

EFFECT OF ELEVATED TEMPERATURES ON BOVINE CORPUS LUTEUM FUNCTION: *HEAT SHOCK PROTEIN 70* AND *90* MRNA, CELL VIABILITY AND HORMONE PRODUCTION IN CULTURED LUTEAL CELLS

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Summer heat stress significantly contributes to low fertility in cattle in hot environments by influencing oocyte quality, follicular activity and progesterone (P4) level in blood plasma. However, the mechanisms by which elevated temperature influences corpus luteum (CL) function remain unclear. Elevated temperature has generally been known to up-regulate the gene expressions of heat shock proteins (HSPs) family including HSP70 and HSP90 in a variety of cell types. To clarify the direct effects of elevated temperature on bovine CL function, we examined the expressions of *HSP70* and *HSP90* mRNA, cell viability and the production of P4 and prostaglandins (PGs) in luteal cells cultured at 37.5 C (normal temperature in our culture system), 39.0 C (moderately elevated temperature) or 41.0 C (severely elevated temperature) for 12 or 24 h. *HSP70* mRNA expression was increased by incubation at 39.0 C for 12 h and at 41.0 C for 24 h. *HSP90* mRNA expression was increased by incubation at 41.0 C for 24 h. The viability of luteal cells cultured for 24 h, measured by flow cytometry with propidium iodide staining, was not significantly affected by temperature. Interestingly, the production of P4 by cultured luteal cells was higher at 39.0 C than at 37.5 C after 12 and 24 h of incubation. The production of PGF2 α was higher at 39.0 and 41.0 C than at 37.5 C after 12 and 24 h of incubation. The production of PGE2 was higher at 41.0 C than at 37.5 C after 24 h of incubation. The overall results using this *in vitro* culture model suggest that elevated temperature does not negatively affect the luteal cells function, and that the low fertility observed during summer is not due to any direct effects of elevated temperature on luteal cells.

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THE EFFECTS OF THE BREEDING- AND NON-BREEDING SEASON ON LUTEAL FUNCTION IN MARES: PROGESTERONE CONCENTRATION AND MRNA EXPRESSIONS FOR PROGESTERONE SYNTHESIS-RELATED FACTORS IN THE CORPUS LUTEUM

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The mare is a seasonal breeder in that daylength (DL) mainly controls the reproductive seasonality. The reproductive activity of the mare reaches a peak during summer, then decreases during autumn and eventually stops through winter as DL decreases. Although there are many reports that investigated the luteal function based on plasma progesterone (P4) concentration during various seasons, the results reported are not consistent. Therefore, the seasonal changes in luteal function remain still unclear. In this study, we examined the effects of the breeding- and non-breeding season on luteal function in mares. Ovaries containing corpus luteum (CL) were collected from Anglo-Norman mares at a local abattoir in Kumamoto, Japan (N327) during spring (April, DL: 12.8-13.3 h), summer (July, DL: 13.8-14.1 h), autumn (September-October, DL: 11.5-12.0 h), early-winter (December, DL: 10.0-10.1 h) and late-winter (January-February, DL: 10.4-10.6 h). CL was classified as the early, mid and regressed luteal stages by macroscopic observation of the CL. P4 concentration (EIA) and mRNA expressions for *steroidogenic acute regulatory protein (StAR)*, *P450 cholesterol side-chain cleavage (P450_{scc})*, *3 β -hydroxysteroid dehydrogenase (3 β -HSD)* and *luteinizing hormone receptor (LHCGR)* (real-time PCR) in the luteal tissues were measured and used as indexes of luteal function. Since P4 concentration in the mid CL was the highest in the estrous cycle, the mid CL was used to evaluate the change of the luteal function in the various seasons. P4 concentration was the highest during the late-winter. mRNA expressions for *StAR*, *P450_{scc}* and *3 β -HSD* were parallel to the DL, whereas *LHCGR* mRNA was the highest during the late-winter. The overall results suggest that luteal function is affected by the seasons and the increase in P4 concentration in the luteal tissue during the late-winter has correlation with the increase in *LHCGR* mRNA expression.

THE INTERACTION OF INTRA-OVARIAN ANDROGEN WITH GROWTH DIFFERENTIATION FACTOR 9 IN FEMALE JAPANESE EEL OVARY.

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Japanese eel (*Anguilla japonica*), a migratory eel migrates from freshwater to seawater environment for spawning. The complete ovarian development has not been observed in wild and cultivated eels. In the past 40 years, the salmon pituitary homogenate (SPH) has been used to induce the eel ovarian growth and maturation. In the other hand, the artificial induction by use SPH combined with MT (17 α -methyltestosterone) was established in our study for many years. However, the ovarian follicles development is asynchronous under SPH injections. In contrast to SPH injection, our data reveal that SPH+MT maintain rather synchronous development and survive more ovarian follicles. In order to study the interactions of exogenous SPH and SPH+MT on early ovarian follicles growth in detail, this study focus on intra-ovarian androgen receptors (AR), FSH receptor (FSHR) and oocyte-specific growth differentiation factor 9 (GDF9). Ovarian development was induced by serial weekly SPH injections and, was examined by histochemistry assay. In results, RT-qPCR data showed both of GDF9 and FSH receptor mRNA was up-regulated in 48 hours after SPH+MT, MT and flutamide (androgen antagonist) treatments. Furthermore, several weeks of SPH+MT serial treatments have better potency to maintain the mRNA expression levels of GDF9 than SPH alone injections. AR α and AR β were also detected in ovary. According to above data, we further propose that intra-ovarian androgen may interact with oocyte-secreted GDF9 during early previtellogenetic stage to maintain synchronous growth between the earliest stage and the most advanced growth ovarian follicle populations. In this regard, GDF9 was shown to stimulate integral folliculogenesis and suppress FSH-dependent follicular cell differentiate to steroidogenesis. The exogenous SPH treatment is prerequisite for complete ovarian maturation. The co-treatment with MT results in more profound growth of previtellogenetic stage and extend vitellogenetic stage.

IN VITRO SURVIVAL OF FOLLICLES IN PREPUBERTAL EWES OVARIAN CORTEX CRYOPRESERVED BY SLOW FREEZING OR NON-EQUILIBRIUM VITRIFICATION.

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The aim of the present study was to compare follicular survival in vitro after non-equilibrium vitrification and conventional slow freezing of ovarian cortex fragments. After dissection, 1mm³ cortex fragments from 3 prepubertal ewes were cultured for 9 days in Waymouth's medium supplemented with ITS-BSA, testosterone (30 ng/ml) and FSH (50 ng/ml) immediately or after cryopreservation in straws. Slow freezing was performed using 30 minutes exposure at 4°C in culture medium supplemented with 10% FCS, 10% DMSO and 0.1 M sucrose followed by conventional slow freezing curve. Vitrification was performed after 5 min exposure to culture medium supplemented with 10% FCS+1X[CP] (CP=5% DMSO, 5% Ethylene Glycol and 0.125 M sucrose), 5 min exposure to culture medium supplemented with 10% FCS+2X[CP] and 2 min in medium supplemented with 10% FCS+ 4X[CP] before being plunged into liquid nitrogen. For thawing, reverse steps were respectively performed for each cryopreservation method. Histology and immunohistological (PCNA and TUNEL) analysis was performed before and after cryopreservation/thawing. If no significant differences in follicle morphology or TUNEL labeling were observed, PCNA labeling was significantly increased in primordial follicles for both cryopreservation methods. After 0 vs. 9 days of culture, histology of fresh fragments showed strong activation of follicular growth characterized by significant decreases ($P<0.05$) in primordial follicle proportions (71.5 vs. 31%) and significant increases of transitory (25 vs. 40%), primary (3 vs. 22.5%) and secondary follicles (0 vs. 5%). These proportions were not affected by slow freezing, whereas non-equilibrium vitrification increased proportions of transitory follicles on D9 (40 vs. 48%, $P<0.05$). Proportions of D9 TUNEL positive follicles was not statistically altered by slow freezing or vitrification in comparison to fresh cultured fragments (24.3 vs. 20.3 vs. 17.8%, respectively, NS). Taken together these data suggest that both non-equilibrium vitrification and slow freezing were efficient for cryoconservation of ovarian cortex fragments.

FIBROBLAST GROWTH FACTORS 16 AND 17 (FGF16 AND FGF17) STIMULATE CUMULUS EXPANSION IN BOVINE CUMULUS-OOCYTE COMPLEXES.

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We have recently detected mRNA encoding FGF-16 and -17 in oocytes and their receptors (FGFR-2c and FGFR-3c) in cumulus cells during in vitro COC maturation, suggesting that these oocyte-derived FGFs may regulate CC differentiation. This study tested the hypothesis that FGF16 and FGF17 enhance cumulus expansion and alter expression of regulatory genes. Groups of 15 immature COCs from 3-8mm follicles were matured for 22 hours with graded doses of FGF16 or FGF17 (0, 1, 10 and 100 ng/ml; n=4). Cumulus expansion was visually assessed (grades 1, 2 and 3) and relative mRNA expression was measured by real time RT-PCR. Effects of treatments were tested by ANOVA and groups were compared by Tukey-Kramer HSD. Both FGF16 (at 10ng/ml) and FGF17 (at 100ng/ml) increased the proportion of fully expanded COCs (grade 3) but did not alter the expression of cyclooxygenase 2 (COX2), hyaluronan synthase 2 (HAS 2), pentraxin 3 (PTX3), tumor necrosis factor-stimulated gene-6 protein (TNFAIP6), epiregulin (EREG) and betacellulin (BTC). However, amphiregulin (AREG) mRNA expression was upregulated by FGF17 at the dose that stimulated expansion, but not by FGF16. In conclusion, FGF-16 and FGF17 can stimulate in vitro cumulus expansion in cattle. The enhancement of AREG expression in CC appears to be involved in the mechanisms by which FGF-17 stimulates expansion.

BID AND BAX ARE INVOLVED IN FOLLICULAR GRANULOSA CELL APOPTOSIS AND SUPPRESSION OF THEIR EXPRESSION BY SMALL INTERFERING RNA DECREASES IN APOPTOTIC RATE IN SAWS

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More than 99% of follicles undergo atresia during follicular development and growth. Follicular atresia is predominantly regulated by granulosa cell apoptosis. However, the intracellular signaling pathway of apoptosis in granulosa cells has not been revealed. We examined changes in the expression of BH3-interacting domain death agonist (Bid) and Bcl-2-associated X protein (Bax), which are considered to promote the cell death ligand and receptor mediated process in mitochondrion dependent type II apoptosis, in porcine granulosa cells during atresia. mRNA and protein levels of Bid and Bax were determined by the reverse transcription polymerase chain reaction (RT-PCR) and Western blotting techniques, respectively. Levels of Bid and Bax mRNA and protein were markedly increase in granulosa cells of early atretic follicles compared to those of healthy follicles. *In situ* hybridization and immunohistochemical staining revealed that mRNA and protein of Bid and Bax were present in the granulosa cells though only negative or traces were found in healthy follicles, but strong staining were noted in atretic follicles. Then, to confirm the proapoptotic activity of Bid and Bax in granulosa cells, we examined the effect of RNA interference of Bid or Bax on apoptosis using an ovarian granulosa cells. By RT-PCR and Western blotting, spontaneous expression of Bid and Bax was detected in the cells. We suppressed Bid and Bax mRNA expression in the cells using small interfering RNA (siRNA). When Bid or Bax mRNA was suppressed, a significant decrease in the apoptotic cell rate was noted. The present results indicate that Bid and Bax appear to be signal transduction factors in granulosa cells during follicular atresia and to play proapoptotic roles, and confirm that porcine granulosa cell is type II apoptotic cell.

A NOVEL *IN VITRO* APPROACH FOR STUDYING THE CELLULAR EFFECTS OF HYPOXIA IN OVINE GRANULOSA CELLS, AND SOME EARLY OUTCOMES.

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It is now widely accepted that as the granulosa cell (GC) compartment in growing follicles expands and becomes more densely populated, the inner layers of cells progressively become more nutrient and oxygen restricted (hypoxic). It is therefore likely that physiological hypoxia has roles critical to follicle development. In this present work we have tested a modified specialised GC culture system as a means to study cellular responses to naturally occurring hypoxia without the need for either chemical agents or low oxygen regimens.

Ovine GC were seeded at either low- (LD) or high-density (HD) and cultured under specialised conditions. The LD cells were either treated with the hypoxia-inducing agent cobalt chloride (LD+CC) or left untreated. The different cell groups were transfected with a Hypoxia-Induced Factor 1 (HIF-1) responsive, luciferase reporter gene and cultured for a further 18 hours. Levels of active HIF-1 were determined by assaying luciferase activity. In parallel experiments the effect of cobalt chloride on the levels of mRNA expression of two prominent markers of hypoxia, HIF-1 subunit alpha (HIF-1alpha) and VEGF, was determined.

The process of forming into dense clusters under our prescribed conditions was accelerated in the HD cells and retarded in the LD cells. It was found that levels of activated HIF-1 were markedly higher in HD cells than in either untreated LD ($P<0.001$) or LD+CC ($P<0.01$) cells. VEGF but not HIF-1alpha mRNA expression could be up-regulated in the LD cells by addition of cobalt chloride but levels of both were markedly higher in untreated HD cells ($P<0.01$) than in untreated LD cells. The results indicate that hypoxic conditions can be induced in cultured granulosa cells by manipulation of plating density and it is concluded that this culture system represents a valuable model for studying the cellular responses to physiological hypoxia in GC during follicle development.

ROLE OF APOPTOSIS INITIATOR FOXO3 IN GRANULOSA CELLS DURING FOLLICULAR ATRESIA IN PIG OVARIES

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Most follicles are lost by atresia before ovulation in mammalian ovaries. It has become apparent that the apoptosis of granulosa cells induces follicular atresia. Forkhead box O3 (FOXO3) is a proapoptotic molecule that belongs to the FOXO subfamily of forkhead transcription factors. *Foxo3*-deficient female mice were reported to be infertile because of abnormal ovarian follicular development, but the precise influences of FOXO3 on follicular atresia of mature ovary have not been determined. Therefore, we examined the expression and function of FOXO3 in porcine ovarian follicles and granulosa-derived cell lines. The full-length mRNA sequence of pig *FOXO3* was determined and the homology of the estimated amino acid sequence was very high, 96.3% with the human sequence. *FOXO3* mRNA levels in granulosa cells of porcine ovaries increased during atresia by RT-PCR, while FOXO3 protein was abundant in granulosa cells of early atretic follicles by Western blotting. By immunohistochemistry, the inner surface area of the granulosa layer in early atretic follicles was strongly stained with anti-FOXO3 antibody. The granulosa cells expressing FOXO3 coincided with apoptotic cells detected by TUNEL staining, indicating a role of FOXO3 as a proapoptotic factor in granulosa cells of porcine ovaries. In porcine (JC-410) and human (KGN) granulosa-derived cell lines, cell death was induced by transfection of FOXO3 expression vectors. Expression of the proapoptotic factors Fas ligand (FASLG) and BCL2-like 11 (BCL2L11) was upregulated by overexpression of FOXO3 in KGN cells. In conclusion, FOXO3 is expressed in porcine ovarian follicles and induces apoptosis in granulosa cells, suggesting that it is an initiator of follicular atresia.

REGULATION OF ANGIOGENESIS-RELATED PROSTAGLANDIN F_{2A} -INDUCED GENES IN THE BOVINE CORPUS LUTEUM

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We recently compared prostaglandin F_{2α} (PG)-induced global gene expression profiles in PG-refractory, collected on day (d) 4 of the estrous cycle, versus PG responsive bovine corpus luteum (CL) (collected on d11). These transcriptome analyses led us to study the regulation of angiogenesis-related genes by PG and their functions in luteal endothelial cells (EC). We found that PG regulated angiogenesis-modulating factors in a luteal stage-dependent way. The two proangiogenic factors, VEGF and FGF₂, exhibited different patterns of expression, whereas VEGF mRNA decreased 4h post PG, in both d4 and d11 CL FGF₂ expression (mRNA and protein) was upregulated. The robust elevation of FGF₂ by PG at an early stage (especially when no FGF₂ inhibitors exist) may act as a survival signal for the CL, causing the gland to become resistant to luteolysis. What was intriguing about our results was not only the prominent PG-induction of FGF₂ expression (mRNA and protein) in d 4 CL, but also pronounced PG induced upregulation of thrombospondin-1 & 2, their receptor (CD36) and PTX3 in d 11 CL associated with luteolysis. Thrombospondins and PTX3 are anti-angiogenic factors shown to inhibit actions of FGF2. These genes were expressed in both dispersed luteal EC and steroidogenic cells, however, thrombospondin-1 and FGF₂ were more abundant in luteal EC. Expression of such genes in vitro and their ability to modulate FGF₂ actions were also investigated. Similarly to its in vivo effect, PG stimulated the expression of thrombospondins and PTX3 genes in several luteal cell models. Importantly, these factors influenced the angiogenic properties of luteal EC. FGF₂ dose-dependently enhanced cell migration and proliferation, whereas thrombospondin-1 and PTX3 inhibited FGF₂ actions in luteal EC. Collectively, the data presented here suggest that by tilting the balance between pro- and anti-angiogenic factors, PG can control the ability of the CL to resist or advance toward luteolysis.

THE PROAPOPTOTIC PROTEIN PUMA REGULATES PRIMORDIAL FOLLICLE NUMBER AND EARLY FOLLICULAR DEVELOPMENT IN MICE

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The number of primordial follicles in the ovary at birth is determined by extensive germ cell proliferation and subsequent cell death during fetal development. Referred to as 'Guardians of the Genome', members of the p53 family, including the oocyte-expressed transcription factor p63, are critical regulators of germ cell death in the mouse ovary. We have identified that PUMA, a proapoptotic BH3-only Bcl-2 family member, is a downstream target of p63, and plays critical roles in regulating germ cell viability and quality. Targeted disruption of the *Puma* gene in female mice revealed an oversupply of germ cells (26,000 in *Puma*^{-/-} vs. 20,000 in WT) as early as embryonic day 15.5. Germ cell numbers remain elevated in early postnatal life, resulting in an excessive endowment of primordial follicles (8400 in *Puma*^{-/-} vs. 4500 in WT) at PN10. These data indicate that PUMA is a key regulator of germ cell apoptosis, and determines the number primordial follicles established in the ovarian reserve. *Puma*^{-/-} ovaries revealed unexpected morphology at PN10, characterised by the presence of many small cells, with dense nuclei, located in clusters within the ovarian stroma and surprisingly within the granulosa cell layers of some secondary follicles. The absence of MVH, FOXL2 and AMH staining indicated that these cells were not of germ cell or granulosa cell lineage, respectively and were unlikely to be apoptotic (as assessed by TUNEL) or of leukocyte origin (CD45-ve). We hypothesise that these cells are normally eliminated by PUMA-mediated apoptosis during ovarian development.

EFFECTS OF TUMOR NECROSIS FACTOR A AND INTERFERON Γ ON THE VIABILITY AND APOPTOSIS SIGNALING IN BOVINE LUTEAL ENDOTHELIAL CELLS.

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In nonpregnant cows, the corpus luteum (CL) regresses after day 17 post ovulation by apoptosis. We previously reported that tumor necrosis factor α (TNF) and interferon γ (IFNG) induce cell death in luteal steroidogenic cells by increasing BCL2 family proteins and caspases. TNF in combination with IFNG induces apoptosis in luteal endothelial cells (LECs) which reduces the number of blood vessels within the CL. However, it remains unclear whether a single treatment with TNF or IFNG affects apoptosis. To clarify the mechanisms of luteolysis, we investigated the apoptosis signal pathways involved in TNF and/or IFNG-induced apoptosis in LECs. To induce cell death, LECs obtained from the mid-stage bovine CL were treated with TNF (0.5 nM) and/or IFNG (0.5 nM) for 24 h. The viability of LECs was reduced by a single treatment with TNF or IFNG (P<0.05). Furthermore, the cell viability was further decreased by TNF in combination with IFNG (P<0.001). A real-time RT-PCR analysis revealed that TNF alone did not significantly affect the expressions of apoptosis-related factors compared with those of untreated cells. On the other hand, IFNG alone increased *caspase-3* mRNA expression. TNF in combination with IFNG increased the expression of *FAS* mRNA (P<0.05), and increased caspase-3 activity (P<0.05) compared with those of untreated cells. Interestingly, a single treatment with IFNG, but not a single treatment with TNF, increased caspase-3 activity. In summary, TNF and IFNG increased cell death in cultured bovine LECs. The different effects between TNF and IFNG on the expression of apoptosis signals suggest that TNF and IFNG activates independent pathways, and that they act synergistically to induce the death of LECs, resulting in loss of luteal blood capillaries during luteolysis.

FOLLICULAR FLUID GLUCOSE AND LACTATE LEVELS ARE ALTERED BY MATERNAL AGE AND OVARIAN RESERVE

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The follicular fluid within the ovarian follicle is important for the development and nourishment of the oocyte. Therefore, this fluid may serve as an indicator of follicular health and consequently, oocyte development. Maternal age and reduced ovarian reserve (low anti-müllerian hormone (AMH) levels) affect the follicular environment and consequently oocyte viability. While previous studies in humans have correlated metabolite concentrations in follicular fluid to IVF outcome, the impact of maternal and age and low AMH have yet to be determined.

Follicular fluid was collected from 30 patients undergoing routine IVF treatment. Patients were classified into three cohorts based on their age and ovarian reserve; <35 years (young maternal age) with normal AMH, >40 years (advanced maternal age) with normal AMH and <35 years with low AMH. In addition to glucose, lactate and pyruvate, follicular fluid was assessed for hormones and selected ions.

There was a significant decrease in glucose concentration in both women with advanced maternal age and in women with low AMH (2.94 ± 0.17 mmol/L) and (2.32 ± 0.23 mmol/L) respectively, compared to young women with normal AMH (3.83 ± 0.20 mmol/L; $p < 0.05$). In addition, pyruvate concentration was increased in women with advanced maternal age and in women with low AMH (0.68 ± 0.01 mM) and (0.69 ± 0.02 mM) respectively, compared to young women with normal AMH (0.62 ± 0.02 mM; $p < 0.05$). Furthermore, women of advanced maternal age and women with low AMH exhibited a significant increase in lactate concentration by 31.3% and 55.1% respectively when compared to women of young maternal age with normal AMH.

This study demonstrates for the first time that concentrations of metabolites in follicular fluid are altered by advanced maternal age and reduced ovarian reserve. Taken together, the results of this study and what has been found previously, suggest that this environment may have an impact on oocyte developmental competence and subsequently embryo development.

ASSESSMENT OF IN VITRO CULTURE OF PRIMARY AND SECONDARY FOLLICLES FROM ADULT SUPEROVULATED FEMALE MICE

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Aim: Preantral mouse follicles have been successfully cultured using α -minimal essential medium (α -MEM) [1] as the basal medium. However, these follicles were obtained from juvenile mice and may not mimic the situation in human primordial/primary follicles from cycling adults. Our aim was to assess primary and secondary follicles (2 layers of granulosa cells) from adult superovulated mice cultured in this medium and 3 other basal media.

Method: Primary and secondary follicles (50-120 μ m diameter) were mechanically isolated from adult superovulated mouse ovaries and cultured in groups (7) for 11 days (2 replicates per condition). Four media were compared; α -MEM, McCoy's 5A, G2 Plus and Ham's F-12. Each medium was supplemented with the same concentration of glutamax, FSH, ascorbic acid, ITS and HSA. For each medium, 2 groups of follicles were cultured in drops under oil and 2 groups in Millicell culture inserts. Follicular diameters were measured on Day 1 and Day 11 to determine growth. On Day 11, follicles were evaluated morphologically and stained with calcein AM and ethidium homodimer-1 to determine viability.

Results: Primary and secondary follicles cultured for 11 days showed no increase in growth in all media. The proportion of intact follicles did not differ between conditions (79-100%). The number of viable follicles in α -MEM and G2 was higher than in the other media; drop culture; α -MEM (33%; 4/12), G2 (69%; 9/13), McCoy's (8%; 1/12), Ham's F-12 (0%; 0/12), and insert culture (45%; 5/11, 50%; 6/12, 15%; 2/13, and 8%; 1/13 respectively). There was no difference between follicles cultured in drops and inserts.

Conclusion: Although no growth was observed, α -MEM and G2 appear to have more potential for culture of adult primary/secondary mouse follicles. McCoy's medium, which has been used for human follicle culture [2], did not appear to be an appropriate medium for adult mouse follicles.

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IS REDUCING OVARIAN VOLUME IN POLY CYSTIC OVARIAN SYNDROME PATIENTS AFTER ADMINISTRATION OF METFORMIN ASSOCIATED WITH IMPROVING CARDIOVASCULAR RISK FACTORS?

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Objective: It is generally thought that women with polycystic ovary syndrome (PCOS) are at increased risk for cardiovascular (CV) and metabolic disorders. It is well known that there is a close relationship between elevated androgen plasma levels and the ultrasound finding of stromal hypertrophy. In randomized trials, the administration of metformin was followed by an improvement in insulin sensitivity and decreasing in androgen levels in most women. In present study we investigated association between reducing ovarian volume in PCOS patient after administration of metformin with improving CV risk factors.

Materials and Methods: This is a randomized clinical trial with before and after design. Twenty eight women with PCOS who referred to infertility clinic were selected. The anthropometric characteristics of the patients and mean ovarian volume and plasma level of Fasting blood sugar (FBS), lipid profile, Luteinizing hormone (LH), Follicle stimulating hormone (FSH), Estradiol, Testosterone, 17OH Progesteron (17OHP),

Dehydroepiandrosterone sulfate (DHEAS), C reactive protein (CRP), Homocysteine (Hcy), were evaluated before and after treatment with 500mg metformin three times a day for 3 months. Statistics were calculated with the aid of the SPSS 16.0 with student paired t test and Pearson's correlation coefficient test. Significance was set as $P < 0.05$.

vs 26.84 ± 4.55 , $p < 0.05$) 4.55 ± 28.11 vs 8.27 ± 3.71 , $p < 0.05$), BMI 4.31 mean ovarian volume ($11.70 \pm$ There is significant reduction in : Results Testosterone (ng/ml) (1.33 ± 0.33 vs 1.26 ± 0.32 , $p < 0.05$), CRP (mg/l) (12.92 ± 2.46 vs 10.56 ± 2.01 , $p < 0.05$) Hcy (μ g/ml) (10.26 ± 1.02 vs 9.15 ± 0.77 , $p < 0.05$), vs 55.15 ± 3.9 , $p < 0.05$) LDL (mg/dl) (101.08 ± 11.15 vs 91.50 ± 11.04 , $p < 0.05$) 5FBS (mg/dl) (94.65 ± 11.32 vs 85.34 ± 10.12 , $p < 0.05$) HDL (mg/dl) (50.84 ± 6.4

After treatment there was positive correlation after treatment. There was positive correlation between mean ovarian volume and waist to hip ratio. .decreasing in CRP, LDL, Hcy, testosterone level and BMI with reduction in mean ovarian volume with
administration of Conclusion: It may be a positive correlation between reducing mean ovarian volume and improvement in CV risk factor after adm
volume, cardiovascular risk, metabolic syndrome min, ovarianKey words: polycystic ovarian syndrome, metformin.

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DIRECT IN VITRO EFFECTS OF ALTERED INSULIN LEVELS AND DEXAMETHASONE-INDUCED INSULIN RESISTANCE ON THE PRODUCTION OF ANDROSTENEDIONE AND OESTRADIOL BY CULTURED BOVINE THECA AND GRANULOSA CELLS

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Lifestyle and pharmacological interventions that increase insulin sensitivity in women with polycystic ovarian syndrome (PCOS) can decrease elevated androgen levels. In this study cultured bovine theca (TC) and granulosa (GC) cells were used to assess direct effects on ovarian steroidogenesis of: (i) altered insulin levels and (ii) metabolic insulin resistance purportedly induced by dexamethasone exposure *in vitro*^[1,2]. Cells from 4-6mm follicles were cultured in serum-free medium^[3]. GC cultures were supplemented with androstenedione (10^{-7} M) as P450arom substrate. Effects of ovine LH (0, 0.015, 0.15 and 1.5 ng/ml) on TC and of FSH (0, 0.03, 0.3 and 3 ng/ml) on GC were tested in combination with insulin (0, 1, 10 and 100 ng/ml) with/without dexamethasone (10^{-6} M). Medium was changed every 48h and cultures ended after 144h. Androstenedione (A4), oestradiol (E2) and progesterone (P4) secretion during the 96-144h time period were determined by ELISA. LH and FSH had biphasic effects on A4 and E2 secretion by TC and GC, respectively. There was a significant interaction between insulin and LH/FSH, with peak A4 and E2 levels achieved with the combination of 10 ng/ml insulin plus 0.15 ng/ml LH (TC) or 0.33 ng/ml FSH (GC). Higher levels of LH (1.5 ng/ml) or FSH (3 ng/ml) inhibited A4 and E2 production, stimulated P4 production and altered cell morphology, reflective of luteinisation. In the absence of LH/FSH, and in luteinised cells, dexamethasone stimulated thecal A4 production ($p < 0.01$) and inhibited granulosa E2 production ($P < 0.05$), whilst having no effect on P4 production. However, at optimal levels of LH (0.15 ng/ml) and FSH (0.33 ng/ml) the effect of dexamethasone was not evident. Collectively, these *in vitro* findings support the hypothesis that follicular insulin resistance may contribute to ovarian hyperandrogenism both by stimulating thecal androgen production and by reducing its conversion to oestradiol by GC.

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EFFECT OF ANTIANDROGEN TREATMENT ON PORCINE FOETAL OVARY AND UTERUS DEVELOPMENT.

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Mammalian folliculogenesis is regulated by many critical genes important for reproductive life span. Recent reports have documented maternally mediated effects of antiandrogens on reproduction. The formation of porcine ovarian follicles starts on day 56 *post coitum*, but follicles surrounded by a single layer of squamous pregranulosa cells are present only on day 106 *post coitum*. Uterine morphogenesis in pigs is initiated prenatally, during embryonic development of the paramesonephric or Mullerian ducts, and completed postnatally. Therefore, there are distinct differences in the cell proliferation and the number of proliferating cells could be assessed using immunohistochemistry by determining Ki-67 expression.

The presence of androgen receptors in the porcine reproductive tract was revealed. Flutamide, well known potent androgen receptor antagonist, was administered to pregnant sows in 7 doses (50 mg/kg body weight, every day) starting from critical days of gestation (43, 83, 100). On days 50, 90 and 108 the foetuses were excised during a surgical procedure. Both ovaries and uteri were fixed in Bouin's fixative for routine histology and immunohistochemistry. Therefore, the objective of this study was to determine whether *in utero* exposure to flutamide leads to changes in androgen-dependent genes expression and cell proliferation during foetal development of the ovary and uterus.

Ki-67, a nuclear protein that is present in all phases of the cell cycle, but is absent in the G0-phase cells. The declined cells proliferation can be revealed by lower Ki-67 expression. The number of positively stained cells varied between flutamide-treated and non-treated animals both in ovaries and uteri. Exposure of gilts to flutamide *in utero* resulted in morphological differences in foetal ovaries and uteri in comparison to control ones.

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EFFECT OF ESTRADIOL ON *IN VITRO* DEVELOPMENT OF PORCINE PREANTRAL FOLLICLES

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In mammalian ovaries, estradiol 17 β (E₂) is produced from androstenedione (A₄) by aromatase activity of the granulosa cells in the follicles and plays a key role in the follicle development. The present study investigated the effects of E₂ and A₄ on *in vitro* antrum formation of porcine oocyte-granulosa cells complexes (OGCs) collected from preantral follicles and the relationship between E₂ secretion from the OGCs and antrum formation of the OGCs during *in vitro* culture. OGCs were dissected from the preantral follicles (250-300 μ m in diameter) and individually cultured for 14 days in medium containing E₂ (0, 0.1, 1 and 10 μ g/ml; Exp. 1) or A₄ (0, 0.1, 1 and 10 μ g/ml; Exp. 2), and antrum formation was examined every 4 days. The highest rate of antrum formation was observed in OGCs cultured in medium containing 1 μ g/ml E₂ (62.6%) and 0.1 μ g/ml A₄ (35.2%), respectively. When OGCs were cultured in medium containing 0.1 μ g/ml A₄ (Exp. 3), antrum formation began on day 8 of culture period. The E₂ concentration in medium collected on day 4 was measured for individual OGCs. On day 14 of culture, the OGCs were divided into two categories; OGCs that formed an antrum and OGCs that did not form an antrum. The E₂ concentration was compared between the two categories. Significantly higher E₂ levels were detected in the medium of OGCs that formed an antrum compared with those that did not form antrum (8.3 vs. 3.2 ng/ml). When OGCs were cultured in medium containing A₄ (0.1 μ g/ml) and fulvestrant (0 or 1 μ g/ml), and fulvestrant drastically inhibited antrum formation. These results suggest that endogenous and exogenous E₂ improves antrum formation of OGCs derived from preantral follicles.

HAEMOGLOBIN MRNA AND PROTEIN EXPRESSION OCCURS WITHIN CUMULUS CELLS OF *in vivo* MATURED, BUT NOT *in vitro* MATURED MOUSE CUMULUS-OOCYTE COMPLEXES.

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In vitro maturation (IVM) of cumulus-oocyte complexes (COC) is associated with reduced developmental competence compared with *in vivo* matured (IVV) oocytes. The function of cumulus cells (CC) is known to be critical for oocyte developmental competence, due to bi-directional communication between CC and the oocyte. We hypothesized that differences between IVV and IVM-derived CC mRNA expression would provide valuable information in identifying CC functions important for oocyte competence. Microarray analysis (Affymetrix GeneChip Mouse Genome 430 2.0) of CC mRNA from CBAB6F1 mouse COCs either eCG-primed IVV (13 hr post-hCG) or IVM (17 hr in α MEM + 5% FBS + 50 mIU FSH at 37 C in 6% CO₂/air) was conducted. Surprisingly, the most differentially expressed gene was haemoglobin A1 (*Hba1*) (3 x 10³-fold increase for IVV vs. IVM), as well as haemoglobin B1 (*Hbb1*) (2 x 10³-fold increase). The decrease in *Hba1* expression within IVM compared to IVV CC was verified by RT-PCR. Furthermore, IVV CC expressed a temporally-regulated profile following hCG. Variable levels of *Hba1* were detected in CC 44 hr post eCG across different animals, but decreased to nearly undetectable levels within 3 h post-hCG. *Hba1* expression was then re-established to pre-hCG levels. *Hba1* mRNA was low to undetectable in CC mRNA levels throughout IVM. Western analysis confirmed expression of a 14 kDa HbA1 band in both CC and COC extracts. HbA1 protein was further identified by immunohistochemistry in both granulosa and CC, with a decrease in levels at 4 h post-hCG in both. In contrast, no HbA1 protein was detected in IVM-derived CC. Our results suggest that haemoglobin may play an as yet unidentified role during ovulation. Known functions of haemoglobin include NO and O₂ sequestering and the concentrations of both will most likely alter during ovulation. We also provide further evidence of the deficiency in cellular physiology during IVM.

CHARACTERISATION OF C-KIT EXPRESSION AND LOCALISATION IN HUMAN OVARIES

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Growth factor kit ligand (KITL) and its receptor c-kit are essential for fertility and normal folliculogenesis in mice. The significance of the KITL/c-kit system in human ovaries remains poorly understood, and previous investigation has been limited to human fetal ovaries. We previously showed KITL protein is present in all follicular compartments throughout follicle development in human adult ovaries, and showed evidence of increased levels in polycystic ovaries (PCO) compared to non-PCO. To identify KITL target cells throughout follicle development, we characterised expression and localisation of c-kit mRNA and protein, and compared immunostaining intensity in non-PCO (n=5) and PCO (n=4).

C-kit immunostaining was performed on paraffin-embedded, premenopausal human ovarian tissues. C-kit protein levels were examined in human ovarian tissue lysates and a granulosa tumour cell line, KGN. Human mural granulosa cells (MGC) and cumulus cells (CC) were used to examine c-kit mRNA levels.

Oocytes at all developmental stages exhibited moderate to strong cytoplasmic and membrane immunostaining, and some preantral granulosa cells (GC) exhibited weak cytoplasmic staining. Antral follicles showed weak to moderate cytoplasmic staining in GC and theca cells, with a distinct absence of staining in the basal lamina. Staining intensity was observed to be no different in PCO compared to non-PCO. Human ovarian lysates contained a 150kD c-kit protein band which was absent in KGN cell lysates. Low levels of c-kit mRNA were detected in MGC and CC. These results indicate that KITL has an autocrine function in human GC, unlike in the mouse. The absence of c-kit protein in KGN cells is consistent with reports of an absence of c-kit expression in most ovarian tumours. While the function of KITL/c-kit in human folliculogenesis is yet to be elucidated, lower mRNA and protein expression in antral follicles suggest a diminished role for KITL in later stages of follicle development.

DEVELOPMENT OF THE RIGHT GONAD TRANSPLANTED FROM OVARIECTOMIZED FEMALE FOWLS TO CASTRATED MALE CHICKENS

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Transplantation experiments provided a unique tool in the study of gonadal development and possible preservation of germline in chicken. Both transplantation of testicular and ovarian tissues into recipient host proceeded to advance development and subsequent production of viable offspring showing germ cells produced maintain their full fertilizing potential. In the present study, right gonad tissues from a 2-month-old ovariectomized female chick of GSP inbred line were transplanted under the skin and inside the abdomen of castrated pre-pubertal GSP male chicks. After 10 months, the host males were killed and the gonad grafts were subjected into histological and genetic analysis. Secondary sex characteristics were observed in the male host such as increase in head furnishings, spur development and male plumage pattern. Around 10% of the total grafts were collected inside the abdominal cavity with 50-60% increase in volume, however there are no gonad grafts that developed under the skin. Histological analysis of the gonad grafts showed a more advance differentiation into testicular tissues and active mitotic division of germ cells compared to intact gonad that developed in an ovariectomized chicken. The seminiferous tubules contain spermatocytes as the most advance germ cells and the sizes of the lumen observed in the gonad grafts are mostly normal with some dilation. FISH analysis revealed numerous spermatids with fluorescent signals bearing the W chromosome indicating that the second meiosis occurred normally, although more advance germ cells were not observed. These results demonstrate that the right gonad obtained from a sex reversed chicken maintains the structural integrity and physiological characters when transplanted into a castrated male host.

LOSS OF ANDROGEN RECEPTOR ACTIONS CAUSES DYSFUNCTIONAL OVARIAN FOLLICLE DEVELOPMENT AND NEUROENDOCRINE CONTROL OF OVULATION

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Recently the androgen receptor (AR) has been proven to play a role in female reproduction. We generated AR knockout (AR^{-/-}) female mice using Cre/LoxP recombination for an in-frame excision of exon 3, deleting the second zinc finger essential for DNA-binding. AR^{-/-} females are sub-fertile, with reduced ovulation rates identified as a key defect. Ovary transplantation studies identified that the sub-fertility is due to both intrinsic ovarian defects and a disruption in extra-ovarian hypothalamic-pituitary regulatory mechanisms. We examined further the intra- and extra-ovarian defects by investigating the natural ovulatory LH surge and late follicle development and health in AR^{-/-} and control (AR^{+/+}) females at the proestrus stage of the estrus cycle. In ovaries collected at proestrus, AR^{-/-} ovaries exhibited fewer preovulatory follicles (AR^{-/-}: 2.25 ± 0.8 ; AR^{+/+}: 5.2 ± 0.9, P<0.05) but showed no difference in antral follicle numbers. AR^{-/-} small antral follicles had a reduced oocyte:follicle ratio (AR^{-/-}: 0.3 ± 0.004 ; AR^{+/+}: 0.4 ± 0.007, P<0.01) , indicating disrupted oocyte and somatic cell communication and an altered growth pattern. AR^{-/-} ovaries also exhibited an increase in unhealthy (more than 10% pyknotic granulosa cells) large antral follicles (AR^{-/-}: 67.9% +/- 11.4 ; AR^{+/+}: 27.9% +/- 11.2, P<0.05). To investigate the role of AR-mediated actions in triggering ovulation, we investigated the natural ovulatory LH surge and observed that serum LH in AR^{-/-} mice measured at proestrus was significantly reduced (AR^{-/-}: 3.96 ± 1.1 ng/ml; AR^{+/+}: 7.95 ± 1.8 ng/ml, P<0.05, n≥10). In conclusion, reduced ovulations observed in AR^{-/-} females are due to both impaired ovulatory LH surge and dysfunctional late follicle development, manifesting as fewer preovulatory follicles. The relative contributions and sequence of effects underlying these observations requires further elucidation to determine the mechanisms for AR-mediated actions in the regulation of the hypothalamic-pituitary-ovary axis.

GONADOTROPINS REGULATE MATERNAL GENE EXPRESSION WHICH POSSIBLY IMPROVE PORCINE OOCYTES MATURATION AND EMBRYO DEVELOPMENT

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In present work we examined the potential roles of maternal genes on porcine oocyte maturation and early embryo development by recombinant FSH and LH supplementation. Cumulus-oocyte complexes were cultured in different groups supplemented with FSH and LH. We observed significant cumulus expansion and nuclear maturation in the groups which the COCs exposed to FSH alone or combination of FSH and LH. We next examined the effect of maternal genes after treatment. The results showed that supplemented with LH and FSH significantly enhanced the polyadenylation of *Gdf9* and *Bmp15* during IVM, and also altered their expression levels. The addition during whole IVM increased mRNA levels of four maternal genes, *C-mos*, *Cyclin B1*, *Gdf9* and *Bmp15* at 28h. Following parthenogenesis, treatments with LH and FSH significantly increased cleavage and blastocysts rates. There is no significant difference in total cell number among different treatments, however, the apoptosis index of control group showed higher than the treatment groups. Moreover, LH and FSH also suppress the autophagy and apoptosis process, showing with down-regulation of *Atg6* and up-regulation of *bcl-xl* mRNA level. In conclusion, our data shows that supplementation of LH and FSH affects porcine oocyte maturation through the regulation of maternal genes expression, and improves competence of embryo development through inhibiting autophagy and apoptosis progress.

CHARACTERISATION OF HEPARAN SULPHATE PROTEOGLYCANS IN THE MATURING CUMULUS OOCYTE COMPLEX

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Many growth factors including the TGF β , FGF and EGF superfamilies signal via interactions with heparan sulphate proteoglycans (HSPGs). The oocyte secreted factors (OSF) growth differentiation factor 9 (Gdf9) and bone morphogenetic protein 15 (Bmp15) are members of the TGF β superfamily that act selectively on cumulus cells. We investigated the possibility that HSPGs contribute to the spatially restricted responses to these growth factors within ovarian follicles.

The expression of Syndecan and Glypican families and Betaglycan in murine COC and GC show selective regulation of some family members in mural vs cumulus cell compartments. Specifically *Syndecan 4* was induced 15-fold in COC after hCG treatment, reaching only half that level in GC. *Glypican 1* and *6* were induced up to 20-fold and 3-fold respectively in COC with no significant change in GC, while *Glypican 5* was induced 20-fold in GC but remained undetectable in COC.

Immunohistochemistry showed predominant Syndecan 1 and Glypican 1 in the COC of follicles in vivo. Shedding of Glypicans can control spatial trafficking of Tgfb family morphogens and intriguingly Glypican 1, which is normally cell-surface anchored, was released into COC matrix in vivo but remained cumulus cell associated in IVM COC as demonstrated by western blot and whole mount immunofluorescence.

In Vitro matured (IVM) oocytes are less competent than those matured *in vivo*. Proteoglycan family members *Betaglycan*, *Syndecan 1* and *4* and *Glypican 1*, *2* and *6* were dysregulated in IVM vs *in vivo* matured COC. This dysregulation of HSPGs in IVM could reduce the signalling capacity of important growth factors including OSFs. We found that cumulus gene expression during IVM with heparin was disrupted, but was restored when exogenous Gdf9 was added demonstrating that Gdf9 signalling is modulated by heparin sulphate interactions.

Combined, this data suggests that HSPGs may be co-receptors for Gdf9 during maturation of oocytes.

PROLIFERATION OF BOVINE LUTEAL STEROIDOGENIC CELLS.

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[Introduction] The normal development of the corpus luteum (CL) and parallel increasing amounts of progesterone production are essential for the establishment and maintenance of pregnancy. The rapid increase in weight of the CL after ovulation is believed to be mainly due to an increase in size of the luteal steroidogenic cells (LSCs) rather than an increase in their number. However, the pattern of luteal cell proliferation during CL formation is not fully understood. To clarify the detail of luteal formation, we examined cellular proliferation of bovine LSCs throughout the estrous cycle and in cultured LSCs. The changes of mRNA expressions of cell proliferation related genes (*Pten*, *cyclinD2* and *p27kip1*) were also examined. [Methods] Luteal tissues were immunostained with a cell proliferation marker ki-67, then co-labelled with a steroidogenic cell marker 3 β -HSD to examine whether luteal cells proliferate. Bovine LSCs isolated from mid stage CL were cultured for 1, 4, 7 and 10 days with or without LH and daily observed, DNA contents were measured at the end of culture. Cell proliferation related genes were examined by real-time PCR. [Results] Ki-67 was expressed at the early, developing, mid luteal stages as well as in the pregnant CL. Interestingly, some of ki-67 positive cells co-expressed 3 β -HSD confirming that LSCs proliferate. Cultured bovine mid LSCs increased DNA contents in a time - dependent manner and this increase in DNA contents were suppressed by LH. *Pten* mRNA level was lower at the early luteal stage than at other luteal stages. *CyclinD2* : *p27kip1* ratio was high in early and developing CL. [Conclusion] The overall results indicate that the bovine LSCs proliferate, and that these proliferation is more active during the early to developing stage than other stages. LH seems to regulate the luteal cell proliferation.

ACTIVIN B IS PRODUCED EARLY IN FOLLICULAR DEVELOPMENT AND SUPPRESSES THECAL ANDROGEN PRODUCTION

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We have previously reported that activin A suppresses theca cell androgen production, and inhibin A blocks these effects. However, little is known about the presence and effects of activin B during folliculogenesis. This study investigated the effects of activin B on thecal androgen production in vitro, activin/inhibin subunit (β A, β B, α) expression in theca (TC) and granulosa cells (GC) by Q-PCR, and activin A, B and inhibin A protein levels in follicular fluid (FF) of developing sheep follicles during anestrus and the estrous cycle using Elisa. Activin B decreased androstenedione production from primary sheep theca cells cultured in vitro and these effects were blocked in the presence of inhibin A. During folliculogenesis, the concentration of FF activin B decreased as follicle diameter increased from 6mm, and varied significantly between stages of estrous/anestrus. Follicles were segregated into groups depending on the concentration of FF estradiol. Estrogenic follicles had reduced amounts of antral activin A and activin B at the pre-ovulatory stage (>6mm). Theca and granulosa cells of estrogenic follicles expressed higher levels of mRNA encoding activin β A at 3-4mm, and theca cells produced more inhibin α mRNA at >4mm stages of development compared to non-estrogenic follicles. In summary, at early antral stages of development, sheep follicles contained high levels of activin B within the antral fluid, and the concentration decreased as the follicle size increased. Although the pattern of expression is different to that observed for activin A, activin B acts similarly to activin A by reducing androgen production in theca cells in vitro, and these effects are blocked in the presence of inhibin. These results show that activin B is produced by follicles at early stages of development at levels able to modulate androgen production.

FACTORS INFLUENCING PLACENTAL MORPHOMETRY IN THE FIRST TRIMESTER OF PREGNANCY IN ANGUS COWS

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The aim of the study was to investigate factors influencing placental development in Angus heifers. This was achieved by analysing data from two years of a nutritional study. In the first year, 16 uteri were collected on Day 90 of gestation, from heifers fed either a diet designed to achieve moderate weight gain (target 500g/day) (n=8) or low weight gain (target 100g/day) (n=8). For the second year, 25 uteri were collected on Day 91 of gestation, from moderate heifers (target 750g/day) group (n=9) and low heifers (target 100g/day) group (n=16). Placental parameters were measured including weight, size and number of the placentomes as well as fetal and maternal weights. Principal component analysis (SAS 9.1), was used to show the pattern of distribution of the placental variables measured for each treatment. There were no significant differences between treatments for both years. Fetal weight was correlated to weight of caruncle while maternal weight was moderately associated with the number of caruncles and cotyledon. The result shows that nutrition may not be a critical factor affecting placental development within the first trimester of pregnancy in beef cattle.

PLACENTAL MESENCHYMAL STEM CELLS MIGRATE IN A PLACENTAL VESSEL PERFUSION MODEL

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Objective: The migratory properties of human term placental mesenchymal stem cells (pMSCs) are not yet fully understood. In order to study the migration of human term pMSCs an *ex vivo* model of pMSCs is required. The hypothesis of this study therefore was that pMSCs migration can be assessed using a placental vessel *ex vivo* perfusion model.

Method: Term placentae from uncomplicated pregnancies were obtained with informed consent. A stem cell line (PRCFP) was created from primary, term pMSCs by hTERT transformation (Prof. S Gronthos, Adelaide). We verified that PRCFP preserves important mesenchymal stem cell characteristics. PRCFPs were >95% positive for surface markers CD105, CD73, and <5% positive for CD45, as expected. PRCFPs were differentiated into adipocytes and osteocytes (n=5 each), and PRCFPs were able to migrate in scratch assays (22±2% scratch closure in 8 hours, n=5). Cells were labelled with live Cell Tracker orange (CMTMR; Invitrogen, CA, USA) Optimization of the CMTMR concentration and staining time was carried out. The perfusate contained $1.6 \times 10^6 - 4.1 \times 10^6$ CMTMR-labelled PRCFPs and incubation times ranged between three hours to four days in 37°C. The perfused vessels were then washed, cryopreserved and sectioned (5µm). Multicolour immunofluorescence was carried out. Endothelium was detected with a fluorescein conjugated anti-vWF antibody (Meridian Life Science, USA). Nuclei were detected by DAPI staining. Our criterion for migration was the detection of CMTMR-labelled PRCFPs that had crossed the endothelial barrier.

Results: In five successful perfusions, assessment of sections revealed CMTMR-labelled PRCFPs had crossed the endothelium barrier. Sectioning of a representative vessel revealed approximately 221 CMTMR labelled PRCFPs per cm of vessel had crossed the endothelial barrier.

Conclusion: Placental vessel *ex vivo* perfusion model can be used to assess pMSCs migration.

PLACENTAL EXPRESSION AND LOCALIZATION OF ADRENOMEDULLIN SYSTEM DURING BOVINE PREGNANCY

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Adrenomedullin (ADM) is a potent vasodilator peptide and also involved in various physiological activities such as angiogenesis and antiapoptosis. In humans and rats, ADM system is found in uteroplacental unit and may responsible for fetal development and maintenance of normal placental function during pregnancy. This study aimed to determine mRNA expression patterns of ADM and its receptor component proteins in bovine uteroplacental unit during pregnancy. We investigated mRNA and protein localization of ADM in bovine placentome. Bovine uteroplacental tissues on Day 60, 100, 150, 200 and 250 of gestation were collected and separated into caruncular areas (CAR), intercaruncular areas (ICAR), cotyledonary villous (COT) and intercotyledonary areas (ICOT). The mRNA expression levels of ADM, calcitonin receptor-like receptor (CRLR), receptor activity modifying protein (RAMP) 2 and RAMP3 were determined by real-time quantitative RT-PCR. In addition, we performed in situ hybridization and immunohistochemistry to investigate cellular localization of mRNA and protein of ADM in bovine placentomes on Day 60 of gestation. ADM mRNA expression in CAR, ICAR and COT were higher at Day 60 and 200 than other days of gestation. CRLR mRNA expression in CAR, COT and ICOT were decreased from Day 60 to 100 followed by increased again at Day 150 and 200. RAMP2 mRNA expression in CAR, ICAR and ICOT were higher at Day 60 than other days of gestation. RAMP3 mRNA expression at Day 100 and 200 were higher than other days of gestation in all four regions. Both mRNA and protein of ADM were only localized in trophoblast binucleate giant cells (BNC) of placentomes. Our results demonstrate that ADM system in bovine uteroplacental unit is activated during placentation and transition from mid to late gestation period. ADM produced in the BNC may play crucial role in regulation of placental vascular and cellular functions during pregnancy.

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DOWNREGULATION OF SPARC EXPRESSION INHIBITS CELL INVASION IN HUMAN TROPHOBLAST CELLS

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Successful pregnancy depends on the precise regulation of extravillous trophoblast (EVT) invasion into the uterine decidua. The implantation process is mediated by molecular and cellular interactions, controlled by the trophoblast and maternal microenvironment. SPARC (secreted protein acidic and rich in cysteine), is a matricellular glycoprotein that modulates extracellular matrix assembly and turnover in many physiological processes. It is a key player in the pathologies associated with obesity and diabetes, and it is a matricellular regulator of tumorigenesis. The objective of this study was to investigate the role of SPARC in blastocyst implantation especially in the process of trophoblast invasion which shares many similarities with invasion of tumor cells. By Western blotting, higher expression of SPARC was found in mouse brain, ovary and uterine compared to other mouse tissues. Furthermore, the expression of this gene was much higher at implantation sites compared to interimplantation sites on day 7.5 of pregnancy. We further investigated the function of SPARC in extravillous trophoblast cell line HTR8/SVneo. Introduction of SPARC-targeted small interfering RNA (siRNA) into trophoblast cells resulted in downregulation of SPARC expression, and the invasiveness was significantly reduced in the cells transfected with SPARC siRNA compared with those transfected with control siRNA. Using gene array analysis to identify SPARC-responsive genes, we found that SPARC depletion up-regulated the expression of IL11, KISS1, COL1A1, APOE, MM9, TIMP3 and down-regulated the expression of CGA, MMP1, GJA1, et al. The gene array result was further demonstrated by RT-PCR and Western blotting. The present data indicate that SPARC plays an essential role in the regulation of normal placentation by promoting trophoblast cells invading into the uterine decidua.

DECIDUAL EXPRESSION OF CANDIDATE MATERNAL PRE-ECLAMPSIA SUSCEPTIBILITY GENES

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The key to pregnancy success is the establishment and growth of the placenta. When this is deficient, complications develop which compromise maternal and fetal health. It is well recognized that the fundamental origin of pre-eclampsia (PE) lies in the placenta, but while deficient placentation is a characteristic feature, the underlying causes of this abnormality are unknown. PE is known to have a familial association, with daughters of women who have PE during pregnancy having more than twice the risk of developing PE themselves. Through genome-wide linkage and genetic association studies in PE-affected families and large population samples, we have identified promising candidate maternal susceptibility genes for PE. The aim of the current study was to determine mRNA expression for these candidate maternal PE susceptibility genes in third trimester decidua tissue obtained from normotensive and severe PE (SPE) pregnancies. RNA was extracted from decidua *basalis* samples collected at Caesarean section from n=8 normotensive and n=7 SPE patients (SPE defined according to (1); mean gestational age normotensive 38.13±0.12 weeks versus SPE 36.43±0.53 weeks, p<0.05, Mann-Whitney U test). Gene expression analyses were performed using inventoried TaqMan[®] gene expression assays (Applied BioSystems) for the following genes: *ERAP1*, *ERAP2*, *LNPEP*, *ACVR1*, *INHA* and *INHBB*. The genes encoding the aminopeptidases *ERAP1* and *LNPEP* showed a significant increase in expression in SPE samples (4.7 and 11.9 fold respectively) compared with normotensive samples (p<0.05, unpaired t test with Welch's correction). The expression of *ERAP2*, *ACVR1*, *INHA* and *INHBB* were not significantly different between the two groups. This data demonstrates differential decidua mRNA expression of two candidate maternal PE susceptibility genes which were identified using a strategy combining quantitative bioinformatics, transcriptional profiling in pregnancy-specific tissue and gene-centric SNP associations with PE. The possible consequence of altered expression of these genes on successful placentation remains to be investigated.

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DOES CYTOKINES AND REPRODUCTIVE HORMONES INFLUENCE 2,3 INDOLEAMINE-DIOXYGENASE EXPRESSION IN CULTURE OF PLACENTA AND EMBRYO CELLS FROM WISTAR RATS?

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Indoleamine 2,3-dioxygenase (IDO) is an enzyme produced by trophoblast cells and due to its ability to catabolize tryptophan, inhibits the proliferation of maternal T cells, thus playing an important role as one of the mechanisms of maternal-fetal tolerance. However, little is known whether the action of IDO is influenced by substances present in the pregnant uterine microenvironment. This study evaluated the behavior of the IDO expression in cultured placental and embryonic cells from rats in face of the addition of estradiol, progesterone, γ interferon, tryptophan and 1-methyl-D-tryptophan, by flow cytometry, at 4, 24 and 48 hours periods.

The results showed that the most significant differences were observed in pregnant females where the addition of progesterone, γ interferon and tryptophan increased the IDO expression in the cultivated cells to 19.24% (4hs period), 11.22% (24hs period) and 23.53% (24hs period), respectively. Considering these results, we may conclude that the expression of IDO by cultured placental and embryonic cells from Wistar rats is indeed influenced by factors present in pregnant uterus, which provides additional information to better understand IDO role in the maternal-fetal tolerance, particularly on its interactions with reproductive hormones and cytokines.

CIRCULATING MICRORNAS AS POTENTIAL PREDICTIVE BIOMARKERS FOR PREECLAMPSIA

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MicroRNAs (miRNAs) are short, non-coding RNAs that post-transcriptionally regulate translation of mRNA. They can be released into the circulation and their abundance is altered in pathologies such as cancer [1]. Circulating miRNAs are also altered during pregnancy (compared to the non-pregnant state) [2] and can be released into the circulation by syncytiotrophoblasts [3]. Thus, the current study aimed to determine if circulating miRNAs could be used as a predictive biomarker of preeclampsia. The exact origins of preeclampsia remain unknown, but evidence suggests that early placental development is impaired. The only current cure for severe preeclampsia is delivery, which is often premature and has associated risks for both the mother and her newborn. In the current study, plasma samples from 12 healthy women and 12 women of matched BMI who developed severe preeclampsia later in pregnancy were collected at 15 weeks gestation, at least 5 weeks before symptoms of preeclampsia can present. Plasma miRNAs were extracted and profiled using Taqman® MicroRNA array cards. Predicted mRNA targets of miRNAs were found using miRecords database [4] and highly represented molecular networks involving targets were identified using Ingenuity® Pathways Analysis (www.ingenuity.com). Nine miRNAs (mir-148a, 30c, 491-5p, 28-5p, 483-5p, 330-3p, 140-3p, 636, 331-3p) varied in abundance in plasma between preeclamptic mothers and BMI-matched healthy mothers ($p < 0.05$). Interestingly, all 9 miRNAs were significantly decreased in the preeclamptic state. Many predicted targets of these miRNAs are involved in the molecular mechanisms of cancer, a process which draws many parallels with trophoblast migration in the early establishment of the placenta. Pathways involved include integrin signalling, SMAD signalling and control of cell cycle progression. Using these findings, we hope to develop an early predictive test for preeclampsia, so preventative measures can be instituted to try and limit the severity of symptoms and to improve neonatal outcomes.

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- (2) Miura, K., et al. 2010. *Clin Chem*, 56, 1767-71.
- (3) Luo, S. S., et al. 2009. *Biol Reprod*, 81, 717-29.
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PRIMITIVE PROGENITOR CELLS DETECTED BY HIGH ALDEHYDE DEHYDROGENASE (ALDH) EXPRESSION ARE PRESENT IN MULTIPOTENT STROMAL CELL PREPARATIONS DERIVED FROM THE HUMAN PLACENTAL BED, AND THEIR NUMBERS ARE SIGNIFICANTLY REDUCED IN PREECLAMPSIA.

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Introduction

Pre-eclampsia (PE) is a serious human pregnancy disorder characterised by shallow invasion of trophoblast cells and failure to remodel maternal spiral arterioles in the decidua [1]. Recently, abnormal multipotent stromal cells (DMSCs) have been associated with PE [2]. Our interest is in primitive progenitor cells, which comprise a small subpopulation of DMSCs. Primitive progenitor cells have high ALDH enzyme activity levels [3], are called ALDH^{br} cells in flow cytometry, usually comprise about 5% of the MSC population but have greater *in vitro* proliferation, colony forming unit and differentiation potential than ALDH^{low} MSCs [4]. Our hypothesis is that the primitive progenitor cell subpopulation (i.e. ALDH^{br} cells) of DMSCs is abnormal in PE compared with controls.

Method: Normal and PE-affected human term placentae were obtained with informed consent (n=6 per group). PE is diagnosed when hypertension arises after 20 weeks gestation and is accompanied by significant proteinuria. DMSCs were isolated from the decidua basalis using mechanical mincing followed by enzymatic digestion. Cultured DMSCs were characterised by flow cytometry and were >95% CD105+, CD73+, CD45- as expected. We utilised the "Aldefluor" system (Aldagen, USA) for detecting ALDH^{br} primitive progenitor cells. The green fluorescent product produced by the ALDH enzyme activity in cells was detected by flow cytometry and the number of viable ALDH^{br} primitive progenitor cells in the DMSC population determined.

Results: Results show the percentage of ALDH^{br} cells was significantly different between control decidual MSCs (5.667 ± 1.724) and PE- decidual MSCs (1.433 ± 0.4310) (n=6 each group, 10^5 representative cells for each sample, p-value 0.0193, Unpaired t-test). These results show a significant reduction in primitive progenitor cell numbers between control decidual MSCs and PE- decidual MSCs. Future work will identify gene and protein expression differences between the two populations of primitive progenitor cells.

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- (2) Hwang, J. H., M. J. Lee, et al. (2010)
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PRESENCE OF THE CELL SURFACE ESTROGEN RECEPTOR GPR30 IN THE APICAL MEMBRANE OF HUMAN PLACENTAL SYNCYTIOTROPHOBLAST.

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Context: Traditionally, estrogen binds to the nuclear receptors, ER α and ER β . Recently, a novel seven transmembrane receptor cell surface receptor for estrogen, called GPR30, was identified in cancer cells.

Objective: We investigated the presence of GPR30 in placenta from pregnant women at 1st trimester, 2nd trimester and term pregnancies.

Methods: Placental tissues were collected from elective caesarean sections and abortions (early termination of pregnancies). Tissues were immediately frozen in liquid nitrogen and stored at -80° C. These tissues were used for assaying GPR30 mRNA by quantitative PCR, for protein extraction and assay by western blotting and also for immuno-histochemistry.

Results: The mRNA for GPR30 was detected in placenta at 1st trimester, 2nd trimester and term placenta. There was no change in expression of mRNA of GPR30 across this gestational age range. Expression of mRNA of GPR30 is higher in term placenta tissue than in amnion, chorion or decidua. Western blot analysis revealed that a native ~38 kDa protein band specific for GPR30 was expressed in placenta. In addition, a mature glycosylated form was also detected at ~120 kDa. Western-blot and fluorescence immuno-histochemistry data also showed that expression of protein of GPR30 is higher in term placenta than amnion, chorion and decidua. Additionally, immuno-histochemistry data revealed that GPR30 is expressed only in outer membrane of syncytio-trophoblasts of term and 2nd trimester placenta.

Conclusion: In this study, we detected the membrane estrogen receptor GPR30 at the protein and message level in human placenta. We demonstrate localisation of the receptor to the apical membrane of syncytiotrophoblasts. We hypothesize that the high molecular weight glycosylated form of GPR30 permits transport to the plasma membrane.

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EXPRESSIONAL CHARACTERIZATION OF BOVINE ENDOGENOUS RETROVIRUS K ENVELOPES IN BOVINE PLACENTA

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Endogenous retroviruses (ERVs) are the remnants of ancestral infection of exogenous retroviruses and inherited as Mendelian laws. Some ERVs form envelopes (gene; *env*, protein; Env), which are also known as syncytins, have crucial roles in trophoblastic cell-to-cell fusion of haemochorial placentae. Meanwhile, ruminants including cow, sheep, goat and deer develop synepitheliochorial placentae and there are fetomaternal cell-to-cell fusion events; however, the precise mechanisms involved in the events have not been revealed. Recently, we identified two novel bovine ERVs, named BERV-K1 and BERV-K2, and they possessed intact *env* sequences (Baba *et al.*, 2011). These genes were transcribed in placenta and BERV-K1 *env* was exclusively expressed there. Furthermore, both BERV-K Envs harbored fusion peptides at the N-termini of transmembrane (TM) subunits. In this study, we focused on the relationship between fusogenic potencies of BERV-K Envs and the fetomaternal cell-to-cell fusion taken place in bovine placenta. We successfully expressed FLAG-tagged BERV-K Env proteins in Cos-7 cells. Immunoblotting analysis revealed that the BERV-K1 but not BERV-K2 Env precursor protein was efficiently cleaved into surface (SU) and TM Env subunits, which is important for fusogenic abilities of retroviruses. Real-time RT-PCR, *in situ* hybridization and immunohistochemistry demonstrated that BERV-K1 but not BERV-K2 Env was specifically expressed in trophoblast binucleate and multinucleate cells through gestation. TSA treatment to MDBK cells up-regulated the expression levels and modified the histone acetylation statuses of both BERV-K *envs*. Additionally, we also identified 3 BERV-K *env*-related sequences and confirmed by real-time RT-PCR that their expression levels were as low as BERV-K2 *env* in almost all tissues including placenta. This study suggested that BERV-K1 Env may possess fusogenicity and play a central role in trophoblastic multinucleation.

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ADENYLATE CYCLASE ACTIVATION ELEVATES MIR-34A AND SUPPRESSES INVASION IN THE TROPHOBLAST CELL LINE BEWO

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In mammals, cytotrophoblasts differentiate into villous cytotrophoblasts or extravillous cytotrophoblasts. The former fuse to form syncytiotrophoblasts responsible for endocrine production and fetal-maternal exchange, while the latter invade the endometrium. Dysfunctional trophoblast is associated with pathological condition such as pre-eclampsia and intrauterine growth restriction. MicroRNAs (miRNAs) are small non-coding RNAs that can silence gene expression by either repressing translation and/or promoting target mRNA degradation. The role of miRNA in trophoblast function is largely unknown. BeWo cells are widely used to study trophoblast physiology. It is well-known that forskolin stimulates BeWo cells to undergo syncytiotrophoblastic cell fusion. Here, we found that miR-34a was significantly elevated upon forskolin challenge in BeWo cells. Transient expression of miR-34a did not affect proliferation but reduce the invasiveness of the cells. In silicon miRNA target prediction indicated that a Notch signaling ligand, Delta-like 1 (DLL1), is a potential target of miR-34a. Western blotting and 3'UTR reporter assay demonstrated that DLL1 expression was regulated by miR-34a in the BeWo cells. Activation of Notch signaling with DLL1 stimulation and transfection of Notch receptor intracellular domain increased while miR-34a forced expression decreased the invasiveness of the cells. To examine whether miR-34a regulates the invasion of BeWo cells through Notch signaling, we force-expressed miR-34a and Notch receptor intracellular domain simultaneously and found that the effect of miR-34a on invasion was partially nullified. It is known that Notch proteins are expressed in placentae and miR-34a is expressed in trophoblasts. Our findings open up a possibility that miRNA regulation may be a mechanism for controlling trophoblast invasion. [The work is supported by GRF grant from Research Grant Council, Hong Kong]

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PLACENTAL OXIDATIVE STRESS, SELENIUM AND PRE-ECLAMPSIA

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There is considerable evidence that placental oxidative stress plays a significant role in the etiology of preeclampsia. Prophylactic use of exogenous anti-oxidants such as Vitamins E and C have proven to be ineffective and potentially dangerous. The current study addresses the role of endogenous anti-oxidant systems in preeclampsia. In particular, data on the seleno-dependent enzymes glutathione peroxidase and thioredoxin reductase will be presented and the role of selenium in preeclampsia will be considered. The aim of these studies was to determine the levels of endogenous antioxidants, selenium, and biological oxidation in normal and preeclamptic placental tissues. Furthermore, animal studies were conducted to assess the impact of selenium depletion on anti-oxidant expression and activity, oxidative stress and symptoms of preeclampsia. Selenium depletion generated placental oxidative stress and produced a preeclamptic like syndrome in pregnant rats suggesting a link between placental oxidative stress, endogenous antioxidant disequilibria and the pathogenesis of preeclampsia that may be linked to insufficient dietary selenium. The selenium status of preeclamptic mothers was also considered and lower levels of selenium were observed when compared to normal controls. Selenium

supplementation improves endogenous anti-oxidant expression in trophoblast cells and might provide an effective method of protecting the placenta from oxidative stress during preeclampsia. Clinical studies are now underway to investigate the benefits of low dose selenium supplementation on the development and progression of preeclampsia.

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A NOVEL HYSTEROSCOPIC TECHNIQUE FOR ACCURATE BIOPSY OF THE *DECIDUA BASALIS* AND *PARIETALIS*: IMPLICATIONS FOR PROGRESS IN RESEARCH ON THE EARLY EVENTS OF HUMAN PLACENTATION

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Introduction: Early pregnancy events at the embryo-maternal interface play a crucial role in determining the success or failure of a pregnancy and a healthy dialogue between trophoblast and decidua is key to prevention of pregnancy pathologies like preeclampsia, and recurrent miscarriage. Research into these interactions is hampered by ethical constraints, poor access and the accuracy of biopsy material. Contemporary methods of collection of first trimester decidua include suction curettage or punch biopsy with/without ultrasonography which are blind in nature and rely on immunostaining for identification. Experts have acknowledged the conflicting results in the published literature due to the imprecise methods of sampling the decidua¹. There is a critical need for a simple yet accurate technique of decidual biopsy.

Methods: The technique is applicable to pregnancies undergoing first trimester surgical termination. Following cervical dilatation, a rigid cystoscope is introduced into the cervical canal. The pressure of the saline distending medium shears the membranes of the gestation sac away from the *decidua parietalis*, leaving the pregnancy suspended at the site of the early placenta. Under direct vision a biopsy forceps is used to sample the *decidua parietalis*, and then the forceps is introduced beneath the gestation sac to sample the *decidua basalis*.

Results: There are no additional risks or adverse outcomes from the technique. Morphological and immunohistochemical studies have confirmed the accuracy, purity and adequacy of the samples, with a high (>40%) myometrial spiral artery presence. Functional studies using explant or single-cell suspension cultures are under way.

Conclusion: This is a remarkably simple and safe novel technique of decidual biopsy under direct vision which allows for accuracy of the site of biopsy. It therefore has the potential to revolutionise research on trophoblast-decidua interactions.

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MARSUPIAL *IGF2* EXPRESSION AND IMPRINTING IS TISSUE AND DEVELOPMENTAL AGE SPECIFIC

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IGF2 is an important regulator of growth, metabolism and differentiation. Abnormal expression level of *IGF2* is associated with Silver-Russell syndrome, Beckwith-Wiedemann syndrome and various types of cancer. We have used a comparative genomics approach to investigate the evolution of *IGF2* control mechanisms, by comparing distantly related mammals including both eutherians and marsupials. In mice and humans, *IGF2* has multiple promoters to maintain the complex tissue and developmental stage specific expression. In marsupials, whilst *IGF2* is imprinted, its promoter structure and regulation still remain to be characterized. In this study, we isolated 3 different transcripts by performing 5'RACE on both placenta and liver samples. Each of the 3 promoters precedes a non coding exon which is orthologous to the exons P1-P3 of the human and mouse *IGF2*. The expression pattern of tammar *IGF2* was similar to that seen in humans, with predominant expression from the tammar P2 promoter. Expression was higher in pouch young (developmentally equivalent to a eutherian fetus) than in adult and imprinting was highly tissue and developmental-stage specific. Interestingly imprinting in the placenta was restricted to the trilaminar layer of the yolk sac, which may be linked the vascularisation of this placental region. In addition, *IGF2* was imprinted in the adult mammary gland while in the liver it switched from monoallelic expression in the pouch young to biallelic in the adult. The conserved imprinting of *IGF2* transcripts in marsupial and eutherian mammals suggests that regulation and expression of this gene originated before the divergence of marsupial and eutherian lineages, from these 3 promoters which have been selectively maintained for at least 130-150 million years.

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THE EFFECT OF MULTIVITAMIN SUPPLEMENTATION AND INCREASING PRE-PREGNANCY BMI ON THE INCIDENCE OF PREECLAMPSIA IN SOUTH EAST QUEENSLAND SUB-POPULATION.

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Adequate maternal health and nutrition have long been considered important to a healthy pregnancy, with use of a pregnancy specific multivitamin widely recommended to prevent disorders such as neural tube defects and support the maternal system to cope with the stresses of pregnancy. Recent studies have suggested that the use of multivitamins during pregnancy may significantly reduce the risk of developing preeclampsia, a complex hypertensive disorder that develops after 20 weeks gestation. Analysis of the effect of multivitamin use on the incidence of preeclampsia in normal weight and overweight/obese women was conducted using data collected as part of the Environments for Healthy Living Project, Griffith University. Data from a total of 1937 pregnancies with complete multivitamin and BMI data were collected between 2006-2010 as part of the study. The incidence of preeclampsia for the cohort was 1.8% a value considered low in the context of reported values for Australia of (2.5-8%). Despite the cohort having a relatively low incidence a significant increase in preeclampsia was observed in the 42.1% of women considered obese/overweight in the cohort (P=0.0063). First trimester multivitamin use was reported by 57.5% of women in the study and was found to significantly reduce the incidence of preeclampsia (0.0194, Or=0.446, RR=0.9853). Analysis of the effect of multivitamin supplementation in women with a BMI greater than 25 demonstrated a significant 61% reduction in preeclampsia incidence (P=0.038, OR=0.3898, RR=0.9746) that appears to negate the increased risk associated with increasing BMI. The results of this study support the hypothesis that multivitamin supplementation may be beneficial in reducing the incidence of preeclampsia during pregnancy

EFFECT OF SELENIUM STATUS ON THE INCIDENCE OF PREECLAMPSIA: A GLOBAL PERSPECTIVE.

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Preeclampsia is a complex multisystem disorder of pregnancy which has a significant impact on a large number of mothers and babies. The aetiology of this disease is only partially described and includes shallow trophoblast invasion, poor placental oxygenation, increased oxidative stress, and an exacerbated immunological reaction to pregnancy. Selenium is an essential micronutrient required for the expression and synthesis of endogenous antioxidants and other selenoproteins. There have been numerous reports demonstrating a significant reduction in selenium status in preeclamptic women. The objective of this study was to map global selenium intakes and plasma selenium status with the incidence of preeclampsia. This study identified peer reviewed journal articles reporting national preeclampsia incidence (%) and matched these with reported values of selenium intake and plasma/serum selenium concentrations ($\mu\text{g/L}$). Matched data was obtained for 45 regions, reporting 6,456,570 births, spanning Europe, Asia, Australasia, Africa, North and South America. Increasing plasma selenium concentration was correlated to a reduction in preeclampsia incidence (Pearson's $r = -0.604$, $P < 0.0001$). Countries with a reported serum/plasma selenium level of $\geq 95 \mu\text{g/L}$ were considered selenium sufficient and a significant reduction in preeclampsia incidence for countries above this value was noted ($P = 0.0007$). Significant reductions in preeclampsia incidence were found to coincide with increases in plasma/serum selenium concentration in the New Zealand ($P = 0.0003$) and Finland (0.0028) populations following Government intervention to instigate Se supplementation. This study supports the hypothesis that selenium status is important during pregnancy and that supplementation may be beneficial in reducing the incidence and severity of preeclampsia.

DIFFERENT STROKES FOR DIFFERENT FOLKS: INTERACTIONS OF ANTIPHOSPHOLIPID ANTIBODIES WITH CELLS OF THE PLACENTA.

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INTRODUCTION: Antiphospholipid antibodies (aPL) predispose women to various diseases of pregnancy such as preeclampsia, intrauterine growth restriction, recurrent miscarriage and stillbirth. This may be due to the ability of aPL to inhibit the function of placental cells (trophoblasts), although exactly how they have this effect is unknown. Identifying how aPL interact with trophoblasts of the villous and extravillous lineages is key to understanding how they can have a detrimental effect on the placenta and ultimately pregnancy. **METHOD:** First trimester placental explants were incubated with the murine monoclonal aPLs IIC5 or ID2 or a control antibody for various times up to 2 hours. Tissues were then frozen and sectioned for immunohistochemistry. Antiphospholipid antibody binding proteins in lysates of villous placenta were examined by western blotting with murine aPL. Extravillous trophoblasts grown on matrigel-coated wells were incubated with fluorescently-labelled aPL IIC5 or ID2 or a control antibody before being visualised live on a Zeiss LSM_710 confocal microscope. **RESULTS:** The aPL, ID2, but not a control antibody was rapidly internalised by the syncytiotrophoblast of the first trimester placenta. After two hours ID2 was also present in the villous stroma but not villous cytotrophoblast. Antiphospholipid antibody binding proteins at 250, 170 and 130kDa were identified by western blotting. Extravillous trophoblasts in contrast, bound but did not internalise aPL. Compared to ID2, IIC5 preferentially bound to the extravillous trophoblast. **CONCLUSION:** Antiphospholipid antibodies reacted differently with villous and extravillous trophoblasts and there were also differences in the behaviour of the individual aPL, suggesting that different women may have aPL that are targeted against distinct trophoblast populations. This could account for the broad range of clinical outcomes associated with aPL. Identifying aPL-binding proteins on the villous placenta is the next step to further characterise these pathological interactions.

PROTECTING TROPHOBLAST CELLS FROM OXIDATIVE STRESS WITH SELENIUM SUPPLEMENTATION

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Oxidative stress is an imbalance between reactive forms of partially reduced oxygen and anti-oxidants. Oxidative stress plays an important role in the pathogenesis of preeclampsia, a serious complication affecting 7% of all pregnancies. Placental cells (trophoblast cells) within placenta are susceptible to oxidative stress which causes increased cell death and release of placental debris into the maternal circulation. Understanding this process and ultimately controlling it will lead to improved therapies for treating this complex disease. In this study trophoblast cells (BeWo) were treated with organic and inorganic forms of selenium to increase the biological activity of key anti-oxidant proteins selenoproteins, Thioredoxin Reductase (ThxRED) and Glutathione Peroxidase (GPx). Addition of 100nM NaSelenite or 500nM Selenomethionine resulted in maximal enzyme activity. Trophoblast cells were then oxidatively stressed with various concentrations of H₂O₂, Cumene H₂O₂ or t-butyl- H₂O₂. The rezsaurin end point assay was used to measure cellular metabolic activity. There was a dose dependent decrease in cellular activity with increasing concentrations of all 3 stressors. There was a relatively narrow window of efficacy where low concentrations of stress failed to generate stress and high concentrations caused irreversible cellular damage. For H₂O₂ concentrations of between 100-200 μM , applied for 24Hrs resulted in approximately 50% decrease in cellular activity, an effect readily reversible by the addition of 100nM NaSelenite or 500nM Selenomethionine. Similar results were found for 30-40 μM Cumene- H₂O₂ and t-butyl H₂O₂. We also used, Auranofin, a selective inhibitor of ThxRED and GPx, to inhibit the up-regulation of these selenoproteins and demonstrated increased susceptibility to oxidative stress. These data clearly show that selenium supplementation is an effective method of up-regulating the expression and activity of key anti-oxidant proteins such as ThxRED and GPx in placental trophoblast cells. Upregulation of these enzyme systems offers protection from oxidative stress in vitro. We are currently examining the selenium status of pregnant women who experience preeclampsia with a view to using selenium supplementation to treat this serious complication of pregnancy.

BETAGLYCAN PROMOTES A NON-INVASIVE PHENOTYPE IN HUMAN GRANULOSA TUMOUR CELLS VIA THE DOWN-REGULATION OF MMP2

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Betaglycan, the type III TGF- β receptor, is a proteoglycan that acts as a co-receptor for TGF β s, inhibins, and certain BMPs. We have previously shown that betaglycan is a suppressor of metastasis in human ovarian granulosa cell tumors (GCTs), and that loss of betaglycan expression in GCTs is associated with a more aggressive, invasive tumor phenotype [1]. The current study tested the hypothesis that betaglycan inhibits invasive GCT behaviours by regulating matrix metalloproteinases (MMPs), which have been shown to enhance cancer progression and tumor invasion through tissue barriers [2]. We investigated by quantitative real time PCR the expression of MMPs in two human cell lines, KGN and COV434 originally isolated from GCT metastases. *MMP-2*, *-9*, *-14*, *-15*, and *-16* expression was detected in the GCT cells lines. Over-expression of wildtype betaglycan in the GCT cells resulted in significant decreases in the basal expression levels of *MMP15* and *MMP16* mRNAs (6- and 2-fold, respectively). Additionally, gel zymography using conditioned media, and Western blot analysis using cell lysates, from control and betaglycan-expressing GCT cultures established that MMP2 expression and activity was significantly down-regulated in betaglycan-expressing GCT cultures. *SMAD2* or *SMAD3* gene silencing abrogated the betaglycan-mediated block in GCT migratory and invasive behaviours and resulted in the up-regulation of MMP2. Chemical inhibition of MMP2 mimicked the betaglycan-mediated block in GCT wound healing, matrix invasion, and increase in cell-substrate adhesion. Collectively, our data indicate that the presence of betaglycan in GCTs reduces the expression of specific MMPs and this regulation is SMAD2/3-dependent. Furthermore, we conclude that specific MMPs may be involved in the spread of GCTs and that retention of betaglycan during GCT progression may hamper tumor progression by limiting MMP expression and GCT invasion.

Supported by the NHMRC of Australia (RegKeys 494802; 441101; 388904) and Victorian Government Infrastructure funds.

(1) Bilandzic et al., 2009, *Mol Endocrinol* 23(4): 539-548

(2). Hotary et al., 2000, *J Cell Biol* 149(6): 1309-23

COMPREHENSIVE MUTATION SCREENING IN MIRNA IN TESTICULAR GERM CELL TUMOURS.

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MicroRNAs (miRNAs) are endogenous small non-protein coding RNAs which regulate basic cellular processes. There is considerable evidence that expression of miRNA genes is deregulated in human cancer, and specific over- or underexpression has been shown to correlate with particular cancer types.

Testicular germ cell tumours (TGCTs) are the most common malignancy affecting males between the ages of 15 and 45 years, and is associated with significant morbidity including infertility. This study is focused on identifying genetic variants in type 2 TGCTs, which is the most frequent testis cancer subtype. These cancers are most commonly formed from undifferentiated fetal germ cells contained within the testis.

There are several methods for identifying variants in DNA. High-resolution melting (HRM) analysis and multiplex ligation dependent probe amplification (MLPA) are two sensitive, cost effective and high throughput techniques for rapidly screening a large number of DNA samples. To identify point mutations we performed HRM analysis on eight miRNA loci implicated in either testis cancer or tumorigenesis in general. To identify deletions and duplications we developed an MLPA mix containing probes that recognize 50 miRNA sequences that have been identified as being deleted or duplicated in tumours.

We have carried out a pilot study using these two approaches on 48 TGCT samples. To date we have identified a number of potential variants that we are currently confirming with independent techniques. Given the success of this initial screen we plan to study an additional 100 DNA samples. Our findings will give a better understanding of the genetic basis of testis cancer, and may lead to improved diagnostic or therapeutic protocols.

OVER-EXPRESSION OF ACTIVIN-BC MODULATES REPRODUCTIVE TUMOR DEVELOPMENT IN INHIBIN KNOCK-OUT MICE

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The inhibin knock-out mouse is the only genetically manipulated mouse in the inhibin/activin family that develops cancer of the gonads and when gonadectomised the adrenals. Tumour formation leads to elevated activin levels, resulting in a cachexia-like wasting syndrome in the liver and stomach leading to severe weight loss from 6-7 weeks and lethality by 17 weeks. Follistatin is a known regulator of activin bioactivity. When crossed with follistatin over-expressing mice, inhibin knock-out mice do not develop activin associated cachexia and tumour differentiation is modulated. We postulate that the activin- β_C subunit is a significant alternate regulator of activin bioactivity and tested this by crossing activin- β_C over-expressing mice with inhibin knock-out mice. Inhibin knock-out mice over-expressing activin- β_C show reduced circulating activin A, no significant weight loss and reduced incidence of testis and ovarian tumors. Our data indicate that like follistatin the activin- β_C subunit is a significant regulator of activin A bioactivity.

FURIN, THE SOLE PROPROTEIN CONVERTASE ASSOCIATED WITH PROGRESSION OF ENDOMETRIAL EPITHELIAL CARCINOMAS

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Background: Proprotein convertases (PCs) are a family of serine proteases that post-translationally modify precursor proteins to their active form and are thus regarded as “master switch” molecules. PCs have been implicated in tumor growth and metastasis in reproductive cancers such as prostate and ovarian cancer.

Aim: To determine which PCs were present in endometrial epithelial cell lines; HES, ECC1, HEC1A, HEC1B, Ishikawa, RL95-2 and AN3CA, and to determine the localization of the relevant PCs by immunohistochemistry in the endometrium of women with various grades of endometrial cancers compared to normal postmenopausal women (control).

Methods and Results: Total RNA from cell lysates were screened for seven PCs (PC1, PC2, furin, PC4, PC6, PACE4 and, PC7) using conventional RT-PCR. Transcripts of furin, PC4, PACE4 and PC7 were detected in all cell lines with no cell lines containing PC2 mRNA. Transcript for PC6 was detected in all cell lines except HES, which was also the only cell line containing PC1 mRNA. Immunohistochemistry determined the level and cellular localization of furin, PC6, PACE4 and PC7 in women with endometrial carcinomas compared to normal postmenopausal women. PC6, PACE4 and PC7 while present at low levels showed no correlation with the grade of endometrial cancer. In contrast, furin was increased with endometrial cancer. A low level of furin was localized in the glandular epithelium of control endometrium, the immunoreactivity of furin increases as the epithelial cells underwent transformation.

Conclusion: These results show that among the seven PCs, four members furin, PC4, PACE4 and PC7 were expressed in all endometrial cancer cell lines examined. However, furin was the only PC to change as endometrial cancer progressed in women. Furin may be a “master switch” for the progression of endometrial cancers and furthermore be useful as a diagnostic marker for their detection.

OVEREXPRESSION OF ACTIVIN-BC IS ASSOCIATED WITH MURINE PROSTATE DISEASE

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Activins are members of the TGF- β super-family. There are 4 activin subunits (β_A , β_B , β_C and β_E) that combine to form functional proteins. The role of activin A ($\beta_A\beta_A$) has been relatively well characterized and is known to be a potent negative growth regulator. Two of the activin subunits (β_C and β_E) were discovered only in the last decade and little is known about their function, however recent work indicates activin C ($\beta_C\beta_C$) is an antagonist of activin A. Activin A inhibits growth under normal conditions however resistance to activin A-induced growth inhibition occurs during tumor progression. This paradox is not currently understood, however, we hypothesize that local expression of activin C antagonizes activin A-dependent growth inhibition and is a key factor mediating insensitivity to the growth inhibitory effects of activin A during prostate disease progression. To test our hypothesis we characterized the prostate in aged (9 and 12 months) transgenic mice over-expressing activin- β_C . Prostate epithelial cell hyperplasia, low grade PIN lesions, alterations in cell proliferation and reduced Smad-2 were evident in aged mice over-expressing activin- β_C . This work indicates that over-expression of activin C antagonizes activin A mediated growth inhibition leading to murine prostate disease.

PIRNA PATHWAY GENES MAY PLAY A ROLE IN THE ORIGIN AND PROGRESSION OF EPITHELIAL OVARIAN CANCER.

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Most cancers show alterations of DNA methylation patterns, retrotransposon derepression and genome instability. Genes of the Piwi-interacting RNAs (piRNA) pathway are well known for their role in protecting genome integrity via the repression of TEs in developing germ cells. Mutations of components in piRNA pathway such as PIWIL1, PIWIL2 and MAEL result in TEs derepression and overexpression in mice testes and Drosophila ovaries. We recently discovered expression of piRNA pathway genes in the mammalian ovary. To investigate if piRNA genes could also play a role in ovarian cancer we investigated the expression of the human TE, *Line-1*, and piRNA pathway genes in epithelial ovarian cancer (EOC). Compared to normal ovary, 32% of malignant EOC (N=19) have elevated *Line-1* expression while more than 70% of the cancer samples have increased expression of *Piwil1* and *Mael*, respectively. *In situ* hybridisation reveals that *Line-1* is overexpressed in the epithelial cells of EOC. Interestingly, in some samples *Line-1* seems to be inversely correlated to *Piwil1* expression in the epithelial cells. In addition, *Piwil2* and *Mael* are expressed in both the epithelial and stromal cells of EOC. The expression pattern of these two genes is similar and seems positively correlated with that of *Line-1* in the epithelial cells. In summary, the expression of piRNA pathway genes and *Line-1* in normal ovary and EOC may reveal new aspects of the origin and progression of ovarian cancer and we currently investigating a direct role of piRNA pathway genes in ovarian cancer progression.

EPITHELIAL AR INACTIVATION MODIFIES INTRAPROSTATIC ANDROGENS IN PTEN KNOCKOUT PROSTATE CANCER MOUSE MODEL

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Inactivation of the phosphatase and tensin homologue (PTEN) pathway has been associated with development of castrate resistant prostate cancer. Inactivation of PTEN in prostate epithelium (pPTENKO) leads to hyperplastic growth as well as suppression of androgen-responsive gene expressions by modulating androgen receptor (AR) activity. Androgen signaling is amplified in the prostate by the conversion of circulating testosterone (T) to the more potent androgen dihydrotestosterone (DHT). Conversion is mediated by the 5 α -reductase (5 α R) enzymes, which are positively regulated by androgens via AR. Therefore, to further analyse the role of AR and PTEN inactivation on prostate pathology and intraprostatic

androgens, we created homozygous PTEN inactivation in pePTENKO and a combined homozygous PTEN and hemizygous AR inactivation (pePTENARKO) using the Cre/LoxP technique, with probasin-cre mice used for prostate epithelial targeting. Cre negative littermates were used as controls (denoted WT).

Prostate growth, histopathology as well as serum and intraprostatic T and DHT (liquid chromatography tandem mass spectrometry) were analysed in young adult males at 9 weeks of age. Compared to WT, the pePTENKO males had heavier ($p < 0.001$) prostate lobes with severe epithelial hyperplasia. The prostate epithelial AR inactivation in pePTENARKO males prevented the PTEN inactivation induced increase in prostate weights, but not the severe epithelial hyperplasia. Compared to WT, the intraprostatic T levels were increased over 3.3-fold in pePTENKO and pePTENARKO males ($p = 0.036$). In contrast, intraprostatic DHT was significantly lower ($p = 0.034$) in pePTENKO but returned to normal in pePTENARKO. Serum T levels were normal.

Genotype	Serum T (ng/ml)	Prostate weight (mg)	Intraprostatic androgens (pg/mg anterior prostate)		Ratio
			T	DHT	
WT	3.3 ± 1.9	34.1 ± 4.5	0.7 ± 0.3	10.6 ± 4.7	15.5 ± 4.8
PTENKO	4.3 ± 3.5	90.8 ± 10.4	2.0 ± 1.0	1.6 ± 0.2	1.3 ± 0.3
PTENARKO	4.3 ± 1.5	30.8 ± 3.9	0.9 ± 0.3	16.6 ± 1.8	23.5 ± 4.5

* Data as Mean ± SE; N=5

In conclusion, we demonstrate that the prostate epithelial inactivation of PTEN tumor suppressor leads to severe prostate epithelial hyperplasia that is not prevented by simultaneous AR inactivation. However, the results suggest that both the PTEN inactivation and the AR inactivation can modify the intraprostatic steroid environment. Further analysis of intraprostatic steroid pathways affected may reveal new targets for prostate cancer treatment.

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ACTIVATION OF PRO-SURVIVAL FACTOR AKT IS ESSENTIAL FOR THE SURVIVAL OF HUMAN GRANULOSA CELL TUMOURS

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The serine/threonine protein kinase AKT is an important regulator of cell survival and proliferation. We have investigated pathways which regulate cell survival in granulosa cell tumours (GCT) using the human KGN cell line. We showed that human GCT samples and KGN cells exhibit lower levels of Betaglycan (BG), a TGF- β accessory receptor; stable expression of BG in KGN cells revealed anti-migratory and anti-invasive roles for BG but no effect on cell survival (Bilandzic et al. 2009). We hypothesised that pro-survival pathways involving AKT were over-activated in KGN cells and that these pathways may be masking the effects of BG and its ligands on cell viability. To test this hypothesis, we first examined the expression levels and activation status of components of the AKT pathway, using Western blot analyses and activation-state specific antibodies. This analysis revealed that in the basal state, KGN cells exhibited constitutive activation of AKT and PDK1, which is one of the upstream activator of AKT. TGF β 2 (1 ng/ml) treatment increased phosphorylation of AKT at residues Ser473 and Thr308 by 2-fold in both wildtype KGN and BG-expressing KGN (KGN BG) cells. The AKT kinase inhibitor LY294002 abolished phosphorylation at both Ser473 and Thr308 in both control and TGF β 2-treated KGN and KGN BG cells. Furthermore, LY294002 also inhibited cellular survival, reducing cell viability in both KGN and KGN BG cells to 40% of the control-treated cells ($p < 0.05$). Exogenous TGF β or neutralization of TGF β s alone or in combination with AKT inhibition did not have a further effect on cell viability. Collectively, these data indicate that the AKT pathway contributes to the survival of the GCT cell line. TGF β s/BG can further activate the AKT pathway but do not have a measurable effect on cell viability in KGN cells. (supported by the NH&MRC of Australia and Victorian Government Infrastructure funds).

(1) Maree Bilandzic, et al. Loss of Betaglycan Contributes to the Malignant Properties of Human Granulosa Tumor Cells; *Mol Endocrinol*, 2009

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HEAT SHOCK PROTEIN 27 EXPRESSION IN EPITHELIAL OVARIAN CANCER: POTENTIAL CORRELATION WITH PREDICTED EPITHELIAL OVARIAN CANCER WITH PERITONEAL METASTASIS

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Introduction: Ovarian cancer is the 3rd most common gynaecologic malignancies and the leading cause of death in gynaecological cancer. Most ovarian cancers are epithelial ovarian cancer. Although the advanced treatment of ovarian cancer increases the five-year survival rate, the peritoneal metastasis of epithelial ovarian cancer is still one of the main causes of death. This is because there is no potential predictor of peritoneal metastasis in ovarian cancer. Heat Shock proteins (HSPs) are a class of functionally related proteins. The expression of these proteins is low in normal conditions but highly expressed in many malignant cancers including ovarian cancer. Studies have suggested that high level of HSP27 is present in serum of patients with ovarian cancer (1). In this study we investigated whether the expression of HSP27 was associated with epithelial ovarian cancer with peritoneal metastasis, and whether it could be a potential predictor of peritoneal metastasis of epithelial ovarian cancer.

Method: Tissues were obtained from 10 cases each of epithelial ovarian cancer with or without peritoneal metastasis. The mRNA level of HSP27 was measured by Real time PCR. In addition the protein level of HSP27 in epithelial cancer tissues was measured by western blotting. The localisation of HSP27 was performed by immunohistochemistry.

Results: Immunohistochemistry images showed that HSP27 was expressed in the plasma of epithelial ovarian cancer cells, and significantly highly expressed in epithelial ovarian cancer with peritoneal metastasis. mRNA level and protein level of HSP27 were significantly increased in epithelial ovarian cancer with peritoneal metastasis.

Conclusion: These data suggested that higher level of HSP27 was associated with peritoneal metastasis of epithelial ovarian cancer. It may be a predictor of poor survives in clinical treatment and may provide a basis for the development of molecular therapeutics modulating this survival pathway.

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RELATIONSHIP BETWEEN EXOGENOUS LEPTIN AND OXIDATIVE STRESS IN ADULT RAT

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Introduction: Previous studies have shown that the percentage of abnormal sperm in the leptin-treated animals was slightly higher when compared with their age-matched controls. Oxidative stress could play a critical role in the induction of sperm abnormalities. Methods: This study assessed the effect of exogenous leptin on the production of ROS in 55 adult Sprague-Dawley rats. The rats were divided into 3 groups and daily intraperitoneal injections of 5, 10 and 30 mg/kg of leptin were administered for a duration of 7, 15 and 42 days. Samples from the rat epididymis were treated with DCFH-A and analyzed using flowcytometry. Samples were also prepared for tissues tests. The results were analyzed using SPSS and 2-way ANOVA with factors dose and time was administered. P-value < 0.05 was considered significant. Result: ROS levels were compared between groups in Flowcytometry assay, the interaction effect of factors dose and time was significant (p < 0.05). The difference between dose 30 with dose 10 in day 7 was significant (p < 0.05). Also these doses had significant differences with dose 5 and control (p < 0.01). The ROS percent in doses 10 and 30 was significantly reduced in day 42 (p < 0.05). Evaluation of tissue showed increase of leptin receptor on 15th day and reduction on 42th day of injection. Conclusions: It seems that exogenous leptin caused an increase in ROS percentage. Up to day 15 the increase of leptin concentration along with the increase of FSH and estrogen concentration stimulated spermatogenesis. But a decrease in the concentration of estrogen from day 15 to 42 due to negative feedback resulted in a decrease of ROS and consequently damage to sperm. Evaluation of the tissue also showed that in groups receiving 15 days of treatment a higher expression of leptin receptors was observed which is in approval of the higher levels of Flowcytometry in these groups.

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HEK-293 CELLS USING AS VEHICLES FOR THE EXPRESSION OF BOVINE RECOMBINANT FOLLICLE-STIMULATING HORMONE RECEPTOR (FSHR) AND LUTEINIZING HORMONE RECEPTOR (LHR).

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The LH receptor (LHR) is a member of the superfamily of G protein-coupled receptors, it has multiple isoforms and it binds with high-affinity to LH and human chorionic gonadotropin (hCG). In cattle, changes in the granulosa cells LHR expression were closely and temporally associated with the dominant follicle selection and, in general, with activation of adenylate cyclase-linked Gs class of heterotrimeric G proteins. The aim of this study was to investigate if cultured HEK-293 cells express the mRNA of FSH and LH receptors and to check the possibility to use this cell type in transfection studies involving the expression of splicing variants of bovine LHR. Moreover the FSHR gene expression was investigated since some ligands as equine chorionic gonadotropin (eCG) can bind in both receptors. For this, HEK-293 cells were cultured using DMEM/F12 medium (Dulbecco's Modified Eagle Medium Nutrient Mixture F-12), supplemented with calf fetal serum and maintained at 37°C in 5 % CO₂ in air during 2 days. Four replicates (1x10⁶ cells/replicate) were submitted for total RNA extraction according Trizol (Invitrogen[®]) reagent protocol. The receptors gene expression was investigated by real time RT-PCR (reverse transcription followed by polymerase chain reaction) with oligo-dT in RT and Sybr Green system in PCR. Two primer sets for each gene were used to cDNA amplification, one bovine-specific and the other human-specific (both for FSHR and LHR). Expression of human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as internal control of PCR. Human or bovine FSHR and LHR genes were not expressed in HEK-293 cells. Thus, the absence of bovine LHR and FSHR transcripts provides the validation to use HEK-293 cells as a vehicle for transfection of these bovine-specific gonadotrophin receptor (*i.e.* LHR) to gain insight about intracellular signaling of its isoforms.

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BMPR-IB MODULATES INTRA-TESTICULAR TESTOSTERONE AND 3 β -HYDROXYSTEROID DEHYDROGENASE (3 β -HSD) ACTIVITY IN MALE MICE.

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In males pituitary gonadotrophin signaling up-regulates testicular steroid production and male germ cell development. Transforming growth factor- β (TGF- β) superfamily members such as bone morphogenetic proteins (BMPs) and their receptors have been shown to modulate this process. Previously, BMPR-IB immunization reduced seminal vesicle weight of eCG-stimulated immature mice and increased basal serum testosterone in mature males. Therefore, in this study we investigated the role of BMPR-IB on intra-testicular testosterone and 3 β -HSD activity in immature and mature mice vaccinated against BMPR-IB.

Male mice aged 21 and 56 days were passively immunized with anti-BMPR-IB in 100ul of PBS by subcutaneous injections with and without equine chorionic gonadotrophin (eCG) to override endogenous gonadotrophins. The preparations were administered daily for six days. On the seventh day mice were sacrificed by asphyxiation with CO₂ and the testes were removed, one was stored frozen and the other immersion fixed in Bouins fixative. Testis tissue was homogenized and assayed for testosterone using an RIA. Fixed tissues were prepared for wax embedding using a histokinetic, wax embedded and cut on a microtome at 3 μ m for histological examination. Sections were de-waxed and stained using 3 β -HSD histochemistry.

In immature mice BMPR-IB immunization markedly reduced eCG-stimulated 3 β -HSD activity in Leydig cells, while in mature mice it significantly increased basal Leydig cell 3 β -HSD activity and intra-testicular testosterone. We conclude that signaling through BMPR-IB enhances gonadotrophin stimulated 3 β -HSD activity in immature mice, while in mature mice it acts to reduce 3 β -HSD activity in Leydig cells. The signaling ligands involved

in these processes and the downstream intracellular mechanisms involved in the developmental differences in BMPR-IB function are currently under investigation.

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MODULATION OF BASAL AND GONADOTROPHIN STIMULATED TESTOSTERONE IN MOUSE TESTIS BY OXYTOCIN *IN VITRO*.

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A discrete number of studies have shown that administered oxytocin regulates testosterone locally in the testis and FSH centrally, and that its endogenous secretion is altered by gonadotrophin stimulation. There have been no reported studies to demonstrate the effect oxytocin has on gonadotrophin-stimulated testosterone *in vitro*. The aim of this research was to conduct an initial pilot study of the affects of different doses of exogenous oxytocin on dose-response gonadotrophin-stimulated testosterone in mature mouse testis *in vitro*.

Mature testicular interstitial cells and testis slices were cultured from male Swiss mice aged 70 days. Cell count and viability were ascertained. Cells were stimulated with eCG and treated with oxytocin. Tissues were stimulated with eCG, hCG, and FSH and treated with oxytocin. Testosterone production was determined with radioimmunoassay.

Oxytocin profoundly upregulated basal and sub-physiological eCG-stimulated testosterone in mature testicular interstitial cells, and had no effect on mildly stimulated cells. Oxytocin reduced testosterone in moderately stimulated cells, and completely abolished the stimulatory affect of maximal eCG stimulation. These effects were observed at 10 IU/mL oxytocin. Testis slice culture showed similar trends with eCG and FSH stimulated tissues, while hCG stimulated tissues were not reflective of results obtained with cell bioassay. Both LH and FSH activity appeared to facilitate the modulating actions of oxytocin on testosterone production in mouse testis while LH activity was sufficient in interstitial cells.

In conclusion, oxytocin has a strong modulating action on basal and gonadotrophin-stimulated testosterone from cells and tissues of the testis in mice. Further research should explore a link between testicular oxytocin and the pituitary gonadotrophins.

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A LOCAL DEVELOPMENTAL ROLE FOR ENDOGENOUS LEPTIN AND ITS RECEPTOR IN BASAL AND GONADOTROPHIN-STIMULATED TESTICULAR TESTOSTERONE PRODUCTION IN MICE.

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The reproductive mechanisms of leptin at the hypothalamic-pituitary-gonadal axis have been thoroughly researched, however the role of leptin in local testosterone production in the testis is not well understood. Leptin receptor RNA and protein are localized in the testis, notably in Leydig cells, suggesting a local role for leptin in steroidogenesis. The aim of this study was to determine the effects of endogenously produced leptin on 3 hour testosterone production in testicular cells by neutralization of leptin and leptin receptor *in vitro*.

Sexually immature and mature testicular interstitial cells were isolated and cultured from male Swiss mice aged 21 and 70 days. Cell count and viability were ascertained. Cells were stimulated with equine chorionic gonadotrophin, and treated with recombinant human leptin, anti-leptin, or anti-leptin receptor. Testosterone production was determined with radioimmunoassay. Leptin receptors in immature and mature testis homogenates were visualized using Western blots.

Western blots showed several receptor bands similar in both age groups. Immature and mature cells responded with massive increases in basal testosterone after leptin neutralization, while leptin receptor neutralization increased basal testosterone in immature cells only. Leptin and leptin receptor neutralization in gonadotrophin-stimulated cells increase testosterone profoundly only in mature cells.

This study gives unique insight into the developmental mechanisms of leptin in the testis. Firstly, endogenous leptin regulates basal testosterone at both stages with partial function through the receptor in immature interstitial cells, and secondly endogenous leptin regulates gonadotrophin-stimulated testosterone via the leptin receptor only in mature interstitial cells.

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DOES CANOLA MEAL SUPPLEMENTATION ADVANCE THE TIMING OF PUBERTY AND SEXUAL MATURITY IN MALE ALPACAS?

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The objective of this research was to determine if canola meal supplementation influences the timing of puberty and sexual maturity in male alpacas. Clinically healthy juvenile males (n=40) were randomly assigned to four groups with similar age (16.7±0.47, 16.1±0.92, 16.8±0.66 and 15.9±0.60 m.o) and live bodyweight (35.8±2.50, 34.45±1.26, 36.05±2.65 and 35.1±1.75 kg). The groups received a standard maintenance diet supplemented with 25, 50, 75 and 100 g/head/day of canola meal. Bodyweight, body condition score, testicle sizes, the degree of the liberation of the penis-prepuce adhesion, and libido were monitored on a monthly basis until puberty and sexual maturity were confirmed. Puberty was physically judged as the penis separation from the preputial attachments and ability to copulate. At the end of the experiment, the juveniles were castrated and their testicular size was measured. Canola supplementation had no significant effect on the bodyweight or body condition score. Dietary treatment also had no effect on the size or volume of testicles, libido or the timing of preputial separation. We conclude that canola meal supplement did not alter growth or reproductive maturity in male alpacas.

Keywords: alpaca, canola meal, puberty

HAPLOTYPE ANALYSIS OF POLYMORPHISMS IN FSH RECEPTOR GENE SUGGESTS ITS ASSOCIATION WITH TYPE OF OVARIAN RESPONSE TO GONADOTROPIN STIMULATION

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The influence of follicle stimulating hormone receptor (FSHR) variant on the type of ovarian response during IVF treatment is extensively studied. There are several reports which indicate the potential role of single nucleotide polymorphisms (SNPs) at position -29 (G⁻²⁹A) and Asp⁶⁸⁰Ser (N⁶⁸⁰S) of FSHR gene in determining poor ovarian response. In the present study, we extend our previous observation and analyze the haplotypes at position -29 and 680 of FSHR gene, to study its association with type of ovarian response. The genotypes at these positions were analyzed by PCR-RFLP technique in proven fertile healthy women (n=100) and women undergoing in-vitro fertilization treatment (n=100). Further, the association between receptor expression at mRNA level in granulosa cells with haplotypes was analyzed by real-time PCR in IVF subjects. The haplotype analysis revealed that the frequency distribution of GA-NS haplotype was more in controls as compared to IVF subjects. Interestingly, AA-NN haplotype did not exist in control subjects and was found to be significantly higher in IVF subjects. The clinical and endocrinological parameters in IVF subjects revealed that subjects with AA-NN haplotype required significantly higher amount of exogenous FSH for ovulation induction as compared to subjects with GA-NS haplotype during IVF treatment (P=0.35). Furthermore, the FSHR expression at mRNA level in granulosa cells was observed to be lower in subjects with AA-NN haplotype as compared to GA-NS haplotype (P=0.36). The above findings indicate that the subjects with AA-NN haplotype could be poor ovarian responders. We conclude that the haplotypes at position -29 and 680 of FSHR gene is associated with type of ovarian response to gonadotropin stimulation during IVF treatment.

ASSESSMENT OF PROGESTERONE CLEARANCE RATE BY COMPARISON OF PROGESTERONE LEVELS @BETWEEN THE JUGULAR VEIN AND THE CAUDAL VENA CAVA IN LACTATING AND NON-LACTATING DAIRY COWS

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We previously showed that lactating cows have higher circulating progesterone (P₄) concentrations and LH pulse frequency than non-lactating cows during the luteal phase at the 103rd SRD meeting in 2010. This study examined the P₄ clearance rate in lactating and non-lactating cows. Six cycling healthy Holstein cows (lactating; n=3, non-lactating; n=3) were catheterized into the caudal vena cava and the jugular vein during the functional luteal phase. Blood samples were taken from both veins simultaneously every 15 min for 12 h to analyze P₄ and luteinizing hormone (LH) on the day after catheterization. Cows were fed one half of the daily diet 6 h after the start of blood sampling. The P₄ clearance rate was calculated by utilizing AUC of P₄ concentration between the jugular vein (JV) and the caudal vena cava (CVC), i.e. (CVC - JV)/CVC ~100. Mean P₄ concentrations during the 12 h sampling period in the jugular vein and the caudal vena cava were 6.8 ± 1.4 and 55.1 ± 29.8 ng/ml for lactating cows, 5.5 ± 0.9 and 57.0 ± 60.0 ng/ml for non-lactating cows, respectively. In lactating cows, P₄ levels in the caudal vena cava significantly increased by 101.3 ± 77.0% 1 h after feeding in comparison with that before feeding, whereas P₄ levels in the jugular vein significantly decreased by 24.2 ± 7.2% 2 h after feeding and the low levels persisted thereafter. The P₄ clearance rate significantly increased during the period from 1 to 3 h after feeding, but LH pulse frequency did not change. In non-lactating cows, one cow showed a P₄ change similar to that in lactating cows together with increased LH pulses after feeding, whereas the remaining two cows showed no change in the P₄ clearance rate and LH pulse. These results suggest that in lactating cows circulating P₄ levels are influenced by increases in P₄ metabolism and ovarian P₄ secretion after feeding.

TRANSGENIC MICE EXPRESSING GFP IN TWO POPULATIONS OF KISSPEPTIN NEURONS IN THE HYPOTHALAMUS

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Kisspeptin, encoded by Kiss1 gene, is expressed in the anteroventral periventricular nucleus (AVPV) and the arcuate nucleus (ARC) of female rodents. AVPV kisspeptin neuronal population is considered to be responsible for female-specific surge mode of gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) release in female rodents. ARC kisspeptin neuronal population would be associated with generation of pulse mode of GnRH/LH release in both sexes. The present study was conducted to generate Kiss1-green fluorescence protein (GFP) transgenic mice to investigate the roles of two populations of kisspeptin neurons. GFP cDNA was inserted into the translational start site of Kiss1 gene in the mouse BAC clone. The DNA construct was microinjected into pronucleus of fertilized one-cell stage embryo of BDF1 mice. Transgenic founder mice were detected by PCR on ear genomic DNA. Kisspeptin and GFP expression in the hypothalamus was validated in ovariectomized mice with/without estradiol-17β implant. Two transgenic lines showed co-localization of GFP- and kisspeptin-immunoreactivities in both AVPV and ARC in female mice. In summary, we generated two lines of Tg mouse model, showing kisspeptin neuron specific-GFP expression in both AVPV and ARC. This will allow us to further elucidate the molecular mechanism regulating Kiss1 gene expression in the AVPV and ARC kisspeptin neurons of female mice. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences of Japan.

ARE THE EFFECTS OF OXYTOCIN IN THE HUMAN PROSTATE DETERMINED BY THE LOCATION OF ITS RECEPTOR IN THE CELL MEMBRANE?

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Background. Oxytocin (OT) is involved in regulating prostate growth. This study investigated why OT, which normally inhibits prostatic growth, may stimulate proliferation in prostate cancer. Caveolae are specialized invaginations in the cell membrane involved in the regulation of signal transduction. With progression of prostate cancer there is a loss of caveolae. This study aims to investigate if the movement of oxytocin receptor (OTR) changes with the progression of prostate cancer and results in altered activation of signal transduction pathways.

Methods. Normal human prostate epithelial cells (PrEC) and androgen-independent (PC3) cancer cells were used. Co-localisation of OTR with lipid rafts and caveolin (cav) proteins was identified using triple labelled immunohistochemistry. Changes in ERK phosphorylation were identified using Western blot analysis. Total internal reflection fluorescence (TIRF) microscopy was used to determine the effects of OT on movement of OTR in living cells.

Results. In PrEC and PC3 cells treatment with OT alone increased co-localization of OTR and lipid rafts. In PC3 cells, treatment with testosterone or R1881 plus OT decreased co-localization of OTR and lipid rafts but no effect was seen with PrEC cells. OT stimulated ERK phosphorylation in PrEC but not in PC3 cells. TIRF analysis showed that in PrEC movement of OTR was confined with many immobile periods. Addition of OT resulted in longer immobile periods and a more confined trajectory. In malignant cells the OTR had a more extensive trajectory and addition of OT resulted in fewer transient immobile periods.

Conclusion. This study provides evidence that changes in localization and movement of the OTR occur with the development of prostate cancer. These changes are accompanied by alteration of ERK phosphorylation and cell proliferation.

SYNCHRONY OF HUMAN MALE AND FEMALE HORMONALLY RELATED TEMPERATURE CYCLES- BIOLOGICAL AND BEHAVIOURAL EFFECTS

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The stimulus for this study followed an investigation relating to perimenopausal women with Professor Jim Brown at the Royal Women's Hospital, Melbourne. Questions subsequently arose about cyclic functions in male physiology. A literature search showed 12 naturally occurring physiological functions recorded in males – including cycles of beard growth !

Results were first reported at the Annual Meeting of Endocrine Societies of Australia & New Zealand in NZ in 1976.

The study involved 25 males, with ages ranging from 20-56 years. Checked thermometers were supplied for them to take oral and rectal temperatures to within .05 ° C, both morning and night from between 6 weeks and 6 months. Standardised conditions were required and records kept of physical, emotional and habit variations. Results were recorded graphically. 20 of the males were cohabiting with a female, 2 males lived together, 3 were living separately. Females also kept detailed records...3 were pregnant, 3 used oral contraceptives and 1 was perimenopausal.

The data showed midcycle temperature peak synchrony with normally cycling females, but not if they were taking oral contraceptives. The 2 gay males had well defined synchronous midcycle temperature peaks. The husband of one of the pregnant females showed a temperature pattern that was thought to predict the time of birth.

Since that study report...boys in a senior boarding school dormitory were found to be cycling together by the end of their 1st Term back from vacation. Male and female children were found to be cycling with their parents. A communal group living together for 3 months were cycling together.

(1) Martha McClintock, Nature Jan 1971, girls living in a dormitory were cycling together. Later showing human pheromones caused the synchrony

(2) Mimi Halpem, circa 1987, showed the vomeronasal organ detects pheromones in animals and is present in humans

(3) Behavioural studies support physiological involvement in bonding - such an important function will not be dependant on one system alone.

Oxytocin is now well known for its role in trusting behaviour.

MODIFICATIONS TO PROTEIN CONVERTASE INHIBITOR NONA-D-ARGININE BY PEGYLATION AND CYCLISATION TO IMPROVE PHARMACOKINETIC PROPERTIES FOR IN VIVO USE

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Uterine expression of proprotein convertase (PC) 6 is critical for embryo implantation in mice and women. Host PCs including PC6 also play a vital role in HIV infectivity. Local administration of PC6 inhibitors through the vagina may protect women from both pregnancy (contraception) and HIV infection. The first step towards proving this hypothesis in an animal model is to develop a potent and serum-stable PC6 inhibitor. Nona-D-arginine (Poly R) is a potent inhibitor of PCs including PC6. In this study, we modified Poly R by PEGylation and cyclisation and determined their biochemical properties *in vitro*, in human uterine stromal cells, and their pharmacokinetic properties in mice *in vivo* following local delivery through vagina. PEGylation at the C-terminus, regardless of PEG size (1.2kDa or 30kDa), did not affect the inhibitory potency of Poly R towards recombinant human PC6 *in vitro*. In contrast, PEGylation at both termini or cyclisation, dramatically reduced the inhibitory activity of Poly R. Docking of Poly R and the cyclised derivative into the structure of the catalytic site of human PC6 *in silico* explained these observations. Two equipotent C-terminally PEGylated Poly Rs containing 1.2kDa or 30kDa PEG (C-1239-PEG Poly R and C-30k-PEG Poly R respectively) were further examined in cells and *in vivo*. While both effectively inhibited primary human uterine stromal cell decidualisation (a cellular differentiation process requiring PC6 and critical for implantation) in culture, they exhibited substantial serum pharmacokinetic differences following vaginal delivery in mice. The peak

concentration reached by C-1239-PEG Poly R was much higher than that of C-30k-PEG Poly R or parental Poly R. These studies provide important insights into future design of Poly R derivatives for in vivo applications.

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GALANIN RECEPTOR SIGNALING MODULATES KISSPEPTIN-INDUCED LUTEINIZING HORMONE SECRETION IN MALE RATS

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Kisspeptin plays a key role in controlling luteinizing hormone (LH) secretion via gonadotropin-releasing hormone (GnRH) secretion. Besides GPR54, GPR147/74 might mediate kisspeptin action to control GnRH release. The present study aims to examine if non-GPR54 G protein coupled receptors are involved in mediating kisspeptin action to control GnRH release. Neuropeptide Y (NPY) Y2 receptor (Y2R) and galanin receptor (GalR) were selected because of their high similarity to GPR54 of amino acid sequences in the ligand-binding region. RF-9, a GPR147/74 antagonist, BIIE0246, a Y2R antagonist, or M15, a GalR antagonist was intravenously injected with or without kp-10 in adult Wistar-Imamichi strain male rats. Among the three antagonists, only M15 significantly enhanced in vivo LH secretion induced by kp-10, but showed no significant effect when injected alone. To examine binding capability of kisspeptin to GalR, CHO cells expressing GalR1, 2 or 3 were treated with kp-10 but showed no response. In addition, mRNA expression of these receptors was not found in GnRH neurons taken from GnRH-GFP transgenic rats. These results suggest that GalR signaling in non-GnRH neuron modulates kisspeptin-induced LH secretion. Therefore, not only direct stimulatory effect on GnRH neurons, kisspeptin may also have indirect inhibitory action on GnRH/LH secretion mediated by GalR.

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INVOLVEMENT OF KISSPEPTIN NEURONS IN REFLEX OVULATION IN THE MUSK SHREW (*SUNCUS MURINUS*)

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Kisspeptin (firstly named metastin) is considered to play a crucial role in reproductive functions. In the present study, we investigated whether kisspeptin neurons involved in controlling reflex ovulation in the musk shrew (*Suncus murinus*). For this purpose, we first investigated the effect of estrogen on the distribution of *Kiss1* mRNA in the brain. *Kiss1*-expressing cells were distributed in the medial preoptic area (POA) and arcuate nucleus (ARC) of hypothalamus. *Kiss1*-expressing cells in the POA or ARC were up-regulated or down-regulated by estrogen, suggesting that POA or ARC kisspeptin neurons were the targets of estrogen positive or negative feedback action, respectively. We then investigated whether mating stimulus activates *Kiss1*-expressing cells in the POA and ARC. The mating stimulus induced c-Fos expression in *Kiss1*-expressing cells in the POA, suggesting that mating stimulus induces the activation of kisspeptin neurons in the POA. The present results indicate that kisspeptin neurons might be a part of neural circuit mediating reflex ovulation in the musk shrew. This work was partly supported by the Program for Promotion of Basic Research Activities for Innovative Biosciences of Japan (PROBRAIN).

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IMPACT OF A CALCIUM CASEINATE-ENRICHED DIET DURING PREGNANCY ON POSTPARTUM GABA RECEPTOR SUBUNIT EXPRESSION.

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After birth, there is an increased risk of maternal anxiety, which can adversely affect the offspring's development and behavior. Low prolactin levels in early pregnancy in mice decrease maternal neurogenesis, which results in increased anxiety and impaired maternal behavior postpartum. We have recently shown that a casein-enriched diet, which did not affect prolactin levels, restored maternal neurogenesis to normal levels, and prevented postpartum anxiety in this mouse model of postpartum anxiety. The aim of this study was to explore potential mechanisms underlying the casein-induced prevention of postpartum anxiety. Large fluctuations in hormones occur throughout pregnancy, which are essential for pregnancy continuance and onset of maternal behaviors. Expression of the subunits that comprise the GABA receptor are influenced by hormonal fluctuations, and subunit plasticity of this receptor has been implicated in the aetiology of mood disorders. Hence, we hypothesized that subunit composition of GABA receptors would be differentially altered in mice with postpartum anxiety in areas of the brain that regulate maternal mood. We further hypothesized that these changes would be prevented in low-prolactin mice fed a casein-enriched diet. Using Western Blotting, we analysed expression of GABAA $\alpha 1$, $\alpha 4$, $\alpha 5$ and GABAB B1 and B2 subunits in the amygdala, BnST, hippocampus, MPOA and subventricular zone (SVZ) – regions that regulate maternal and anxious behaviours. There were no significant differences in GABA receptor subunit expression between control and anxious mice in the SVZ or hippocampus. However, in the BnST and amygdala, levels of GABAA receptor subunit $\alpha 4$ and GABAB receptor subunits B1 and B2 were decreased in anxious postpartum mice. The decrease was prevented in low-prolactin mice fed the casein diet. The data suggest that a casein-enriched diet restores normal GABA receptor subunit expression in mice, despite aberrant hormone levels. How casein alters GABA receptor subunit composition remains to be investigated.

DEVELOPMENT OF THE FETAL OVINE HPG AXIS IS PERTURBED BY ENVIRONMENTAL CONCENTRATIONS OF ENDOCRINE DISRUPTING CHEMICALS

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BACKGROUND: Fetuses, exposed in utero to environmental chemicals in sewage sludge, exhibit hepatic accumulation of DEHP and PCB congeners (101+118). We exposed pregnant ewes to these endocrine disrupting chemicals (EDC's), separately or combined, throughout pregnancy.

METHODS: Scottish Blackface ewes (n=110) were AI using semen from a single Suffolk ram. Individually penned ewes were randomly allocated to 1 of 4 groups (Control, DEHP, PCBs, DEHP+PCBs) in a 2x2 factorial design. Ewes were exposed daily to 450 µg DEHP, 375 ng PCB101 and 625 ng PCB118 (environmental levels) applied to the morning meal. Pregnancies were terminated at Day 140 (term = Day 147) and fetal gonads and pituitaries processed for histological (follicle density, Sertoli and Leydig cell staining), qPCR, microarray (testis, ovary) and proteomic (ovary) analysis. Data were analysed using REML mixed linear models.

RESULTS: Gonad weight was unaffected by treatment in either sex. In male fetuses, pituitary *GnRHR* expression increased (P=0.03) in PCB relative to control and DEHP exposed mothers. Relative to controls, Sertoli, but not germ cell, numbers were reduced (P=0.004) by all treatments and testis *CYP17A1* immunostained area was reduced by DEHP and PCBs separately but not combined. Both PCBs and DEHP reduced testis *CYP11A1* expression (P<0.05) and PCB reduced *CYP17A1* expression (P<0.001) in singletons only. In female fetuses, pituitary *GNRHR* expression and ovarian follicle density were unaffected by treatment. EDC exposure induced differential expression (≥1.2-fold, P<0.05) of 19 (PCBs), 522 (DEHP) and 60 (DEHP+PCBs) transcripts coding for proteins involved in cell cycle, cell death, RNA metabolism or cytoskeleton organization. By proteomics, 94 of 776 spots were differentially expressed (≥1.2-fold, P<0.05) and proteins identified represented stress response/detoxification, cell cycle, cell structure and differentiation and metabolism pathways.

CONCLUSIONS The sensitivity of the developing fetal gonad to environmental concentrations of DEHP and PCB's has implications for both male and female fertility.

THE RELATIONSHIP BETWEEN STEROIDOGENIC ULTRASTRUCTURAL FEATURES AND PROGSTERONE RECEPTORS IN THE GREEN SEA TURTLE CHELONIA MYDAS

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Freshly laid green turtle (*Chelonia mydas*) eggs were collected at random from different nests at Ras-Al-Hadd Reserve and placed in incubators set at 30 °C. Under this incubation temperature, it revealed that high temperature produce 100% female hatchlings. Prior to day 20 of incubation the gonads were undifferentiated. Progesterone receptors (PR) is an indicator of steroidogenesis and gonadal development. Immunohistochemistry was used to localize and monitor the expression of PR. No PR expression was found in the gonads. At day 20 of incubation the gonads started to differentiate and continue to express PR until post hatching day 38. A stable expression of PR was observed suggesting stable steroid activity. The finding of this investigation is of value in estimating the effect of PR expression in gonadal differentiation and also evaluating steroidogenesis during the developmental stages of the embryo.

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TESTOSTERONE REPLACEMENT MODIFIES THE PROSTATIC MICROENVIRONMENT AND ALTERS THE ANDROGEN RECEPTOR IMMUNOREACTIVITY IN A MODEL OF ETHANOL-PREFERRING RATS (UCHB).

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Chronic ethanol intake causes hormonal imbalance by decreasing circulating testosterone (T), inducing disorders in reproductive tissues, including the prostate. Studies assessing the effects of hormone replacement associated with chronic alcoholism are rare and their results are inconclusive. This study evaluated the influence of testosterone replacement on the histopathology and androgen receptor (AR) immunoreactivity in the prostate of alcoholic UChB rats. Rats aged 90 days were divided into two experimental groups (n=20/group). UChB: drinking water; UChBEtOH: drinking ethanol 10% (v/v) at 2-6 ml/100g/day + water. At 150 days old, ten rats from each group received subcutaneous injections of testosterone cypionate (5mg/kg b.w.) diluted in corn oil during 4 weeks in alternating days, constituting UChBT and UChBEtOHT groups, while the other twenty (10/group) males received corn oil. At 180 days old, the animals were euthanized by decapitation and ventral prostates were collected, weighed and processed for histopathological and stereological analysis. Tissue sections were stained by HE and reticulin. AR immunohistochemistry was performed. Body weight gain was lower in the ethanol treated groups. Testosterone increased prostate weight but it had no influence on the ethanol consumption in alcoholic animals. There was decrease in epithelial and increase in luminal compartments in UChBEtOH. T replacement enhanced epithelial compartment in UChBEtOH. Atrophic prostatic acini and basement membrane folding in UChBEtOH were observed. However, this pattern was changed after T replacement, resembling the UChB and UChBT groups. Inflammation foci were noticed in 60% (UChB) and 100% (UChBEtOH) of animals. T treated animals did not present inflammation. There was impairment in AR immunoreactivity in atrophic acini of UChBEtOH. In

UChB/OHT, despite the presence of epithelial atrophy in some acini, there was increase in the AR immunoreactivity, but less homogenous than UChB. Therefore, testosterone replacement appears to reverse some effects caused by ethanol in the prostate.

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LUTEINIZING HORMONE PULSE AND SURGE IN FEMALE ICR MICE

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Mice have become more important as a genetically-modified model animal for the analysis of physiologic functions. The establishment of a frequent blood sampling system in conscious mice would provide a powerful tool for a better and more detailed understanding of the physiological status of circulating hormonal changes, such as pulse or surge modes of luteinizing hormone (LH) secretion. Frequent blood sampling, however, is considered problematic in mice because of the limited blood volume for their small body size. The present study, therefore, aims to establish a blood sampling protocol to determine the pulse and surge modes of LH secretion using intra-atrial cannulation and frequent blood sampling in free-moving conscious mice. Female ICR strain mice were ovariectomized and were bled every 3 min for 1.5 h to detect LH pulses. Obvious LH pulses were observed in ad lib-fed ovariectomized mice. The mean frequency of LH pulses was 4.64 ± 0.65 pulses/1.5 h (mean \pm S.E.M.), which seemed much higher than in other animal species undergoing ovariectomy. Other groups of mice were also bled after 24-h fasting to discover whether the present sampling protocol was capable of detecting suppressed LH pulses. The mean LH levels and frequency of LH pulses decreased significantly ($P < 0.05$) after 24-h fasting in ovariectomized mice. Thus, the present study clearly demonstrated that the current sampling protocol was suitable for detecting physiological changes in pulsatile LH secretion in mice. In addition, 1-h-interval blood collections in proestrous mice between 13:00 and 22:00 h revealed that individual preovulatory LH surges occur on the evening of proestrous days. In summary, the present study has developed a blood sampling protocol to detect individual profiles of pulse and surge modes of LH secretion in mice.

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ANNUAL PATTERNS OF THYROID HORMONES AND BLOOD BIOCHEMICALS, AND THEIR RELATIONSHIP WITH AMBIENT CONDITIONS IN IRANIAN DALLAGH EWES

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The aim of the study was to determine the pattern of annual changes in T_3 , T_4 , glucose and total protein levels in an Iranian native ewe and their relationship with ambient temperature and daylength environment.

Five healthy, mature and fertile Dallagh ewes, approx 3 years of age (weighing 61.2 ± 7.7 kg) were randomly selected from a herd in the north of Iran (latitude $36^{\circ}9'$ N and longitude $54^{\circ}75'$ E). The study was conducted under natural conditions. Throughout the study, ewes were maintained separately from rams and received a standard maintenance ration. Blood samples were obtained weekly via jugular vein (between 0800 and 1000 h). Sera assayed for T_3 , T_4 , glucose and total protein. Local daily temperatures and daylength were recorded.

The greatest T_3 , T_4 , glucose and total protein levels were observed at spring (2.12 ± 0.06 nmol/L), winter (141.31 ± 5.25 nmol/L), autumn (3.55 ± 0.06 nmol/L) and spring (9.09 ± 0.06 g/dL), respectively, while lowest for T_3 , T_4 and glucose levels were in summer (1.85 ± 0.06 , 72.95 ± 5.25 and 2.91 ± 0.06 nmol/L, respectively), and for total protein in autumn (7.52 ± 0.15 g/dL).

In conclusion, results showed that there is a negative relationship between ambient temperature and daylength with T_3 , T_4 and glucose levels, but a positive relationship with total protein is revealed.

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EFFECT OF TRANSIENT HYPOTHYROIDISM INDUCTION IN PUBERTAL ROOSTERS

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Thyroid hormones have marked roles in reproduction of many species whereas thyroidectomy causes some defects in reproductive tract. Induction of transient hypothyroidism in birds and rats showed an increase in testosterone levels, and in sperm production (Villar et al., 2002; De Cuypere et al., 2005). Thus, the aim of the study was to evaluate the effect of transient hypothyroidism in pubertal rooster on testosterone level and sperm production.

Thirty pubertal rooster (19 week-old) were assigned for two groups receiving the same diet with or without polythiouracil (PTU). Birds were adapted to abdominal massage for 3 weeks. Blood and semen samples were collected weekly for 13 consecutive weeks (3 weeks pre-treatment, 7 weeks treatment, and 3 weeks post-treatment periods) to determine serum thyroxine (T_4), Triiodothyronine (T_3) and testosterone levels, and semen volume and characteristics.

Results showed that PTU caused a significant decline in serum T_3 and T_4 levels, while an increase in T_3/T_4 ratio in treatment period that those continued to post-treatment period. Supplementation of PTU led to a slight decrease testosterone level in treatment period while it significantly risen in post-treatment period. There were not any significant differences in semen volume, sperm mortality and mobility between groups.

Previous studies in rats showed the different results regarding the effect of transient hypothyroidism on testosterone level (Chiao et al., 2002; Kala et al., 2002), but akhlaghi et al. (2002) in agreement with our findings, reported that PTU administration to prepubertal chickens caused an increase in serum testosterone level and also in sperm production.

In conclusion, it seems that transient hypothyroidism induction in roosters can alter testosterone secretion without affecting on sperm Characteristics.

Key words: Hypothyroidism- Testosterone- Sperm Characteristics -Rooster

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INVOLVEMENT OF NEUROKININ B AND DYNORPHIN IN THE ONSET OF PUBERTY IN FEMALE RATS

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Kisspeptin neurons in the ARC coexpress neurokinin B (NKB) and dynorphin (Dyn), and are referred to as KNDy neurons. The KNDy neurons are suggested to be involved in gonadotropin-releasing hormone (GnRH) pulse generation. We hypothesized that during the prepubertal period an inhibitory neuronal signal suppresses the activity of KNDy neuron and that during the subsequent maturation of the hypothalamus this prepubertal inhibition is removed to allow the activation of KNDy neuron and the adult pattern of pulsatile GnRH / LH secretion. In this study, we examined the role of NKB and Dyn on puberty onset in female rats. After weaning on postnatal day 20, female pups were infused with senktide, a NKB receptor agonist, or nor-BNI, a Dyn receptor antagonist, at 0.067 nmol/min for 2 weeks by osmotic minipump placed in the abdominal cavity. Animals were weighed and vaginal opening was examined as an indicator for pubertal onset. After the vaginal opening, daily vaginal smears were taken to monitor estrous cyclicity. Vaginal opening was slightly earlier in senktide-treated rats than vehicle-treated controls. The day of the first estrus was significantly earlier than vehicle-treated rats. On the other hand, nor-BNI treatment significantly accelerated both vaginal opening and first estrus compared with vehicle-treated control rats. These results suggest that the increase in Dyn tones might inhibit KNDy neuronal activity before the onset of puberty in female rats.

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KISSPEPTIN STIMULATES LUTEINIZING HORMONE SECRETION AND FOLLICULAR DEVELOPMENT IN JAPANESE BLACK BEEF COWS

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The present study aimed to investigate whether kisspeptin stimulates follicular development via gonadotropin-releasing hormone (GnRH)/gonadotropin release in Japanese Black beef cows. In Experiment 1, cows were intravenously injected with kisspeptin C-terminal decapeptide [Kp-10 (0.02, 0.2 or 2 nmol/kg)] or saline during mid-luteal phase to determine the effects of kisspeptin on gonadotropin secretion. Blood samples were collected every 10 min for 8 h beginning 4 h before the injection. Injections of Kp-10 at 0.2 and 2 nmol/kg increased plasma LH concentrations but not FSH concentrations. In 0.02 nmol/kg of Kp-10- and saline-treated groups, neither LH nor FSH secretion were affected after injection. These results indicate that kisspeptin stimulates gonadotropin secretion in a dose-dependent manner and injection of kisspeptin at more than 0.2 nmol/kg is effective to stimulate LH secretion. In Experiment 2, to determine whether kisspeptin facilitates development of first dominant follicle, cows were intravenously injected with full-length bovine kisspeptin [Kp-53 (0.2 or 2 nmol/kg)] or saline 5 days after PGF2 α -induced estrus. Blood samples were collected every 10 min for 8 h beginning 4 h before the injection. Follicular diameter was measured with ultrasonography once daily at 1800 h during experimental period, and additionally at 0600 h for 3 days after the injection. Injection of Kp-53 at 2 nmol/kg increased plasma LH concentrations, but not FSH concentrations. In 0.02 nmol/kg of Kp-53- and saline-treated groups, neither LH nor FSH secretion were affected after injection. One out of 4 cows ovulated within 30 h after Kp-53 injection at 2 nmol/kg. In non-ovulatory cows, size of dominant follicles in the first follicular wave increased after Kp-53 injection at 2 nmol/kg compared with saline injection. These results suggest that follicular development would be stimulated by actions of kisspeptin-induced GnRH/gonadotropin in Japanese Black beef cows. This research was supported by PROBRAIN of Japan.

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FAT SUPPLEMENTATION, FEED INTAKE, WEIGH GAIN AND PROGESTERONE CONCENTRATION DURING A ESTROUS CYCLE IN NELORE HEIFERS

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Fat supplementation effect on estrous cycle was evaluated in 18 Nellore heifers sorted randomly in two groups Fat or Control heifer kept in individual paddocks for daily feed intake evaluation. The tested hypothesis was that fat supplement increases leptin secretion, consequently LH concentration and increase CL activity reflecting in progesterone production. Diet composition was 12kg of sugar cane bagasse and 2kg of growth phase food, Fat group was supplemented with 100g/animal/day of Megalac-E®. All heifers had their estrous synchronized by progesterone device (Cronipress®, single use 0.5mg) estradiol benzoate (Sincrodiol®, 2 mg, I.M.) and PGF2 α (Sincrocio®, cloprostenol, 50 mg, I.M.) at device insertion (Day 0- Fat supplementation started). After 8 days the device was removed and eCG (Novormon®, 300 UI, I.M.) and PGF2 α injected, heifers were daily evaluated for ovulation (U.S.) blood samples were collected daily for 26 days after ovulation. Experimental protocol was repeated after 30 days

inverting the treated animals by control to minimize individual effects. Progesterone was quantified by Siemens Coat-a-Count kit (intrassay CV was 7.89% for 22.03 ng/ml and 0.12% for 0.56 ng/ml; sensitivity 0.0072ng/ml). Data were analyzed by SAS, PROC GLM repeated measures. Progesterone curves were evaluated by Prima and analyzed by Instat (GraphPad). Body weight was not different ($P=0.83$) either before ($F=393\pm 51\text{kg}$; $C=389\pm 55\text{kg}$) or after fat supplementation ($F=400\pm 54\text{kg}$; $C=391\pm 58\text{kg}$), individual feed consumption did not varied ($P=0.63$) between groups ($F=15.8\pm 5\text{kg/day}$; $C=15.0\pm 5\text{kg/day}$) and within intervals in the group ($P=0.50$). An expected decrease on feed intake and body weight consequence of fat supplementation was not observed. Average progesterone concentration did not varied ($P=0.61$) between Fat ($2.6\pm 2.5\text{ng/mL}$) and Control ($2.6\pm 2.5\text{ng/mL}$). Total progesterone production area did not differ ($P=0.96$) between groups ($F=22.1\pm 3.9$ (ng/ml)day; $C=22.0\pm 3.9$ (ng/ml)day), also total peak area was similar ($P=0.89$) between Fat (13.6 ± 2.4 (ng/ml)day) and Control (13.7 ± 2.5 (ng/ml)day). The expected effect of fat supplementation on weight gain and on progesterone production was not observed in Nellore heifers ingesting 100g fat/day.

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MALE KISSPEPTIN NEURONAL NETWORK AND ITS INTERACTION WITH GnRH NEURONS IN GOATS

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Kisspeptin has been thought to play pivotal roles in the control of both pulse and surge modes of gonadotropin-releasing hormone (GnRH) secretion. To clarify loci of kisspeptin action on GnRH neurons, the present study examined morphology of the kisspeptin system and the associations between kisspeptin and GnRH systems in gonadally intact and castrated male goats. Kisspeptin-immunoreactive (ir) and Kiss1-positive neurons were found in the medial preoptic area (MPOA) of intact but not castrated goats. Kisspeptin-ir cell bodies and fibers in the arcuate nucleus (ARC) and median eminence (ME) were fewer in intact male goats compared with castrated animals. Apposition of kisspeptin-ir fibers on GnRH-ir cell bodies was very rare in both intact and castrated goats, whereas the intimate association of kisspeptin-ir fibers with GnRH-ir nerve terminals was observed in the ME of castrated animals. Neurokinin B immunoreactivity colocalized not only in kisspeptin-ir cell bodies in the ARC but also in kisspeptin-ir fibers in the ME, suggesting that a majority of kisspeptin-ir fibers projecting to the ME originates from the ARC. A dual immunoelectron microscopic examination revealed that nerve terminals containing kisspeptin-ir vesicles made direct contact with GnRH-ir nerve terminals at the ME of castrated goats. There was no evidence for the existence of the typical synaptic structure between kisspeptin- and GnRH-ir fibers. The present results suggest that the ARC kisspeptin neurons act on GnRH neurons at the ME to control (possibly pulse mode of) GnRH secretion in males. This research was supported by PROBRAIN of Japan.

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SUPEROVULATORY RESPONSE TO FSH LEVELS IN KOREAN NATIVE CATTLE (HANWOO)

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This study was performed to investigate the FSH levels for superovulation procedure in *Korean Native Cattle (Hanwoo)*. The effectiveness of 200 mg and 400 mg of FSH to initiate superovulation was examined in Hanwoo. Donors, at random stages of the estrous cycle, received a CIDR. 7 days later, 200 mg FSH group were treated with 40, 30, 20, 10 mg FSH levels in declining doses twice daily by intramuscular injection for 4 days. Also, 400 mg FSH group were treated with 80, 60, 40, 20 mg FSH levels. On the 3rd day administration of FSH, 25 mg PGF_{2α} was administered and CIDR was withdrawn. Donors were artificially inseminated twice at 12 hr intervals. The donor cattle received 250 ug GnRH at time of 1st insemination and embryos were recovered 8 days after the 1st insemination. As a results, Treated group of 200 mg FSH level increased ($P<0.05$) the number of embryos recovered per procedure compared to 400 mg FSH level (22.0 ± 2.35 versus 13.8 ± 0.48 , respectively). When treatment of 200 mg FSH was performed, average transferable embryos/ova increased ($P<0.05$) to 20.3 ± 2.25 from 7.3 ± 0.48 of treated of 400 mg FSH. Group of 200 mg FSH increased ($P<0.05$) to 11.3 ± 3.07 from 2.0 ± 0.41 in morula stage and to 6.5 ± 4.19 from 3.8 ± 0.25 in blastocyst stage embryos compare to 400 mg FSH group. Mean of total expanded blastocyst stage embryos was similar ($P<0.05$) between the 200 mg and 400 mg FSH levels group (2.5 ± 0.87 vs. 1.5 ± 0.29). These results suggest that 200 mg FSH level-based superovulation protocol may be effectively used for production of superior embryos in Hanwoo. In other words, the less level of FSH may be effectively applied for Hanwoo (Korean Native Cattle), because Hanwoo was smaller body size than beef or dairy cow. This work was carried out with the support of Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ907008052011), Rural Development Administration, Republic of Korea.

Key words : FSH level, Superovulation, Korean Native Cattle(Hanwoo), Embryo recovered

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DO AUSTRALIAN MERINO SHEEP SHOW SEXUAL PREFERENCE?

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The MHC appears to be submitted to natural and sexual selection. The variability of MHC genes is maintained by some sort of parasite-driven balancing selection. Odours have been shown to play a role in mate choice. Sexual selection includes mate choice and maternal selection and could lead to a MHC-heterozygous offspring. Consumers worldwide are demanding drugs-free products. Therefore, mate choice by odour and MHC variability may be an interesting strategy to increase productive and reproductive efficiency.

Twenty pairs of two-years-old sheep twins kept together since birth were tested to verify if sheep present sexual preference for the opposite sex other than her/his twin. Animals were divided into two groups: 1- males with horns and their sisters; 2- males without horns and their sisters. Oestrus was synchronized with intravaginal progesterone sponge and oestradiol benzoate. Females were previously tested for sexual proceptivity and receptivity.

Males and females were adapted to indoor tests and submitted to 16 sexual proceptivity tests. Each individual was assessed for three minutes in each test. The animals were tested in the pen divided into six imaginary squares. Opposite squares 1 and 3, through the fence it was kept two animals of opposite sex into the cage, with a space (2) between them. Each animal was always tested in the group comparing its twin and another animal. The sexual preference consisted of visual and olfactory contact. Proceptivity was considered when the animal preferred approach to one of them, into squares 1 or 3. The time was recorded to each square. There was no sexual preference ($p > 0.05$). The animals preferred to stay in the squares near the entrance (4) or the exit (6). However, when the animals remained together in the paddock and observed for 30 minutes, males mounted other females, but never mounted their twin.

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DIFFERENTIATING THE SITES OF ACTION OF TESTICULAR STEROIDS IN THE REGULATION OF GONADOTROPHIN RELEASING HORMONE SECRETION AND MATING BEHAVIOUR IN RAMS.

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In males, testosterone acts in the brain to regulate reproduction, through inhibition of gonadotrophin-releasing hormone (GnRH) secretion, and through stimulation of mating behaviour. In both cases, the main site of action is within the brain, but the precise location is largely unknown. The aim of this study was to differentiate the site(s) of these two actions of testosterone in the hypothalamus of the ram. Much of the action of testosterone is through the metabolites, 5 α -dihydrotestosterone (DHT) and/or oestradiol 17 β . Adult Merino wethers ($n=5$ /group), castrated as lambs, were injected (im) twice daily for 32 days with peanut oil, 8mg DHT benzoate (DHTB), 25 μ g oestradiol benzoate (EB) or both DHTB and EB. Jugular blood samples collected every 15 minutes for 10h revealed that all 3 treatments significantly ($P < 0.001$) suppressed plasma LH concentrations (mean LH: EB 0.80 \pm 0.35, DHTB 0.17 \pm 0.15, EB+DHTB 0.38 \pm 0.23, Oil 1.55 \pm 0.33 ng/ml \pm SEM). Mating tests with oestrous ewes repeated 4 times over 9 days revealed that wethers treated with oil or DHTB showed little or no mating behaviour, while EB treated wethers consistently exhibited courtship-type behaviour (genital sniffing, tongue flick, nudge/foreleg kick and flehmen, but generally without mounting). By contrast, all the wethers treated with both EB and DHTB exhibited all the above behaviours but with frequent mounting, intromission and ejaculation. Following this the sheep were killed and the brains perfusion-fixed with 4% paraformaldehyde. Cryostat sections (40 μ m) were used for immunohistochemistry for cFos/Fos-related antigens (FRA), a marker for longer term neuronal activation. Several hypothalamic nuclei showed activation of cFos/FRA, indicating involvement of neurons in those nuclei in the regulation of GnRH secretion and/or mating behaviours. From this study, we have a model that can be used to examine the brain areas involved in the actions of testosterone in the brain of males.

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STIMULATORY EFFECTS OF NUTRITIONAL SUPPLEMENTATION ON METABOLIC AND OVARIAN ENDOCRINOLOGY IN AUSTRALIAN CASHMERE GOATS.

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In sheep, nutritional supplements increase the concentrations of leptin and insulin and these hormones are thought to reduce the production of oestradiol and inhibin by ovarian follicles, thus allowing increases in FSH secretion, follicular development and ovulation rate (1). We tested whether this concept applies to goats. The Control group ($n = 14$) was fed to maintain live weight and the treated goats ($n = 14$) received twice their requirement for maintenance. Oestrous cycles were synchronized via an 18-day treatment with intravaginal progestagen pessaries (CIDR). The nutritional treatments lasted for 21 days, beginning 10 days before CIDR withdrawal on Day 0. Bucks were placed with the does for Days 1-5. Live weight and body condition were measured every 2 weeks and blood was sampled every 2 days. The concentrations of insulin and leptin were increased immediately ($p < 0.001$) by supplementation and declined immediately after supplementation ended. These responses were independent of body condition and live weight. During the pre-ovulatory period, supplementation significantly increased the concentrations of inhibin ($p < 0.05$), but oestradiol and FSH concentrations remained unaffected. Ovulation rate, determined by laparoscopy on Day 12 after CIDR removal, did not differ between the Treated (2.14 \pm 0.14) and Control does (1.79 \pm 0.19). Thus, in goats, supplementation elicits robust responses in leptin and insulin concentrations, indicating a major impact of the diet on the energy status of the animals. The lack of effect of the supplement on ovulation rate needs to be confirmed with a larger study. However, the increase in inhibin concentrations is notable because oestradiol levels remained normal so there should have been more inhibitory feedback, despite which FSH concentrations were maintained. These observations suggest that the reproductive systems of goats and sheep do not respond to acute nutritional supplements in the same way.

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DELAYED EFFECTS OF LOW-DOSE 17 α -ETHINYLESTRADIOL TREATMENT ON NEONATAL FEMALE RATS

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In order to evaluate long-term effects of exposure to small amount of 17 β -ethinylestradiol (EE) during the critical period for sexual differentiation of the brain, female Sprague-Dawley rats were treated with EE subcutaneously on postnatal day (PND) 1 (Exp. 1) or orally (Exp. 2) for 5 days from PND 1 at dose levels of 0 (vehicle control), 0.08, 0.4 and 2 f \dot{E} g/kg BW or 0, 0.4 and 2 f \dot{E} g/kg/BW/day, respectively. Their estrous cycles were monitored periodically for two weeks at two-week intervals from postnatal week (PNW) 8 until 33 in Exp. 1 and until PNW 21 in Exp. 2. Then, the

THE NEUROTROPHIC FACTOR, NEURTURIN, REGULATES TISSUE ANDROGEN SENSITIVITY

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Neurturin (NTN) is a neurotrophic factor and member of the glial cell line-derived neurotrophic factor (GDNF) family of ligands and signals through GFR α 2 (GDNF family receptor alpha-2) and the ret tyrosine kinase. In adult male mice NTN is expressed in the endocrine system including pituitary and in reproductive system including testes, while GFR α 2 is present in hypothalamus, developing pituitary and testes. The functional role of NTN signalling in endocrine and reproductive organs is not clear but is suggested by impaired autonomic innervation and epithelial atrophy of reproductive organs in NTN-deficient mice. Therefore, we determined the role of NTN signalling in the hormonal regulation of hypothalamic-pituitary-testicular axis of male mice. Homozygous NTN (NTNKO) and GFR α 2 knockout (GFR α 2KO) males were compared to wild-type (WT) littermates. Serum steroid hormones were analysed using liquid chromatography tandem mass spectrometry and serum luteinizing hormone (LH) using a mouse-specific immunoassay. Serum testosterone and LH were reduced ($p < 0.05$) in NTNKO and GFR α 2KO males leading to increased ($p \leq 0.001$) androgen sensitivity (defined by $1/(T \times LH)$) when compared WT males. Increased androgen sensitivity was supported by normal weights of androgen dependent prostate and seminal vesicles (SV) despite low serum testosterone. In order to test androgen sensitivity, we used "DHT (dihydrotestosterone) clamp" (castration plus DHT implant) to create fixed circulating levels of a non-aromatisable androgen allowing evaluation of androgen sensitive endpoints. Under DHT clamp, serum LH was significantly lower and prostate and SV weights significantly higher in NTNKO compared to WT, further supporting increased androgen sensitivity in NTNKO. Testicular and pituitary responsiveness were normal in NTNKO males, as determined by the response to the human chorionic gonadotropin (hCG) or gonadotrophin releasing hormone (GnRH) analog Lucrin, respectively. In conclusion, our results suggest that the neurotrophic factor NTN regulates tissue androgen sensitivity with the inactivation of NTN or its respective receptor GFR α 2 leading to increased sensitivity.

THE ROLE OF THE GDF9 PRODOMAIN IN THE BIOACTIVITY OF MATURE HUMAN GDF9

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Oocyte-derived GDF9, a member of the TGF- β superfamily, is essential for mammalian ovarian folliculogenesis. GDF9 controls both early follicular maturation and the number of ovulating follicles in each oestrus cycle. Knockout mice and sheep with inactivating mutations in GDF9 are infertile, due to a block in the progression of primary follicles to the secondary follicular stage. In addition, human GDF9 mutations are associated with both premature ovarian failure and dizygotic twinning. GDF9 is synthesised as a homodimer comprising an N-terminal prodomain and the biologically active C-terminal mature domain. Our data indicates that mouse GDF9 (mGDF9) is secreted in the active form, whereas human GDF9 (hGDF9) is secreted in a latent pro-mature complex. Five other human TGF- β ligands are also secreted in this latent form due to high affinity interactions between the mature growth factors and their respective prodomeins. Therefore it is hypothesised that hGDF9 must be liberated from its prodomain in order to regulate folliculogenesis. The aim of this study was to determine sequence regions within the GDF9 prodomain which confer latency. Our studies have demonstrated that prodomain removal by heat or extremes of pH, similar to other TGF- β ligands, will activate hGDF9 in an adrenocortical cell luciferase reporter assay. Sequence comparison revealed mouse and human prodomeins are only 64% homologous, whereas mature domains are >90% homologous. By site-directed mutagenesis substituting both single and multiple amino acids from human to mouse GDF9, key residues that confer latency were identified. *In vitro* bioactivity of the mutant constructs is currently being confirmed by thymidine uptake in a granulosa cell bioassay. It is concluded that hGDF9 is synthesised in a latent form conferred by specific sequence regions in the GDF9 molecule. Therefore, it is predicted that a novel activation mechanism for hGDF9 must be required *in vivo*.

THE PLASMA LUTEINIZING HORMONE RESPONSE IN ANESTRUS EWES FOLLOWING THE INTRODUCTION OF MALES REQUIRES KISSPEPTIN SIGNALLING

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In sheep, the introduction of a novel male stimulates the hypothalamic-pituitary-gonadal axis of females in seasonal anestrus, leading to ovulation. The means by which this cue leads to gonadotropin releasing hormone (GnRH)/luteinizing hormone (LH) secretion is unknown. We hypothesised that the stimulus activates kisspeptin neurons whose product is critical for the stimulation of GnRH neurons and fertility. During the anestrus season (December) female Corriedale sheep were prepared for lateral ventricle (LV) infusion and allocated to 3 groups: no male + artificial cerebrospinal fluid (aCSF) treatment; male exposure + aCSF treatment; male exposure + kisspeptin antagonist treatment ($n=4$ per group). The ewes had been isolated from males for 2 months and they received progesterone priming for 7 days. On the day of experimentation, blood was sampled every 10 min for 3 h prior to and following male exposure. A kisspeptin antagonist (p271) or aCSF (flow rate 200 μ l/h) was infused into the LV for 4 h (300 μ g/h with an initial 200 μ g bolus), beginning 1 h before male exposure. Plasma LH levels (which reflect GnRH secretion) were measured. Male introduction increased LH levels (mean LH: 0.53 ± 0.06 ng/ml) compared to ewes not exposed to males (0.07 ± 0.06 ng/ml; $P < 0.05$). Moreover, regular

LH pulses commenced in females exposed to males (pulse interval: 82 ± 8 min vs no male control 270 ± 57 min; pulse amplitude: 1.23 ± 0.20 ng/ml vs control 0.22 ± 0.22 ng/ml; both $P < 0.05$). The kisspeptin antagonist completely blocked the effect of male exposure on LH secretion in females (mean LH: 0.08 ± 0.04 ng/ml; pulse interval: 255 ± 45 min; pulse amplitude: 0.21 ± 0.13 ng/ml). We conclude the stimulus from male sheep that activates reproductive centres in the brain to elicit GnRH secretion is dependent on kisspeptin signalling.

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IMPRINTING OF THE *IGF2-H19* DOMAIN EXTENDS TO THE MARSUPIAL MAMMARY GLAND

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Genomic imprinting is an epigenetic mechanism that results in the monoallelic expression in a parent-of-origin specific manner. Imprinted expression of several genes that regulate nutrition and growth of the developing mammalian fetus has been detected in the placenta of both eutherian and marsupial mammals. However, in marsupials, the placental attachment is short lived and the majority of growth and development of the young occurs post-natally, supported by a complex and extended period of lactation. Two genes that are imprinted in the placenta in eutherian mammals, and insulin-like growth factor 2 (*IGF2*), are also important for the initiation and maintenance of lactation. In particular insulin is an absolute requirement to induce milk protein synthesis in the majority of mammals, including the tammar wallaby. We therefore examined whether these genes also imprinted in the mammary gland of the marsupial tammar wallaby (*Macropus eugenii*). Direct sequencing confirmed that both *INS* and *IGF2* were tissue-specifically imprinted in the tammar mammary gland. Surprisingly, *INS*, which was thought to be imprinted only in the placenta in eutherian mammals, was also imprinted in the tammar liver. Bisulphite sequencing showed that the promoter region of *INS* was highly methylated, but that it did not differ between parental alleles, whereas there was very little methylation at each of the *IGF2* promoters. Therefore, as in eutherians, imprinted expression of these genes in the marsupial, is probably regulated by the *H19* imprinting control region. This is the first study to identify *INS* imprinting outside the yolk sac and suggests that genomic imprinting also plays a role in regulating growth and development of the post-natal young. These results support the maternal-infant co-adaptation hypothesis and suggest that imprinting in the mammary gland may be as critical for post-natal survival in mammals, as genomic imprinting in the placenta is for pre-natal development.

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DIFFERENTIAL EFFECTS OF CONTINUOUS EXPOSURE TO THE INVESTIGATIONAL METASTIN/KISSPEPTIN ANALOG TAK-683 ON PULSATILE AND SURGE MODE SECRETION OF LUTEINIZING HORMONE IN OVARIECTOMIZED GOATS

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The aim of the present study was to determine if estradiol-induced LH surge is influenced by the continuous exposure to metastin/kisspeptin analog, TAK-683, under conditions that had been established to completely suppress pulsatile LH secretion. Ovariectomized goats were used and the treatment group ($n=6$) received TAK-683 at the rate of 500 nmol/kgBW/week by continuous using a subcutaneous osmotic pump. The control group ($n=6$) received vehicle in the same way. On 5 days after the start of the treatment, estradiol was infused for 16 h at the rate of 6 $\mu\text{g/h}$ in both groups to evaluate the effects of TAK-683 treatment on the LH surge. Blood samples were collected at 6 min intervals for 4 h prior to the initiation of either the TAK-683 treatment or the estradiol infusion, respectively, to determine the profiles of pulsatile LH secretion. They were also collected at 2 h intervals from - 4 h to 32 h after the start of estradiol infusion for analysis of LH surge. The frequency and mean concentrations of LH pulses in the treatment group were significantly ($p < 0.05$) suppressed on 5 days after the start of TAK-683 treatment as compared to those of the control group (frequency: 0.8 ± 1.3 v.s. 5.8 ± 1.6 pulses/4h, mean concentration: 0.3 ± 0.2 v.s. 1.7 ± 0.9 ng/ml). However, LH surge was observed in all animals of both groups. Peak LH concentration during the surge in the treatment and control group was 23.8 ± 18.4 and 34.2 ± 11.3 ng/ml, respectively. There was no significant difference in the peak time between the two groups (15.3 ± 1.0 and 14.7 ± 1.0 h). These findings indicate that while continuous exposure to TAK-683 strongly suppresses pulsatile LH secretion it does not suppress the occurrence of the estradiol-induced LH surge under current settings for ovariectomized goats. These findings suggest that effects of continuous exposure to metastin/kisspeptin or its analog on the mechanism(s) that regulates the pulsatile and surge mode secretion of GnRH/LH might be different in goats.

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CHANGE IN EXPRESSION OF TRANSFORMING GROWTH FACTOR- β 3 DURING SEASONAL GROWTH AND REGRESSION IN THE PROSTATE OF THE ADULT BRUSHTAIL POSSUM (*TRICHOSURUS VULPECULA*)

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The prostate gland in the brushtail possum is structurally similar to the human. Moreover, the adult possum prostate grows and regresses seasonally providing a novel animal model for the study of the regulation of prostate growth (Martyn et al., 2011). Transforming growth factor- β 3 (TGF- β 3) has been implicated in the regulation of prostate growth and *in vitro* can inhibit epithelial cell proliferation and induce apoptosis (Massague et al., 1992; Zhu & Kyprianou, 2005). This study investigates the localisation and expression of TGF- β 3 in the possum prostate during seasonal growth and regression. Prostate tissue was collected every 2 months over a 12 month period ($n = 3-10$ per month). Each prostate was weighed and separated into periurethral and outer glandular regions. Half of the tissue was frozen for Western blot analysis and the remaining tissue was fixed for immunohistochemistry. TGF- β 3 immunoreactivity was identified in both stromal and epithelial cells of the prostate but immunoreactivity was more intense in the epithelial cells of periurethral and outer glandular areas. Western blot analysis showed a 15 kDa band representing TGF- β 3 and 50, 70 and 90 kDa bands representing TGF- β latency-associated peptides (LAP).

Expression of TGF- β 3-LAP complex in the outer glandular part of the prostate was highest in July at the end of the main breeding season and immediately prior to prostate regression. In the periurethral area there was no significant change in TGF- β 3 expression during the year. The data

suggest that *in vivo* TGF- β 3 may be involved in controlling growth of the glandular tissue in the possum prostate and may be involved in initiating prostate regression.

Key words: TGF- β ; prostate

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EPIGENETIC REGULATION OF THE KISS1 GENE EXPRESSION IN THE MOUSE HYPOTHALAMUS

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Kisspeptin is a hypothalamic neuropeptide encoded by Kiss1 gene and is focused as a major regulator of gonadotropin-releasing hormone secretion from the hypothalamus. Kiss1 gene expression is detected in the anteroventral periventricular nucleus (AVPV) and hypothalamic arcuate nucleus (ARC) in the rodent brain. In the present study, we investigated the involvement of epigenetic mechanism in the regulation of the Kiss1 gene expression in both nuclei. To determine the Kiss1 promoter region functioning in hypothalamus, we carried out luciferase reporter assays with a mouse hypothalamic immortalized neuronal cell line. Several constructs containing Kiss1 upstream region showed a significant increase in luciferase activity, but a shortest construct showed highest activity, indicating that this region has strong promoter activity in the hypothalamus. Next, as one possibility of the transcriptional regulatory mechanism, we predicted the presence of CpGs, which is differentially methylated between Kiss1-expressing and unexpressing cells, in the core promoter region of Kiss1 gene. Kiss1 neurons visualized by *in situ* hybridization (ISH) were isolated from AVPV and ARC based on the ISH signals. Bisulfite sequencing analysis revealed that the CpGs upstream of the gene appeared hypermethylated in all samples examined. On the other hand, we further investigated the effects of TSA, an inhibitor of histone deacetylation and/or 5-aza-dC, an inhibitor of DNA methylation, on Kiss1 expressions in hypothalamic cell lines. TSA induced Kiss1 expression in all cell lines, whereas 5-aza-dC alone had no effect on Kiss1 repression, suggesting that among epigenetic regulatory mechanisms, histone acetylation plays a major role in the regulation of Kiss1 expression in the hypothalamus. This work was supported in part by PROBRAIN of Japan.

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LONG-TERM NEONATAL ESTROGEN EXPOSURE SUPPRESSES PULSATILE LH RELEASE BY AN IRREVERSIBLE SUPPRESSION OF ARCUATE KISS1 GENE EXPRESSIONS THROUGH ER ALFA IN RODENTS

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Developing brain is highly sensitive to estrogen. Neonatal exposure to estrogen during the critical period causes defeminization of neuroendocrine mechanism regulating gonadotropin-releasing hormone (GnRH)/luteinizing hormone (LH) releasing system, but a long-term exposure causes a permanent suppression of reproductive functions. In the present study, we examined the effects of long-term neonatal estrogen treatment on pulsatile LH secretion, gonadal functions and hypothalamic kisspeptin expressions in both sexes of adult rodents. Daily injection of estrogen benzoate (EB) from the day of birth to 10 days postpartum caused a significant suppression of pulsatile LH release in both female and male rats. The EB treatment caused persistent vaginal diestrus in females and significant suppression of testosterone release in male rats. Kiss1 mRNA and kisspeptin expressions were significantly inhibited in the arcuate nucleus (ARC) of gonadectomized female and male adult rats. On the other hand, tyrosine hydroxylase (TH) expression in the ARC and GnRH immunoreactivity in the median eminence were not inhibited by the neonatal EB treatment. Exogenous administration of kisspeptin significantly increased LH release in animals with the neonatal EB treatment, suggesting that the responsiveness to kisspeptin remains intact in the neonatally EB-treated rats. The long-term neonatal EB treatment showed significant suppression of ARC kiss1 mRNA expressions in wild-type and estrogen receptor (ER) beta-KO mice, while considerable number of Kiss1-expressing cells remained in the ARC in ER alpha-KO mice. Taken together, the present results suggest that the long-term neonatal estrogen acts on ARC kisspeptin neurons via ER alpha to cause an irreversible suppression of ARC kisspeptin expression later in their life, resulting in GnRH/LH pulse suppression, and then reproductive dysfunction in both male and female rodents. The present study also provides evidence for the involvement of ARC kisspeptin neurons in the GnRH/LH pulse generation. This study was supported in part by PROBRAIN.

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MORPHOLOGICAL AND FUNCTIONAL INTERACTIONS BETWEEN KISSPEPTIN AND GNRH NEURONS AT THE MEDIAN EMINENCE IN FEMALE RATS

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Kisspeptin neurones are a gatekeeper of puberty onset and have a critical role in the regulation of reproduction in mammals. The present study was conducted to determine the morphological and functional interactions between kisspeptin and gonadotropin-releasing hormone (GnRH) neurons at the median eminence in female rats to clarify a possibility that kisspeptin directly stimulates GnRH release from the GnRH neuronal terminals. To this end, dual immunoelectron microscopy for kisspeptin and GnRH, and retrograde tracing with intraperitoneal injection of FluoroGold (FG) were performed. In addition, exogenous kisspeptin was challenged on median eminence tissues containing GnRH fibers, to determine stimulatory action of kisspeptin on GnRH neuronal terminals. Kisspeptin-immunoreactive fibers were found over the ARC and internal layer of the median eminence region of female rats. GnRH fibers were densely located in the external layer of the median eminence. Immunoelectron microscopy showed that the kisspeptin-immunoreactive nerve element directly abutted the GnRH-immunoreactive nerve element in the internal layer of the median eminence. No obvious synaptic structure was found between these contacts. No FG-immunoreactivities were found in kisspeptin-immunoreactive cells in the whole

THE TESTICULAR CYCLE OF THE HOUSE GECKO *H. FLAVIVIRIDIS* IN OMAN IN RELATION TO STEROID HORMONAL LEVELS, ULTRASTRUCTURAL STEROIDOGENIC FEATURES AND PROGESTERONE RECEPTORS

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The annual testicular cycle of the house gecko *H. flaviviridis* in Oman was examined. Plasma testosterone, estradiol, and progesterone levels were measured using the sensitive HPLC/MS-MS technique. The ultrastructural steroidogenic features of Sertoli and Leydig cells, which are the major source of steroidogenic activity, were examined using transmission electron microscopy. These features were characterized by the presence of smooth endoplasmic reticulum (SER) in the form of cisternal whorls and tubular cisternae, presence of swollen vesiculated mitochondria and also association between SER, mitochondria and lipid droplets. The rise in plasma steroid levels was closely associated with the development of the ultrastructural features. In addition, progesterone receptors (PR) were monitored throughout the testicular cycle using an immunohistochemical technique. The PR expression was closely related with the development of ultrastructural steroidogenic features and the hormone levels. During the active phase (Nov–May), there was a significant rise in steroid levels associated with well developed steroidogenic features and strongly expressed PR. During the inactive phase (Jun–Aug), there was a significant drop in steroid levels, underdeveloped steroidogenic features and weakly expressed PR. During spermatogenesis PR was strongly expressed and the ultrastructural steroidogenic features were well developed in the Leydig and Sertoli cells.

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DEVELOPMENTAL EXPRESSION OF BMPR1A, BMPR1B AND BMPR2 GENES IN THE POSTNATAL MOUSE TESTIS

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Transforming growth factor- β superfamily members such as the bone morphogenetic proteins (BMPs) and their receptors have been shown to be important in male reproductive physiology including modulation of steroid production and germ cell development. *Bmpr1a* and *Bmpr2* knockouts have proven to be embryonically fatal and there are limited inconsistent reports on BMP receptor expression in the postnatal mouse testis. Therefore, in this study we clarify the developmental expression patterns of BMP receptors in the testis directly to achieve a greater understanding of these genes and to gain insight into their likely functions.

To determine the relative expression patterns of *Bmpr1a*, *Bmpr1b* and *Bmpr2* in mouse testis we obtained fresh tissues at specific developmental stages – immature, pubertal and mature and performed total RNA extraction and RNA quantification followed by reverse transcription polymerase chain reaction (RT-PCR). Gene amplification results were analysed using beta-actin as the control.

Bmpr1a was expressed at all developmental stages while *Bmpr1b* and *Bmpr2* had differential developmental patterns of expression. *Bmpr1b* was only present at maturity and *Bmpr2* was expressed in immature and mature testis but not pubertal testis. As *Bmpr2* has been detected in human testis and prostate, and altered expression of *Bmpr1b* has been implicated in numerous cancers including testicular, prostate and breast cancer, this murine model for normal BMP receptor expression will help to improve our understanding of normal testicular physiology and may be of importance in the future development of therapeutic agents used to target cancer.

FETAL POPULATION OF LEYDIG CELLS DETERMINES THE DEVELOPMENT OF ADULT POPULATION IN RATS

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The fetal Leydig cells (FLCs) develop in embryo and involute after birth. However, the function after birth is unclear. In rats, adult Leydig cells (ALCs) develop as the appearance of fibroblast-like progenitor Leydig cells (PLCs) at postnatal day (PND) 11. The objective of the present study is to determine how FLCs affect the development of ALCs. Ethane dimethane sulfonate (EDS), an alkylating agent that specifically kills FLCs, was injected intraperitoneally at a dose of 100 mg/kg to male pup at PND2. At PND6, control testis contained only clusters of FLCs, identified by histochemical staining of Leydig cell biomarker 3β -hydroxysteroid dehydrogenase (3β HSD), while there were no 3β HSD positive cells in EDS-treated testis. Gene expression profiling and Q-PCR confirmed all FLCs were destroyed at PND6. At PND9, control testis still contained clusters of 3

β HSD positive FLCs, while EDS-treated testis contained many scattered 3 β HSD positive fibroblast-like PLCs. The expression levels of Leydig cell biomarkers in EDS-treated testis including *Star*, *Cyp11a1*, *Hsd3b1* and *Cyp17a1* were 5-10 folds higher than those in control testis, and maintained the higher levels until PND35. At PND56, testis and seminal vesicle weights in EDS-treated rats were significantly reduced compared to control, so was the serum testosterone level. At PND56, ALCs were well developed and mature, while those Leydig cells in EDS-treated testis were still immature, as identified by the delayed expression of 11 β -hydroxysteroid dehydrogenase 1 and *Cyp2a1*, the biomarkers of ALCs. In conclusion, FLCs determines the fate of adult population of Leydig cells postnatally.

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AN IN-DEPTH PROTEOMIC ANALYSIS OF HUMAN TESTIS PROTEOME

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As the male gonad, testis is the location of spermatogenesis and production of androgen in men. Delayed protein translation is an important regulatory mechanism in spermatogenesis. It is important to characterize testis proteome profile. Till now, only a few proteins have been identified in human testis due to limitations of the technology. Herein, using an advanced proteomics platform, we identified 7346 unique proteins from human testis with high confidence. Immunohistochemistry analysis of 20 identified proteins in human testis confirmed the validity of the human testis proteome. Annotation of the adluminal compartment germ cell proteins, which contains proteins from germ cells protected by blood-testis barrier including spermatocytes, spermatids and sperm, showed hyperrepresentation of metabolic pathways including pyruvate metabolism, fatty acid metabolism, and oxidative phosphorylation. 42 of the 50 identified CT genes (84%) were found to be expressed in germ cells in adluminal compartment. While only 43.5% (30 of 69) X-linked CT genes were identified in germ cells in adluminal compartment. This phenomenon may be caused by meiotic sex chromosome inactivation (MSCI). As the largest reproductive organ proteome profile, it will provide rich resources to study spermatogenesis and androgen production, and provide potential targets for contraceptive drugs.

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INHIBIN ALPHA NULL AND HAPLOINSUFFICIENT MICE: MODELS FOR INVESTIGATING ACTIVIN/HORMONE CROSSTALK IN SERTOLI CELL MATURATION AND TUMOURIGENESIS

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Background. Activin A, a dimeric cytokine formed from inhibin- β A subunits, regulates testis development and fertility by acting locally on testicular cells and distally, promoting pituitary production of follicle stimulating hormone. The closely related inhibin, a heteromeric inhibin- α /inhibin- β dimer, antagonizes activin production and activity [1]. Mice lacking inhibin (*Inha*) develop somatic cell (Sertoli/granulosa) gonadal tumours, identifying inhibin- α as a tumour-suppressor [2]. Pituitary gonadotropins [3], androgens [4] and estrogens [5] contribute to Sertoli cell tumours, yet how hormones and activin interact to promote tumourigenesis is not understood.

Methods. Histological and immunohistochemical assessment of Sertoli cell proliferation and maturation were performed using testis sections from 16 and 28 day *Inha*^{+/+}, *Inha*^{+/-} and *Inha*^{-/-} mice.

Results. Sertoli cells in *Inha*^{-/-} tumours exhibited mislocalized Connexin43 (blood-testis barrier protein) and intense Anti-Mullerian Hormone (AMH) immunoreactivity (marker of immaturity). The predominantly cytoplasmic Androgen Receptor localization indicated impaired androgen signalling. Clusters of Sox9-positive cells within the tubule lumen suggested Sertoli cells had detached from the basement membrane. Unexpectedly, *Inha*^{+/-} mice also had a testicular phenotype, despite being fertile and apparently healthy. Most tubules appeared normal, with advanced spermatogenesis indicated by the presence of round spermatids at 16 dpp, correlating with apparently advanced Sertoli cell maturation. Other regions were tumour-like, with mislocalized Connexin43 and Androgen Receptor and increased AMH immunostaining in Sertoli cells. No Sertoli cells were observed in the lumen.

Conclusion. *Inha* haploinsufficiency results in a mixed testicular phenotype of advanced maturation and tumour-like areas, with impaired androgen signalling a feature of aberrant *Inha*^{+/-} and *Inha*^{-/-} Sertoli cells. This differs to the advanced testis maturation and increased androgen responsiveness in *Smad3*^{+/-} mice, which have altered activin signalling but do not develop focal Sertoli cell lesions [6]. These mice with moderate (*Inha*^{+/-}) versus excessive (*Inha*^{-/-}) activin production and altered signalling (*Smad3*^{+/-}) provide models to reveal outcomes of altered activin/hormone crosstalk.

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ISCHEMIC/REPERFUSION INJURY IN THE RAT TESTIS: EFFECTS OF OXIDATIVE STRESS DURING SPERMATOGENESIS ON DNA DAMAGE AND TELOMERE LENGTH

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In humans, testicular torsion is a relatively common urological emergency in newborns, children and adolescents (an incidence of 1 in 4000 per year of men under 25) that usually requires urgent surgical treatment to return blood flow to the testis. Prolonged testicular torsion is highly painful and can result in permanent damage to the torqued testis (requiring removal) while the subsequent blood reperfusion can lead to further damage in both the torqued and non-torqued testes, resulting in a loss spermatogonia and impaired lifelong fertility. Due to the serious harm that can occur in the human testis when it is torqued, we have used a surgical method of ischemia/reperfusion (I/R) injury as an animal model of testicular torsion.

Using temporary clamping of blood flow to one testis per Wistar rat to induce I/R injury, we have been able to examine the post operative consequences in both clamped and non-clamped testis. In particular, we have focussed on the effects of I/R induced oxidative stress on the DNA of spermatozoa produced 1 or 2 weeks post surgically, especially in the repetitive DNA sequences (telomeres) at the end of the chromosomes.

Understanding how such temporary I/R injury can cause such long term alterations to the germ-line DNA and consequentially any offspring may have repercussions for humans who experience testicular torsion.

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INTRINSIC IMPORTANCE OF BETA-CATENIN FOR MAMMALIAN MALE GERMLINE DEVELOPMENT

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Male infertility is a worldwide health problem with increasing incidence. Disruptions in the development of male germ cells and their supporting somatic cells are considered to underpin many cases of idiopathic disease. We have established that testicular cell communication via the Wnt signalling pathway is required for normal germ cell development. Mice in which mutations are acutely induced in the key Wnt signalling components, APC and β -catenin, to increase and decrease Wnt signalling levels, respectively, exhibit varying degrees of disrupted spermatogenesis. We have identified that Wnt signalling is normally activated in some pachytene spermatocytes and all round spermatids, as assessed by nuclear localisation of β -catenin protein through immunohistochemistry and Western blot analyses. Profiling of the maturing postnatal testis (birth to 56 day postpartum) has identified a cohort of developmentally regulated transcripts encoding Wnt receptors and ligands. Our findings present the first comprehensive delineation of beta-catenin/ canonical Wnt signalling in developing and adult mouse testes, demonstrating that Wnt signalling is intrinsic to germ cells and essential for adult spermatogenesis.

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DIFFERENTIAL EFFECTS OF EXTRACELLULAR MATRIX ASSOCIATED WITH CELL ADHESION AND SELF-RENEWAL OF BOVINE GONOCYTES CULTURED *IN VITRO*

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Gonocytes that reside in neonatal mammalian testis have a germline stem cell potential and generate the cascade of spermatogenesis. In the testis of domestic animals, *N-acetylgalactosamine* (GalNAc) that are found on the surface of gonocytes and/or spermatogonial stem cells may involve in cell adhesion and behavioral feature of germ cells. The lectin DBA (*Dolichos biflorus agglutinin*) recognizes GalNAc residue on the surface of germ cells. This study focused on the relationship between self-renewal and adhesion of gonocytes in culture under different extracellular matrix (ECM) components such as gelatin (GN), laminin (LN), Poly-L-Lysine (PLL) and DBA. We also analyzed expression profiles of transcription factors (*NANOG*, *POU5F1*, *SOX2*, *c-Myc*) which have been known to be essential for maintaining pluripotency of stem cells. At 4 days after plating of gonocytes, mouse embryonic stem (mES) like cell colonies were observed on pre-coated plate with different ECM components (GN, PLL and DBA), but colonies were not formed on LN plate. Interestingly, the colony formation rates significantly increased on the DBA (126.5 \pm 10.0) compared to other ECM components (GN (72.5 \pm 0.7, $p < 0.05$), PLL (33 \pm 18.38, $p < 0.01$), LN (0, 0.001)). In addition, the expressions of pluripotency-related transcription factors were observed on the colony formed ECM plate (GN, PLL and DBA), but the transcripts of *POU5F1* and *SOX2* were not observed on the LN plate during the same culture period. Interestingly, the levels of *POU5F1* and *SOX2* transcripts were increased on the DBA pre-coated plate. These results suggest that the GalNAc residue on the surface of gonocytes can recognize pre-coated lectin DBA, which induce cell adhesion and pluripotential gene expressions of gonocytes by the formation of GalNAc-DBA complex. These glycan complexes may offer microenvironment for self-renewal and proliferation of spermatogenic stem cells by reconstructing a tube-like structure of the seminiferous tubules.

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IMPAIRED SPERMATOGENESIS AND FERTILITY IN MICE CARRYING A MUTATION IN THE *SPINK2* GENE EXPRESSED PREDOMINANTLY IN TESTES

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Spermatogenesis is a complicated process involving an intrinsic genetic program composed of germ cell-specific and -predominant genes. In this study, we investigated the mouse *Spink2* (serine protease inhibitor Kazal-type 2) gene, which belongs to the SPINK family of proteins characterized by the presence of a Kazal-type serine protease inhibitor-PSTI (pancreatic secretory trypsin inhibitor) domain. We found that his-tagged mouse SPINK2 has inhibitory activity against trypsin. Tissue distribution analyses demonstrated that *Spink2* is detected strongly in the testis and weakly in the epididymis, but is not transcribed in other mouse tissues. Expression of *Spink2* is specific to germ cells in the testis and is first evident at the pachytene spermatocyte stage. Immunoblot analyses revealed that SPINK2 protein is present in male germ cells at all developmental stages, including in testicular spermatogenic cells, testicular sperm, and mature sperm. In addition, immunohistochemical analysis revealed that SPINK2 protein is present in the cytoplasmic region of round spermatids and in acrosomal regions as round spermatids differentiate into spermatozoa. To elucidate the functional role of SPINK2 *in vivo*, we generated mutant mice with reduced levels of SPINK2 protein using a gene-trap mutagenesis approach. Gene-trap mice carrying a mutation in the *Spink2* gene exhibit significantly impaired fertility; further phenotypic analyses revealed that testicular integrity is disrupted, resulting in a reduction in sperm number. Moreover, we found that testes from mutant mice show abnormal spermatogenesis and germ-cell apoptosis accompanied with elevated serine protease activity independent on caspase-3, an executioner of caspase-dependent apoptosis. Our studies thus provide the first demonstration of SPINK2 roles which are required for maintaining normal spermatogenesis, and potentially SPINK2 regulates serine protease-mediated apoptosis in male germ cells.

GROWTH HORMONE IS SYNTHESIZED IN THE TESTIS AND STIMULATES SPERMATOGONIAL PROLIFERATION IN JAPANESE EEL, *ANGUILLA JAPONICA*.

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Growth hormone (GH) plays important roles in development, somatic growth and gametogenesis in vertebrates (1-3). In this study, to evaluate the biological function of GH in spermatogenesis in teleosts, the expression of genes encoding GH and the two GH receptors (GHRs) in the Japanese eel (*Anguilla japonica*) testis and the action of GH *in vitro* were examined using the eel testicular organ culture system (4, 5). Our results showed that mRNAs encoding GH and GHR-1 and -2 are present during spermatogenesis, with GHR-1 and -2 mRNA detectable in the germ cells by *in situ* hybridization. Moreover, we investigated the presence of GH protein in the testis during spermatogenesis using an antibody against eel GH. Western blot analysis showed that testicular eGH protein was present in testis extracts of eels with germ cells consisting of proliferating spermatogonia, but was not detectable in testis at more advanced-stage. Immunohistochemistry indicated that eGH protein was localized in Sertoli cells surrounding the germ cells in early spermatogenesis. To understand the direct function of GH in spermatogenesis, the eel testicular culture system was used. Treatment of eel testicular fragments with recombinant eel GH (r-eGH) induced spermatogonial proliferation, an effect that was independent from the production of steroid hormones or IGF-1. Long term *in vitro* treatment with r-eGH alone induced mitosis of type-A spermatogonia only, which are renewal-proliferated primary gonial cells undergoing mitosis, but not of proliferated type B spermatogonia awaiting entry into meiosis. The results of these studies lead to the discovery of a new action of GH on germ cells, wherein GH produced by Sertoli cells plays an important paracrine role in spermatogenesis, promoting the mitotic phase of eel spermatogenesis by interacting with GHR on germ cells that is not mediated by either steroid hormones or IGF-1 production.

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TGFB2 PARTIALLY RESCUES CORD FORMATION IN THE BETAGLYCAN KNOCKOUT MOUSE TESTIS *IN VITRO* AND NEGATIVELY REGULATES LEYDIG CELL GENE EXPRESSION

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Betaglycan, the TGF β family co-receptor, binds TGF β s with high affinity enhancing their association with their signalling receptors. We have previously demonstrated that betaglycan knockout testis at 12.5-13.5 dpc displays poorly delineated seminiferous cords and disrupted Leydig cell development (Sarraj et al., 2010). As betaglycan is particularly important for high-potency TGF β 2 action, we hypothesised that disruption to TGF β 2 function underlies the defects observed in betaglycan knockout fetal testis. To test this hypothesis, testis/mesonephros complexes from 13.5 dpc mouse embryos were cultured in the absence or presence of recombinant TGF β 2 (2.5-5 ng/ml) to determine if exogenous TGF β 2 could rescue the betaglycan knockout fetal testis phenotype. Following 48 hours of culture, the testes were processed for morphological evaluations. From each embryo, one gonad was used as a control, the other was treated. *In vitro* development of the control knockout testis recapitulated the aberrant structural phenotype observed *in vivo*. TGF β 2 treatment partly rescued cord formation in two of three betaglycan knockout testes *in vitro*, with testis morphology evaluated by laminin immunostaining. We then tested the effect of TGF β 2 *in vitro* on steroidogenesis using quantitative real time PCR (n=3-4 single gonads/group). Cultured wildtype mouse testes exhibited a significant 43% (p<0.0001) decrease in the expression of the Leydig cell marker steroidogenic factor 1 (*Sf1*) after treatment. Genes encoding proteins involved in the steroidogenic pathway such as *Star* (47.5%), *Cyp11a* (46.7%), *Hsd3b1* (65%), and *Cyp17a1* (74.4%, p<0.0001) were significantly decreased in response to TGF β 2. The expression of these genes in the betaglycan knockout testis did not significantly change after treatment. Collectively, our data suggest that TGF β 2 acts via betaglycan to regulate cord formation during foetal testis development. In addition, our results indicate that a high dose of TGF β 2 is sufficient to reduce Leydig cell steroidogenic gene expression independently from betaglycan.

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ANDROGEN RECEPTOR SIGNALING IN PERITUBULAR MYOID CELLS IS ESSENTIAL FOR NORMAL DIFFERENTIATION AND FUNCTION OF ADULT LEYDIG CELLS

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Testosterone synthesis depends on normal Leydig cell (LC) development but the mechanisms controlling this development remain unclear. We recently demonstrated that androgen receptor (AR) ablation from testicular peritubular myoid cells (PTM-ARKO) did not affect LC number but resulted in compensated LC failure [1]. The current study has extended these investigations, demonstrating that PTM AR signalling is important for normal development and function of adult LCs. Notably mRNAs for LC markers (e.g. steroidogenic factor 1, desert hedgehog, insulin-like growth factor 1, and insulin-like factor 3) were significantly reduced in adult PTM-ARKOs but not all LCs were similarly affected. Two LC sub-populations were identified, one apparently 'normal' sub-population which expressed adult LC markers and steroidogenic enzymes as in controls, and another 'abnormal' sub-population which had arrested development and only weakly expressed *Ins3*, leutinising hormone receptor, and several steroidogenic enzymes. Both sub-populations expressed AR. Furthermore, unlike 'normal' LCs in PTM-ARKOs, the 'abnormal' LCs did not involute as expected in

response to exogenous testosterone. Differential function of these LC sub-populations is likely to mean that the 'normal' LCs work harder to compensate for the 'abnormal' LCs to maintain normal serum testosterone. These findings reveal new paracrine mechanisms underlying adult LC development, which can be further investigated using the PTM-ARKO model.

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INSTABILITY AND STRUCTURAL PROPERTY OF A HUMAN TESTIS-SPECIFIC HISTONE H3T NUCLEOSOME

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Eukaryotic genomic DNA is compacted into the chromatin structure. Nucleosome, which is the fundamental unit of chromatin, is composed of a histone octamer and a 146 bp DNA. The histone octamer contains two each of the core histones, H2A, H2B, H3, and H4. Eight nonallelic histone H3 variants, H3.1, H3.2, H3.3, H3T, H3.5, H3.X, H3.Y and CENP-A, have been identified in human. H3T and H3.5 are specifically expressed in testis. During spermatogenesis, most histones are replaced by protamine, which is small arginine-rich protein, and the global chromatin reorganization is occurred. Since H3T and H3.5 are testis specific histone H3 variants, they may be involved in this chromatin reorganization. To analyze biochemical and structural properties of testis specific histone variants, we individually purified H3T, H4, H2A, and H2B, as recombinant proteins, and the histone complexes were formed *in vitro*. Nucleosomes were reconstituted with the purified histone complexes by the salt-dialysis method. Biochemical experiments showed that H3T-nucleosome is significantly unstable compared to canonical H3.1-nucleosome. To reveal structural basis for reduced stability of H3T-nucleosome, we crystallized H3T-nucleosome, and performed X-ray crystallographic analysis. The crystal structures revealed structural differences at both ends of the central $\alpha 2$ helix between H3T and H3.1 within the nucleosomes. The H3T-specific residues (Met71 and Val111) are responsible for the structural difference observed between H3T and H3.1. A mutational analysis revealed that the unstable property of the H3T-nucleosome may be mainly due to the Val111 residues of H3T. These physical and structural properties of the H3T-nucleosome may provide the basis of chromatin reorganization during spermatogenesis.

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TESTIS-SPECIFIC ARGONAUTE PROTEIN MIWI MEDIATES TRANSLATIONAL SILENCING IN A GW182-INDEPENDENT MANNER

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Mammalian spermatogenesis is a highly specialized process of male germ cell differentiation to produce spermatozoa from stem cells. A large number of mRNAs undergo posttranscriptional and translational regulations in the haploid phase of spermatogenesis, spermiogenesis.

The mouse PIWI subfamily Argonaute proteins, MIWI, MILI, and MIWI2, are specifically expressed in germ cells. The loss of any one of these three proteins results in the arrest of spermatogenesis. Although MILI and MIWI2 are responsible for transcriptional silencing of retrotransposons, the molecular function of MIWI remains largely unknown. Here, we show that MIWI associates with various mRNAs by directly interacting with the cytoplasmic poly(A)-binding protein PABPC1. MIWI was barely recruited into a cap-binding complex but rather associated with poly(A)-binding complex. When MIWI was artificially tethered to the 3'-untranslated region of a luciferase reporter mRNA in cultured cells, translation was significantly inhibited. The N-terminal and PIWI domains of MIWI were found to induce translational silencing. These two silencing domains of MIWI failed to inhibit the interaction between PABPC1-poly(A) and PABPC1-EIF4G, despite direct binding of MIWI to the RRM domain of PABPC1 responsible for the interaction. Although GW182 is required for translational repression mediated by AGO proteins, MIWI and GW182 did not interact. These findings suggest that MIWI, together with factor(s) other than GW182, represses translation during spermatogenesis.

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DIFFERENTIAL EXPRESSION OF *SMOC1* AND *SMOC2* IN FOETAL AND NEONATAL MURINE GONADS

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SMOC1 and SMOC2 (SPARC related modular calcium binding 1, 2) are two newly-described matricellular proteins thought to influence growth factor signalling, migration and proliferation. SMOCs have been shown to be expressed in the somatic cells of early developing mouse gonads (1). In order to better understand the role of these proteins in murine gonadogenesis, we conducted an mRNA expression study of *Smoc1* and *Smoc2* in wildtype murine gonads from 12.5 *dpc* until birth (D0), using quantitative real time PCR. Expression was examined in separately-dissected gonads and mesonephroi (n=3-4 pairs per age). *Smoc1* was found to be expressed in the testis from 12.5 *dpc* until D0, with the expression decreasing by 70% at D0. In the ovaries, *Smoc1* expression was highest from 12.5-14.5 *dpc* before decreasing by 42% at D0. In contrast, *Smoc2* expression did not peak until 18.5 *dpc* in the testis and remained low in the ovaries until birth. These data suggest a dynamic role for SMOCs in the developing testis, particularly SMOC2. We further investigated *Smoc* expression in mice null for betaglycan, a TGF β co-receptor, which show testis dysgenesis, somatic cell defects and compromised Leydig cell function (2). *Smoc1* and *Smoc2* expression was analysed from 11.5-14.5 *dpc* in betaglycan null and wildtype testis and ovaries (n=3 per age/genotype). At 14.5 *dpc*, a significant down-regulation in *Smoc1* was observed in both null ovary (p<0.05) and testis (p<0.01), while *Smoc2* was significantly down-regulated only in the null testis (p<0.05). Our data show that in the absence of betaglycan, expression of *Smoc1* is decreased in both the foetal testis and ovary, while *Smoc2* is only decreased in the foetal testis. Our data indicate that *Smoc1* and *Smoc2* are differentially expressed during foetal gonadal development and may be downstream of betaglycan or betaglycan-mediated changes in foetal murine gonads.

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CHARACTERISATION OF REC8, A MEIOTIC-SPECIFIC COHESION SUBUNIT, AND ITS ROLES IN GONADOGENESIS AND GAMETOGENESIS IN ZEBRAFISH

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Meiosis is critical for sexual reproduction, wherein genetic variation in offspring is provided by the exchange of genetic material between parental chromosomes during homologous recombination at meiotic prophase I. A secondary function of homologous recombination is the repair of double stranded DNA breaks (DSB), which is governed by the Fanconi anaemia (FA) and the Rad21/Rec8 gene families. Recently, it was reported in zebrafish that loss-of-function mutations in *Fancl* and *BRCA2*, members of the FA gene family, produced all male populations (Rodriguez-Mari et al. 2010; 2011) and that *BRCA2* mutants were also infertile. Further studies in zebrafish with defective meiotic prophase I (Saito et al. 2011) demonstrated the inability of spermatogenic cells in these mutants to complete spermatogenesis, leading to infertility. Taken together, these studies indicate the importance of the DSB repair and meiotic prophase I progression in zebrafish gametogenesis and germ cell fates. In this study, we describe the characterisation of the expression of zebrafish *Rec8* (a meiotic-specific component of the sister chromatid cohesion complex). *Rec8* regulates sister chromatid segregation during meiotic prophase I and is also involved in DNA DSB repair. Given the crucial role of meiosis in the gonadal fate decisions in zebrafish, we hypothesise that *Rec8* may be a regulator of sex differentiation in zebrafish. Additionally, since sex specific differences in the timing of onset of meiosis in germ cells have been observed, the temporal and spatial expression patterns of *Rec8* in the gonads of transgenic vas:egfp zebrafish were investigated. To examine whether *Rec8* activity is modulated by sex hormones, we also studied the expression patterns of *Rec8* in sex hormone exposed zebrafish. As most prior studies on meiosis have focused on mammalian systems, our study of the function of *Rec8* in zebrafish gonadogenesis broadens substantially our understanding of the role of meiosis in germ line differentiation in non-mammalian vertebrates.

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GONAD IS ONE OF THE CONTROL ORGANS FOR GROWTH IN FISH

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It has been hypothesized that there is a relationship between growth and reproduction, thus it is believed that gonad is an important organ for growth (1, 2, 3, 4). However, there is no evidence that supports this assumption. In this report, we show the direct evidence confirming gonad as the endocrine organ equal to pituitary for controlling body growth in both male and female using Mozambique tilapia (*Oreochromis mossambicus*) as an experimental model. In RT-PCR and western blot analysis, mRNA and protein of growth hormone (GH) are expressed not only in pituitary but also in both testes and ovaries in tilapia. In histochemical observation, the expression sites of GH in gonads were in Sertoli cells surrounding spermatogonia in male and follicle cells layer surrounding oocyte in female. To understand the direct function of gonad on growth, the experiments for gonadal loss-of-function, gain-of-function and growth rescue were performed. Gonadectomical experiments were carried out in 40 days old post-hatching juvenile male and female fish. Gonadectomy significantly delay growth of these fish compared to sham operated fish. However, this delay was rescued by implantation of surgically removed gonads into the gap between skin and muscle. The gametogenesis of these implanted testes and ovaries progressed into the transplanted ectopic site. In this transplanted experiments, serum growth hormone levels were measured by ELISA system. The results showed that GH serum level of gonadectomised fish were lower than those of sham operated fish and those of gonad transplanted fish in both male and female, significantly. This result indicated that gonadal GH expression influenced serum GH levels. These investigations collectively proved that gonad is an integral player in the dynamic regulation of growth in teleosts.

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A NOVEL SRY MISSENSE MUTATION IN A 46,XY FEMALE PATIENT WITH BILATERAL GONADOBLASTOMA AND DYSGERMINOMA

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Introduction: Disorders of Sex Development (DSD) are congenital conditions of incomplete or disordered gonadal development leading to discordance between genetic sex, gonadal sex, and phenotypic sex. Patients with gonadal dysgenesis and hypovirilization are at risk of developing malignant type II germ cell tumors (GCTs) (seminoma/dysgerminoma and nonseminoma). SRY mutations residing in the HMG (High Mobility Group) domains are found in 10 -15% of the 46,XY gonadal dysgenesis cases and affect binding to and bending of DNA. This domain also contains two nuclear localization signals (NLSs).

Methods: Immunohistochemical staining was performed on formaline - fixed specimens, using antibodies directed against OCT3/4, c-KIT, SCF, TSPY, SOX9 and FOXL2. Sequencing of the SRY gene on peripheral blood DNA of the patient was also performed.

Results: A unique case is here reported of a phenotypical normal woman (age 22), who had primary amenorrhoea at presentation, later diagnosed as hypergonadotropic hypogonadism on the basis of 46,XY gonadal dysgenesis with a missense mutation in the SRY gene just before the second NLS

(K128R). Laparoscopic examination revealed streak ovaries and a normal, but small, uterus. Pathological examination showed the presence of bilateral gonadoblastoma and dysgerminoma, confirmed by immunohistochemistry including OCT3/4, c-KIT, Stem Cell Factor (SCF), TSPY, SOX9 and FOXL2. The patient had a severe kidney impairment and (treatment-resistant) osteoporosis.

Discussion & conclusion: Most likely the mutation results in reduced transcription activation or binding and bending of the DNA, currently under investigation. This would result in decreased SOX9 expression, i.e., absence of Sertoli cell development, and a maturation block of embryonic germ cells, increasing the risk for malignant transformation. In addition, it might be linked to the osteoporosis and kidney anomalies found. This further supports the importance of proper diagnosis of DSD patients, especially those with an increased risk for GCTs.

CHEMOKINE GENE EXPRESSION PROFILES IN ARTERIAL AND VENOUS VSMC

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During uterine decidual spiral arteriole remodelling vascular smooth muscle cells (VSMC) secrete chemokines, facilitating the migration and invasion of trophoblast cells (TC) into the arterial walls. This remodelling increases luminal diameter, allowing increased blood flow to the placenta and growing fetus. TC invasion is limited to the uterine spiral arterioles, with limited or absent invasion in uterine decidual veins. Although oxygen is a known regulator of spiral arteriole remodelling, its effect on chemokine gene expression profile is yet to be investigated. The aim of this study was to determine chemokine gene expression profiles of arterial and venous VSMC and investigate the effect of oxygen on these profiles.

The human aortic smooth muscle cell (HASMC) and the saphenous vein smooth muscle cell (SVSMC) lines were used to model arterial and venous VSMC respectively. RNA was extracted using the RNeasy Micro Kit (Qiagen) and a PCR was performed using a human chemokine & receptor gene array (SABiosciences). Low oxygen conditions were generated using Billups-Rothenberg hypoxia chambers gassed with 5% O₂, 5% CO₂, and 90% N₂ for 15mins with cells incubated for 48hrs.

The chemokines which showed the highest HASMC gene expression compared to SVSMC are listed based on fold change (FC) differences in Table 1. mRNA for chemokines which have known associations with TC function are highlighted in grey and were also selected for representation. The results also indicate decreased chemokine gene expression by SVSMC at 20% O₂, when compared with HASMC.

Chemokines	Arterial		Venous	
	20% O ₂	5% O ₂	20% O ₂	5% O ₂
CXCL12	40.7	2.04	0.122	477.0
CXCL8	6.87	169	0.0125	17.2
CX3CL1	0.909	1.45	0.0012	352.0
CCL2	2.01	4.02	0.0012	104.8
CCL7	0.038	1.17	0.00017	ND
CCL16	5.78	ND	0.000441	ND
CCL11	1.66	30.0	0.00125	104.8

Table 1: Chemokine profile in HASMC and SVSMC expressed in FC (n=6)

ND= Not determined

This is the first study to screen for chemokine mRNA expression in HASMC and SVSMC at different O₂ environments. Higher chemokine production by HASMC when compared to SVSMC reveals a possible mechanism which contributes to the specific targeting of TC towards the arterial network during first trimester TC invasion.

GENES CONTROLLING PHALLUS DEVELOPMENT

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The external genitalia of males and females are indistinguishable in early development until androgen exposure causes virilisation in males, including differentiation of the phallus. How androgens affect gene expression in the developing phallus has not been well examined in any mammal because the critical developmental stages occur in utero. However, in tammar wallabies early virilisation of the phallus occurs while the young is in the pouch and easily accessible. In this study we examined the influence of androgen administration on expression of SHH, GLI2, FGF8 and BMP4, genes that are crucial for the correct patterning and growth of the external genitalia in mice. Normal expression in the phalluses of males and females was examined throughout differentiation. We also examined expression in females treated with androgen either from day 20 or day 30 post-partum or following castration of males at day 25. Treated animals were killed at day 50 pp for gene expression analysis using qPCR. Interestingly, expression of these key genes was not significantly changed in males after castration at day 25, suggesting that androgen exposure before this age is sufficient to prime subsequent gene expression at day 50. Our preliminary results suggest that in androgen treated females SHH, FGF8, BMP4 and GLI2 expression were upregulated in the phallus which suggests that the phallus remains sensitive to androgens after day 30. This is the first identification of androgen-regulated gene expression in the developing marsupial phallus, and provides a model to investigate how androgens mediate the molecular pathways of phallus development.

DETECTION OF BOVINE PREGNANCY-SPECIFIC MILK PROTEINS USING NEWLY SYNTHESIZED ANTIBODIES

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Early pregnancy diagnosis is essential for reproductive planning in dairy cattle, because long-term absence of pregnancy results in a longer dry period and low milk productivity. However, currently available tests are inconvenient and require either experience or costly instrumentation. The present study sought to identify early pregnancy-specific milk proteins in cows, via comparison of milk samples collected from pregnant cattle (on days 30 and 50) and non-pregnant animals, and to confirm pregnancy using synthesized antibodies. Proteomics analysis was used to identify 39 proteins differentially expressed in pregnant bovine milk samples compared to non-pregnant samples, of which 8 were pregnancy-specific, 11 were down-regulated in pregnant milk, and 20 were up-regulated. Antibodies were developed against peptide sequences identified via proteomic analysis of milk whey proteins specifically expressed during pregnancy such as lactoferrin, lactotransferrin, the carrier organic anion transporter (similar to the MAK31-like protein), alpha-1G, cystatin C and the AMPK gamma subunit. Synthesized peptides were injected into rabbits; three boosts were given; antibodies were isolated from serum. The antibodies were tested in Western blotting to analyze protein expression during pregnancy in milk. Western blotting showed that expression of lactoferrin, lactotransferrin, and alpha-1G increased specifically during pregnancy in milk. The present work suggests that proteomics analysis facilitates to identify specific proteins of which antibodies could reliably serve as predictors of pregnancy.

PROTEOMIC CHARACTERIZATION OF PLASMA FROM MEN WITH KLINEFELTER'S SYNDROME

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This study aimed to identify protein-based plasma markers in men with Klinefelter's Syndrome (KS) using a proteomics approach. KS is a common sex chromosomal disorder (47,XXY) associated with infertility, hypoandrogenism, metabolic syndrome, cardiovascular disease and osteoporosis. The infertility cannot be reversed by hormone therapy implicating other causes. Plasma from KS and age-matched control men (n=10 and 11/group, respectively) were subjected to immunodepletion of 12 most abundant proteins, prior to differential fluorescent dye labeling, 2D SDS-PAGE (DIGE) and quantitative image analysis using Progenesis software. Differentially expressed proteins were identified by LC-MS and 2D Western blotting. Ficolin-3, α -1-antichymotrypsin (AACT, acidic forms) and retinol-binding protein (RBP-4) showed significant increases (1.6- 2.7-fold) compared to controls while acidic forms of vitamin D binding protein (VBP) showed a significant 2.0-fold decrease in KS. As assessed by multiple linear regression, ficolin-3, AACT and RBP-4 were independently correlated with age and circulating reproductive hormone levels and thus these changes are attributed to the variable age/endocrinology between subjects. In contrast, VBP did not correlate with age or endocrine parameters. We therefore postulate that the decrease in VBP levels reflects aspects of the 47,XXY genotype not related to age or reproductive endocrine status. Functionally, the changes in these four proteins in men with KS suggest that the immune system and/or inflammatory responses are compromised.

AUTOPHAGY AND APOPTOSIS ACT AS PARTNERS TO INDUCE GERM CELL DEATH AFTER HEAT STRESS IN MICE

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Testicular heating suppresses spermatogenesis which is marked by germ cell loss via apoptotic pathways. Recently, it is reported that autophagy also can be induced by heat treatment in somatic cells. In this study, the status of autophagy in germ cells after heat treatment, as well as the partnership between autophagy and apoptosis in these cells was investigated. The results demonstrated that besides initiating apoptotic pathways, heat also induced autophagic pathways in germ cells. Exposure of germ cells to hyperthermia resulted in several specific features of the autophagic process, including autophagosome formation and the conversion of LC3-I to LC3-II. Furthermore, the ubiquitin-like protein conjugation system was implicated as being likely responsible for heat-induced autophagy in germ cells since all genes involving this system were found to be expressed in the testes. In addition, the upstream protein in this system, Atg7 (Autophagy-related gene 7), was found to be expressed in all types of spermatogenic cells, and its expression level was positively correlated with the level of autophagy in germ cells. As a result, Atg7 was selected as the investigative target to further analyze the role of autophagy in heat-induced germ cell death. It was shown that down expression of Atg7 protein resulted in the notable decrease in the level of autophagy in heat-treated germ cells, and this down-regulation of autophagy caused by Atg7 knockdown further reduced the apoptotic rate of germ cells. These results suggest that autophagy plays a positive role in the process of germ cell apoptosis after heat treatment. In conclusion, this study demonstrates that heat triggers autophagy and apoptosis in germ cells. These two mechanisms act as partners, not antagonist, to induce cell death and lead to eventual destruction of spermatogenesis.

EARLY LIFE EXPOSURE TO A BACTERIAL MIMETIC ALTERS REPRODUCTIVE FUNCTION IN FEMALE RATS

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Exposure to infection in early life produces long-term neuroendocrine and immune alterations. Our previous findings indicated that neonatal exposure to lipopolysaccharide (LPS) results in increased anxiety-like behaviour, altered HPA axis activity, increased central cytokine levels and microglial

activation. We have also provided evidence that exposure to LPS impairs sexual behaviour and reproductive success in adulthood. The current study assessed reproductive development and ovarian morphology in female rats from prepubertal to adult ages.

Methods: Wistar rats were administered either LPS (0.05mg/kg, i.p.) or saline on postnatal days (PND) 3 and 5. Four developmental time-points were chosen: PND14, day of pubertal onset, adolescence and adulthood. From weaning (PND 22), rats were monitored for vaginal opening. Vaginal smears were taken to determine estrous cyclicity. All tissue samples were collected during proestrous. Ovaries were collected for morphological assessment.

Results: Neonatal LPS exposure resulted in a significantly advanced age of pubertal onset ($p < 0.05$) and a significant increase in body weight at that time-point ($p < 0.05$). While no significant difference was observed in the timing of the 1st proestrous, LPS-treated females gained significantly more weight at this age ($p < 0.05$).

LPS-treated females exhibited significantly increased corticosterone levels in puberty and adolescence ($p < 0.05$)

Assessment of follicular development revealed significantly fewer primordial and primary follicles present in the ovaries of LPS-treated females across all time points ($p < 0.05$). Significantly fewer primordial follicles were observed in LPS-treated females on PND14 as compared with saline-treated controls ($p < 0.005$).

Discussion: Preliminary results indicate that neonatal LPS exposure altered reproductive development in female rats. LPS-treated females exhibited increased weight gain and advanced puberty onset. Increased baseline corticosterone levels suggest a programming effect of neonatal treatment on the HPA axis functioning. Importantly, a significant decrease in the pool of primordial follicles at 2 weeks of age indicate a potential impact of neonatal immune activation on reproductive fitness in later life.