

## **Environment, testicular dysgenesis and carcinoma in situ testis**

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The testicular dysgenesis syndrome (TDS) hypothesis proposes that a proportion of the male reproductive disorders - cryptorchidism, hypospadias, infertility and testicular cancer - may be symptoms of one underlying developmental disease, TDS, which is most likely a result of disturbed gonadal development in the embryo. TDS may be caused by genetic factors, environmental/life-style factors, or a combination of both. Some rare disorders of sex development of genetic origin are among the best-known examples of severe TDS. Among the environmental and life-style factors that are suspected to influence the hormonal milieu of the developing gonad are the endocrine disrupters. A prenatal exposure to commonly used chemicals, e.g. phthalates, may result in a TDS-like phenotype in rats. Currently, this animal model is the best model for TDS. In humans the situation is much more complex, and TDS exists in a wide range of phenotypes: from the mildest and most common form, in which impaired spermatogenesis is the only symptom, to the most severe cases, in which the patient may develop testicular cancer. It is of great importance that clinicians in different specialties treating patients with TDS are aware of the association.

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## **Studies using a mouse model of mild scrotal heat stress revealed increased hypoxia, DNA damage and germ cell death in the testis and early embryonic loss after fertilisation**

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In men problems with spermatogenesis remain the most common cause of infertility. In most mammals the testes are kept 2 to 8°C below core body temperature and men with scrotal temperatures above the normal range can exhibit fertility problems with an increased incidence of abnormal and immature sperm (1). A number of animal models of heat stress have been established either by a transient exposure of the testes (2), or whole animal (3), to heat or by surgically inducing cryptorchidism (4).

In the present study we have explored the impact of heat stress on testicular function and fertility using a mouse model. Anaesthetised mice were subjected to a mild scrotal heat stress for 30 minutes by positioning them on a raft such that the rear third of their body was submerged in a water bath held at 38°C, 40°C or 42°C. Controls were anaesthetised but not placed in a bath. In order to determine the impact of stress on testicular architecture mice were killed hours (3, 6, 24, 48) or days (7, 14, 28) later. For fertility tests males heat stressed 23-28 days previously were paired with females (n=3) or sperm were recovered from epididymes and used to perform IVF.

The impact of heat stress on testicular architecture and gene expression was temperature dependent with minimal effects at 38°C but alterations in testis function as a result of exposure of testes to 40° or 42°C. These included increased expression of the hypoxia-dependent protein Hif1 $\alpha$ , increased numbers of DNA strand breaks in spermatocytes, increased numbers of apoptotic germ cells and increased DNA abnormalities in sperm. Heat stress at 40°C was associated with detection of increased embryonic resorptions at e14.5 and stress at 42°C resulted in a drastic reduction in pregnancy rate. Embryos resulting from IVF using sperm from males stressed at 42°C were compromised between the 4-cell and blastocyst stage suggesting that though sperm with DNA damage are still capable of fertilisation, the paternal DNA was introducing genomic instability to the embryo and having fatal effects on development.

In conclusion, using a mouse model of mild, transient heat stress we have documented temperature dependent changes in testicular function with associated germ cell loss and compromised fertility. These results may give clues as to the causes of reduced fertility in men with raised scrotal temperatures.

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## **Time window for masculinisation and its implications for male reproductive disorders**

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Gender is a critical defining feature, and becoming a male is ultimately determined by androgen-induced masculinization. Disorders of fetal masculinization, resulting in hypospadias (abnormal penis development) or cryptorchidism (undescended testes), are the commonest congenital disorders in humans, but their cause remains unclear. Together with adult onset disorders (low sperm counts, testicular germ cell cancer) they can constitute a testicular dysgenesis syndrome (TDS), with a proposed common fetal origin that may involve deficiencies in androgen production or action; TDS disorders are probably increasing in incidence. Masculinization is well studied, but no unifying concept has emerged that explains normal male reproductive development and its abnormalities, and which encompasses the origin of the TDS disorders. This talk will report on our studies in rats using two 'models' that involve perturbation of normal testis development/function (TDS animal model) or blockade of androgen action during selective fetal time windows (flutamide model). Using these models, we show that masculinization of all reproductive tract tissues is programmed by androgen action during a common fetal time window ('programming window') which is earlier than was thought, as it precedes morphological differentiation of the same tissues, when androgen action is, surprisingly, unnecessary. Androgen-driven masculinization of females is confined to the same programming window. Blocking androgen action only within the programming window induces hypospadias and cryptorchidism and alters penile length in males; these all correlate with anogenital distance (AGD). As AGD provides a non-invasive, lifelong read-out of androgen exposure in the programming window (but not later in gestation) and is measurable in human neonates, it could predict adult onset TDS disorders as well as providing clinically important insights into reproductive tract masculinization and its disorders. We show in our TDS animal model that AGD may predict fetal and postnatal testis size, consistent with a key role for androgens in regulating fetal Sertoli cell proliferation. However, we also show that the time window for androgen-induced regulation of Sertoli cell proliferation extends beyond the 'masculinisation programming window'. Our findings reinforce the view that impaired fetal androgen action may be a central feature of TDS, and we suggest that delayed onset of fetal testosterone production may be important.

## Development of an in vitro Leydig cell function toxicology test

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Within the Reprotect consortium, one of the Work Packages is aimed at the development of in vitro tests for the toxic effects of compounds on different cell systems involved in reproduction. We have used the mouse Leydig tumor cell line BLT1\* as the starting material to develop such as a test. BLT1 cells were isolated from a Leydig cell tumor from a transgenic mouse line that expressed T-antigen under the control of the  $\alpha$ -inhibin promoter. Although these cells initially expressed Leydig cell-specific proteins such as the LH receptor and also produced testosterone under control of LH or hCG, they never had these characteristics in our laboratory. BLT1 cells produced progesterone instead of androgens and were completely non-responsive to hCG. Therefore, we constructed BLT-L17 cells by transfecting and constitutively expressing the LH receptor, cyp17 and 17betaHSD3 genes (human).

BLT-L17 cells show an excellent testosterone response to hCG (minimally 20-fold) and this response stays constant during a minimum of 15 passages (7 weeks of culture). After the construction of the BLT-L17 cells, a protocol was developed to determine the effects of compounds on hCG-stimulated testosterone production in vitro. Cells were plated (medium, 10% FBS; 24 well plates), and 24 hours later were incubated for 17 hours with increasing concentrations of the tested compound in medium, 0.1% BSA and 100 ng/mL hCG. After treatment media were removed and testosterone was determined in the media using a standard testosterone radio-immuno-assay (DPC). The cells were subsequently used in an MTT test to determine cell survival. Comparing the IC50s for testosterone and MTT the specificity of the tested compound for steroid hormone production can be determined.

\*:kind gift from dr. Ilpo Huhtaniemi

## **Sertoli cell based assays for identifying chemicals with a potential to induce testicular damages.**

**A. Tilloy**

Male fertility can be impaired by various toxicants and some of them may target the Sertoli cells. Since the mechanism of action of chemicals provoking testicular damages is not well known, the effect of known testicular toxicants was investigated in two culture systems of rat Sertoli cells. Sertoli cells isolated from immature rats and a rat Sertoli cell line (SerW3) were treated with 24 chemicals. 21 compounds were known to induce testicular damages and 3 compounds were without testicular effect in vivo. 13 chemicals known to be toxic in vivo were also detected to be severely cytotoxic, using the MTT assay, in both cellular models. Five reference chemicals were cytotoxic within 24h of exposure with an  $EC_{50} \leq 50 \mu\text{M}$  and one compound was cytotoxic within 48h with an  $EC_{50} \leq 50 \mu\text{M}$ , the other one having an  $EC_{50} \leq 50 \mu\text{M}$  at 72h. Three chemicals (dieldrin, bisphenol A and zardaverine) were cytotoxic in the SerW3 cell line only. Among the 8 Pfizer proprietary compounds that produced testicular damage in vivo, 6 grouped with the chemicals being cytotoxic with an  $EC_{50}$  below  $150 \mu\text{M}$  after 48h of culture. Six compounds, reference and Pfizer proprietary chemicals, that did not affect the viability of the Sertoli cells, did reduce the level of transferrin and inhibin B secreted in culture media of isolated Sertoli cells. The effect of these chemicals was further studied in evaluating the expression of the proteins involved in the cellular communication (occludin, ZO-1, N-cadherin and connexin 43). Some of the chemicals altered the Sertoli-Sertoli cell direct interaction by delocalizing ZO-1, N-cadherin, and connexin 43 in the cytoplasm.

In conclusion, the Sertoli assays allows the identification of compounds that may insult the Sertoli functions, in evaluating their cytotoxic effect, their potential to alter the inhibin B secretion and their effect on the junctional proteins.

## Clinical Approach to Compounds with Potential or Known Testicular Toxicity

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Compounds with possible testicular toxicity are problematic for clinical development when the risk-benefit comparison is not readily apparent as for oncology drugs. The presentation will discuss the clinical approach for evaluation of 2 compounds: a) dutasteride, a potent 5-alpha reductase inhibitor which raised concerns over possible testicular effects because of marked suppression of dihydrotestosterone, and b) a compound under consideration with established testicular toxicity. Considerations for study designs to further assess injury to the seminiferous epithelium and possible risk to reproductive function will be discussed.

# Chromatin partitioning in mouse sperm

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During the final stage of spermatogenesis in mammals, sperm chromatin undergoes a complete reorganisation that includes the displacement of histones by protamines. Protamines are low molecular weight and highly basic proteins that enable a higher degree of compaction of the sperm DNA, required for its protection from damage in the female reproductive tract. However, in human and mouse, respectively, 15% and 2% of sperm DNA remains packaged by histones. Gatewood and colleagues (Gatewood *et al*, 1987) showed that the packaging of human sperm DNA into nucleosomes and nucleoprotamines was not random but organised in a sequence-specific manner. This epigenetic information may play a role not only in the reorganisation of paternal DNA after fertilisation but also in the development of the early embryo, by enabling the proper expression of paternal developmental genes immediately after fertilisation.

To understand the purpose of chromatin partitioning into protamine and histone bound domains, chromatin from murine spermatozoa was fractionated using limited micrococcal nuclease digestion of decondensed nuclei. Each DNA fraction (MNase sensitive/soluble and MNase resistant/insoluble) was subjected to whole genome amplification before hybridisation onto CGH microarrays. Histone and protamine distribution within the spermatozoa was visualised by immunofluorescence.

The analysis of the composition of DNA sequences in each fraction revealed an amplification bias of the Phi29 DNA polymerase towards gene coding sequences, enhancing the observed difference of gene composition between each compartment. The MNase soluble fraction appeared gene enriched compared to the insoluble fraction and contained clusters of genes essential for embryonic development, such as *Hox* genes. Immunofluorescence of decondensed sperm nuclei showed a striking localisation of acetylated histone H4 at the anterior part of the nucleus while protamines were distributed uniformly over the nucleus.

Although the chromatin fractionation was only partial, this study showed the presence of MNase sensitive sequences, mostly gene rich and probably localised to the histone-rich domain of the anterior nucleus. This is the first clear evidence of chromatin compartmentalisation in murine spermatozoa relating to the packaging of gene rich sequences.

**The relationship between germ cell damage, integrity of the blood-testis barrier and leakage of proteins from seminiferous tubules in the rat; implications for detection of compound-induced damage to spermatogenesis**

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Spermatogenesis takes place in a unique microenvironment created by the supporting Sertoli cells; this protects the post-meiotic germ cells from immune cell attack and toxic molecules brought by blood and lymph. A barrier is created by tight junctions between adjacent Sertoli cells (part of the blood-testis barrier). Under normal conditions, inter-Sertoli cell tight junction dynamics change to allow spermatocytes to pass through (late stage VIII- early stage IX) during spermatogenesis. However, after toxicological insult, we have previously established that certain germ cell specific proteins can leak out of the seminiferous tubules into the interstitial fluid of the testis, and possibly from there into the bloodstream. This leakage could be a direct consequence of germ cell degradation/damage or could result from loss of integrity of the Sertoli cell tight junctions. Both have implications for the early detection of compound-induced damage to spermatogenesis.

We have therefore investigated the relationship between germ cell degradation and integrity of the Sertoli cell tight junctions and the appearance of germ cell derived proteins in testicular interstitial fluid (IF) in the adult Wistar rat using three approaches. The first involves administration of cadmium chloride, a testicular toxicant that is thought to disrupt the Sertoli cell tight junctions as well as inducing damage to spermatogenesis. The second involves administration of the toxicant methoxyacetic acid (MAA) which induces widespread degeneration of pachytene spermatocytes, but is not thought to disrupt tight junctions. The third approach, which is physiological, involved study of rats at ages that span the period before (d10), during (d15) and just after (d25) formation of the Sertoli cell tight junctions. In all instances, the presence/integrity of Sertoli cell tight junctions was assessed by the co-localisation of two tight junction component proteins, occludin and zonula occludens 1 using confocal microscopy/immunofluorescence, whilst integrity of spermatogenesis was assessed by immunohistochemistry using antibodies to germ cell specific proteins (Dazl, Vasa, FABP9).

The same antibodies and Western blotting were used to detect if these proteins were present in IF collected from the same animals.

Our results show that significant damage to the gross organisation of the seminiferous tubules caused by high doses of cadmium chloride (3mg/kg) causes leakage of some small molecular weight germ cell proteins into IF, coincident with disruption of the Sertoli cell tight junctions. Our results also show the presence of germ cell proteins in IF at 10 days of age, before the Sertoli cell tight junctions have formed, but not at day 15, as the junctions are forming, suggesting that loss of integrity of the junctions could be related to leakage of germ cell proteins from the tubules into IF. Whether such proteins leak out into IF after experimentally induced germ cell degeneration (using MAA), in the absence of tight junction disruption, is currently under investigation.

# Impaired male sexual development in rats exposed *in utero* to diisobutyl phthalate

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Certain phthalate esters, such as di-*n*-butyl phthalate (DnBP), are known to disrupt the androgen-dependent male reproductive development in rats following *in utero* exposure. Diisobutyl phthalate (DIBP, C3 backbone) is the branch analogue of DBP and it has the same industrial applications, mainly as a plasticiser. Recent studies have shown that DIBP exposure during gestation has adverse effects on the male reproductive system in rat fetuses. Our aim was to investigate the long term effects of DIBP in rats, when administered during the major period of prenatal male sexual differentiation.

Pregnant Sprague-Dawley rats (n=11-14 per group) were dosed with olive oil or DIBP (125, 250, 500, or 625 mg/kg body weight/day), by daily gavage, from gestation day 12 to 21. DnBP (500 mg/kg) was used as positive control. Male offspring were examined for androgen-dependent endpoints until necropsy on postnatal week 11-12 or 16-17.

Viability of offspring was unaffected at any dose level. Dose-related effects included decrease in anogenital distance on PND 1, areola/nipple retention on PND 13 and in adulthood, delayed preputial separation, undescended testes (25 and 77 % at 500 and 625 mg/kg, respectively), and reduced testis and epididymis weights in mature males. Malformations of the external genitalia consisted of hypospadias (11 and 56 %, at 500 and 625 mg/kg, respectively), exposed os penis, and cleft prepuce.

Thus, DIBP caused severe and permanent alterations in the male reproductive development, with a profile similar to that of DnBP. However, it appeared to be slightly less potent than DnBP in inducing malformations.

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## **Histone and Protamine compartments of Human spermatozoa**

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During the differentiation of mammalian spermatozoa, spermatozoal DNA is re-packaged by arginine-rich protamine although some histone bound DNA remains. In the human, 15% of the genome remains packaged by nucleohistone and 85% by nucleoprotamine. The reason for this unusual partitioning remains obscure. To investigate the differential packaging phenomenon of spermatozoal chromatin, DNA samples isolated from salt and micrococcal nuclease (MNase) soluble and insoluble chromatin compartments were analysed on Agilent 44K and 244K CGH micro-arrays. The localisation of histones was also evaluated by immunohistochemistry and Western blotting. A strong preference in sequence composition of DNA preferentially associated with either histones or protamines was evident along the full length of all chromosomes. Indeed, a striking preference for the packaging of developmental gene clusters into histone-rich DNA was observed while genes encoding Zinc finger proteins, olfactory receptors and regions displaying copy number variations were preferentially packaged by protamines. Immunohistochemistry indicated distinct histone-rich domains localised to the posterior of the nucleus while western blot analysis of histones in the soluble and insoluble fractions showed acetylated histones H3 and H4 in both. Protamines were absent or present in trace amounts in the soluble fractions. In conclusion, we have shown a clear selective partitioning of gene sequences across all chromosomes into protamine and histone enriched compartments. We also observed a strong trend for gene sequences in the soluble fractions to follow the known gene density profiles of individual chromosomes.

## **Studies on dissociated populations of human fetal testicular somatic cells**

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Germ cells within the human fetal testis are supported by somatic cells. Between 6 and 8 weeks gestation germ cells migrate into the genital ridge and become enclosed by the differentiating Sertoli cells (SC). Peritubular myoid cells (PTM) migrate into the testis from the adjacent mesonephros and surround the Sertoli cells to form the testicular cords (at ~8-10 weeks). The interstitial compartment between the cords contains fetal Leydig cells (site of steroid biosynthesis), fibroblasts and blood vessels. Some progress has been made in establishing explant cultures of human testes however the aim of the present study was to determine whether isolated populations of human somatic cells could be maintained in vitro as a prelude to evaluating regulation of gene expression in these cell types.

Human fetal testes were obtained following termination of pregnancy during the 2<sup>nd</sup> trimester (14-20 weeks). Tissues were either fixed for immunohistochemical analysis or dissociated using collagenase IV, DNase 1 and trypsin EDTA. Dissociated cells were cultured in serum-supplemented DMEM with DHT (n = 4). Confluent cells were passaged using trypsin EDTA up to passage 6 (P6). Quantitative RTPCR (Taqman) and Western analysis was performed.

Immunohistochemical analysis confirmed that anti-Mullerian hormone (AMH) and SOX9 were only expressed in SC; 3 $\beta$ HSD and side chain cleavage p450 (SCC p450) were expressed in the Leydig cells and smooth muscle actin (SMA) was restricted to PTM. Cultures of dissociated cells contained a population of cells with mixed morphology that reached confluency every 6-9 days. Taqman analysis of the cultures detected low levels of expression of AMH and desert hedgehog (DHH) only in initial cultures (passage 1). In contrast, the SC specific transcription factors SOX9 and GATA 4 were expressed in P2-P4 cultures although levels did decline by P6. Leydig cells appeared to be rapidly lost from the cultures, as 3 $\beta$ HSD and SCC p450 mRNA were expressed at low levels at P1 but not thereafter. Expression of SMA (PTM), vimentin and fibronectin (fibroblasts) was increased in cultures after repeated passage (P3-P6). Activin receptor II $\beta$  mRNA was maintained throughout the culture period.

Our initial characterization has shown that enzymatic digestion human fetal testis results in survival of a heterogenous population of cells in vitro. Expression of proteins by SC populations is variable and by P6 overgrowth by fibroblasts appears likely. We are currently optimising the cultures by using serum free media and supplementing with peptides such as activin. Our long term aim is to use these cells for the study of somatic-germ cell interactions.

## **Time window for androgen-dependent Sertoli cell proliferation in fetal life**

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Androgen action is essential for the masculinisation of the developing embryo, and recent evidence suggests that the masculinisation of reproductive tract tissues occurs during a specific fetal time window ('programming window'). Evidence also suggests that androgens play a role in regulating Sertoli cell proliferation in fetal life, although it is not yet known whether androgens act to modulate Sertoli cell proliferation during specific time windows in development, or throughout the whole of the later stages of gestation.

Standard (e13.5-21.5) fetal exposure to di(n-butyl) phthalate induces a testicular dysgenesis syndrome (TDS)-like spectrum of disorders in the male offspring, including suppressed fetal intratesticular testosterone (ITT) levels and reduced Sertoli cell number. In the present studies, fetuses were exposed to DBP during different time windows of gestation: early (e13.5-15.5), middle (e15.5-17.5), late (e19.5-20.5) and extended (e11.5-20.5), in addition to the standard dose window, and ITT levels, anogenital distance (AGD) and Sertoli cell number were assessed. ITT levels and Sertoli cell and peritubular myoid cell numbers were also assessed at e17.5 and e21.5, in animals that had been exposed to DBP from e13.5, until the day before kill. The potential relationship/correlation between AGD and testis weight was investigated at e21.5, postnatal day 25 and in adult animals (control and DBP data were combined).

Exposure to DBP during the standard, extended and late treatment windows caused a reduction in ITT of between 70-77%, and exposure during the early and middle windows caused a 30-36% reduction in ITT. AGD was significantly reduced in the standard, extended and middle treatment window groups, and Sertoli cell number was significantly reduced in the standard, extended and late treatment windows.

In animals exposed to DBP from e13.5 until the day prior to kill, at either e17.5 or e21.5, the magnitude of ITT reduction (53-61%) was similar at both ages, as was the reduction in Sertoli cell number (49-52%). Peritubular myoid cell number was unaffected by DBP exposure. A positive correlation between AGD and testis weight was identified at e21.5, and surprisingly, this relationship persisted into adulthood (though the correlation was weaker).

We conclude that androgens act to regulate Sertoli cell proliferation throughout the whole of the later stages of gestation. Since androgen-regulated Sertoli cell proliferation occurs over a broader time window than the masculinisation of reproductive tract tissues, this could explain why low sperm counts (hypothesised to occur as a result of reduced Sertoli cell proliferation/number) is the most prevalent of the TDS disorders, since the window of opportunity for disrupted androgen action is much longer.

Sertoli cell numbers are diminished concurrently with suppressed ITT levels, consistent with the hypothesis that androgens play a role in Sertoli cell proliferation. However, peritubular myoid cell number and the number of AR positive non-Leydig cell interstitial cells are unaffected, implying that this is a Sertoli cell selective effect.

Although a correlation between AGD and testis weight was expected at e21.5, since androgens regulate both AGD and Sertoli cell number (the latter contributing significantly to testis volume at e21.5) during fetal life. The persistence of a vague relationship between AGD and testis weight in adulthood raises the possibility that androgen action during fetal life modulates the Sertoli cells or other cell types in such a way that this affects how they respond to androgens in later life.

## **Use of rat fetal testis explants for study of mechanisms of chemical-induced disruption of testis development and function: studies using Mono Butyl Phthalate (MBP) and Cyclopamine**

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There is increasing concern that certain environmental chemicals may cause aberrant development of the fetal testis in humans and thus give rise to testicular dysgenesis syndrome disorders. Animal models can be used to investigate this possibility, but in vivo chemical treatment studies are potentially complicated by maternal effects, fetal metabolism and/or the fetal endocrine system itself. We are therefore assessing if explants of fetal testes can be used to determine the mechanistic pathways via which such chemicals work and to identify appropriate endpoints of such effects. The present studies report on the use of two such chemicals, monobutyl phthalate (the main in vivo metabolite of Di Butyl Phthalate) and cyclopamine, an inhibitor of the hedgehog pathway which has been implicated in testicular dysgenesis in mouse knockout studies.

Fetal rats were collected at e14.5 and the male gonad and attached mesonephros were micro-dissected and then incubated in 400 $\mu$ L culture medium for up to 48hours, supplemented with either 25  $\times 10^{-3}$ M Cyclopamine, or 1 $\times 10^{-3}$ M MBP and the respective control vehicle of Ethanol or DMSO. After in vitro culture, cord structure, Leydig cell number and function (steroidogenesis), germ cell differentiation, cell proliferation and apoptosis were investigated immunohistochemically. The testis explants retained cord structure and underwent cell proliferation and germ cell differentiation comparable to in vivo testes, but with increased incidence of apoptotic cells. In addition, cyclopamine treatment resulted in a visible reduction in germ cell number without apparently reducing Sertoli or Leydig cell number. In contrast, MBP treatment produced a massive reduction in Leydig cell number, and studies are currently in progress to determine if Sertoli or germ cell number is affected and what effects there are on testosterone production. The in vitro effects of MBP on testis explants are also being compared to testes from animals exposed in vivo to DBP to establish if comparable changes occur. Finally, the possible mechanism via which cyclopamine-induced Hedgehog inhibition is affecting the germ cell numbers is also under investigation. These results suggest that in vitro culture of testis explants may provide a useful tool to investigate fetal testis development and its susceptibility to disruption by chemicals.

## **Inhibitors of steroidogenesis studied in R2C cells in vitro**

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Primary cell cultures have traditionally been a preferred model for the study of compounds which affect steroid hormone synthesis. To be more cost and time effective, a continuous cell line such as R2C cells can be a useful alternative model. Here we report as R2C cell responses to compounds (10 $\mu$ M) known to modulate gonadal steroidogenesis. Ketoconazole (K) inhibited production by estradiol (E2) by 96% and of progesterone (P4) by 90% while testosterone (T) levels were low in both control and K-treated cultures. Aminoglutethimide (AG) inhibited production of E2 by 96% and of P4 by 60% while enhancing media T concentration by 640%. Anastrozole (AZ) inhibited E2 production by 92%, had no significant effect on P4 production and increased media T levels by 900%. In the presence of 10  $\mu$ M AZ, the high level of T production was substantially lowered by co-treatment (10 $\mu$ M) with AG (22%) or K (99%), relative to AZ alone. These results suggest that aromatase inhibition has a profound influence on T production by R2C cells.

## Boar Sperm Express the Oxytocin Receptor

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Oxytocin is a nonapeptide hormone which is known to cause contraction of smooth muscle by mobilising intracellular calcium ions. Oxytocin receptor expression has been found throughout the male and female reproductive tracts. Oxytocin has been found in human and horse semen and is thought to have a role in sperm transport. However, direct effects of oxytocin on spermatozoa have not previously been demonstrated. The aim of the study was to investigate whether the oxytocin receptor was expressed on boar sperm cells and to measure the effect of oxytocin on sperm motility. Fresh boar sperm cells were provided by the Pig Improvement Company. The sperm cells were washed and resuspended in Earle's Balanced Salt Solution containing bicarbonate. Cell suspensions were incubated with a primary rabbit polyclonal antibody to human oxytocin receptor followed by a secondary goat polyclonal anti-rabbit antibody fluorescently labeled with FITC. A rabbit IgG isotype antibody was used as a negative control. Following washing, the cells were mounted on slides and fluorescence microscopy and confocal microscopy were used to detect fluorescence. Flow cytometry was used to measure fluorescence of labeled cells in suspension. An objective measure of sperm motility, involving measuring the progress of fluorescently labeled sperm cells through a 0.5% agarose gel, was used to investigate the effect of oxytocin in a 0.1 nM – 1  $\mu$ M concentration range. Boar sperm cells expressed oxytocin receptors mostly towards the front of the head, on the acrosomal membrane. Flow-cytometry showed a significant ( $P < 0.05$ ) increase in fluorescence due to binding to oxytocin receptors on sperm cells. Oxytocin, at higher concentrations, significantly ( $P < 0.05$ ) increased sperm motility. Oxytocin in semen could have a paracrine effect on sperm function.

## Studies on the impact of adenoviral infection on Sertoli cell function

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The adult testis consists of two compartments, the seminiferous tubules containing the germ and Sertoli cells (SC) and the interstitium that contains Leydig cells (Lc), peritubular myoid cells, macrophages and blood vessels (1). The proliferation rate of SC declines after birth and at puberty SC they 'differentiate' and the blood-testis barrier is established. SC make up a small proportion of the total testicular volume but perform an essential role in supporting germ cell maturation. Recent studies have determined that expression of androgen receptors by SC is essential for maintenance of male fertility (2). We have previously used a cell line prepared from SC isolated from 10-day-old mice; at 34°C cells are mitotically active but at 39°C the cells lose mitotic activity and undergo a change in cell morphology (3). Other investigators have claimed that introduction of adenovirus into the testis results in SC-specific infection without any significant inflammatory response (4). The aim of the present study was to investigate the impact of adenoviral infection on SC function in vitro and in vivo. Studies in vitro were to establish if this type of construct might be more efficient than transient transfection and those in vivo were to investigate whether adenovirus might be used to target shRNAs for cell-selective knockdown.

SK11 cells (34°C or 39°C) were infected with 50 to 250 viral particles per cell (MOI) for 4 to 48h using an adenovirus containing a LacZ construct; infection rates were assessed with a  $\beta$ -galactosidase assay. Expression of interleukin-1 $\beta$  and 6 was determined by qRT-PCR and ELISA. Adenovirus containing a GFP construct was injected into a adult mouse testes via the efferent ducts ( $1 \times 10^5$  to  $4 \times 10^8$  particles/testis) using methods previously optimised for introduction of germ cell populations. The contra-lateral testis served as a control (no virus); 2 to 4 days after injection testes were removed and fixed. The histology of the testes as well as expression of proteins associated with apoptosis, hypoxia and immune cell populations were investigated.

Infection of SK11 cells with 50 MOI of the LacZ adenovirus resulted in 100% of cells expressing  $\beta$ -galactosidase regardless of temperature. There was no detectable cytotoxic effect at 50 MOI but at higher concentrations (100, 250 MOI) some cells died. There was no significant increase in expression of interleukins. Injection of adenovirus into the testis resulted in SC-specific expression of GFP but the number of tubules expressing GFP was low even when high concentrations of viral particles were introduced. At  $4 \times 10^8$  particles/testis testicular architecture was disrupted, a marked increase in the numbers of neutrophils and macrophages and an increased expression of markers of hypoxia (HIF1 $\alpha$ ) and apoptosis (cleaved-caspase 3 and apotag). At lower concentrations ( $1 \times 10^5$  or  $1 \times 10^7$  particles/testis) there was disruption of the seminiferous epithelium consistent with loss of germ cells at specific stages of the spermatogenic cycle and disturbances in expression of junctional proteins such as espin.

In conclusion, SC are readily infected with adenovirus in vivo and in vitro. Adenoviral constructs appear to be a good way of introducing genes/reporters into SC in vitro but our studies suggest that they will not be useful for studies in vivo.

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## **The interaction between age and cyclophosphamide in the induction of genetic damage in male mouse germ-cells**

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The anti-cancer agent cyclophosphamide (CP) is widely used as a positive control substance in mutagenicity studies, especially those in the germline. It is known to induce dominant lethal mutations in sperm and is a male-mediated teratogen. It can induce DNA damage in sperm and induces testicular germ-cell apoptosis. We have therefore used it to investigate the effect of age on susceptibility of germ cells to genetic damage.

A single intra-peritoneal injection of 150 mg/kg was administered to young (2-month) and aged (17 month) animals (n=10 per group). After 24 hours testes and epididymal sperm were collected. Sperm were quantified and subjected to sperm chromatin structure assay (SCSA) which measures sperm chromatin damage. The terminal deoxyuridine nick end-labelling assay was used to determine levels of testicular germ-cell apoptosis.

Testes weight as a proportion of bodyweight was approximately 30% lower in old animals than in young (p<0.001) but sperm counts were unchanged. No significant effects on raw testes weight or sperm counts was observed as a result of either age or treatment. Treatment with 150 mg/kg CP produced a significant increase (p<0.01) in the level of SCSA detected DNA fragmentation in young animals and a comparable increase in old animals (p<0.05). The total number of apoptotic germ-cells was 68% lower (p<0.001) in old control, compared with young control animals. CP-treatment induced increases in 80% and 70% in apoptotic cell numbers in young and old animals respectively (both p<0.05) but because the baseline levels were so low in old animals, the numbers seen were dramatically lower than in young animals.

Although testis weight declines with age, sperm numbers are maintained, suggesting that the decline is not the result of germ cell losses. This is supported by the finding that the level of germ cell apoptosis was in fact significantly lower in old animals than young. Following CP treatment, the proportionate increase in germ-cell apoptosis in old, treated animals is comparable to that in young, treated animals, but the numbers seen are (as with controls) much lower. Together, these data suggest that while the response to induced genetic damage may not be compromised by age, the ability of the germ cells to undergo apoptosis *per se* declines dramatically. Since the induction of SCSA-detectable damage by CP was comparable in young and old animals, it is plausible that the levels of genetic damage in testicular cells would also be comparable in the two groups. Further investigations are therefore required to determine a) the reason why the level of germ-cell apoptosis is so low in aged males; and b) the consequences of the reduced levels of apoptosis for the persistence of genetic damage in the germ line.