohistochemistry and western blot analysis were performed using an antibody (020) raised to the third intracellular loop of the sheep oxytocin receptor [3]. RT-PCR of total prostatic RNA using primers designed to the Tammar mesotocin receptor generated a 337bp amplicon. On sequencing this demonstrated 92 % sequence homology with the Tammar receptor. Western blot analysis confirmed the presence of a single ~60 kDa protein. Immuno-reactive receptor protein was identified in all of the prostates examined and was localised predominantly to the epithelial cells of the glandular acini of both the cranial and caudal regions of the prostate. Little receptor was detected in the stromal tissue surrounding the glands. Significant changes ($P < 0.05$) in prostate weight occurred during the year, being greatest in March ($26.52 \pm 8.25\text{g}$) and lowest in January ($7.54 \pm 1.48\text{g}$). The reduction in weight was paralleled by a decrease in glandular tissue. No alteration in the localisation or the intensity of receptor staining in the prostate was seen during the year. In conclusion the possum prostate express a mesotocin receptor that is similar to that found in the Tammar wallaby. The localisation of the receptor is similar to that seen in eutherian mammals.

1. Nicholson HD et al 1996 Reviews Reprod 1, 69-72.
2. Parry LJ & Bathgate RAD 1998 Biol Reprod 59, 1101-1107.
3. Whittington K et al 2001 Reprod 122, 317-325.

54. TYROSINE PHOSPHORYLATION AND EPIDIDYMAL MATURATION IN MURINE SPERMATOZOA

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During transit through the female reproductive tract spermatozoa undergo capacitation, a maturational event accompanied by a unique signal transduction cascade leading to phosphorylation of multiple sperm proteins on tyrosine residues. Capacitation, and hence tyrosine phosphorylation, can be induced *in vitro* by incubation of murine caudal epididymal sperm in a well-defined medium. Tyrosine phosphorylation of sperm proteins increases under conditions that support cAMP metabolism. The aims of this study were to determine 1) if immature spermatozoa from the caput epididymis can be induced to tyrosine phosphorylate and 2) whether phosphorylated caput cells can then undergo functional endpoints of capacitation, namely the acrosome reaction and zona pellucida binding. Spermatozoa isolated from the caput and cauda epididymis of mice were incubated for 90 minutes in either complete BWW or calcium-free medium containing pentoxifylline (ptx) and dibutyryl-cAMP (dbcAMP) to increase cAMP levels. Tyrosine phosphorylation was assessed by Western blotting using a monoclonal anti-phosphotyrosine antibody. Calcium-ionophore induced acrosome reactions were monitored by staining with *Arachis Hypogaea* lectin, and the ability of spermatozoa to bind to solubilized mouse zona pellucida was examined. When incubated in complete medium caudal spermatozoa displayed tyrosine phosphorylation of a broad range of proteins (M_r 37-200kDa) that was further enhanced by addition of ptx and dbcAMP. In contrast, caput spermatozoa in complete medium showed only a constitutively phosphorylated 116kDa protein, identified as hexokinase, and elevation of cAMP levels did not influence this lack of response. However, omission of calcium from the medium resulted in high levels of phosphorylation comparable to that seen in cauda cells, which could be further stimulated by addition of ptx and dbcAMP. In contrast to cauda sperm, caput cells did not undergo an ionophore-induced acrosome reaction, even under conditions that promote phosphorylation. In addition, preliminary results indicate that tyrosine phosphorylated caput spermatozoa do not bind to the zona pellucida. These results show that caput spermatozoa contain a functional cAMP signalling pathway that is silenced in the immature gamete via a calcium-dependant mechanism. Whilst tyrosine phosphorylation of sperm proteins is associated with the acquisition of fertilising ability, it alone is not sufficient to render caput spermatozoa functionally mature. Further maturation must occur during epididymal transit before spermatozoa have the competence to become capacitated. These results contribute to understanding of the cellular mechanisms involved in sperm maturation.

55. USE OF THE YEAST TWO-HYBRID SYSTEM TO CHARACTERISE THE FUNCTION OF SPERM PROTEIN, TPX-1

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Tpx-1 is a testis-specific member of the cysteine-rich secretory protein (CRISP) family which becomes incorporated into both the acrosome and outer dense fibres of the sperm. Despite the high expression of this 25-27kDa protein, its function and that of all mammalian CRISPs remains largely unknown. In order to ascertain the function of tpx-1, a yeast two-hybrid approach was employed to identify the molecular interactions and subsequent biochemical pathways with which tpx-1 is involved during spermatogenesis. The cDNA encoding the mature tpx-1 protein was used as bait to screen a mouse testis expression library. One hundred and fifty two yeast colonies expressing putative tpx-1 binding partners were detected. Of these positive clones, two putative interacting proteins, called ran binding protein-9 (ranbp-9) and mixed lineage kinase-3 (mlk-3) were most highly represented. Ranbp-9 is a poorly characterized GTPase binding protein and mlk-3 is a serine-threonine kinase previously shown to be involved in the inflammatory response. Northern blot and immunolocalisation data provides evidence for the co-expression of these proteins with tpx-1, both temporally and spatially. Further, co-immunoprecipitation data indicate an interaction of tpx-1 with ranbp-9 and tpx-1 with mlk-3. Deletion experiments, also utilising the yeast two-hybrid system, indicate that the cysteine-rich carboxy terminus of tpx-1 is crucial for both interactions. The significance of tpx-1 binding these two proteins, ranbp-9 and mlk-3, is yet to be clarified. Ranbp-9 has, however, a likely role in calcium channel function, due to the presence of a ryanodine receptor domain and as such may be involved in either the calcium influxes known to be involved in the acrosome reaction or tail movement. At present, functional roles for tpx-1 are speculative, however the combination of molecular biological and biochemical approaches, will provide insight into the role of tpx-1 in sperm function and/or development and will have implications for the treatment of both male infertility and the development of novel contraceptives.

56. GENE EXPRESSION OF ADENYLYL CYCLASE IN RAT TESTIS AND CAUDAL EPIDIDYMAL SPERM

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We have demonstrated a transient Ca^{2+} -dependent increase in intracellular cAMP during the first 60 seconds of motility activation in caudal epididymal sperm (CES) from the rat. This suggested that the sperm adenylyl cyclase (AC) involved in motility activation may be directly activated by Ca^{2+} . Currently, 9 transmembrane forms of AC (tmAC) and one soluble AC (sAC) have been characterized in mammalian cells. Three of these, AC I, III and VIII are stimulated by Ca^{2+} /calmodulin. AC III has been located to the acrosomal membrane in developing spermatids. However, no AC has been identified in mammalian sperm flagella. In this study we have analysed the gene expression of AC in testis and CES, as an initial step in the characterization of AC in sperm flagella. Specific primers were designed against non-conserved regions of all known AC enzymes recorded in the GenBank database. Total RNA from rat brain, testis, germ cells and caudal epididymal sperm was used as a template to amplify cDNA. RT-PCR was performed. Amplicons of correct size were purified, cloned and sequenced to confirm identity. This is the first study to report a systematic analysis of mRNA expression of AC in testis and CES using RT-PCR. Prior to this analysis only AC III, V, VI and VII gene expression had been reported in testis. AC II, IV and IX had not been examined and the expression of the AC VIII gene was ambiguous. We have shown that AC II, III, IV, V, VI, VII, VIII and sAC genes are expressed in rat testis, germ cells and in rat brain (control). The AC 1 gene was expressed in brain but not testis or germ cells. AC IX is still under investigation. Further, we have demonstrated that mRNA from the AC III gene is present in caudal epididymal sperm. In light of our demonstration of a transient, Ca^{2+} -dependent increase in cAMP in sperm undergoing motility activation the future challenge is to determine whether AC III protein is expressed in the sperm flagella membrane.

57. SPERM SURFACE PROTEIN PH-20 EXPRESSION DURING SHEEP SPERMATOGENESIS

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The sperm surface protein PH-20 exhibits dual functions, having hyaluronidase activity that enables sperm to penetrate the cumulus and also being involved in zona pellucida binding. The aim of this study was to determine the site of ovine PH-20 expression during spermatogenesis in sheep testes. Fresh testis samples from foetal (n=3), new-born (n=4), 4 month-old lambs (n=8), 5 month-old lambs (n=12), and 4-6 year-old rams (n=6) were collected and frozen for total RNA extraction or fixed in 10% phosphate buffered formaldehyde for histological analysis. A fragment of ovine testis PH-20 cDNA (282 nt) was identified by reverse transcription and polymerase chain reaction (RT-PCR) in testis RNA, but not gDNA extracts. The cDNA exhibited 77% identity to human PH-20 and 93% identity to red fox PH-20 cDNA and this sequence was submitted to GenBank (AF174691). Ovine PH-20 expression was detected using RT-PCR and western blotting. The developmental stage of the 4-5 month ram testes was assessed histologically. Ovine PH-20 protein was localised in the seminiferous tubules using immunohistochemistry with an anti equine PH-20 antibody. Study of PH-20 mRNA expression using RT-PCR showed that PH-20 mRNA is only expressed in ram testis and not in the other sheep tissues including ovary, brain, liver, lung, heart, epididymis, spleen, or kidney. Western blotting (under reducing conditions) revealed a single 74 KDa immunoreactive band in ram testis but not in liver tissue. PH-20 mRNA and protein were identified only in testes where spermatids were present in the seminiferous tubules and in 4-5 month-old ram testes undergoing the first wave of spermatogenesis, but not in foetal, new-born or prepubertal testicular tissues. Immunohistochemistry for ovine PH-20 protein in adult ram testis confirmed the cellular localisation in the initial spermatogenic wave in developing rams. We conclude that ovine PH-20 is expressed post-meiotically in haploid round and elongate spermatids.

58. OVARIAN FOLLICULAR DEVELOPMENT IS COMPROMISED IN TRANSGENIC (mREN-2)27 RATS AND ANGIOTENSIN II-INFUSED SPRAGUE DAWLEY RATS

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Angiotensin II (ANG II) is implicated in ovulation, vascularisation and atresia, and may influence these processes by a local ovarian renin-angiotensin system (RAS), which we have previously localised in rat ovarian stroma and its blood vessels. The aim of this study was to determine the role of ANG II in follicular development. Three groups of 12 week old normally cycling, female rats were used: 1) untreated Sprague Dawley (SD), 2) SD infused with ANG II (145 ng/kg/min for 12-14 days), and 3) hypertensive homozygous (HMZ) (mRen-2)27 transgenic rats, which display amplified extra-renal tissue renin and ANG II. Ovaries were collected at proestrus for histological analysis of follicular type and number, immunolocalisation of renin and ANG II, *in situ* hybridisation for AT1a receptor (AT1a-R) mRNA and radioimmunoassay of ovarian renin content. Systolic blood pressure (SBP; tail cuff) was highest in HMZ Ren-2 followed by SD+ANG II and SD. HMZ Ren-2 and SD+ANG II had more antral follicles than SD, but fewer large antral and pre-ovulatory follicles. Litter size was reduced in HMZ Ren-2 compared to SD. Consistent with the high RAS of the HMZ Ren-2, ovarian renin content and renin and ANG II immunolabelling were increased and AT1a-R mRNA reduced. SD+ANG II exhibited a reduction in ovarian renin content and AT1a-R mRNA gene expression, indicating a negative feedback on the RAS.

N = 4-6 rats per group	SD	SD+ANG II	HMZ (mRen-2)27
SBP (mmHg)	125±1	199±12*	233±5*†
Antral Follicles: 150-390µm (% Total)	37.5±4.5	53.5±6.3*	52.8±2.2*
Large Antral Follicles: 390-500µm (%Total)	7.7±1.8	2.4±1.1*	2.4±0.9*
Pre-ovulatory Follicles: >500µm (% Total)	17.1±3.6	3.8±1.5*	4.5±1.9*
Litter Size	13.7±0.7	NA	10.3±0.6*
Ovarian Renin Content (mGU/g)	4.8±0.5	2.7±0.3*	18.8±2.1*†
AT1a-R mRNA (Relative Optical Density)	75.3±1.8	64.0±2.4*	60.8±2.3*

*Values are mean ± sem. *p<0.05 compared to SD, †p<0.05 compared to SD+ANG II. NA: not analysed.*

These findings indicate that ANG II compromises follicular development, by the accumulation of antral follicles and a reduction in large antral and pre-ovulatory follicles. The presence of RAS on stromal blood vessels suggests that follicular development and litter size are influenced by an ovarian RAS, possibly through effects on ovarian vascularisation and local blood flow which are essential for follicular growth, antral formation, enlargement and subsequent ovulation.

59. EFFECT OF SUB-BURSAL FAS ANTIBODY ADMINISTRATION ON FOLLICULAR CASPASE-3 ACTIVATION AND OVARIAN VOLUME

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Fas is a transmembrane receptor complex belonging to the TNF- α superfamily which, when ligated by its naturally produced ligand (FasL), activates downstream caspases resulting in the execution of apoptosis. Studies have recently demonstrated that caspase-3, an apoptotic effector enzyme, is present and active in granulosa cells and oocytes of atretic follicles (1). To investigate the possible involvement of FasL in the initiation of atresia *in vivo*, an anti-Fas monoclonal antibody was injected beneath the ovarian bursa in peripubertal mice (n=9). Animals were killed and the ovaries removed after 1, 3, and 5, days following surgery. Ovaries were compared with saline treated counterparts (n=8) and examined immunohistochemically for evidence of caspase-3 activity and the volume determined for each ovary using the Cavalieri method. Histological observation revealed that corpora lutea had disappeared in 7/9 anti-Fas treated animals and that cystic structures had developed in 3 ovaries. No significant differences in ovarian volume were found within or between treatment groups. An increase in the number of atretic antral follicles was apparent, as evidenced by active caspase-3 immunoreactivity in granulosa cells. Also, a larger number of degenerating oocytes from preantral follicles were observed in anti-Fas treated ovaries. Results from this study suggest that the Fas signal is a potent inducer of caspase-3 activity and follicular atresia *in vivo*, and that the cell type governing the mechanism of atresia could be dependent on the stage of follicle development. Furthermore, overstimulation of the Fas/FasL pathway may lead to aberrant cyst formation in the ovary.

1. Fenwick, M.A. and Hurst, P.R. (2002) Reproduction (submitted)

60. GRANULOSA CELLS MATURE BEFORE DEATH IN BASAL ATRESIA

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Most ovarian follicles undergo atresia, yet this process is still poorly characterised. Recently we described a previously unrecognised type of atresia (1). Within the membrana granulosa cell death first occurs near the basal lamina and progresses antrally (basal atresia), the opposite of that found in classic atresia (antral atresia). Basal atresia is rarely seen in follicles >5 mm in diameter and occurs in approximately 20% of follicles <5 mm. Follicular fluids from atretic small bovine follicles reportedly have elevated progesterone concentrations (2, 3). We investigated the hypothesis that only basal atretic follicles have elevated follicular fluid progesterone.

Antral follicles were harvested from the ovaries (one per cow) of young non-pregnant *Bos taurus* cows. Follicles (2-5 mm, n = 24, 2 per ovary) were dissected and snap-frozen in OCT compound. Frozen follicles were bisected and one half immersed in 2.5% glutaraldehyde for histological classification. Cholesterol side-chain cleavage cytochrome P450 (SCC) (0/11 follicles examined) and 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (0/15) were not expressed in the granulosa cells of healthy follicles or antral atretic follicles. On atresia where the basally-located granulosa cells died first, surviving antrally-situated granulosa cells expressed SCC (3/8) and 3 β -HSD (8/8) suggesting that these enzymes had been switched on during the process of atresia.

A further 32 ovaries were collected as above. Follicles (3-5 mm) were dissected (n = 111, 1-4 per ovary) and snap frozen. A portion of the follicle wall was immersed in 2.5% glutaraldehyde and the remaining follicle stored at -70°C for subsequent collection of follicular fluid. Healthy follicles had either rounded or columnar basal cells (4). Follicular fluids from healthy (rounded basal cells, n = 15; columnar basal cells, n = 13) and atretic (antral, n = 9; basal, n = 15) follicles were measured by RIA. Follicular fluid progesterone was significantly greater (468 \pm SEM 49 nmol/ml, $P < 0.001$, ANOVA and SNK) in basal atretic follicles compared to antral atretic follicles (96 \pm 21) or healthy follicles with either rounded (112 \pm 23) or columnar basal cells (137 \pm 26).

Thus in basal atretic follicles granulosa cells undergo maturational changes before dying.

Reproduction 2001: 122, 761-775

Biol Reprod 2001: 65, 726-732

J Reprod Fert 1984: 72, 39-53

J Reprod Fert 2000: 118, 221-228

61. ESTROGEN PROMOTES ANGIOGENESIS IN ER α -EXPRESSING HUMAN MYOMETRIAL MICROVASCULAR ENDOTHELIAL CELLS BY UPREGULATING VEGF RECEPTOR-2

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Angiogenesis is the growth of new blood vessels from pre-existing vessels and involves proliferation of microvascular endothelial cells (MEC). VEGF is a major promoter of angiogenesis and mediates angiogenic effects primarily through interaction with VEGF receptor-2 (VEGF-R2/KDR) present on the surface of MEC. Estrogen promotes bFGF-induced angiogenesis, but its effect on VEGF-mediated angiogenesis is unknown. We have demonstrated that MEC derived from human myometrium constitutively express estrogen receptor- β (ER β), while ER α varies between subjects and is only expressed in approximately 60% of MEC isolates (1). The aims of the present study were to determine whether (a) estrogen increases VEGF-R2 expression in ER α positive and ER α negative myometrial MEC, (b) ER mediates this effect and (c) estrogen promotes VEGF-induced MEC proliferation. Myometrial MEC were isolated from hysterectomy tissue obtained from ovulating women, purified with UEA-1-coated Dynabeads, cultured and used between passages 1-3 (purity >98% CD31+ cells) (1, 2). ER α and ER β mRNA were determined by RT-PCR (3) and protein by flow cytometry or Western blotting. VEGF-R2 expression was measured by flow cytometry using either VEGF-R2 antibody or biotin-rhVEGF₁₆₅ binding. MEC proliferation was determined by MTS bioassay (2). Estrogen (1 and 10 nM) significantly increased rhVEGF₁₆₅ binding and VEGF-R2 receptor expression in ER α positive (P<0.05, n=4) but not in ER α negative MEC samples (n=6). There was a significant association between ER α mRNA and protein expression in myometrial MEC and the ability of estrogen to upregulate VEGF-R2 (P=0.03 and P=0.025 respectively). ER mediated the effect of estrogen on VEGF-R2 expression, since the ER antagonist ICI 182,780 blocked this effect (P<0.05, n=3). Similarly, estrogen augmented VEGF-induced proliferation of ER α -expressing MEC (P<0.05, n=5), which was blocked by ICI 182,780 (P<0.01, n=5). These observations suggest that estrogen promotes proliferation of human myometrial MEC by increasing VEGF-R2 expression, an effect that varies between subjects and appears to be mediated primarily by ER α . [1]Gargett CE, Bucak K, Zaitseva M [2]Chu S, Taylor N, Fuller PJ, Rogers PAW (2002) *Molec Hum Reprod* 8 (in press).

62. DIETARY ESTROGENS HAVE SELECTIVE EFFECTS ON THE REPRODUCTIVE AXIS IN ESTROGEN DEFICIENT ARKO MICE

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Phytoestrogens, or isoflavones such as genistein, are a significant component of many diets including commercial rodent chow that contains soya meal. Estrogen deplete Aromatase knockout (ArKO) mice were used to test the capacity of these sources of estrogen activity to influence components of the female reproductive axis. Wildtype (Wt), Heterozygote (Het) and ArKO mice (n =7-14/group) were maintained from birth (and *in utero*) until 16 weeks of age on either (i) standard commercially available pelleted rat chow containing 14% Soya meal (0.146mg/g isoflavones: genistein and diadzein) (Soy plus), (ii) a soya free (Soy minus) rodent chow with undetectable levels of isoflavones, or (iii) a soy minus diet to which we added 250mg/kg of Genistein (Gen). Diets were corrected for amino acids, vitamins and minerals per kg of feed. ArKO mice displayed increased body weight compared to Wt and Het littermate controls when maintained on either S- or Gen diets (P<0.001), and no effect was observed in S+ mice. ArKO-Gen mice had increased body weight compared to ArKO S- (P=0.005). ArKO mice had reduced ovarian and uterine weights compared to Wt and Het littermates when maintained on any of the diets (p<0.001) and no effect of diet within genotype (WT, Het or ArKO) was noted. ArKO mice had significantly elevated serum LH and FSH compared to Wt and Het controls, when maintained on Gen or Soy minus diets (P<0.001 and P= 0.003 respectively). ArKO-Gen mice had decreased LH (p=0.046) and FSH (p<0.001) compared to ArKO Soy minus mice, an effect not observed in Wt and Het littermates. In summary, ArKO mice were heavier than their littermate controls and possessed hypoestrogenic uteri and ovaries even when maintained on estrogenic diets. The addition of Genistein to the diet reduced serum LH and FSH in ArKO mice. In conclusion, in states of hypoestrogenicity isoflavones can selectively alter different components of the female reproductive axis which may have implications for hormone replacement therapy for menopausal symptoms, and in experimental animals consuming a diet containing phytoestrogens.

63. A LONG TERM FOLLOW-UP OF A FEMALE WITH AROMATASE DEFICIENCY **Yoko Murata¹, József Kovács^{2,4}, Julia Crone², Kurosh Paya³, Gabriele Amman³, Kara Britt¹, Evan R. Simpson¹, Franz Waldhauser²**

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In humans, natural mutations in the *CYP19* gene are a rare occurrence. In the last decade, ten patients with different mutations were described. All mutations translated into inactive forms of aromatase and consequently a lack of estrogens in these patients. In both sexes, epiphyseal closure was delayed, osteopenia and reduced bone mineral density and bone age were observed. Females were presented with pseudohermaphroditism at birth and progressive virilization at puberty, whereas males were presented with normal pubertal development.

We describe here an aromatase-deficient female, who was followed for 18 years since birth. She has never showed elevated levels of androgens or progressive virilization during puberty. The patient had ambiguous genitalia at birth. Her parents are first cousins. Basal estradiol was consistently undetectable, while LH followed the typical pattern with an elevation during infancy and a drop in the following years. FSH was consistently elevated. The explorative laparotomy at age 5 years revealed a normal appearance of the uterus, fallopian tubes and ovaries. Histology of both ovaries showed fibrotic stroma with a remarkably decreased number of cells surrounding primordial, primary, secondary and tertiary follicles. Often there was a discrepancy between the large size/morphology of the germ cells and the flat granulosa cells with reduced cytoplasm. At age 5, the bone age was delayed by one year. Despite high levels of gonadotropins at age 13.5 years, breast development and other signs of puberty were absent. The biopsy of the left ovary showed fibrotic ovarian stroma with many antral follicles with loss of adequate germ cells and an increased number of atretic follicles. Bone maturation was delayed, corresponding to a 10 year old. DNA analysis revealed a homozygous point mutation in exon X, resulting in R457X and deletion of 47 amino acids at the carboxyl terminus. The same mutation was described in an unrelated female infant by Portrat-Doyen (1). *In vitro* activity of this truncated aromatase was zero. With oestrogen therapy, bone maturation has improved and regular menstruations have been established confirming the aromatase deficiency.

Portrat-Doyen 1996, Horm. Res. 46(2): 4.

64. LIVER RECEPTOR HOMOLOGUE-1 REGULATES AROMATASE EXPRESSION IN PREADIPOCYTES

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Estrogen biosynthesis from C₁₉ steroids is catalysed by the enzyme aromatase cytochrome P450. Aromatase is expressed in breast adipose tissue through the use of a distal, cytokine-responsive promoter (pI.4). In the presence of breast tumours, however, aromatase is over-expressed in response to tumor-derived factors that induce the proximal, cAMP-responsive promoter II (pII). In other tissues, transcription from promoter II requires the presence of the Ftz-F1 homologue Steroidogenic Factor-1 (SF-1, NR5A1). Adipose tissue, however, expresses little or no SF-1. We have explored the hypothesis that in adipose tissue, an alternative Ftz-F1 family member Liver Receptor Homologue-1 (LRH-1, NR5A2) can substitute for SF-1 in driving transcription from pII. 3T3-L1 preadipocytes were cotransfected with a fusion gene comprising -516/-17 bp of human *CYP19* pII 5'-flanking DNA linked to the luciferase reporter, and a mouse LRH-1 expression construct. Cells were then incubated in the presence or absence of forskolin (FSK) and phorbol ester (PMA) for 16 hours. In the absence of exogenous LRH-1, FSK+PMA increased luciferase activity 3-fold. In the presence of LRH-1, basal activity increased 2.5-fold, and FSK+PMA increased activity to over 30-fold. This stimulatory effect of LRH-1 required the presence of a nuclear receptor half-site within promoter II, to which LRH-1 was shown to bind in gel shift analysis. To explore the relevance of LRH-1 induced pII activity in preadipocytes we quantified LRH-1 mRNA in various tissues by real-time PCR. LRH-1 mRNA levels in whole adipose tissue were low - approximately 10% that of liver. However, levels in isolated preadipocytes were approximately 2.5 times that of liver, suggesting that LRH-1, like aromatase, is expressed specifically in preadipocytes but not in mature adipocytes. To test this hypothesis, cultured human preadipocytes were differentiated into lipid-laden adipocytes by incubation in an adipogenic medium for 12 days. Differentiating cells displayed a time-dependent induction of PPAR γ with concomitant loss of LRH-1 and aromatase expression. We conclude that LRH-1 is expressed at high level in human preadipocytes, and is a likely regulator of aromatase transcription from pII. Alterations in LRH-1 expression and/or activity in adipose tissue could therefore have considerable effects on local estrogen production and breast cancer development.

65. MECHANISMS UNDERLYING THE REDUCED SENSITIVITY TO PROLACTIN NEGATIVE FEEDBACK DURING LACTATION

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Prolactin negative feedback maintains normal, low prolactin concentrations in non-lactating individuals. Prolactin stimulates the tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus to release dopamine which suppresses prolactin secretion from lactotrophs. During lactation, however, the sucking stimulus reduces the sensitivity of TIDA neurons to prolactin feedback and consequently high prolactin levels are observed. The mechanisms involved in this loss of negative feedback are unknown, but may involve changes in prolactin signal transduction pathways. Activation of prolactin-receptors normally results in phosphorylation and nuclear translocation of Stat proteins, particularly Stat5, so we investigated Stat5 signalling in TIDA neurons of lactating rats. Lactating rats with or without pups (removed for 16h) were given a sc injection of either oPRL (250µg) or saline vehicle (n=4/treatment), and after 1h were killed and perfused. Hypothalamic sections were immunostained for tyrosine hydroxylase (Chemicon, MAB318) and Stat5 (Santa Cruz, Sc835) and confocal images of the dorsomedial arcuate nucleus were captured for quantitation. Stat5 intracellular distribution in TIDA neurons was determined by the ratio of Stat5 fluorescence intensity in the nucleus expressed as a proportion of that in the cytoplasm (N/C ratio). In lactating rats with pups removed for 16h, oPRL injection significantly ($P < 0.05$) increased Stat5 N/C ratio (2.37 ± 0.52) compared to vehicle treated mothers (1.14 ± 0.04). In contrast, oPRL injection did not increase Stat5 N/C ratio in lactating mothers with pups (oPRL 1.41 ± 0.04 vs vehicle 1.37 ± 0.10). These results demonstrate that the TIDA neurons exhibit reduced Stat5 signalling in response to exogenous prolactin when the mother is being suckled. To investigate possible mechanisms involved in this reduced prolactin signalling, we examined the expression of SOCS (suppressors of cytokine signalling) proteins that negatively regulate prolactin signalling through the Jak2/Stat5 pathway. Northern analysis on whole hypothalamus showed that CIS (cytokine-inducible SH2 protein), but not SOCS-1, -2 or -3, mRNA expression was upregulated in suckled lactating rats. Moreover, using dual label immunofluorescence, we found that CIS is localised to TIDA neurons of lactating rats. Together these results support the hypothesis that diminished sensitivity to prolactin negative feedback during lactation is a result of increased expression of CIS in TIDA neurons.

66. THE NEUROENDOCRINOLOGY OF STRESS

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The stress system coordinates the adaptive responses of the organism to stressors of any kind. The main components of the stress system are the CRH and Locus Coeruleus-Norepinephrine (LC/NE)-Autonomic systems and their peripheral effectors, the pituitary-adrenal axis, and the limbs of the autonomic system. Activation of the stress system leads to behavioral and peripheral changes that improve the ability of the organism to adjust homeostasis and increase its chances for survival. The CRH and LC/NE systems stimulate arousal and attention, as well as the mesocorticolimbic dopaminergic system, which is involved in anticipatory and reward phenomena, and the hypothalamic β -endorphin system, which suppresses pain sensation and, hence, increases analgesia. CRH inhibits appetite and activates thermogenesis via the catecholaminergic system. Also, reciprocal interactions exist between the amygdala and the hippocampus and the stress system, which stimulates these elements and is regulated by them. CRH plays an important role in inhibiting GnRH secretion during stress, while, via somatostatin, it also inhibits GH, TRH and TSH secretion, suppressing, thus, the reproductive, growth, and thyroid functions. Interestingly, all three of these functions receive and depend on positive catecholaminergic input. The end-hormones of the hypothalamic-pituitary-adrenal (HPA) axis, glucocorticoids, on the other hand, have multiple roles. They simultaneously inhibit the CRH, LC/NE and β -endorphin systems and stimulate the mesocorticolimbic dopaminergic system and the CRH peptidergic central nucleus of the amygdala. In addition, they directly inhibit pituitary gonadotropin, GH and TSH secretion, render the target tissues of sex steroids and growth factors resistant to these substances and suppress the 5' deiodinase, which converts the relatively inactive tetraiodothyronine (T_4) to triiodothyronine (T_3), contributing further to the suppression of reproductive, growth, and thyroid functions. They also have direct as well as insulin-mediated effects on adipose tissue, ultimately promoting visceral adiposity, insulin resistance, dyslipidemia and hypertension (metabolic syndrome X) and direct effects on the bone, causing "low turnover" osteoporosis. Central CRH, via glucocorticoids and catecholamines, inhibits the inflammatory reaction, while directly secreted by peripheral nerves stimulates local inflammation. Antalarmin, a novel CRH receptor type 1 antagonist, decreases the activity of the HPA axis, suppresses neurogenic inflammation and blocks CRH-induced skin mast cell degranulation, in addition to blocking the development and expression of conditioned fear and stress-induced colonic hyperfunction. Chronic administration of antalarmin is not associated with glucocorticoid deficiency. These data suggest that such antagonists may be useful in human pathologic states, such as melancholic depression and chronic anxiety, associated with chronic hyperactivity of the stress system, along with predictable behavioral, neuroendocrine, metabolic and immune changes, based on the interrelations outlined above. Conversely, we will need potentiators of CRH secretion/action to treat atypical depression, postpartum depression and the fibromyalgia/chronic fatigue syndromes, all characterized by low hypothalamic-pituitary-adrenal axis and LC/NE activity, fatigue, depressive symptomatology, hyperalgesia and increased immune/inflammatory responses to stimuli.

67. SIGNALLING MECHANISMS DURING APOPTOSIS

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Physiological cell death (apoptosis) is fundamental to almost every aspect of normal tissue development, function and homeostasis in multi-cellular organisms. In reproductive physiology, cell death plays an active role in (1) Müllerian and Wolffian duct regression; 2) ovarian germ cell endowment and depletion; 3) spermatogenesis; 4) embryo implantation; 5) parturition; 6) non gonadal tissues such as prostate, uterus and breast; and 7) reproductive cancer. Despite this wide diversity, recent data suggest there are many common features associated with the regulation of cell death throughout the male and female reproductive systems. One of the most obvious is the apparent requirement by cells for a cell-type specific survival factor(s), generally in the form of hormones or growth factors. These survival factors mediate their action via several proteins. Among the rapidly growing list of such proteins, evidence derived from gene expression analyses implicates members of the *ced9/bcl-2* and *ced3*/interleukin-1 β -converting enzyme (*ICE*), gene families and signalling molecules such as TNF, Fas/FasL, sphingolipids as primary determinants of cell survival or death in reproductive tissues. Therefore, by keeping the complexity of this process in mind, and by applying data derived from other tissues, great strides should be made as we attempt to characterise the signalling mechanisms and regulation of apoptosis in development and function of the female reproductive systems.

68. DROSOPHILA AS A MODEL SYSTEM TO STUDY STEROID HORMONE REGULATED CELL DEATH

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Many hormones are known to regulate cell survival and cell death both in embryonic and adult tissues. One of the important models of hormone regulated cell death is *Drosophila melanogaster*. The advantages of using *Drosophila* as an experimental system to study regulation of apoptosis are multifold: the genetics of *Drosophila* is well understood, the genome sequence and a large number of mapped mutants are available, *Drosophila* is amenable to sophisticated genetics to study *in vivo* interactions between various molecules, and a single steroid hormone (20-hydroxy ecdysone) regulates cell death during metamorphosis. Pulses of ecdysone are produced at various times during fly development which temporally and spatially regulate cell proliferation, differentiation and death. During larval/pupal metamorphosis ecdysone mediates deletion of obsolete tissues, such as salivary glands and midgut. Ecdysone binds to its heterodimeric EcR/Usp receptor and regulates a number of primary response genes that encode transcription factors. These transcription factors in turn, regulate several secondary response genes. Recent data suggest that ecdysone and ecdysone induced genes including beta *FTZ-F1*, *BR-C*, *E74*, *E75* and *E93* play a key role in ecdysone-mediated cell death in salivary gland and midgut. For example, in salivary glands of the *E93* mutants *rpr*, *hid*, *dark* and *dronc* mRNA levels are severely reduced. Currently, we are studying the regulation of caspase transcription by ecdysone. There are seven caspases in the fly, of which the initiator caspase *dronc* is transcriptionally regulated by ecdysone in salivary glands and midgut during larval-pupal metamorphosis. *Dronc* is activated early following death signals and once activated, can process downstream caspases such as *Drice*. RNA interference studies indicate that *Dronc* is essential for normal cell death. Our studies suggest that *BR-C* and *E93* regulate *dronc* transcription during salivary gland cell death. In addition to *dronc*, *drice* transcription also appears to be regulated by ecdysone during cell death. These data indicate that transcriptional regulation of death effectors plays a key role in hormone-mediated apoptosis during *Drosophila* development and argues that similar mechanisms may control developmentally programmed cell death in mammals.

69. NEGATIVE REGULATION OF PROLACTIN-RECEPTOR SIGNALLING BY SOCS PROTEINS: A NEW MECHANISM IN LUTEOLYSIS?

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Prolactin (PRL) and placental lactogen (PL) play essential roles in maintaining the rodent corpus luteum (CL) through pregnancy by inducing transcription of some genes (e.g. ER α and ER β , LH-receptor, prolactin-receptor) and repression of others (e.g. 20 α -hydroxysteroid dehydrogenase). These effects are mediated by the PRL-receptor, which signals through several pathways although it is the JAK2/STAT 5 pathway that plays the central role in the CL. At the end of pregnancy, luteolysis is induced by prostaglandin F2 α (PGF2 α), which reverses many of the effects of the PRL-receptor signalling pathway on gene expression. Our recent research on day 19 pregnant rats has shown that one way in which PGF2 α could block PRL-induced gene expression is through inhibition of the JAK2/STAT 5 pathway. Following treatment of rats with cloprostenol, a PGF2 α analogue, there is rapid loss of active STAT 5 from ovarian nuclear extracts and this effect persists for at least 8 h. Moreover, during this period, treatment with prolactin does not induce activation of STAT 5, despite the continued presence of PRL-R. The recently discovered Suppressors Of Cytokine Signalling (SOCS) have been shown to decrease cell sensitivity to cytokines, including PRL, and so we determined whether the negative regulation of PRL-receptor signalling by PGF2 α could be due to upregulation of SOCS proteins. In Northern blots performed on ovaries collected 0.5-16 h after cloprostenol, SOCS-1 showed a transient increase at 0.5 h while SOCS-3 mRNA increased 3-fold at 0.5 h and remained elevated until 4 h after injection. Western blots confirmed that SOCS-3 protein was increased over the same period and, in ovaries collected 4 h after cloprostenol, we confirmed by immunohistochemistry that SOCS-3 was localised predominantly within CLs. In conclusion, induction of SOCS-3 and to a lesser extent SOCS-1 by PGF2 α may be an important element in the initiation of luteolysis, via rapid suppression of luteotrophic support from PL.

70. PARACRINE SIGNALLING WITHIN THE OVARIAN FOLLICLE: POTENTIAL FOR TGF- β SUPERFAMILY MEMBERS TO INFLUENCE OOCYTE AND GRANULOSA CELL INTERACTIONS

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Signalling between the germ cell and somatic cell compartments of the ovarian follicle is a critically important process for growth and differentiation of the oocyte and the granulosa cells. Communication occurs via paracrine and gap-junctional signalling between the two cell types, and indeed both forms of communication are essential for normal oogenesis and folliculogenesis. Traditionally, research has been focused on one side of this communication axis, that is, on the role of the granulosa cells in nurturing the growth and development of the oocyte. Recent studies demonstrate the importance of a bi-directional communication axis, and it is now becoming clear that the oocyte is a pivotal regulator of ovarian function. Folliculogenesis fails in the absence of oocyte paracrine signalling, whether lacking due to genetic deficiencies or from experimental ablation of the oocyte. Oocytes regulate a broad range of granulosa cell functions by modulating fundamental control elements; for example, oocytes regulate expression of FSH and LH receptors, kit ligand, inhibin subunits and expression of extra-cellular matrix molecules. As such, oocytes not only promote growth of granulosa cells and the follicle, but also regulate differentiation processes such as steroidogenesis and physical remodelling of the follicle. The primary recipients of oocyte paracrine signalling are the cumulus cells, which are the specialised granulosa cells immediately surrounding the oocyte. Cumulus cells have a distinct phenotype from the mural granulosa cells (MGC) lining the wall of the follicle, and in fact maintenance of the cumulus cell phenotype is dependent on oocyte-secretions. In the absence of these oocyte signals the cumulus cells will spontaneously differentiate toward the MGC phenotype. Oocyte paracrine signalling therefore establishes a functional morphogenic gradient across the ovarian follicle. The exact identity of these oocyte factors remains elusive, but members of the transforming growth factor-beta superfamily are the prime candidates as some of these growth factors are able to mimic the effects of oocytes on granulosa cells in vitro. Two family members, which are exclusively expressed in gametes, growth differentiation factor-9 (GDF-9) and bone-morphogenic factor-15 (BMP-15), are critical factors as their absence leads to failed folliculogenesis and complete sterility. Interestingly these molecules have more subtle effects on fertility with high or low expression of these oocyte factors leading to perturbations such as ovarian cysts, anovulation, disrupted oocyte maturation and even increases in ovulation rate and litter size. There is now an emerging appreciation that not only does paracrine signalling from the oocyte allow the oocyte to control its own microenvironment, but oocyte signalling also has profound effects on ovarian function, endocrine well being and fertility.

71. EXPRESSION OF TGF- β SUPERFAMILY MEMBERS AND THEIR SIGNALLING COMPONENTRY BY THE RAT OVARY

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An increasing number of transforming growth factor- β (TGF- β) superfamily members are being shown to exert effects on ovarian function. Most of these factors, which include activin, the bone morphogenetic proteins (BMPs) and TGF- β , act via transmembrane serine and threonine kinase receptors which activate pertinent Smad proteins, complexes of which translocate to the nucleus to mediate gene transcription. Despite understanding the molecular basis of these signalling pathways, little has been done to characterise these pathways in the ovary. We've sought to elucidate which elements of these signalling pathways are present in ovarian follicles of the postnatal rat, as an initial step toward identifying active pathways in follicle populations. Activin/BMP receptors (ActRIA, ActRIB, ActRIIA and ActRIIB), β glycan and Smad 1-8 mRNAs were expressed by the postnatal rat ovary. Activin receptor and Smad proteins were present in oocytes at all stages of follicular development; granulosa cells of primary-antral follicles and theca cells. β glycan protein was present in oocytes, granulosa cells and theca cells at all stages of folliculogenesis. The co-localisation of receptors and Smads supports the notion that activin/TGF β and BMP signalling pathways may be functional in the cellular compartments of the follicle. We hypothesise that follicle populations express elements of the TGF- β superfamily (and their signalling pathways) in different combinations during development and thus it is the individual follicle 'blueprint' that determines the response to ligands and ultimately the biological outcomes. These concepts are currently under investigation in our laboratory.

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72. DIFFERENTIAL GENE EXPRESSION IN INTERLEUKIN-11 RECEPTOR α DEFICIENT MICE COMPARED TO WILD TYPE DURING DECIDUALISATION

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Differentiation of endometrial stromal cells into decidual cells is essential for successful embryo implantation. Female interleukin-11 receptor α chain (IL-11Ra) null mice are infertile due to disrupted decidualisation, suggesting a critical role for IL-11 and its target genes in implantation. The molecular mechanisms by which IL-11 acts through its receptor to mediate decidualisation are unknown, but it is hypothesised that the expression of genes encoding other regulatory molecules will be modified by IL-11 signalling. This study aims to identify genes regulated by IL-11 during decidualisation in mouse uterus by DNA microarray analysis, and to examine the expression and functional significance of these genes during early pregnancy. Mice heterozygous for a targeted mutation of the IL-11Ra gene (IL-11Ra^{+/-}) were interbred to produce wild type (IL-11Ra^{+/+}) and null mutant (IL-11Ra^{-/-}) mice. Decidualisation was induced in pseudopregnant (plug = day 0) IL-11Ra^{+/+} and IL-11Ra^{-/-} littermates (n = 8/genotype) by oil injection into the uterine lumen on day 3. Whole uterus was removed at 0, 18, 24 or 48hrs after decidualisation (n = 2/group) and total RNA prepared by acid-phenol/chloroform extraction. Reference RNA was isolated from wild type unstimulated uterus (n = 16). Total RNA (10 μ g/sample) was reverse transcribed to fluorescent-labelled cDNA by indirect Cy3 or Cy5 coupling. NIA 15K microarrays were probed with experimental (IL-11Ra^{+/+} or IL-11Ra^{-/-}) and reference Cy3/Cy5-cDNA and scanned using an Axon GenePix 4000B microarray reader and GenePix Pro 3.0 software. Data was analysed using R 1.4.1, including normalisation for labelling efficiencies and scanning properties of Cy3 and Cy5, print-tip and spatial effects. At 18 hours after decidualisation, 18 genes were found to be upregulated and 6 genes downregulated >2-fold in IL-11Ra^{-/-} mice compared to wild type. These included expressed sequence tags (ESTs), genes with unknown function in the endometrium, and genes associated with transcription/chromatin, matrix/structural proteins and angiogenesis. Data analyses for further time points are underway. Genes identified as strongly regulated by IL-11 during decidualisation are likely to be important modulators of implantation, and will be further verified and characterised. By elucidating the role of IL-11 regulated genes in murine decidualisation, these studies may identify potential new targets for the manipulation of human fertility.

73. MANIPULATION OF BOVINE OOCYTE-CUMULUS CELL GAP JUNCTIONAL COMMUNICATION DURING IN VITRO MATURATION BY MODULATING CELL-SPECIFIC cAMP LEVELS

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Oocyte maturation is associated with the loss of oocyte-cumulus cell gap junctional communication, preventing entry of meiotic-modulating factors such as cAMP into the oocyte. Prolonging communication during *in vitro* maturation (IVM) may improve post-maturation outcomes, such as embryo development, through improved cytoplasmic maturation. We have previously shown that oocyte and cumulus cell (CC) cAMP levels can be independently regulated using selective phosphodiesterase (PDE) inhibitors [1]. The objectives of this study were to examine the effects of cell type-specific PDE inhibitors on the maintenance of oocyte-CC gap junction communication and oocyte meiotic progression. Cumulus-oocyte complexes (COC) were aspirated from antral follicles of abattoir-derived ovaries. Oocyte-CC gap junction communication during oocyte maturation was measured using the fluorescent dye, calcein-AM. COCs were cultured in the presence of specific PDE inhibitors; milrinone (MR, oocyte PDE3 inhibitor) or rolipram (RP, CC PDE4 inhibitor), and were pulsed with calcein-AM to allow dye transfer between the two cell types. Following CC removal, fluorescence in denuded oocytes was measured by microphotometry and meiotic progression assessed. In control COCs, dye transfer from CC to the oocyte fell progressively from 0 to 8h, after which oocyte-CC gap junction communication was completely lost. From 3-6h of maturation, loss of gap junction communication was significantly ($P<0.05$) attenuated by increasing oocyte cAMP with MR, but not by increasing CC cAMP with RP. Forskolin - a stimulator of adenylate cyclase that increases cAMP - further prolonged gap junction communication ($P<0.05$), while treatment with MR and forskolin together actually increased the level of dye transfer from 1-3h above that of control and forskolin alone. Importantly, all treatments that prolonged gap junction communication also delayed meiotic resumption, with meiosis generally resuming when fluorescence had fallen to ~35% of initial levels. These results demonstrate that meiotic resumption can be delayed by manipulating oocyte cAMP levels and oocyte-CC gap junctional communication. More importantly, maintaining/elevating intra-oocyte cAMP levels is associated with the maintenance of communication with surrounding cumulus cells and has the potential for altering the capacity of an oocyte to undergo meiotic and cytoplasmic maturation. Supported by NH&MRC [1] Thomas, RE, *et al* (2002) *Dev Biol*, 244: 215-225.

74. LEPTIN DYNAMICS IN THE PREGNANT RAT: CHANGES IN CLEARANCE RATE AND TRANSPLACENTAL PASSAGE LATE IN GESTATION

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Leptin has recently been implicated as an important player in the hormonal regulation of fetoplacental growth. The rat placenta expresses all three major isoforms of leptin receptor (Ob-Ra, Ob-Rb and Ob-Re), and expression of the truncated forms (Ob-Ra and Ob-Re) increases markedly in late pregnancy. Because Ob-Re is a soluble form of the receptor and binds to leptin in circulation, we hypothesised that the metabolic clearance rate (MCR) of leptin may fall during pregnancy. Since Ob-Ra facilitates transport of leptin across physiological barriers, we also hypothesised that the transfer of leptin from the maternal to the fetal compartment might increase late in gestation. Leptin MCR was measured in rats before and during pregnancy (days 16 and 22; term = day 23), as was the transplacental passage of leptin in pregnant rats. Anaesthetized rats received 1.5×10^6 cpm (~10ng) of ^{125}I -leptin via a jugular cannula and 7 sequential plasma samples were obtained over 90 min. Placentas and fetuses were collected immediately following the final plasma sample. TCA-precipitable radioactivity was determined in plasma and tissue homogenates, and the MCR of leptin calculated from the area under the bi-exponential disappearance curve. The MCR of leptin was 0.61 ± 0.04 ml/min/kg in non-pregnant rats, 0.51 ± 0.03 ml/min/kg at day 16 of pregnancy, then fell to 0.39 ± 0.02 ml/min/kg by day 22 ($P<0.01$). An index of transplacental passage of leptin was provided by the concentration of ^{125}I -leptin in the fetus relative to maternal plasma. This increased 3-fold ($P<0.05$) between day 16 to 22 of pregnancy, but was reduced ($P<0.05$) by almost 50% at day 22 following maternal dexamethasone treatment from day 13. In contrast, suppression of endogenous glucocorticoid synthesis by metyrapone increased ($P<0.05$) transfer of maternal leptin to the fetus by 29% at day 22. These effects of dexamethasone and metyrapone are consistent with the effects of glucocorticoids on maternal and fetal plasma levels of endogenous leptin. In conclusion, our data show that the MCR of leptin falls during rat pregnancy, likely due to increased placental secretion of Ob-Re. Transplacental passage of maternal leptin also increased from day 16 to day 22, an effect likely facilitated by the increase in placental Ob-Ra expression.

75. SEMINAL CYTOKINE CONCENTRATION AND HUMAN REPRODUCTIVE OUTCOME

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Seminal plasma has been thought to function simply as a transport and survival medium for spermatozoa. However, data in several species now suggest that seminal cytokines act to invoke an inflammatory response and immunological changes in the female reproductive tract following insemination [1,2]. Lack of exposure to seminal plasma and sperm may result in less than optimal reproductive outcome [1]. The aim of this study was to investigate the relationship between immunoregulatory molecules in semen and pregnancy outcome in fertile and infertile couples at the Reproductive Medicine Unit, Adelaide. The concentration of transforming growth factor beta (TGF β)1 and TGF β 2, cytokine X* and endotoxin were measured in seminal plasma samples obtained from normal (n=27) couples, or couples with male infertility (n=14), female infertility (n=6), combined male/female infertility (n=11), recurrent spontaneous abortion (RSA) (n=6) and infertility of unknown origin (n=7) using commercially available ELISA kits and limulus amoebocyte lysate assay. Repeated samples (n=5-8) recovered over a period of approximately 12 months from fertile men were also assessed. TGF β 1 and TGF β 2 were present in normal seminal plasma at high concentrations (mean \pm SD = 50.9 \pm 17.4 ng/ml and 1.3 \pm 0.6 ng/ml respectively), of which free TGF β 1 and TGF β 2 comprised <3% and ~20% respectively. In the subfertile groups, mean concentrations were not significantly different from normal samples. Cytokine X was detected in only 1/27 (3.7%) of samples from fertile men (12.8 pg/ml), but in 5/6 (83%) from RSA subjects (11.8 \pm 3.3 pg/ml) and 5/11 (45%) of combined male/female infertility subjects (11.1 \pm 3.7 pg/ml). Endotoxin was detected in all seminal plasma samples (22.8 \pm 34.3 EU/ml in fertile men versus 2.3 \pm 1.3 EU/ml, p= 0.11). In fertile men, there was less than 15% variation in the content of each of the immunoregulatory molecules amongst repeated samples. These results show there is no significant difference in seminal TGF β concentrations between fertile and infertile men. Importantly however, male partners of women suffering from RSA have higher levels of seminal plasma cytokine X. Since cytokine X acts to antagonise the pro-inflammatory and immune-deviating effect of TGF β , the abundance of this cytokine in semen may have implications for the quality of the female immune response to seminal antigens.

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2. Robertson, S. A., D. J. Sharkey, et al. (2000). *Proceedings of the Fertility Society of Australia*, Canberra.

*Not specified for commercial reasons, pending patent lodgement.

76. THE EFFECT OF P53 DELETION ON EMBRYO VIABILITY

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The culture of preimplantation embryos *in vitro* causes a reduction in their pregnancy potential. This is largely caused by apoptosis of embryonic cells by the blastocyst stage. This indicates that stressors associated with culture may activate the apoptotic pathway. A key sensor of cell stress is P53. We hypothesize that the absence of *p53* will reduce apoptosis and lead to improved embryo viability. Zygotes or 8-cell embryos were collected from *p53*^{+/-} X *p53*^{+/-} or wild-type crosses and cultured *in vitro* for 96h or overnight, respectively, in modified-HTF medium with 3mg BSA/ml. All mice were of C57BL/6 background. Morphologically normal blastocysts were transferred to Day 3 pseudopregnant mice. Autopsy was performed 9 days after embryo transfer. Total implantation sites, number of viable fetuses and the genotype of fetuses were recorded. In *p53*^{+/-} X *p53*^{+/-} crosses the number of viable fetuses produced by zygotes cultured *in vitro* for 96h was lower than observed following natural mating (24% vs 91%, p<0.001). There was no difference in the implantation rate for embryos cultured from the zygote compared to 8-cell stage (47% vs 58%). However, more viable fetuses resulted from 8-cell embryos than from zygotes (77% vs 24%, p<0.001). Furthermore, compared to wild-type zygotes, cultured zygotes from *p53*^{+/-} X *p53*^{+/-} produced a higher number of viable fetuses (2.4% vs 24%, p<0.01), and this difference was largely due to significantly increased survival of *p53*^{-/-} embryos. This survival advantage of *p53*-null embryos was not observed following natural mating nor following transfer of embryos cultured overnight from the 8-cell stage. The results confirm (1,2) that *in vitro* culture of zygotes significantly decreased embryo/fetal viability. The absence of *p53*, a tumor suppressor gene, caused a significant improvement in the likelihood of embryos forming fetuses when embryos were cultured from the zygote, but not the 8-cell stage. The results show that stresses produced by *in vitro* culture cause loss of embryo/fetal viability and that this is at least partially P53-dependent. The study also shows that *in vitro* culture of embryos creates a positive selection pressure for loss-of-function mutations to *p53*. [1] Lane M, Gardner DK. (1994) *J Reprod Fertil*, **102**, 305-312 [2] Bowman P, McLaren A. (1970) *J Embryol Exp Morphol*, **23**, 693-704.

77. INSEMINATION AND UTERINE LYMPHOCYTE TRAFFICKING IN MICE

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Lymphocyte populations facilitating embryo implantation and early placental development have immunosuppressive phenotypes, similar to those mediating immune tolerance in other mucosal tissues. It is unclear how and when these populations become activated, but they have some similarities to those primed earlier in pregnancy at insemination. The mechanisms defining lymphocyte phenotype and homing patterns occur at activation, and lymphocytes primed in specific tissues have a propensity to recruit back to those tissues [1]. Moreover, paternal and other antigens are shared by semen and the conceptus. Thus we hypothesise that mating comprises the 'priming' event for activation of lymphocytes required for embryo implantation [2]. The aim of this study was to investigate whether insemination leads to the activation of lymphocytes that can be recruited preferentially into the implantation sites of pregnant mice. We employed cell tracking techniques, utilising [¹²⁵I]-iodo deoxyuridine (¹²⁵IdUR), to label lymphocytes recovered from the para-aortic lymph nodes (PALN) which drain the uterus, in virgin mice or 4 days after insemination. Labelled cells were transferred to day 6 pregnant recipients via the lateral tail vein, and recruitment into a range of tissues was assessed by γ counting 24 hours after transfer. Tissue specific patterns of lymphocyte recruitment were identified, with preferential recruitment of PALN lymphocytes into lymphoid and uterine tissues. PALN lymphocytes from mated donors showed a pattern of recruitment into the uterus, preferentially into implantation tissues as opposed to inter-implantation tissues ($p=0.01$), which was not explained simply by the increased vascularity of this site. The distinction was not evident when PALN lymphocytes were recovered from virgin donors. PALN lymphocytes from virgin donors also showed a greater propensity to recruit back to the PALN and other lymphoid tissues ($p<0.05$). These data show that insemination causes activation and expansion of lymphocyte populations that can be preferentially recruited into the site of embryo implantation and early placental development, supporting the notion that insemination is involved in the programming of cells to home to implantation sites. Virgin derived PALN cells showed a significantly increased recruitment back into the PALN, consistent with these cells not having been activated to recognise the homing addressins or paternal antigens required for recruitment into implantation sites. If a causal linkage between exposure to semen and beneficial immunity to the conceptus can be proven, novel therapeutic strategies exploiting this connection may be established to assist in treatment of pregnancy pathologies having an immune aetiology, such as implantation failure and recurrent miscarriage. (1)Butcher, E.C. and L.J. Picker, *Science*, 1996. 272: p. 60-66. (2).Robertson, S.A., et al., *Am J Reprod Immunol*, 1997. 37(6): p. 438-42.

78. GENERATING PITUITARY CELLS FROM EMBRYONIC STEM CELLS – A ROUTE TO PITUITARY CELL THERAPY?

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Embryonic stem cells are derived from the early mammalian embryo and have a unique capacity to differentiate into all tissues within the body. The availability of human ES cells presents an unprecedented opportunity for the development of cell therapies for human disorders, based on the replacement of defective tissue with functional tissue generated in vitro from directed differentiation of ES cells. Using mouse ES cells as a model system, we are exploring the therapeutic potential of ES based therapy for pituitary diseases. A key requirement of ES cell therapies is the efficient conversion of ES cells into the desired cell type. Using a range of culture conditions and growth factors, we have shown that for the first time that ES cells are capable differentiating in vitro into cell types that express progenitor (*Prop1*, *Hesx1*) and differentiated (*GH*, *POMC*) pituitary cell markers. We are currently developing methodologies which will allow us to produce pituitary cell types with maximum efficiency, and, in the long term, aim to test the function of these cells using mouse models of pituitary disorders.

79. THERAPEUTIC APPLICATIONS OF STEM CELL TECHNOLOGIES

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Stem cell therapies have the potential to provide therapeutic solutions to many currently intractable human diseases. While a number of different sources of therapeutic stem cells have been proposed, including adult stem cells and stem cells from the cord blood, the most advanced methodologies are based around utilisation of stem cells isolated from the pluripotent founder cell population of the mammalian embryo. These embryonic stem (ES) cells have a number of properties which make them well suited to therapeutic application: (1) They have an unrestricted capacity for renewal in vitro which allows expansion of effectively unlimited numbers of precursor cells. (2) They retain pluripotency in vitro and can therefore be differentiated to any embryonic or adult cell type. (3) They are genetically tractable such that the genome can be altered experimentally to achieve site-specific additions, precise deletions or sophisticated modifications. Taken together these properties mean that a population of ES cells can be regarded as an unlimited number, of any kind of cell, with any genotype. Cells obtained by differentiation of ES cells can potentially be used to treat diseases caused by cell loss, damage or dysfunction, or in conjunction with genetic modification to achieve gene therapy. Transplantation of undifferentiated ES cells leads to the formation of teratocarcinomas so the development of methodologies for the production of partially or fully differentiated cells from ES cells is likely to be critical for therapeutic application. Our approach is to control the formation of somatic cell types from ES cells in a manner that recapitulates embryogenesis. Controlled differentiation of ES cells to specific lineages, including neural and myogenic lineages, has been demonstrated, providing promise that extensive, well-characterised populations of transplantable cells will become available. The most suitable cell candidates for therapy are being tested by transplantation and the integration of ES-derived cells has been reported for several tissues. In some cases, transplantation of differentiated ES cells has been shown to restore function to damaged tissue, providing proof of concept for the technology.

80. OOCYTE-DERIVED GROWTH FACTORS AND OVULATION RATE IN MAMMALS

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Ovulation rate in mammals is determined by a complex exchange of hormonal signals between the pituitary gland and the ovary, and by a localised exchange of hormones within ovarian follicles including the oocyte and its adjacent somatic cells. Studies from mice lacking certain growth factor genes and sheep with naturally occurring genetic mutations show that the oocyte plays an essential role in regulating ovarian follicular growth and ovulation rate. If the oocyte has such a major effect on ovulation rate, then it can be hypothesised that the relative importance and indeed actions of certain oocyte-derived growth factors may vary between mammals with a high ovulation rate phenotype (e.g. mice, rats, dogs and pigs) and those with a low ovulation rate phenotype (e.g. humans, cattle and sheep). The evidence from studies of two oocyte-derived growth factors, namely bone morphogenetic protein 15 (BMP15; otherwise known as growth differentiating factor 9B or GDF9B) and GDF9 in mice and sheep suggest that this hypothesis may have some validity. For example, mice lacking the GDF9 gene are infertile with ovarian follicular growth blocked at the primary stage of development. However, mice lacking a functional BMP15 gene are fertile with normal numbers of preovulatory follicles. Mice heterozygous for either gene have apparently normal ovulation rates. By contrast, sheep that are heterozygous for inactivating mutations in BMP15 have higher ovulation rates than normal. Moreover, sheep with modest antibody responses to BMP15 or GDF9 can have higher than normal ovulation rates (i.e. 2 to 12 versus 1 or 2). Sheep that are homozygous for inactivating mutations in BMP15 or with high antibody responses to BMP15 or GDF9 are infertile with completely impaired follicular development beyond the primary stage of development. Also passive immunisation studies in sheep with specific antiplasma to either BMP15 or GDF9 show that both are essential for normal follicular development before ovulation. The molecular forms of BMP15 and GDF9 in biological fluids (e.g. monomers, homodimers, heterodimers etc) as well as the specific receptors to these growth factor remain to be determined. With these caveats in mind, the current evidence is consistent with the hypothesis that both follicular growth and ovulation rate are profoundly influenced by the dose of BMP15 or GDF9 delivered to the somatic cells of the follicle in mammals with a low ovulation rate phenotype, whereas in those with a high ovulation rate phenotype, the follicular somatic cells are relatively insensitive to changing the doses of BMP15 but have an absolute requirement for GDF9.

81. INTRAOVARIAN REGULATION OF FOLLICULOGENESIS: SIGNAL MOLECULES AND THEIR CELLULAR SOURCES

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Folliculogenesis in mammals is a continuous and dynamic process that begins during embryonic development in utero and continues until the end of adult reproductive life. The overall process is regulated by a complex array of interacting chemical signals originating both within and external to the ovaries. The identities and relative importance of specific signals change as follicle growth progresses through a series of morphologically distinct stages, beginning with initiation of growth of the dormant primordial follicles, and ending in either atresia or ovulation. The initiation of follicle growth occurs when a primordial follicle is activated by as yet unknown mechanisms and enters a pool of growing follicles. Recent evidence has established the essential role of the oocyte as the driving force in promoting follicle growth throughout pre-antral and early antral stages, mediated via recently identified members of the TGF β superfamily of growth factors, GDF9 and GDF9B, which have essential roles in both proliferation and differentiation of granulosa cells. The follicular somatic cells, including both granulosa and thecal cells, also contribute to regulation of follicle growth during these stages, through secretions of several other peptide growth factors, in particular bFGF, IGF-I, TGF β , activin, and inhibin. Upon reaching antral stage of development, follicles acquire responsiveness to follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and these gonadotrophins then assume primary importance in regulation of growth to the pre-ovulatory stage, with growth factors continuing to play supportive and modulating roles. The actions of FSH and LH are mediated in part via androgens and oestrogens secreted by the thecal and granulosa cells, respectively. With the approach of ovulation, migratory cells derived from the circulation, including macrophages and to a lesser extent other leukocyte subsets, infiltrate the ovarian stroma and thecal layer of the pre-ovulatory follicle, where they play essential roles in the tissue remodelling that leads to rupture of the follicle and its transformation to a corpus luteum at ovulation. Their actions are mediated via pro-inflammatory molecules including cytokines, chemokines and eicosanoids secreted by the macrophages and by the follicle cells themselves. Recent evidence implicates signals initiated in the endometrium in response to semen exposure, in enhancing this leukocytic infiltration of the ovary, and influencing their pattern of cytokine secretion, with important consequences in enhancing ovulation and luteinization.

82. OESTROGEN TARGET SITES IN HUMAN TESTIS

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It has been proposed that oestrogens play a role in regulating germ cell function in adulthood and during fetal life. Oestrogen action is mediated via high affinity intracellular receptors expressed in target tissues [1]. Two subtypes of oestrogen receptor known as ER α (NR3A1) and ER β (NR3A2), have been cloned and hER β variant isoforms identified. In target cells these receptors can exist as homo- or heterodimers [1]. We have used immunohistochemistry to examine the patterns of expression of ERs in human fetal and adult testis to determine the cellular targets for oestrogen action. In parallel studies we have prepared ER constructs and used these to examine steroid binding to ER homo- and heterodimers in transfected cells.

In human testicular tissues (fetal or adult) we have never detected ER α mRNA or protein. With a polyclonal antibody we detected ER β protein in multiple testicular cell nuclei including those of Sertoli cells, Leydig cells and peritubular myoid cells as well as some germ cells [2]. Additional studies using monoclonal antibodies capable of discriminating between human wild type ER β and the human ER β cx/ β 2 [3] splice variant revealed discrete patterns of expression of the subtypes. In adult testis immunoeexpression of wtER β was most intense in pachytene spermatocytes and round spermatids, whilst low levels of expression were detected in Sertoli cells, spermatogonia, preleptotene, leptotene, zygotene and diplotene spermatocytes. Expression of ER β cx/ β 2 protein was highest in Sertoli cells and spermatogonia with low/variable expression in preleptotene, pachytene and diplotene spermatocytes [4]. In the fetal testis wtER β was undetectable in gonocytes whereas these cells expressed the highest levels of ER β cx/ β 2 compared with other testicular cell types. Both ER β 1 and ER β cx/ β 2 were detected in some but not all Sertoli cells, peritubular cells and Leydig cells. Transfection studies with ER α and ER β constructs confirmed that wtER β and ER α were activated following exposure to oestrogenic ligands but that the ER β cx/ β 2 variant did not bind oestrogens and was not capable of activating an ERE-containing reporter construct.

The testicular cells most likely to be targets for oestrogens in adulthood are round spermatids in which levels of expression of wtER β 1 are high. In contrast, expression of ERcx/ β 2, an isoform that may act as a dominant negative inhibitor of ER action, in adult Sertoli cells and spermatogonia, and in fetal gonocytes could prevent these cells responding to oestrogens.

1. Nilsson, S., et al., *Phys Rev*, 2001. **81**: 1535. 2. Saunders, P.T.K., et al., *Mol Hum Reprod*, 2001. **7**: 227. 3. Ogawa, S., et al., *NAR*, 1998. **26**: 3505. 4. Saunders, P.T.K., et al., *JCEM*, 2002. **87**: 2706.

83. MALE CONTRACEPTION – APPROACH TO HORMONAL METHODS

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Worldwide, men participate actively in contraception through natural methods, condom usage and vasectomy but no new, acceptable, safe and reversible methods have become available. Male hormonal contraception (MHC) is the most promising avenue of research. Testosterone (T) treatment suppresses pituitary LH and FSH secretion, the primary endocrine signals required for spermatogenesis. Large multicentre WHO-sponsored trials have shown that high doses of T reduce sperm counts to zero (azoospermia) in ~70% Caucasian men while over 90% have sperm counts < 1 million/m (severe oligospermia), a level at which contraceptive efficacy appears adequate. But the need for frequent injections and androgenic side effects (acne, mood changes, reduced HDL-cholesterol) demand alternative methods of T administration. Testosterone implants provide the prototype for more physiological T treatment but pellet insertion/extrusion limit their wide application. New long acting intramuscular preparations, such as T undecanoate in oil, are most promising and may allow 8-12 weekly injection intervals.

Co-administration of other gonadotropin-suppressing agents, such as GnRH antagonists or progestins, enhance spermatogenic suppression and permit the use of lower T doses. In MHC, progestin action is probably via gonadotropin suppression but direct actions within the testis have not been excluded. A range of progestins have been used including levonorgestrel, desogestrel and depot medroxy-progesterone acetate, and studies have shown that ~75% and ~95% of men become azoospermic or severely oligospermic, respectively. The only T plus progestin efficacy study to date has reported excellent contraceptive efficacy over a 1 year exposure (Turner et al, ESA 2002, abstract). The stage is set for pharmaceutical company involvement, sadly lacking in the past, in order to bring the first MHC regimen to market. About 5% of men fail to suppress adequately and understanding the reason(s) for their failure to respond is important in gaining the widest acceptance of MHC.

We have explored the MHC effects on spermatogenesis in men using stereological techniques for germ cell quantification and showed that type Apale→B spermatogonial development and sperm release (spermiation) are the key sites of action. The spermatogonial defect probably results from FSH withdrawal but the subtypes affected and their mechanism of action are unknown. Spermiation failure is a feature of both acute (accounting for dramatic falls in sperm output within 3 weeks) and chronic MHC treatment. Both FSH and T regulate rodent spermiation but nothing is known in man. Cell-cell adhesion/communication and regulation of gene expression in later germ cell types can now be explored using laser capture microdissection and *in vitro* culture. From an endocrine regulatory viewpoint, MHC suppresses serum LH to <0.3% of control and intra-testicular T levels to 2% but dihydrotestosterone (DHT) levels are maintained – a possible role may exist for DHT and the up-regulation of testicular 5 α -reductase type 1 in supporting some degree of spermatogenesis. FSH levels are suppressed to 1-3% but remain detectable in most MHC-treated men and again may support some degree of continued spermatogonial development.

84. FOLLICULAR DEVELOPMENT IN WALLABY OVARIAN TISSUE XENOGRAFTS IS NOT ALTERED BY THE HORMONAL ENVIRONMENT OF THE RECIPIENT

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The xenografting of ovarian tissue provides a viable alternative to *in vitro* culture methods for oocyte maturation as well as a strategy for studying follicular development in endangered and threatened wildlife. In the wallaby, follicular development appears to proceed normally in both immature and mature ovarian tissue when xenografted to an immunocompromised recipient (1-2). In most xenografting and allografting reports, bilaterally ovariectomised recipients have been utilized for their elevated gonadotrophin levels that are believed to be beneficial for follicular development (3). In the wallaby, data has indicated that mature oocytes may be more readily collected from xenografts to intact recipients than ovariectomised recipients (1). Similarly, in the mouse follicular development within immature ovarian allografts is influenced by the gonadal status of the recipient, with an ovariectomised recipient required for complete follicular development to occur within immature mouse allografts. The aim of this study was to investigate follicular development in immature wallaby ovarian tissue xenografted to female mice with varying hormone environments. Ovarian tissue was collected from d75-90 female tammar wallaby pouch young (n=5) and xenografted under the kidney capsule, 1 piece per kidney, of 6-8 week old NOD-SCID female mice (n=12). At the time of surgery recipients were either bilaterally (n=4) or unilaterally (n=4) ovariectomised or left intact (n=4) producing three treatment groups. Three months after surgery grafts were recovered and histologically assessed for the number and type of follicles present. Grafts containing viable follicles were recovered from each of the treatment groups (bilaterally ovariectomised, n=7/8; unilaterally ovariectomised, n=5/8; intact, n=7/8). The number of follicles contained within the grafts was highly variable and there were no significant differences between the three treatment groups (p>0.05). In all treatment groups primordial follicles were the most abundant follicle type, however primary and secondary follicles were also observed in grafts from all treatment groups. This study confirms that xenografting supports follicular development within wallaby pouch young ovarian tissue (1-2). Interestingly, follicle survival and follicular development was sustained in all treatment groups, which indicates that the hormonal environment of the recipient may not play as crucial a role as previously believed. [1] Matiske *et al* (2001) *Proc Aust Mamm Soc* 47:35. [2] Matiske D *et al* (2002) *Reproduction* 123:143-153. [3] Cox S-L *et al* (2000) *Fertil Steril* 74:366-371.

85. DEVELOPMENT OF ANTRAL FOLLICLES IN RABBIT OVARIAN XENOGRAFTS IN IMMUNODEFICIENT MICE

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The primordial follicle pool is established in mammalian ovaries in very early life. From this time forward the population of primordial follicles progressively decreases as a result of either atresia or the initiation of growth and development. Very little is understood about the processes that regulate normal follicle recruitment and survival in the rabbit. This report describes an initial trial in the use of ovarian xenografting as a means to investigate follicular dynamics in juvenile rabbits. Fresh ovarian tissue from NZ White rabbits aged 2, 4, 8 and 12 weeks old were grafted under the renal capsule of ovariectomised NOD-SCID mice (n=35). Grafts were recovered 8 days, 2 weeks, 4 weeks or 8 weeks later, assessed macroscopically and subjected to histological evaluation. All grafts were present and had become vascularised at the time of autopsy. Grafts from two-week-old donor rabbits recovered 2 and 4 weeks after grafting did not contain any follicles. In contrast four-week-old donor rabbit tissue (grafted for 4 weeks) contained healthy looking primordial and preantral follicles, resembling folliculogenesis normally observed in 8-week-old rabbit ovaries. Similarly four-week-old donor tissue (grafted for 8 weeks) contained follicles at all stages of development including antral follicles, resembling 12-week-old normal rabbit ovaries. Whilst tissue from 8 week old donors (grafted for 4 weeks) displayed apparently normal primordial, preantral and antral follicles (diameters of approximately 1mm), tissue grafted for only 8 days and 16 days contained primarily degenerating preantral follicles and healthy primordial follicles. Interestingly, corpora lutea like structures were detected in a number of grafts from donors aged 12 weeks though "ovulation" of normal oocytes remains unconfirmed. In general the development and morphology of follicles within the xenografted rabbit ovarian tissue resembled the normal pattern of folliculogenesis. Thus ovarian xenografts in NOD-SCID mice can serve as an experimental model for juvenile rabbit follicular growth and development. We intend to use this technique in conjunction with an *in vitro* culture system we have developed for rabbit ovarian cortical explants to investigate factors regulating folliculogenesis.

86. ACUTE FSH WITHDRAWAL IMPAIRS SERTOLI AND GERM CELL DEVELOPMENT IN THE IMMATURE RAT

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Pituitary follicle stimulating hormone (FSH) is required for normal testicular development, affecting both Sertoli and germ cells (1) through its receptors on Sertoli cells, and thereby determining the potential for sperm production in adulthood. The impact of FSH on *in vitro* Sertoli cell proliferation alters during maturation (2), though the mechanisms underpinning these actions and their development are not understood. To establish an *in vivo* model for examination of the role of FSH at discrete developmental stages, we assessed the impact of acute FSH withdrawal on the Sertoli and germ cell populations during early postnatal life. Sprague Dawley rat pups (n = 7/group) were passively immunised against FSH using an ovine polyclonal antibody to rat FSH (10 mg/kg by subcutaneous daily injection) for 2 and 4 days prior to death on days 3, 9 and 18 days postpartum. Control animals received a normal sheep immunoglobulin (10 mg/kg). Testes were immersion fixed with Bouin's fluid and embedded in resin for determination of cell number using the optical disector stereological technique, and paraffin for assessment of proliferation and apoptosis using immunohistochemistry. Sertoli and germ cell numbers per testis were unchanged following 2 days of FSH withdrawal at day 3, 9, and 18 compared to controls. In response to 4 days of FSH withdrawal in day 9 rats, Sertoli cell and type A/intermediate spermatogonial number were decreased to 73% (P<0.05) and 76% of controls (P=0.1), respectively. While 4 days of FSH withdrawal in day 18 rats had little effect on Sertoli cell number (90% of control), the number of type A/intermediate spermatogonial, type B spermatogonia/preleptotene spermatocytes and leptotene/zygotene spermatocytes were reduced, being 76% (P=0.06), 75% (P=0.03) and 80% (P=0.06) of control, respectively. Cell loss in response to FSH withdrawal at day 9 and 18 was associated with a 50% (P=0.004) and 40% (P=0.06) decrease in cell mitosis, respectively compared to controls. This study demonstrates FSH supports Sertoli and germ cell development, in part by promoting cell proliferation. The contribution of apoptosis to this cell loss is under investigation. Establishment of this experimental paradigm will enable further investigations of the mechanisms by which FSH regulates normal testicular development and function. (1) Meachem et al 1996 *Biology of Reproduction* 54:36; (2) Boitani et al 1995 *Endocrinology* 36(12):5438.

87. EVALUATION OF TESTICULAR COOLING FOR GERM CELL TRANSPLANTATION

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To deplete endogenous germ cells before male germ cell transplantation, recipients have normally been prepared by administration of busulfan. Because busulfan exerts systemic toxicity, alternative ways of depleting the recipient's germ cells are desirable. In this study we examined testicular cooling, long known to selectively kill germ cells in the rat testis. Testes of adult rats were removed from the scrotum in the intact tunica vaginalis and placed in either cold (1°C; treated group, n=63) or room temperature (control group, n=63) Ringer's solution for 60 min. Seven rats per treatment were killed at different times after cooling for blood and testis collection. Circulating inhibin B, FSH and T were assayed by radioimmunoassay. One testis of each animal was fixed in Bouin's for histological analysis by H&E and TUNEL. RNA extracted from the other testis was examined with an RNase protection assay (RPA) to monitor changes in expression of apoptotic mediators. Direct testicular cooling depleted most germ cells and was mediated by apoptosis, as detected by TUNEL. 46-67% of tubules in the cooled testes had only Sertoli cells and spermatogonia surviving 3-10 weeks after cooling. Germ cell loss started 8-24 hours after cooling, with stage XII-I spermatocytes most sensitive to cooling. RPA detected no significant change in apoptosis mediators caused by cooling, though individual animals showed a distinct up-regulation of FAS at 24 hours, in accordance with the stimulation by cooling of germ cell apoptosis. Associated with the germ cell loss, circulating inhibin B concentrations declined, FSH increased, but plasma T was not significantly changed. To assess the utility of cooling as a means of generating recipient testes, donor germ cells from *lacZ* transgenic mouse testes were transferred into testes of nine additional rats 25-35 days after cooling. At 64-92 days after transplantation, rats were killed and the testes stained with X-gal to assess mouse germ cell colonization and development in the rat testes. The functional assay using germ cell transplantation demonstrated that testes in which germ cells had been depleted by cooling were not suitable as recipients since none of the transplanted mouse germ cells survived. This suggests that cooling permanently disrupts the niche required for stem cell survival and colonisation.

88. DELIVERY OF ANTIBODIES FOR MALE IMMUNOCONTRACEPTION

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Although there are claims of immunocontraception in males there is little evidence that sufficient antibody can be delivered to the reproductive ducts to be effective. Consequently, we have assessed antibody delivery using microsurgical methods to collect luminal fluids from animals immunized with tetanus toxoid. Determinations of IgG in rats immunized sub-cutaneously showed that it is delivered in the secretions of the seminal vesicles and prostate gland respectively at 0.2% and 0.3% of the concentration in blood. It enters the lumen of the rete testis to achieve 0.7% the concentration in blood, and is concentrated (due to fluid reabsorption) 1.7-fold in the efferent ducts and a further 1.5-fold in the ductus epididymidis. Halving fluid reabsorption by the efferent ducts (by estradiol administration) did not significantly reduce reabsorption of IgG, and the ductus epididymidis compensated for the reduced fluid reabsorption by the efferent ducts. Immunization protocols that raised mucosal and systemic responses were compared in the mouse. Although intranasal immunization achieved the highest titer of IgG in serum, subcutaneous immunization achieved the highest titers in the sperm ducts. Rectal immunization achieved the highest titer in prostatic fluid (5% of serum), and is interpreted as a local production. Nasal immunization produced the highest titers of IgA in the rete testis and prostate, the titers in the prostate being 3.4-times the titre in blood. Strong PCR product for pIgR primers were demonstrated in the prostate indicating the presence of secretory component. It is concluded that a better understanding of mechanisms may lead to immunocontraception against male reproductive antigens.

89. IMMUNOCONTRACEPTIVE EFFECT OF RECOMBINANT PORCINE ZONA PELLUCIDA C PROTEIN IN RABBITS

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Despite the success of biocontrol methods of population control (such as calicivirus) rabbit populations within Australia remain a significant problem particularly in temperate zones. An alternative is to induce sterility in the female rabbit population via immunisation with a viral vector containing an ovarian antigen. Fertility trials using total porcine zona pellucida as an immunogen have been shown to induce infertility in a number of diverse species. Here we describe the production of recombinant porcine Zona Pellucida C (pZPC) protein and its use as an immunocontraceptive immunogen in small scale fertility trials in domestic rabbits. Recombinant protein was produced by expression of the full length porcine ZPC cDNA in a vaccinia virus/T7 system in a co-infected monkey kidney cell line (CV1) and purified using lectin affinity chromatography. Two adult male and ten adult female laboratory rabbits were inoculated with 100µg recombinant pZPC in Freund's Complete adjuvant and boosted twice with 50µg in Incomplete Freund's adjuvant on Days 28 and 56. Sera were collected prior to immunization and following boosting (Days 14, 44, 70). Four male rabbits of proven fertility were used to mate both immunized and untreated control rabbits (n=4). The immunized rabbits were sacrificed 9 days post mating and the reproductive tracts examined for corpora lutea and implantation sacs. Ovaries from rabbits immunized with recombinant pZPC were fixed in Bouin's solution. Tissues were paraffin embedded and sectioned prior to staining with hematoxylin and eosin. By Day 70 sera from all animals contained antibody capable of detecting recombinant pZPC protein by Western blotting. Immunoperoxidase staining was detected on the zona pellucida of early primary and more mature follicles when the antisera from immunized animals were used to probe normal ovarian sections. At autopsy all ten immunized animals had visible corpora lutea but only two were pregnant with 10 and 2 viable implantations respectively. The remaining eight females displayed signs of pseudopregnancy only. In contrast all control females (n=4) had normal sized litters (mean±sem. 7.25±1.3). Histological evaluation showed no difference in ovarian folliculogenesis between treated animals and age matched control specimens. Serum antibody titres measured using ELISA showed no difference between males and females immunized with pZPC and no correlation with pregnancy. As antibody was not detected bound to the ZP in vivo the mechanism by which recombinant pZPC induced infertility remains to be elucidated.

90. EFFECT OF ANGIOGENESIS INHIBITORS ON OESTROGEN-MEDIATED ENDOMETRIAL ENDOTHELIAL CELL PROLIFERATION IN THE OVARIECTOMISED MOUSE

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It has been suggested that endometrial angiogenesis in response to the sex steroids oestrogen (E) and progesterone (P) is mediated at a local level via compounds such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF), acting through their respective tyrosine kinase receptors (RTK). The aim of the present study was to use RTK angiogenic inhibitor compounds to determine if VEGF, FGF or PDGF play a role in mediating endometrial endothelial cell (EC) proliferation following administration of E and P. Endometrial EC proliferation was induced in adult ovariectomized mice by either E alone for 1 day (E1), or P with low dose E followed by P with high dose E (PE). Each angiogenesis inhibitor compound was injected daily for four days (100mg/Kg/day, s.c.) before endometrial tissue collection. We also evaluated the effect of VEGF antiserum (0.2ml i.p.) on EC proliferation at E1. All the angiogenic inhibitor compounds significantly reduced EC proliferation activity at E1 and PE. The greatest reduction in EC proliferative index was at E1 in the group treated with a VEGF receptor inhibitor (2.5 ± 0.7% versus 27.9 ± 1.1%, $P < 0.001$), with a reduction of similar magnitude in the group treated with VEGF antibody. At the PE stage, all the inhibitors significantly reduced EC proliferation to a similar degree, suggesting that VEGF, FGF and PDGF may all be involved. These data demonstrate that endometrial angiogenesis following acute E treatment is primarily mediated by VEGF, but that under the influence of combined E and P, FGF and PDGF may also be involved.

91. MOLECULAR RESPONSE OF MYOMETRIAL MICROVASCULAR ENDOTHELIAL CELLS TO VEGF AND PROGESTERONE ASSESSED BY MICROARRAY

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There is evidence that the endothelial cells of different organs display heterogeneity of function in response to different growth factors and circulating hormones. Further, little is known of direct effects of progesterone on endothelial cells. The aim of this study was to use cDNA gene expression microarrays to analyse changes in gene expression following stimulation of myometrial microvascular endothelial cells (MMECs) with vascular endothelial growth factor (VEGF) (1), or progesterone plus and minus an inflammatory stimulus. Primary isolates of MMECs were obtained from fresh hysterectomy specimens, and immunopurified. For the VEGF study, MMEC were stimulated with 15 ng/ml VEGF for 3, 6 and 12 hours with 2 unstimulated experiments to act as a control. For the progesterone study immunohistochemistry and RT-PCR were used to confirm progesterone receptor expression in cultured MMEC. 10.5K cDNA microarrays were used to examine gene expression responses in MMECs to progesterone, as well as alteration of two inflammatory stimuli (IL-1 β and TNF α) by progesterone. One hundred and ten genes were identified as upregulated by VEGF, 19% of which (21) have previously been reported. Among the novel genes to be upregulated by VEGF were brain derived growth factor (BDNF), oxytocin receptor, and estrogen sulfotransferase. Progesterone receptor was expressed in MMECs, but no change in gene expression was detected following progesterone (100 nmol/L) stimulation. Progesterone reduced the ability of the MMECs to express E-selectin protein in response to TNF α as measured by flow cytometry, but neither cDNA microarray nor Northern blot could demonstrate regulation at a transcriptional level. Our VEGF data suggests that in addition to genes commonly upregulated in endothelial cells in response to VEGF, some genes specific to MMEC's may exist. The progesterone data shows that stimulation by progesterone alone does not have any detectable effects on gene transcription despite the presence of progesterone receptor. However, progesterone appears to have a non-genomic effect on TNF α -induced E-selectin expression. (1) Weston GC, Haviv I, Rogers PAW (2002) Microarray analysis of VEGF-responsive genes in myometrial endothelial cells. *Mol. Hum. Reprod.* (in press).

92. ANALYSIS OF REPRODUCTIVE PHENOTYPE AND EXPRESSION OF CALCIUM BINDING PROTEINS IN CABP-D28K NULL MICE

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The uterine endometrium is hostile to embryo implantation except during the "window of receptivity". Receptivity depends on changes in gene expression. Differential display PCR shows calcium binding protein (CaBP) d9k mRNA is upregulated in early mouse interimplantation sites. CaBP-d28k also displays differential regulation in the mouse endometrium. Both proteins are localised in endometrial epithelium during early implantation with subsequent downregulation at the trophoblast/endometrial interface. CaBP-d28k null mice (d28k^{-/-}) show severe ataxia in complex tasks, and were anecdotally reported to have an abnormal reproductive phenotype. We aimed to establish in d28k^{-/-} mice: (1) uterine expression of CaBP d9k & d28k, (2) whether d28k^{-/-} mice have reproductive abnormalities, (3) sources of such abnormality. To characterize reproductive behaviour 6 d28k^{-/-} males and 4 (W/T) C57^{bl6} males (age matched) were mated with 20 W/T females. Mating was detected by checking vaginal plugs (plug=day 0). Uteri and ovaries were examined on day 5 for implantation (imp) sites and corporal lutea (CL) respectively. Reproductive phases were determined in age matched d28k^{-/-} and W/T mice over 21 days by examination of vaginal smears. No differences were observed between d28k^{-/-} and W/T in mean time (days) before plugging (W/T 2.9 \pm 0.4, ^{-/-} 2.8 \pm 0.4) and number of imp sites (W/T 7.4 \pm 0.8, ^{-/-} 6.8 \pm 1.4). CL numbers matched number of imp sites (W/T 8.3 \pm 1.1, ^{-/-} 7.4 \pm 1.5). Significant differences (p<0.01) in the d28k^{-/-} female cycle were observed, in terms of number of estrous (W/T 3 \pm 0.5, ^{-/-} 1.7 \pm 0.2), metestrous (W/T 2.3 \pm 0.2, ^{-/-} 1.1 \pm 0.2) and pro-estrous stages over 21 days (W/T 2.4 \pm 0.2, ^{-/-} 1.89 \pm 0.4). Days spent in each estrous (W/T 5.1 \pm 0.8, ^{-/-} 3.3 \pm 0.8), metestrous (W/T 7.8 \pm 1.0, ^{-/-} 12.1 \pm 0.9), diestrous (W/T 3.2 \pm 0.6, ^{-/-} 1.8 \pm 0.4) and proestrous stages (W/T 4.8 \pm 0.3, ^{-/-} 3.3 \pm 0.8) were also different. Thus, in spite of poor motor co-ordination, d28k^{-/-} males were able to mount normally. Embryo's fertilized by d28k^{-/-} males implant normally. However d28k^{-/-} females have a longer cycle than W/T with less time in estrous, but prolonged metestrous stages, This provides an explanation for observed overall lower fertility of d28k^{-/-} mice. Acknowledgements: supported by the Monash Faculty of Medicine and PHIMR.

93. MAST CELL NUMBER AND SPATIAL LOCATION IN THE VAGINAL CUL-DE-SAC OF THE BRUSHTAIL POSSUM AFTER ADMINISTRATION OF EXOGENOUS OESTRADIOL

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Mast cells are present in large numbers in the vaginal cul-de-sac, a component of the reproductive tract in female Brushtail possums. Their presence does not appear to be an immunological response to the presence of microflora, as established in a previous study of mast cell number and spatial location in cul-de-sac tissues (1). In this study mast cell numbers and spatial location were determined in seasonally anoestrus possums and those treated with sesame oil or oestradiol 17 β implants for 6 days.

Cul-de-sac tissue was collected aseptically for microbiological analysis, and the Fractionator and Optical Disector stereological methods were used to quantify mast cell populations in tissue sections.

In all groups (n=6/group), microflora populations were consistently low (<4.0 x 10⁴ organisms gram⁻¹). Mean weight and volumes of epithelial and connective tissues were significantly greater in oestradiol treated animals. Mean numbers of mast cells in epithelial and connective tissues did not differ significantly amongst groups. Mean mast cell density was therefore significantly greater in the seasonally anoestrus and control animals than the oestradiol treated. Although previous empirical studies may have reported a decrease in mast cell number when circulating oestrogen levels were elevated (2, 3) this study has highlighted the need for careful interpretation of both tissue volume and cell number (4).

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94. IMMUNOLocalISATION OF INTERLEUKIN 11 AND ITS RECEPTOR IN CYCLING ENDOMETRIUM AND IMPLANTATION SITES OF THE RHESUS MONKEY

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Interleukin 11 (IL-11) signalling is essential for stromal cell decidualization and embryo implantation in the mouse¹ and is involved in decidualization in the human². IL-11 action is mediated via binding to its specific IL-11 receptor alpha (IL-11R α). The aim was to examine the temporal and spatial location of IL-11 and IL-11R α in cycling endometrium and implantation sites of the Rhesus monkey. Immunohistochemistry was performed on cycling monkey tissue from proliferative phase day 13 (one day before ovulation) and secretory phase days 19, 24 and 29 (5, 10, and 15 days respectively after ovulation) (n=4 per day), and from implantation sites between days 24 and 35 of pregnancy (n=8). Data was expressed as relative intensity units. IL-11 immunoreactivity was elevated in the secretory phase of the menstrual cycle compared to the proliferative phase. At day 24 of the cycle, both IL-11 (2.8 \pm 1.2, mean \pm SEM) and IL-11R α (3.4 \pm 0.8) staining were most intense in glandular epithelium. Staining in the stroma for IL-11 was minimal (0.4 \pm 0.1) and moderate for IL-11R α (1.3 \pm 0.3). By day 29 of the cycle staining for IL-11 was higher in stroma compared to glandular epithelium (3.4 \pm 0.8 vs 1.7 \pm 0.6, P<0.05) however staining for IL-11R α was higher in glandular epithelium compared to stroma (1.4 \pm 0.6 vs 0.3 \pm 0.1). Luminal epithelium immunostaining for IL-11R α was moderate and minimal for IL-11 at days 24 and 29 of the cycle. In implantation sites, immunoreactive IL-11 was seen in syncytiotrophoblast and cytotrophoblast cells and IL-11 staining was seen in glandular epithelial cells. IL-11R α staining was observed in decidual cells, smooth muscle cells, and both syncytiotrophoblast and cytotrophoblast cells of the floating villus. However cytotrophoblast cells present in the trophoblast shell did not stain. Very little staining was seen in glandular epithelial cells. These data suggest a role for IL-11 in the preparation of a receptive endometrium and in placentation, both of which are essential for the establishment of pregnancy. Supported by NH&MRC, CONRAD/CICCR and Sylvia and Charles Viertel Charitable foundation. [1]Robb L, Li R, Hartley L, Nandurkar HH, Koentgen F, Begley CG (1998) *Nat Med* 3:303-308. [2] Dimitriadis E, Robb L, Salamonsen LA (*in press*) *Mol Hum Reprod*.

95. REPEATED MATERNAL GLUCOCORTICOID TREATMENTS IN SHEEP IMPAIR PLACENTAL STRUCTURAL MATURATION

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Maternal treatment with antenatal glucocorticoids improves neonatal outcome following preterm birth. In sheep, maternal intramuscular betamethasone injections (0.5 mg/kg ewe weight) and direct fetal intramuscular betamethasone injections (0.5 mg/kg estimated weight) improve preterm lung function but fetal growth restriction accompanies maternal but not fetal treatment, suggesting that placental function may be affected. We assessed placental structural development in sheep following single (1-beta) or repeated (3-beta) maternal or fetal intramuscular injections of betamethasone. Forty-one ewes were injected with 150mg medroxy-progesterone acetate on day 98 of pregnancy (term is 150 days) and randomised to maternal or fetal saline (104, 111, 118 days), 1-beta (104 days, followed by saline at 111, 118 days) or 3-beta (104, 111, 118 days) treatment groups. At 146 days, ewes were killed and the fetus and all placentomes were weighed. Each placentome was classified (types A-D), fixed and processed for image analysis. Fetal weight was reduced in only the maternal 3-beta group ($p=0.031$). The fetal:placental weight ratio was reduced by single and repeated maternal betamethasone treatments ($p=0.038$) but total placental weight was unaffected. The proportion of placentomes which were of the 'immature' A-type was greater in maternal than fetal treatment groups ($p=0.022$). The proportion of type B placentomes was increased in the maternal 3-beta group ($p=0.006$). The area of feto-maternal syncytium (FMS) was increased in maternal, versus fetal, treatment groups ($p=0.05$).

	Mat. saline	Mat. 1-beta	Mat. 3-beta	Fetal saline	Fet. 1-beta	Fet. 3-beta
Fetal Weight (kg)	5.19±0.31*	4.8±0.36*	4.04±0.2*	5.44±0.3	5.34±0.2	5.15±0.2
Fetal:Placental Wt	13.4±0.8	11.2±0.7*	11.1±0.4*	13.1±0.5	12.9±0.8	12.3±1.2
Prop. Type A	0.57±0.16**	0.66±0.14**	0.49±0.13**	0.18±0.1	0.19±0.1	0.55±0.2
Prop. Type B	0.04±0.02	0.04±0.02	0.2±0.05**	0.09±0.06	0.17±0.06	0.13±0.05
Area FMS (μm^2)	2668±154**	3042±764**	3321±436**	2574±216	2480±349	2241±393

These results suggest that treatment of the pregnant ewe with betamethasone alters placental structural maturation. However, when compared with previous findings of reduced placental weight at 125 days gestation after maternal betamethasone, the smaller effect on placental weight at 146 days suggests recovery. Discordance between placental findings and fetal growth restriction indicate that the role of the placenta in the mechanism by which glucocorticoids alter fetal growth requires assessment of placental transport capacity.

96. TREATMENT WITH A GLUCOCORTICOID RECEPTOR ANTAGONIST PREVENTS BIRTH IN TAMMAR WALLABIES

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In tamar wallabies (*Macropus eugenii*) the fetus provides the signal that initiates parturition (1). This signal may be cortisol from the fetal adrenal. Near term the fetal adrenal matures and there is a dramatic increase in fetal cortisol levels in fetal blood and yolk sac fluid (2). Treating tammars on day 24 of pregnancy with the synthetic glucocorticoid desamethasone induces birth 23 hours later, about 1 day prematurely (3). In this study we treated tammars on day 24 with the drug RU486, which in tammars binds to the glucocorticoid receptor (unpublished data) but not the progesterone receptor (4). RU486 (5 mg/kg) $n=9$ or vehicle ($n=9$) was administered daily starting on the morning of day 24, and the animals were checked 3 times per day for signs of birth or mating. Five of the 9 control animals gave birth, all in the normal time period (day 26.4±0.3). One RU486 treated animal gave birth on day 26. Another had a mating plug on day 29 consistent with a non-pregnant cycle. The rest had not given birth by day 29. Six of the seven were investigated by laparotomy. One showed no signs of pregnancy. One carried a malformed fetus at a developmental stage approximately equivalent to day 22. The remaining 4 had dead large postmature fetuses which weighed 744±31 mg, significantly more than the weight of normal neonates (400-500 mg). The death of these embryos may have resulted because the placenta was inadequate to support the postmature fetus. The inhibition of birth by RU486 treatment supports the hypothesis that glucocorticoid hormones are an essential part of the endocrine cascade that triggers birth. (1) Tyndale-Biscoe CH, Hinds LA, Horn CA. (1988) Fetal role in the control of parturition in the tamar, *Macropus eugenii*. *J Reprod Fert.* **82**:419-28. (2) Ingram JN, Shaw G, Renfree MB. (1999) Cortisol in fetal fluids and the fetal adrenal at parturition in the tamar wallaby (*Macropus eugenii*). *Biol Reprod.* **60**: 651-5. (3) Shaw G, Renfree MB, Fletcher TP. (1996) A role for glucocorticoids in parturition in a marsupial, *Macropus eugenii*. *Biol Reprod* **54**: 728-33. (4) Lim-Tio SS, Keightley MC, Fletcher TP, Fuller PJ. (1996) The molecular basis of RU486 resistance in the tamar wallaby, *Macropus eugenii*. *Mol Cell Endocrinol.* **119**: 169-74.