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**Early Life Programming
of
Adult Leydig Cell Function**

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Declaration

The experimental work described in this thesis, is the sole work of the author, except where acknowledgement has been made. These studies have not been submitted in support of another degree or qualification at the University of Edinburgh, or any other institute.

Karen Kilcoyne

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Sin é, Buíochas le Dia.

Abstract

Early life programming of adult Leydig cell function

There is increasing evidence to suggest that fetal events can predetermine reproductive health and general wellbeing in adulthood, a process termed 'fetal programming'. This refers to the association between altered fetal growth/development and health disorders in adulthood e.g. the metabolic syndrome, which is linked to low male testosterone levels. Studies from both Europe and the USA have shown that adult male testosterone levels have been declining, independent of age. As low testosterone levels in aging men are associated with increased morbidity and mortality, this highlights the importance of investigating how testosterone levels are determined or potentially 'programmed' during fetal development.

Evidence from human and rodent studies have shown that reduced fetal androgen exposure results in lower adult testosterone levels, although the mechanism(s) is unknown, to date. One way to explain how a fetal insult (e.g. androgen deficiency) could affect (testosterone producing) adult Leydig cells in adulthood, is if their progenitor cells were present during fetal life and were thus affected by such an insult. This hypothesis has been unexplored to date, due to the lack of a unifying/defining marker for adult Leydig progenitor cells. An earlier study promoted the hypothesis for the studies in this thesis, namely that chicken ovalbumin upstream promoter transcription factor-II (COUP-TFII) might constitute such a marker, as inducible knockout of COUP-TFII in pre-pubertal male mice results in failure of adult Leydig cells to develop. Therefore, the hypothesis which was explored in this thesis was that 'fetal programming' of COUP-TFII⁺ adult Leydig progenitor cells prior to their differentiation into adult Leydig cells, would explain how fetal events could predetermine adult testosterone levels.

To investigate whether adult Leydig cells (ALC) develop from COUP-TFII⁺ interstitial cells, firstly an adult Leydig cell ablation/regeneration model was used, which involved a single injection of ethane dimethane sulphonate (EDS).

This identified that in rats, ALC derive from COUP-TFII⁺ interstitial cells which do not express any other phenotypical adult Leydig or interstitial cell markers prior to differentiation. Secondly, COUP-TFII⁺ adult Leydig progenitor cells are abundant in the fetal testis and conserved across species, including man. Thirdly, fetal interstitial cells which differentiated into ALC, as evident from an ALC lineage tracer model, also expressed COUP-TFII. Overall, these findings suggest that the COUP-TFII⁺ interstitial cells which differentiate into ALC are 'adult Leydig progenitor cells'.

The findings from this thesis also show that the identified adult Leydig progenitor cells express the androgen receptor (AR) in fetal life. Furthermore, experimental reduction of androgen action in fetal life in transgenic mice (AR knockout) or chemical manipulations to reduce fetal testosterone levels (di (*n*-butyl) phthalate; DBP exposure) resulted in a similar reduction (~40%) in progenitor cell numbers from birth through to adulthood. A parallel reduction of adult Leydig cell numbers across postnatal development was found in mice, but not rats, but as a result of altered fetal androgen action, both models showed evidence for compensated adult Leydig cell failure. This is defined as normal/low testosterone and elevated luteinising hormone (LH) levels. Cell-selective knockout of AR in peritubular myoid (PTM) cells (PTM-ARKO) or Sertoli cells (SC-ARKO) did not affect the numerical development of adult Leydig progenitor cells. To manipulate testicular testosterone action in postnatal life, rats were exposed to a potent AR antagonist, flutamide, which reduced the number of adult Leydig progenitor cells but did not affect ALC number/function. However, the combination of fetal DBP+postnatal flutamide exposure reduced adult Leydig progenitor cells and resulted in compensated ALC failure. Overall, these studies highlight the importance of fetal androgens for the normal development of adult Leydig progenitor cells and for the subsequent development of normally functioning adult Leydig cells.

As fetal deficits in androgen exposure resulted in adult Leydig cell dysfunction, this thesis also investigated three separate models to determine whether increased fetal androgen exposure could increase/enhance adult Leydig

progenitor cell development, resulting in a 'gain of adult Leydig cell function'. In the first model to increase fetal androgen exposure, pregnant dams injected with testosterone propionate (TP; 20mg/kg/day e14-21.5) were discarded, due to confounding factors including fetal growth restriction and aromatisation of TP. The second model utilised dihydrotestosterone (DHT; 10mg/kg/day), administered to pregnant dams, but there were no effects found in adulthood to male offspring. It was concluded that the administered dose was not sufficient to increase intratesticular testosterone levels in the fetus. The third model utilised an inducible nitric oxide synthase knockout (iNOS^{-/-}) mouse model, for which previous evidence showed increased testis weight, Leydig and Sertoli cell number (~50%), and normal testosterone but low LH levels in adulthood. Stereological quantification showed an increase in the number of adult Leydig progenitor cells in postnatal, but not fetal life, which resulted in the conclusion that the observed changes were a consequence of postnatal effects.

Finally, a potential mechanism to explain how DBP-induced androgen deficiency in fetal life, could result in adult Leydig cell dysfunction in adulthood was investigated. Analysis of testicular genes in adulthood, involved in the steroidogenic pathway, showed a reduction in *3b-hsd* and *StAR*. The reduced *StAR* expression was associated with increased repressive histone methylation (H3K27me3) in its proximal promoter region, as demonstrated by a chromatin immunoprecipitation (ChIP) assay, qPCR, and densitometrical analysis. Accordingly, adult Leydig cells were shown to express increased H3K27me3 by immunohistochemistry, a change also evident in adult Leydig progenitor cells in the fetal testis. This would provide a potential mechanism to explain how fetal events can 'programme' adult Leydig cell testosterone production, namely via an epigenetic change to adult Leydig progenitor cells. In summary, the results in this thesis show how fetal events, including androgen action on progenitor cells, can potentially programme adult Leydig cell function and thus determine testosterone levels. As testosterone is crucial to man, the findings reported in this thesis may have important implications for the general health and longevity of man.

Publication and Presentations Relating to this Thesis:

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- Awarded a prize for poster presentation at BES conference, Harrogate, UK (2013). 'Perinatal origins of adult Leydig cells and function: role of developmental androgens'.
- Poster presentation at 7th European Testis Workshop on Endocrine Disruptors, Copenhagen, Denmark (2013). 'Perinatal origins of adult Leydig cells and function: role of developmental androgens'.
- Awarded best student oral presentation at the Society for Reproduction and Fertility conference, Edinburgh, UK (2012). 'Perinatal origin of putative adult Leydig progenitor cell: role of COUP-TFII and androgens'.
- Selected in the top ten best poster presentations at the 7th European Congress of Andrology, Berlin, Germany (2012). 'Perinatal origins of adult Leydig cells and function: role of developmental androgens'.
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List of Abbreviations

3 β -HSD	3 beta hydroxysteroid dehydrogenase
AGD	Anogenital Distance
AIS	Androgen insensitivity syndrome
ALC	Adult Leydig cells
AMH	Anti Müllerian Hormone
AMHR2	Anti-Müllerian hormone receptor 2
aP2	Adipocyte protein
AR	Androgen Receptor
ARE	Androgen response element
ARKO	Androgen receptor knockout
bp	base pairs
BSA	Bovine serum albumin
BSA	Bovine serum albumin
CAH	Congenital adrenal hyperplasia
CAIS	Complete androgen insensitivity syndrome
cAMP	cyclic adenosine monophosphate
CD	Cluster of differentiation
ChIP	Chromatin Immunoprecipitation
CO ₂	Carbon dioxide
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor II
CpG	Cytosine-guanine dinucleotide
Cre	Cyclisation recombination
CREB	cAMP response element (CRE)-binding protein)
CRF	Corticotrophin releasing factor
DAB	3,3'-diaminobenzidine
DAPI	4',6- Diamidino-2-Phenylindole dihydrochloride

DBP	Di- <i>n</i> -butyl phthalate
DES	Diethylstilboestrol
Dhh	Desert hedgehog
DHT	Dihydrotestosterone
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DNMT	Deoxyribonucleic acid methyltransferase
DSD	Disorders of sexual development
E	embryonic
EDS	Ethane dimethane sulphonate
EED	Embryonic ectodermic development
EGFR	Epidermal growth factor receptor
ER	Oestrogen receptor
EZH	Enhancer of zeste
FGF9	Fibroblast growth factor 9
FSH	Follicle stimulating hormone
FSHRKO	Follicle stimulating hormone receptor knockout
GATA4	GATA-binding protein 4
GFP	Green fluorescent protein
GnRH	Gonadotrophin releasing hormone
GREAT	G-protein-coupled receptor affecting testis descent
GTP	Guanosine-5'-triphosphat
H	Histone
H3K27me3 (H3)	Trimethylation (me3) of lysine (K27) at histone 3
H3K4me3 (H3)	Trimethylation (me3) of lysine (K4) at histone 3
hCG	Human chorionic gonadotrophin
HDL	High density lipoprotein

HMT	Histone methyltransferase
HPG	Hypothalamic-pituitary-gonadal
HRP	Horseradish peroxidase
HSP	Heat-shock chaperone proteins
IGF1	Insulin-like growth factor 1
IL-1	Interleukin-1
iNOS	Inducible nitric oxide synthase
InsI3/RLF	Insulin-like 3/Relaxin like factor
ITT	Intratesticular testosterone
IUGR	Intrauterine growth retardation
LBD	Ligand binding domain
LDL	Low density lipoprotein
LH	Luteinising Hormone
LHR	Luteinising hormone receptor
LHX9	LIM homeobox 9
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
LSD1	Lysine-specific demethylase 1
LuRKO	Luteinising hormone receptor knockout
MDF/25-HC	Macrophage derived factor/25-hydroxycholesterol
miRNA	Micro ribonucleic acid
MPW	Masculinisation programming window
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NChS	Normal chicken serum
NO	Nitric oxide
NOS	Nitric oxide synthase
NP	Natriuretic peptides

NRS	Normal rabbit serum
NTD	N-terminal domain
PACAP-27	Pituitary adenylate cyclase-activating polypeptide
PAIS	Partial androgen insensitivity syndrome
PAIS	Partial androgen insensitivity syndrome
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR2	Polycomb-repressive complex 2
PDGFR α	Platelet derived growth factor receptor α
PDGFs	Platelet derived growth factors
PGK	Phosphoglycerate kinase
PI	Protease inhibitor
Pip2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase 2
Pnd	Postnatal
PRC2	Polycomb repressive complex 2
PTCH1	Patched 1
PTM	Peritubular Myoid
PTMARKO	Peritubular myoid knockout
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCARKO	Sertoli cell androgen receptor knockout
SCP-2	Sterol carrier protein-2
SF1	Steroidogenic factor 1
SH2	Src homology 2 domain
SHBG/ABP	Sex hormone binding globulin/Androgen binding protein

SMA	Smooth muscle actin
SREBP	Sterol regulatory element binding proteins
StAR	Steroid Acute Regulatory Protein
T3	3,5,3-triiodo-L-thronine
T4	Thyroxine
TBR/TSPO	Peripheral benzodiazepine receptor/transporter protein
TBS	Tris buffered saline
TDS	Testicular Dysgenesis Syndrome
Tfm	Testicular feminisation
TP	Testosterone Propionate
TR α 1	Thyroid hormone receptor α 1
TSH	Thyroid stimulating hormone
VEGF	Vascular Endothelial Growth Factor
VIP	Vasoactive intestinal peptide

Chapter 1 Literature Review

Normal testosterone level/function is fundamental for man, firstly during fetal life to become male and secondly during adult life for reproductive function and overall health (Verhoeven et al 2010). Perturbations to fetal androgen level/function can cause a variety of disorders, which can manifest at birth e.g. cryptorchidism and/or hypospadias, or in young adulthood e.g. testicular cancer and low sperm counts, all of which are thought to be consequences of abnormal fetal testicular somatic cell function. These disorders, which are believed to originate in fetal life comprise a 'testicular dysgenesis syndrome' (TDS) (Sharpe & Skakkebaek 2001, 2003, 2008). This proposes that abnormal development of the testis results in malfunctioning testicular cells, thus increasing the risk of reproductive disorders or TDS. The precise mechanisms behind abnormal testicular fetal development of individuals with TDS remain to be identified.

1.1 Testicular dysgenesis syndrome

Investigating how fetal events i.e. abnormal androgen level/action predetermines adult reproductive function would ideally be carried out in the human male. However, as there is limited access to fetal testes or to fetal blood to measure testosterone levels, the evaluation of normal/abnormal testis development in humans is difficult. In order to examine the effects of altering fetal androgen action/levels on male reproductive development, rodents provide a very useful model. In line with this, fetal di-*n*-butyl-phthalate (DBP) exposure in rats induces a TDS like syndrome in male offspring, resulting in cryptorchidism, hypospadias, impaired spermatogenesis, altered hormone levels, focal areas of dysgenesis and Leydig cell aggregation and dysfunction (Mylchreest et al 2000, Fisher et al 2003, van den Driesche et al 2012). These disorders, which are comparable to the human TDS phenotype, may arise as a result of androgen insufficiency during the critical masculinisation period (Welsh et al 2008, Wohlfahrt-Veje et al 2009). The rodent model for human TDS provides a useful model for investigating potential mechanisms behind 'fetal programming' of adult male reproductive function and disease risk.

TDS is believed to have a foetal origin as a result of genetic, environmental, lifestyle factors or a combination of all three (Skakkebaek et al 2001, Sharpe and Skakkebaek, 2008). Cryptorchidism may serve as a marker for TDS or a risk factor for infertility (Toppari et al 2010), and its incidence appears to be increasing both in the UK (Acerini et al 2009) and Europe (Main et al 2010). From the mid 1900's to the present date, studies have reported an increasing secular trend in reproductive abnormalities e.g. hypospadias and/or cryptorchidism, decreased spermatogenesis (Giwerzman, 1995, Paulozzi et al 1999, Skakkebaek et al 2001, Acerini et al 2009, Main et al 2010, Toppari et al 2010) and an increase in testicular cancer in Europe (Richiardi et al 2004, Bray et al 2006) and the USA (Shah et al 2007). Furthermore, there is evidence for declining semen quality (Andersen et al 2000, Jorgensen et al 2002, 2006, Nordkap et al 2012) and sperm quality (Jorgensen 2002, 2006, Aitken, 2013). An indication/read-out of fetal androgen exposure is provided by the anogenital distance (AGD) (Eisenberg et al 2012, Dean & Sharpe 2013). Semen quality and infertility were shown to be associated with reduced AGD (reduced fetal androgen exposure) (Mendiola et al 2011, Eisenberg et al 2011). Overall, there appears to be an international increase in the prevalence of male reproductive disorders (Serrano et al 2013). Recent studies from the US (Travison et al 2007) and Europe (Andersson et al 2007, Perheentupa et al 2013) have also shown that adult male testosterone levels are gradually declining, independent of age. One proposed hypothesis for declining testosterone levels is 'fetal programming' i.e. fundamental events during fetal development are pre-determining testosterone levels in adult life (Vanbillemont et al 2010, Traish et al 2011). The importance of investigating this phenomenon is highlighted by the association between low adult testosterone and increased risk of the metabolic syndrome i.e. hypertension, cardiovascular diseases, type 2 diabetes (Muraleedharan et al 2010, Traish et al 2011, Tsujimura et al 2013) and mortality (Laughlin et al 2008, Tivesten et al 2009).

1.2 Abnormal androgen action or levels in fetal life

Abnormal androgen action during fetal life can result in male infertility, as evident from patients with complete or partial androgen insensitivity syndromes (CAIS and PAIS) (Hughes et al 2012). This manifests as under-masculinisation of the external genitalia e.g. hypospadias and/or cryptorchidism and are the most common congenital disorders in humans (Boisen et al 2004, 2005). AGD is reduced in boys presenting with cryptorchidism or hypospadias (Thankamony et al 2013), thus highlighting androgen insufficiency as one of the primary causes. Complete and partial AIS most frequently occur due to mutations in the androgen receptor gene or due to a deficit in androgen production or exposure. CAIS individuals are genetically male (XY) but present with a female phenotype and often with cryptorchidism (Hughes et al 2012). Rodent models are extremely useful to investigate human CAIS or PAIS and include transgenic mice (Cre/loxP technology) with mutation in the *AR* gene, essentially rendering the model insensitive to androgens e.g. the ubiquitous androgen receptor knockout (ARKO) mouse model or cell-specific ARKO (De-Gendt et al 2005, Verhoeven et al 2010) and will be discussed later in this review. Abnormal fetal testosterone production, either as a result of genetic mutations (as above) or environmental e.g. exposure to phthalates (e.g. DBP) can affect adult reproductive function (Jensen et al 2004, Kristensen et al 2011, Toppari et al 2010).

1.3 Clinical studies of compensated Leydig cell failure

A large study that investigated serum hormone levels in 318 fertile and 357 infertile men, demonstrated that on average, the 'infertile group' exhibited a 19% increase in luteinising hormone (LH) and an 18% reduction in testosterone serum level, in comparison to the 'fertile' group of men. This is indicative of compensated adult Leydig cell failure whereby increased LH is necessary to maintain a low-normal testosterone level (Andersson et al 2004). Patients with TDS commonly display abnormal/compensated adult Leydig cell failure, as evident from increased LH and decreased testosterone (Joensen et al 2008,

Lardone et al 2012). In long-term follow up studies of males with disorders of sexual development (DSD), analysis of their serum hormone levels demonstrated an increase in LH and FSH with low-normal testosterone, indicative of compensated adult Leydig cell failure (Kojima et al 2009, van der Zwan et al 2013). This phenotype is comparable to the ARKO murine model which also demonstrates compensated adult Leydig cell failure (Yeh et al 2002, DeGendt et al 2005), thus providing a useful model to investigate the mechanisms involved in abnormal adult Leydig cell function. Understanding the mechanisms via which genetic or lifestyle/environmental exposures lead to TDS or CAIS e.g. by affecting Leydig cells is pivotal to our understanding of testicular disorders.

In order to investigate whether fetal events, namely androgen action/level, contribute to testicular cell dysgenesis and thus determine adult Leydig cell function, it is firstly important to consider how the testis forms during fetal life, the organisation of testicular cells and factors which may be involved in adult Leydig cell development.

1.4 The bipotential gonad

The bipotential gonads develop from within the urogenital system of the developing embryo, comprising of separate regions; pronephros, mesonephros and metanephros. The kidneys arise from the metanephros while the gonads develop from the medial side of the mesonephros (Byskov, 1986). As a result of proliferation of the coelomic epithelium which overlies the mesonephroi, an 'outgrowth' is formed, termed the 'genital ridge' and is first evident in mice at embryonic day (e) 10.5 (Merchant, 1975, Schmahl et al 2000). There is no difference in the morphology between XX and XY genital ridges and at this point, the genital ridges can develop into either testis or ovary, depending on the specific genes and cellular activity (Sekido and Lovell-Badge 2013).

1.4.1 Sex determination

Sex determination of the embryo is generally known to be controlled by the presence or absence of the 'Y' chromosome in mammals. As most studies involving gonadal development and regulation have been undertaken in the rodent, due to its accessibility and the generation of knockout models for investigation of specific genes, the rodent model will be referred to most frequently in this review. The expression of the Y-linked gene, *Sry*, initiates male (XY) development and was coined the "testis-determining" gene (Gubbay et al 1990, Sinclair et al 1990, Berta et al 1990, Koopman et al 1991, Basciani et al 2010). In the absence of the testis-determining gene, *Sry*, the gonad develops into an ovary, evident from deletion/mutation of *Sry* (Gubbay et al 1992, Hawkins et al 1992) leading to the original assumption that the female pathway was simply the "default". In the presence of *Sry* and/or *Sox9*, female (XX) gonads, can be induced to develop as testes, while gonads in *Sox9*^{-/-} XY males develop as females (Koopman et al 1991, Vidal et al 2001, Sekido and Lovell-Badge 2013). Heterozygous mutations in *Sox9* in the human have been associated with XY male sex reversal (Gordon et al 2009).

However, rather than the simplistic view of the female XX pathway of development being the 'default', it is now clear that in females, male sex development is actively repressed by female factors, namely *Foxl2*, which encodes a forkhead transcription factor. Conditional deletion of this factor in adult mouse ovaries, leads to gonadal sex reversal, specifically ovarian granulosa and theca cells change their cell fate into Sertoli and Leydig cells respectively. Moreover, testosterone levels in XX female adult *Foxl2*^{-/-} mice were similar to adult control XY mice (Uhlenhaut et al 2009). The granulosa cells also expressed *Sox9* which highlights the opposing roles of *Sox9* and *Foxl2*. Wingless type MMTV integration site family, member 4 (*Wnt4*), is regulated by R-spondin 1 (*RSP01*) which suppresses male sexual differentiation and promotes Müllerian duct differentiation in the female. Loss of both *Wnt4* and *Foxl2* leads to XX-XY sex reversal (Ottolenghi et al 2007). Although development and regulation of female gonadogenesis is out of the scope of this review, it is

important to note that sex determination is now viewed as an active process, involving several supporting and opposing regulatory genes from both XX and XY gonads (Piprek et al 2010). The development of the testis and the timing of differentiation of its comprising cells are described below and summarised in Figure 1.1 (page 10).

1.5 Male Reproductive Development

1.5.1 Testis formation and development

The adult testis is the site of spermatogenesis and steroidogenesis and thus its formation and organisation is essential for normal male functioning. This organised structure is a source of testosterone producing interstitial Leydig cells which this review will focus on. Sertoli and germ cells are contained within seminiferous cords and enveloped by peritubular myoid cells. The testis first begins to form when germ cells migrate from the yolk sac into the undifferentiated/bipotential gonad, which causes the coelomic epithelial cells to proliferate. The increased proliferation is evident from the increase in gonad size which is indicative of a 'masculinised' or male gonad (Schmahl et al 2000). The testis structure is in place at approximately e13 in the mouse and 6 weeks in the human (O'Shaughnessy et al 2011).

1.5.2 Coelomic epithelium

The coelomic epithelium describes the layer of coelomic epithelial cells which form in both XY and XX genital ridges and which encapsulate the gonad (Bullejos and Koopman 2001, Rey and Grinspon 2011). These cells proliferate and increase the size of the XY gonad at e11.2 in the mouse in response to the male specific fibroblast growth factor 9 (FGF9) (Schmahl et al 2000, 2003) as a result of *Sry* expression. Coelomic epithelial cells which express steroidogenic factor-1 (SF1⁺) give rise to the Sertoli (Schmahl et al 2000) and fetal Leydig cells (Defalco et al 2011).

1.5.3 Mesonephros

The gonad develops on the medial surface of the mesonephros. Following *Sry* expression, migrating cells from the mesonephros lead to the formation of testis cords (Buehr et al 1993, Tilmann and Capel 1999). It has been suggested that the migrating mesonephric cells can give rise to interstitial cells (Buehr et al 1993, Merchant-Larios et al 1993) and specifically Leydig cells (Merchant-Larios and Moreno-Mendoza 1998, Nishino et al 2001, Val et al 2006). The mesonephroi also contain a sex-specific ductal system, the mesonephric Wolffian ducts and the paramesonephric Müllerian ducts in males and females respectively. In males, the Wolffian ducts develop into the epididymis, seminal vesicles and vas deferens. Anti-müllerian hormone (AMH) acts to regress the female ductal system in males, which is discussed later in this review.

1.5.4 Primordial germ cells

Primordial germ cells are first evident in the genital ridges at e11 in the mouse and 4 weeks' gestation in humans (Culty et al 2009). Unlike the other testicular cells, as described above, which migrate from the mesonephros or coelomic epithelium, the primordial germ cells proliferate during and after migration from the hindgut into the genital ridge (McLaren, 1991). Once present in the genital ridge, the primordial germ cells mature into fetal germ cells and continue to proliferate until e15.5-e16.5 in the mouse or e17.5-e18.5 in the rat and then enter quiescence (Culty et al 2009). The germ cells become mitotic again between postnatal (pnd) 1-4 in the mouse and pnd 4-6 in the rat (Ferrara et al 2006) and migrate toward the basement membrane of the seminiferous cords and differentiate into spermatogonial stem cells (Boulogne et al 1999, Culty et al 2009). Spermatogenesis describes the postpubertal progression of germ cells from spermatogonia, to spermatocytes, to spermatids and is maintained by testosterone stimulation from Leydig cells acting via Sertoli cells (Svingen and Koopman 2013).

1.5.5 Sertoli cells

Following *Sry* expression at e10.5 in the mouse gonad, gonadal somatic cells begin to differentiate into Sertoli cells, arising from the coelomic epithelium (Cool et al 2008), which serve three significant fetal functions. Firstly, Sertoli cells aggregate in a layer around germ cells, thus forming seminiferous cords, where they continue to proliferate and elongate the seminiferous cords (Nel-Themaat et al 2011). It is unlikely that germ cells are the focal point for Sertoli cell aggregation into seminiferous cords, as in XY gonads where germ cells are absent, seminiferous cords are formed nonetheless (Escalante-Alcalde and Merchant-Larios 1992). Secondly, Sertoli cells also produce and secrete Desert hedgehog (Dhh) (Pierucci-Alves et al 2001) (detailed in section 1.7.9) which induces fetal Leydig cell differentiation (Bitgood et al 1996, Yao et al 2002, Griswold and Behringer 2009). Thirdly, Sertoli cells also function to secrete AMH (detailed in section 1.5.8.1) which is necessary for regression of the Müllerian ducts. Sertoli cells do not express AR during fetal life (Majdic et al 1995, Williams et al 2001, Sharpe et al 2003) but during postnatal life, androgen signalling via the Sertoli cells is essential for spermatogenesis (De Gendt et al 2004). Each adult Sertoli cell supports a fixed number of germ cells and this number is reached post-puberty when Sertoli cells are no longer mitotic (Sharpe et al 2003). Specialised tight junctions between each Sertoli cell, forms the "blood-testis-barrier" at puberty (Cheng and Mruk 2010).

1.5.6 Seminiferous cord formation

The formation of seminiferous cords is a crucial event, marking testis differentiation (Svingen and Koopman 2013). Testis cords separate Sertoli cells and gonocytes from the interstitium to create a separate environment for spermatogenesis, which occurs from puberty onwards. Seminiferous cords are first evident in the mouse at e12 (Combes et al 2009). Vascular endothelial cells have been shown to play a role in testis cord formation as interstitial cells respond to vascular endothelial growth factor, (VEGF), signalling from migrating cells and proliferate forming testis cords (Cool et al 2011). Together

with vascular endothelial cells, migrating endothelial cells from the mesonephros are also involved in cord formation, as evident from the abnormal/disrupted cord formation following blockage of cell migration and vascular formation via VE-cadherin (Combes et al 2009).

1.5.7 Peritubular myoid cells

At e13 in the mouse, a single layer of spindle shaped elongated cells, termed peritubular myoid (PTM) cells, form around the layer of Sertoli cells (Skinner et al 1985). Earlier studies suggested that PTM cells arose from the mesonephric layer (Merchant-Larios et al 1993, Martineau et al 1997), however more recent studies demonstrate that migrating mesonephric cells contribute rather to the endothelial cell population and testis vasculature, than to the PTM cells (Cool et al 2008, Combes et al 2009). The precise ontogeny of PTM cells remains unknown although some authors (Combes et al 2009) propose the coelomic epithelium is a likely origin whereby PTM cells arise by "epithelial-to-mesenchymal" transformation. PTM cells express AR during fetal and postnatal life (You and Sar 1998, Welsh et al 2009, 2012) and in relation to Leydig cells, specific AR knockout in PTM cells results in altered adult Leydig cell function, although their numbers were unchanged (Welsh et al 2012). This suggests a role for PTM cells in regulating normal adult Leydig cell function via androgen signalling.

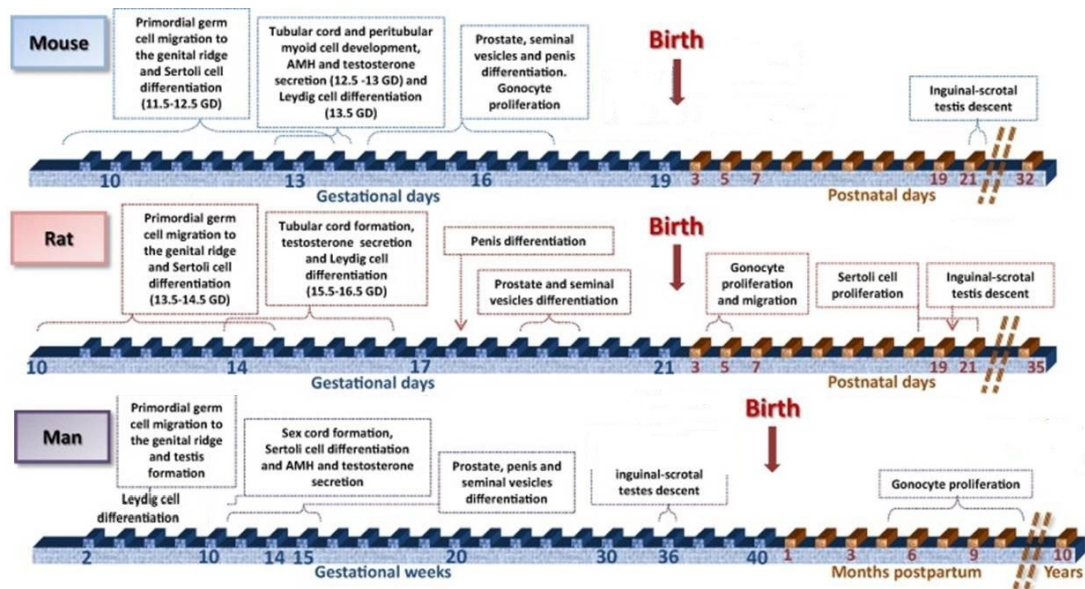


Figure 1.1 Timing of reproductive development in rodents and man (Adapted from Wilhelm and Koopman 2006).

1.5.8 Masculinisation

Masculinisation of the fetus is an androgen-dependent process (Zirkin, 2010, Swrdloff et al 2010, Svehnikov et al 2014) and occurs from e15.5-17.5 in the rat and during weeks 8-12 in the human during a critical period termed the 'masculinisation programming window' (MPW) (Welsh et al 2008, Scott et al 2009). This involves differentiation and development of the internal (epididymis, vas deferens, seminal vesicles and prostate) and external (penis, scrotum and perineum) genitalia (Svehnikov et al 2014). Fetal masculinisation is driven by three crucial hormones, AMH, *Insl3* and testosterone (Rey et al 2012), which are described in more detail in the following sections.

1.5.8.1 Anti-Müllerian hormone

Anti-müllerian hormone (AMH) or müllerian inhibitory substance (MIS) is secreted by fetal Sertoli cells as a 140kDA glycoprotein to cause regression of female müllerian reproductive derivatives (Ingraham et al 2000). This occurs between e14-17 in the rat whereby AMH binds to its receptor AMHR2 on

mesenchymal cells of the müllerian duct to induce their apoptosis (Mishina et al 1996). Its potential role during adult Leydig cell differentiation will be discussed later (Section 1.7.4).

1.5.8.2 Insulin-like factor 3

Insulin-like factor 3 (Insl3 or relaxin-like factor, (RLF)) is produced by fetal Leydig cells (Ivell and Bathgate 2002, Foresta et al 2008) and is involved in the first (trans-abdominal) phase of testis descent. This occurs when Insl3 binds to its receptor (G-protein-coupled receptor affecting testis descent, GREAT) present on the gubernacular ligaments, which links the testis to the inguinal region of the abdominal cavity (Ivell and Hartung 2003, Park et al 2008). Testosterone induces the regression of the cranio-suspensory ligament, which is required to release the testis from the abdominal wall for descent into the inguinal region. Without functional Insl3, as evident from the disruption of *Insl3* in transgenic mice, testes remain intra-abdominal (Figure 1.2) (Zimmermann et al 1999, Ivell et al 2009). The second phase of testicular descent (inguino-scrotal) is predominantly androgen-dependent and occurs due to shortening of the gubernaculum cord which pulls the testis through the inguinal canal and into the scrotum (Hughes and Acerini 2008). This occurs between pnd 10-14 in the rat completing testis descent (Amann and Veeramachaneni, 2007). In humans, the process of testis descent is normally completed prior to birth (Hutson et al 2013). However, incomplete descent is a common finding in boys at birth, although the incidence varies between countries, it has generally been shown to occur in 2-9% of boys at birth and the majority of cases result from a failure of the inguino-scrotal phase of descent (Foresta et al 2008). In contrast, incomplete testis descent in rats and mice is extremely rare (<0.5%) (Amann and Veeramachaneni 2007). Patients with AIS commonly display undescended testes due to lack of androgen-induced inguino-scrotal descent (Hughes and Acini 2008). Fetal rats exposed to DBP during the masculinisation programming window, demonstrate a significant reduction in the production of Insl3 and testosterone by fetal Leydig cells, which results in cryptorchidism (Mylchreest et al 2000, Foster et al 2001, van den Driesche et al 2012).

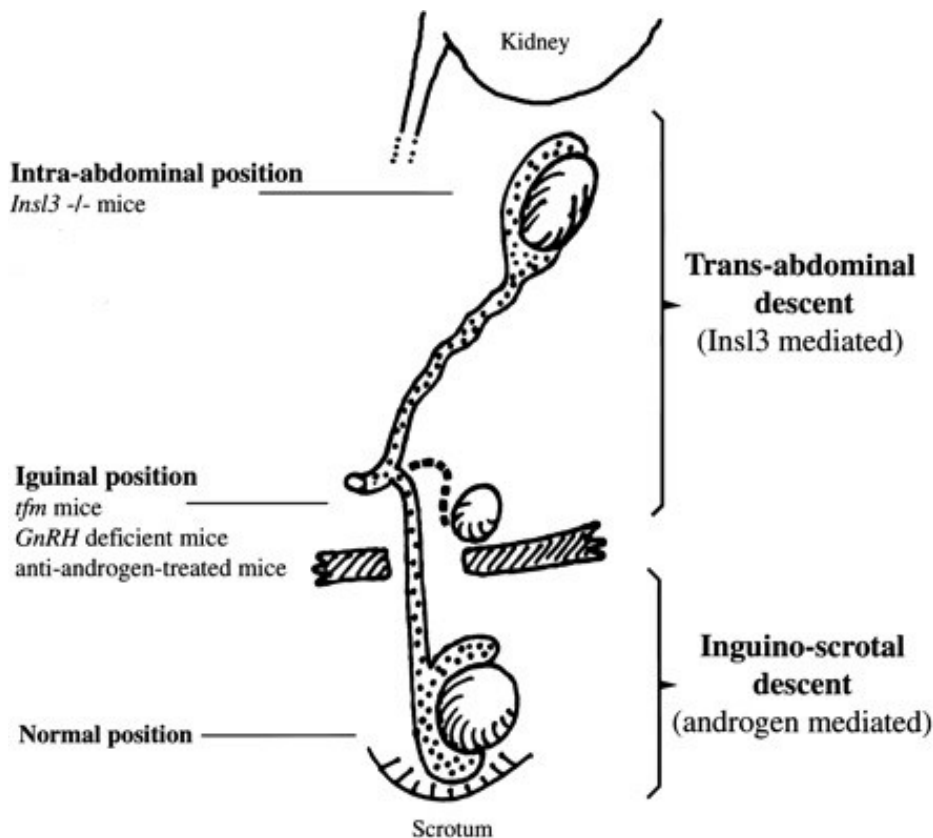


Figure 1.2 Testis Descent. The schematic depicts testis descent and the role of *Ins13* and androgens in this process. (Adapted from Nef and Parada et al 2000).

1.5.8.3 Testosterone and fetal masculinisation

Testosterone produced by fetal Leydig cells is crucial for the differentiation of the internal reproductive organs (epididymis, vas deferens, seminal vesicles, prostate) and the external genitalia (penis, scrotum) and perineum (anogenital distance) (Imperato-McGinley et al 2002, Welsh et al 2008, Murashima et al 2011). Testosterone is converted into dihydrotestosterone (DHT) by 5 α -reductase and signals through the androgen receptor in peripheral target tissues, as mentioned above, to masculinise the external genitalia and the prostate (Svechnikov et al 2014). Testosterone is also important in testis descent, as mentioned above.

Anogenital distance (AGD) is twice the length in males compared to females in humans (Salazar-Martinez et al 2004, Swan et al 2005, 2008, Thankamony et al 2009, Sathyanarayan et al 2010, Eisenberg, 2011) and in rats (McIntyre et al 2001, Zhang et al 2004, Welsh et al 2008), and provides a permanent read out of androgen exposure during fetal life (Welsh et al 2008, MacLeod et al 2010, Dean and Sharpe 2013). AGD is decreased following fetal exposure to DBP in male rats, as a result of decreased androgen exposure (Foster et al 2000, Mylchreest et al 2000, Barlow and Foster 2003, van den Driesche et al 2012).

1.6 Identification of Leydig cells

Interstitial cells of the testis were coined 'Leydig cells' following their discovery in 1850 by Franz von Leydig, however it was not until the 1880's and early 1900's that researchers began to realise the endocrine function of Leydig cells. The potential for endocrine factors in the testis was introduced in 1849 with Arnold Berthold's experimental transplantation of testes into castrated roosters, which resulted in the roosters exhibiting male sexual characteristics. This suggested that a hormone from the blood circulation was causing the 'male traits' as the nerve supply to the testis was severed. Berthold's study, a year prior to the identification of Leydig cells, has been widely accepted as the start of endocrinology. The controversial studies of Brown-Séguard in 1889 who claimed a 'beneficial effect' from self-injection with animal testes extracts, further highlighted a role for the testis as an endocrine gland. The hypothesis that it was in fact the Leydig cells which had an endocrine function, was shown by Bouin & Ancel in 1903-1905. Finally in 1958, the first direct evidence that Leydig cells were steroidogenic and could produce testosterone came from an enzymatic activity experiment demonstrating the expression of the steroidogenic enzyme 3β -HSD in Leydig cells (Wattenberg, 1958).

1.6.1 Separate Leydig cell lineages

As previously described, Leydig cells are essential testicular cells for endocrine function, producing the steroid hormone testosterone. Leydig cell development in the human is considered a 'triphasic event', evident with separate lineages of Leydig cells developing during fetal, neonatal and pubertal periods (Prince 2001). The developmental timing of testosterone production in the rodent is substantially faster than the human, with only two separate lineages of Leydig cells, 'fetal' and 'adult', evident during fetal and postnatal life respectively (Rosen-Runge and Anderson 1959, Lording and De Kretser 1972, Ariyaratne and Mendis-Handagama 2000, Habert et al 2001, O'Shaughnessy et al 2002, Chen et al 2009). 'Fetal Leydig cells' are required for masculinisation of the fetus whereas 'adult Leydig cells' are essential from puberty onwards for spermatogenesis, sex drive and overall male health. There is continuing debate over Leydig cell origin, and various hypotheses have been proposed e.g. that both fetal and adult Leydig cell populations arise from a common precursor cell, fetal Leydig cells then regress after birth and adult Leydig cells differentiate from the common precursor which lies dormant until puberty (Barsoum et al 2013, Svingen and Koopman 2013). Both generations will be discussed in this review.

1.6.2 Fetal Leydig cells

The first population of Leydig cells, the fetal generation, are evident at e12.5 in the mouse (Baker et al 1999), and 7-8 weeks in human, having differentiated from 'mesenchymal-like stem cells' (Byskov, 1986, O'Shaughnessy et al 2006, Ostrer et al 2007). The fetal Leydig cells have also been postulated to arise from the adrenogenital primordium, which gives rise to steroidogenic cells in the adrenal and gonads (Hatano et al 1996), with some gonad steroidogenic cells retaining "adrenal-like" properties (Val et al 2006). More recent studies describe two origins for fetal Leydig cells, namely the coelomic epithelium and the gonad-mesonephric border (DeFalco et al 2011). As steroidogenesis requires cholesterol as a substrate and cholesterol is not evident in the testis

prior to testosterone production (Budefeld et al 2009), it suggests that cholesterol might be exogenously derived during this period, likely from the coelomic blood vessel or vascular plexus near the gonad-mesonephric border.

The expression of steroidogenic factor 1 (SF1) (as a result of *Sry*) increases in differentiating Leydig cells which initiates Leydig cell steroidogenesis (Barsoum et al 2013), evident by testosterone production at e13 in the mouse (O'Shaughnessy and Fowler 2011) and 7 weeks of gestation in the human (O'Shaughnessy et al 2007, Scott et al 2009). The fetal population of Leydig cells regress after birth or are superseded by adult Leydig cells (Baker et al 1999, Chen et al 2009, Scott et al 2009, O'Shaughnessy and Fowler 2011). Both cell generations differ in their morphology, function and regulation (O'Shaughnessy et al 2002, Dong et al 2007, Scott et al 2009, Pinto et al 2010, Kotula-Balak et al 2012) and will be discussed in this review.

1.6.3 Function and regulation of fetal Leydig cells

In the rat, morphological (Jost et al 1981) and functional differentiation of Leydig cells occurs at e15.5, coincident with testosterone production (Warren et al 1973, Habert & Picon 1984). Fetal Leydig cells produce androgens which induce the formation of the epididymis, vas deferens and seminal vesicles from the Wolffian ducts, and development of the penis and scrotum (Scott et al 2009) along with the production of *Insl3*, which is crucial for testis descent (Ivell et al 2009). Testosterone produced by fetal Leydig cells is converted by 5α -reductase into dihydrotestosterone (DHT) in certain target peripheral tissues such as the external genitalia, which virilises these tissues.

Fetal Leydig cell development and initial steroidogenesis occurs in a gonadotrophin-independent manner (Habert and Picon 1982, O' Shaughnessy et al 1998, 2002, Baker et al 2001, Migrenne et al 2001), as evident from their ability to produce androgens required for fetal masculinisation prior to the establishment of the hypothalamic-pituitary-gonadal (HPG) signalling axis

(Huhtaniemi 1989, O'Shaughnessy and Fowler 2011). There is some evidence to suggest that factors secreted by Sertoli cells including Dhh (Byskov 1986, Koopman 2001, Pierucci-Alves et al 2001, Yao et al 2002) and/or the extracellular matrix (Jost et al 1988) may be involved in this process. Other paracrine factors in the testis e.g. vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP-27) have also been shown to stimulate fetal, but not adult Leydig cell steroidogenesis (El-Genahi et al 1998). Natriuretic peptides (NP) e.g. brain and C-type (BNP and CNP) have also been shown to stimulate steroidogenesis in the fetal rat testis (El-Genahi et al 2001) and adult mouse testis (Khurana and Pandey 1993).

Chicken ovalbumin upstream promoter transcription factor II has been recently identified as a molecular target for DBP-induced testicular dysgenesis (van den Driesche et al 2012). This study demonstrated the abundant expression of COUP-TFII in fetal Leydig cells, interstitial, and PTM cells. In control animals, COUP-TFII is down-regulated in fetal Leydig cells during differentiation. However in DBP-exposed animals, COUP-TFII expression is maintained in the fetal Leydig cells which results in decreased fetal Leydig cell maturation and a 70% reduction in testosterone production (van den Driesche et al 2012). This study proposed that down-regulation of COUP-TFII in fetal Leydig cells may be an important regulatory mechanism to promote testosterone production. The role of COUP-TFII in adult Leydig cell differentiation is described later in this review.

1.6.4 Adult Leydig cells

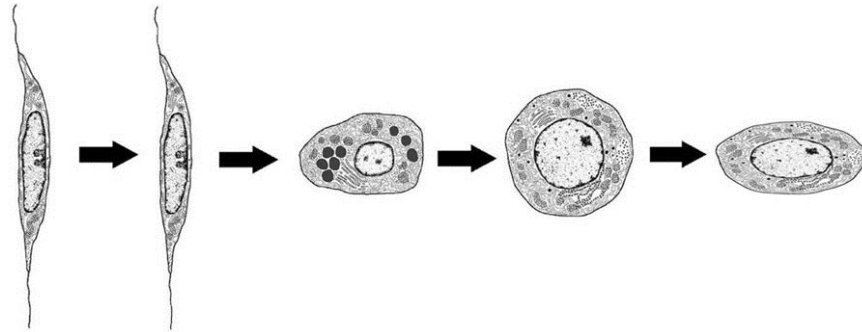
Adult Leydig cells gradually decline in number and function with increasing age in the human testis (Neaves et al 1984, 1985, Zirkin and Tenover 2012). In ageing rodents, adult Leydig cells become less responsive to LH and thus produce less testosterone with increasing age (Chen et al 2009). Adult Leydig cells have been proposed to arise from a multitude of sources; interstitial mesenchymal stem cells located in close proximity to peritubular cells (Mendis-

Handagama and Ariyaratne 2001, Ge and Hardy 2007), pericytes in the vasculature (Davidoff et al 2004) or peritubular myoid cells (Landreh et al 2013), which supports the notion of a multifocal origin of adult Leydig cells (Haider, 2004, Habert et al 2001).

Four stages of adult Leydig cell development have been identified in the rat; stem, progenitor, immature and adult (Hu et al 2009, Chen et al 2010) (Figure 1.3) with similar stages evident in humans, over an extended time frame (Prince et al 2001). The adult Leydig 'progenitor' cell is the first evidence of a committed interstitial cell to the adult Leydig cell lineage, found in the rat testis between pnd 10-14 and the mouse at pnd 7-10 (Wu et al 2010). Progenitor Leydig cells express 3 β -HSD and CYP11A1 and mainly produce androsterone and androstenedione (Haider et al 1986, Shan et al 1993, Ge et al 1998, O'Shaughnessy et al 2000, Chen et al 2009, Stanley et al 2011). Progenitor cells also express a truncated form of the LH receptor (Hu et al 2010). Progenitor Leydig cells continue to proliferate and their cytoplasm enlarges, and becomes rounded in shape, which marks their differentiation into immature Leydig cells, evident at pnd 28-56 in the rat (Ge et al 1998, Stanley et al 2011). Immature adult Leydig cells have a high lipid content and high expression of androgen-metabolising enzymes, namely 3 α -HSD and 5 α -reductase, which causes androstenediol to be the most abundant androgen at this stage of Leydig cell development (Chen et al 2009, Wu et al 2010). The immature adult Leydig cells differentiate into large rounded cells with abundant smooth endoplasmic reticulum, few lipid droplets and high steroidogenic enzyme expression to produce testosterone (Chen et al 2009, Stanley et al 2011). This final differentiation of immature cells (between pnd 28-56) results in the testosterone producing, adult Leydig cell population (Stanley et al 2011).

It has long been a question of debate as to whether or not progenitor Leydig cells differentiate from a pool of undifferentiated self-renewing 'stem Leydig cells', which has largely remained unanswered due to the lack of a unifying specific marker. It is widely accepted that stem cells are capable of self renewal,

commitment, amplification and differentiation. If there are adult Leydig stem cells, these would be present in the fetal and postnatal testis and their numbers would be maintained by cell renewal divisions throughout postnatal life. One or both of the adult Leydig stem cell progeny would be expected to commit to the Leydig cell lineage, resulting in an increase through amplification of cell divisions, thus giving rise to progenitor Leydig cells (Zirkin et al 2009). Prior to the appearance of progenitor Leydig cells (3β -HSD⁺), undifferentiated (3β -HSD⁻ and LHR⁻) spindle shaped interstitial 'stem Leydig cells' are found at pnd 7 in the rat (Ge et al 2006). Isolation of these stem cells, which express PDGFR α , GATA4, c-kit and LIFR (3β -HSD or LHR negative) followed by cell culture in expansion medium, demonstrated the identified interstitial cells were capable of maintaining a stable phenotype for 6 months (Ge et al 2006). If these cells were further cultured in medium containing thyroid hormone, IGF1 and LH, 40% of the cells expressed steroidogenic enzymes CYP11A1, 3β -HSD, CYP17A1, LHR, and STAR and were capable of producing testosterone. In line with this, the isolated stem cells were also capable of differentiating into 3β -HSD⁺ adult Leydig cells in an ALC ablation/regeneration rat model (Ge et al 2006).



<u>CELLULAR ATTRIBUTES</u>	STEM LEYDIG CELL	PROGENITOR LEYDIG CELL	IMMATURE LEYDIG CELL	ADULT LEYDIG CELL	AGED LEYDIG CELL
Mitotic activity	Low	high	Once	Probably none	Probably none
Morphology	Spindle	Spindle	Round with large lipid droplets	Round with small lipid droplets	Round with small lipid droplets
Smooth ER	Low	Low	High	Highest	High
Testosterone Metabolism	None	High	Highest	Low	Low
Testosterone Synthesis	None	Low	Intermediate	High	Intermediate
Age when cell is typical	7 days	21 days	35 days	90 days	20 months
LH Receptor Number	None	Low	Intermediate	High	Intermediate
Androgen Receptor Number	?	High	High	Low	?

Figure 1.3 Development of adult Leydig cells The development of adult Leydig cells occurs in four stages consisting of stem, progenitor, immature and adult Leydig cells (Chen et al 2009).

1.7 Factors involved in adult Leydig cell differentiation

There are several factors implicated in adult Leydig cell differentiation, including endocrine hormones and paracrine factors produced locally in the testis. Some of the key hormones and factors involved in adult Leydig cell development are described below.

1.7.1 Luteinising hormone

Luteinising hormone (LH) is a key regulator of male reproductive function, specifically the production of testosterone by Leydig cells (Rey and Grinspon 2011). LH is a gonadotrophin which is released from the anterior pituitary gland in response to the hypothalamic hormone GnRH (gonadotrophin releasing hormone) secreted from the hypothalamus. LH is a glycoprotein hormone, composed of an alpha and a beta subunit, which binds to its protein receptor, the LHR (McFarland et al 1989). Due to its protein structure, it is unable to diffuse through the membrane and instead binds to LHR on the cell membrane of Leydig cells, and thus initiates the intracellular signalling pathway for steroidogenesis (Huhtaniemi 1996). Once bound, the LH receptors stimulate a signalling cascade involving an increase in adenylyl cyclase activity via GTP binding proteins which results in the production of cAMP (Cooke 1996) and also increased cytoplasmic Ca²⁺ release following LH stimulation (Abdou et al 2013). Other second messengers include phospholipids and arachidonic acid which are activated in Leydig cells following LH stimulation (Wang et al 2000). Although LH is the natural ligand for the LHR in most adult mammals, hCG produced by the primate placenta can also bind to and activate the LHR (Rey and Grinspon 2011).

LH stimulation is critical for the functional development of adult Leydig cells, as demonstrated in mice lacking circulating LH, in which the number of adult Leydig cells only reached 10% of that in control mice (Baker and O'Shaughnessy 2001). This is also evident in transgenic mice with a mutated LH receptor (LuRKO) which, at birth, resulted in normal testis development and androgen levels but during postnatal life resulted in hypogonadism, blocked development of sex accessory organs, and an arrest in spermatogenesis (Zhang et al 2001). In relation to adult Leydig cells, LHR knockout resulted in a reduction in the size and number of adult Leydig cells (Zhang et 2001, 2004, Ma et al 2004) which was not restored by testosterone administration (Lei et al 2004). Conversely, fetal Leydig cell number and testosterone levels were normal in the aforementioned studies, highlighting the gonadotrophin-independent

development of the fetal Leydig cell lineage in the mouse (O'Shaughnessy et al 1998, Baker et al 2001). However, while LH is crucial for their functional maturation, it does not appear to be involved in the onset of adult Leydig cell differentiation, as adult Leydig cells in rats express steroidogenic enzymes prior to any LHR expression (Ariyaratne et al 2000). This demonstrates the ability of adult Leydig cells to differentiate without initial LH stimulation. Human males with inactivating mutations in the *LHCGR* gene have lower testosterone levels, with elevated LH and mature adult Leydig cells are absent or scarce (Latronico and Arnhold 2012, 2013). Earlier studies of severe inactivating *LHR* mutations in XY humans resulted in a female phenotype (female external genitalia) and Leydig cell hypoplasia. Milder *LHR* mutations resulted in micropenis and hypospadias (Themmen, 2005). Conversely, activating mutations in *LHR* in humans leads to precocious puberty and Leydig cell hyperplasia (McGee and Narayan 2013). While LuRKO mouse models have proved useful for investigating the effects of inactivating *LHR* mutations on Leydig cell development in humans, transgenic mouse models for activating *LHR* mutations are not as well developed. One potentially useful mouse model with an activating *LHR* mutation has been described recently, which displays a similar phenotype to humans with this condition e.g. precocious puberty, increased testosterone and hyperplasia of adult Leydig cells (McGee and Narayan 2013).

1.7.2 Follicle stimulating hormone

Follicle-stimulating hormone (FSH) is one of the gonadotrophins released from the pituitary in response to hypothalamic stimulation by gonadotropin-releasing hormone (GnRH). FSH binds to its receptor (FSHR) in Sertoli cells pre-pubertally, to stimulate Sertoli cell proliferation. In adults, the binding of FSH and testosterone production maintain spermatogenesis (Rey and Grinspon 2011). The product 'inhibin' produced by Sertoli cells is involved in negative feedback regulation of the HPG axis, specifically of FSH (Themmen and Huhtaniemi 2000). FSHRs are first evident in the mouse testis at e16.5

(Rannikki et al 1995), before development of the HPG axis, thus indicating Sertoli cell function, prior to establishment of the HPG axis in mice, is FSH-independent (O'Shaughnessy and Fowler 2011). In line with this, FSHR knockout (FSHRKO) mice did not display any altered Sertoli number or protein expression markers (e.g. Dhh, AMH, PDGF α) from birth to pnd 5 (Johnston et al 2004). In postnatal life, testosterone or DHT act alongside FSH via Sertoli cells to stimulate and maintain spermatogenesis (O'Shaughnessy et al 2012).

An important role for FSH in stimulating Leydig cell development has been shown from FSHRKO^{-/-} mice. The FSHRKO mouse was first reported by Dierich et al 1998, which resulted in oligospermia, reduced fertility, normal LH but a 3-fold increase in serum FSH with decreased testosterone. A later study demonstrated that FSHRKO^{-/-} male mice were fertile although displayed increased serum LH and FSH levels (Abel et al 2000). There is a failure of normal adult Leydig cell development in FSHRKO^{-/-} mice with evidence for compensated Leydig cell failure, evident from increased LH (Abel et al 2008) and decreased testosterone (one third of the wild-type control value) in adult FSHRKO^{-/-} mice (Baker et al 2003). In a more recent FSHRKO^{-/-} study, the number of adult Leydig cells was significantly reduced at pnd 20 and in adulthood (O'Shaughnessy et al 2012), which was exacerbated in a double knockout of FSHRKO-ARKO (O'Shaughnessy et al 2012). To date, there have only been 5 cases of human FSHR mutations reported (Siegel et al 2013). The FSHR gene mutation was reported in five Finnish men who presented with normal-small testis size, abnormal sperm parameters, normal-elevated FSH and LH, with normal testosterone levels (Tapanainen et al 1997), which seems similar to the phenotype of the FSHRKO^{-/-} mouse, making this a useful model.

1.7.3 Insulin-like Growth Factor 1

Insulin-like growth factor 1 (IGF1) and its receptor are expressed in progenitor and immature Leydig cells (Hu et al 2010). IGF-1 has been shown to stimulate steroidogenesis and increase StAR expression at both the mRNA and protein

level in adult Leydig cells via hCG stimulation (Lin et al 1998). IGF-1 treatment of cultured rat Leydig cells in vitro from pubertal and adult animals demonstrated an increase in intracellular steroidogenesis and testosterone production, with a greater response from the pubertal cells than adult, suggesting a role for IGF-1 in Leydig cell maturation (Gelber et al 1992). IGF-null mice have a significant reduction in body weight, adult Leydig cell number and an 80% reduction in serum testosterone levels, compared to wild-type (Baker et al 1996, Wang et al 2003, Hu et al 2010), implicating a role for IGF-1 in adult Leydig cell development and function. IGF-1-null mice express higher levels of 5 α -reductase-1 in adulthood and as a result, testosterone is reduced. Furthermore, mRNA levels of StAR, CYP11A1 and CYP17a1 are also reduced, which suggests that the developing adult Leydig cells are arrested at the immature stage (Wang et al 2003). In a recent study of IGF-1 null mice, the proliferative index of progenitor and immature adult Leydig cells was reduced in *Igf1*^{-/-} mice, and this deficit could be rescued by recombinant IGF-1 (Hu et al 2010). Interestingly, this deficit in proliferative capacity was not evident in 'stem' Leydig cells, in which proliferation was comparable to wild-type controls. Overall, this suggests IGF-1 is involved in the proliferation and differentiation of adult Leydig progenitor cells, at least at pnd 14, in the rat (Hu et al 2010).

1.7.4 Anti-Müllerian Hormone

Anti-Müllerian hormone (AMH) is expressed at high levels pre-pubertally in males (Grinspon and Rey 2010) and its receptor, AMHR2, is found on adult Leydig cells (Lee et al 1999, Fynn-Thompson et al 2003). AMH expression decreases during puberty, coincident with the increasing intratesticular testosterone concentration at this time (Lukas-Croisier et al 2003). This inverse relationship occurs in central precocious puberty and gonadotrophin-independent precocious puberty (Lukas-Croisier et al 2003), demonstrating androgens down-regulate AMH, independent of age and gonadotrophin levels. As testosterone increases at puberty, AMH decreases, which is coincident with

adult Leydig cell differentiation (Grinspon and Rey 2010). A failure for androgens to stimulate the normal reduction in AMH levels at puberty results in hypogonadotrophic hypogonadism, in which patients present with increased AMH and low testosterone levels (Young et al 1999). Furthermore, patients with AIS have high levels of AMH (Rey et al 1994). In rodents, binding of AMH to its receptor on Leydig cells, can inhibit the proliferation of adult Leydig progenitor cells pre-pubertally, and reduces steroidogenesis, potentially via down-regulation of *Cyp17a1* (Racine et al 1998, Trbovich et al 2001). This 'repression' is lifted during puberty with increasing testosterone levels (Grinspon and Rey 2011). Overexpression of AMH in a mouse model, resulted in blockage of adult Leydig cell differentiation at the progenitor stage (Racine et al 1998). This was shown to occur via its receptor (AMHR2), on adult Leydig progenitor cells in both rats and mice (Lee et al 1999).

1.7.5 Oestrogens

Oestrogens are highly expressed within the testis and are produced from the aromatisation of testosterone via P450aromatase/CYP19 (de Ronde et al 2003). Mature adult Leydig cells produce oestrogen and express oestrogen receptors (ER- α and β), as evident in the mouse and rat (Saunders et al 1998, Zhou et al 2002) with mainly ER β expression in human adult Leydig cells (Saunders et al 2001). *ER β* knockout mice have increased adult Leydig cell numbers but not cytoplasmic volume, with comparable testosterone levels to wild-type (Gould et al 2007). In *ER α* knockout mice, the number of adult Leydig cells is unchanged, however there is an increase in adult Leydig cell volume, testosterone levels and steroidogenic gene expression e.g. *Star* and *Cyp11a1* (Gould et al 2007). A study investigating oestrogen-mediated fetal testis dysgenesis in the rat, via administration of oestradiol and diethylstilboestrol (DES) during fetal life, showed significant reductions in mRNA levels of *StAR*, *Insl3*, *Cyp17A1*. This oestrogenic response was found to be mediated via ER α , as evident in *ER α* knockout mice, which displayed comparable mRNA levels of genes encoding

steroidogenic enzymes, compared to wild-type control (Cederroth et al 2007). In a more recent study, transgenic male mice expressing aromatase (Arom+ mice) crossed with *ERα* knockout mice showed a reduction in steroidogenic gene expression (*StAR* and *Cyp17a1*) and abnormal adult Leydig cell protein expression e.g. *StAR*, *Insl3* (Strauss et al 2009). This suggests that the imbalance of oestrogens to androgens (high oestrogen, low androgen) affects adult Leydig cell development. There appears to be species and age differences in oestrogen receptor expression within the human testis, as there is inconsistency in the literature regarding the localisation of *ERα* and *β*. One study reported *ERα* in fetal Leydig cells of the human testis (Shapiro et al 2005) while most report it is absent (Boukari et al 2007, Mitchell et al 2013, Svechnikov et al 2014). *ERβ* expression was reported in human fetal Leydig cells (Shapiro et al 2005) and human adult Leydig cells (Cavaco et al 2009). Aromatase is expressed in various cell types in the human including adipose tissue, the brain and adult Leydig cells (Simpson et al 2002). There have been very few reports of aromatase deficiency; those reported relate to seven men in whom aromatase is non-functional due to a mutation in the *CYP19a1* gene, encoding aromatase (Jones et al 2006). This results in the absence of oestrogen, normal-high testosterone levels and gonadotrophins, tall stature, osteoporosis and impaired reproductive function (Jones et al 2006). The aromatase knockout (ArKO) mouse is a very useful model as it displays a similar phenotype to that reported in the human. Male mice deficient in aromatase display elevated testosterone, DHT and LH serum levels, while FSH levels were unchanged (Fisher et al 1998, Robertson et al 1999). While the aromatase deficient mouse model (ArKO) is useful to explore the role of aromatase and oestrogens, the balance between androgen and oestrogens may be more important than just the level of oestrogens per se (Seralini and Moselemi 2001).

1.7.6 Thyroid hormone (Thyrotrophin)

Thyroxine (T₄), a thyroid hormone, can be deionised to 3,5,3-triiodo-L-thyronine (T₃), the bioactive thyroid hormone, where it binds to its receptors TR α and TR β in target tissues (Mendis-Handagama and Ariyaratne 2004, 2005, Martin and Tremblay 2010), including the human and rat testes (Jannini et al 2000, Buzzard et al 2000). Thyroid hormones stimulate and maintain mouse Leydig cell function via stimulation of StAR and intracellular steroidogenesis in adult Leydig cells (Manna et al 2001, Mendis-Handagama and Ariyaratne 2005). Thyrotrophin stimulates thyroid stimulating hormone (TSH) release and receptors for TSH are present in interstitial cells, PTM and germ cells across development (Buzzard et al 2000). Thyrotrophin mRNA levels, protein expression and its receptor localisation are all found specifically in adult Leydig cells (Mendis-handagama and Ariyaratne 2005). In pre-pubertal hypothyroid rats, intratesticular testosterone and Leydig cell activity, as measured by 3 β -HSD and 17 β -HSD enzyme expression, were reduced both in vivo and vitro. The abnormal adult Leydig cell function could be reversed by thyroxine (T₄) supplementation (Antony et al 1995). Acute stimulation of Leydig cells in culture with thyronine (T₃) resulted in an increase in testosterone production (Maran et al 2000). The same authors later investigated neonatal onset hypothyroidism which resulted in altered Leydig cell development, reduced steroidogenesis and compensated adult Leydig cell failure, evident by increased LH and decreased testosterone (Maran et al 2001). The role of thyroid hormone in developing adult Leydig cells (during puberty) and regenerating adult Leydig cells (post-EDS injection), was investigated in rats via dietary induced hypothyroidism. This resulted in a delay in the development of adult Leydig cells in the two experimental hypothyroid rat groups, at puberty and during adult Leydig cell regeneration post-EDS (Rijntjes et al 2009, 2010). Overall, thyroid hormone appears to play a role in adult Leydig cell differentiation. Interestingly in relation to potential adult Leydig stem/progenitor cells, an isoform of the thyroid hormone receptors, (TR α 1) was found abundantly expressed in unidentified spindle shaped interstitial cells in the developing

mouse testis (Pnd 0-5) and in adult testis (Pnd 63) (Buzzard et al 2000). Young boys with hypothyroidism have elevated serum FSH, but normal LH and testosterone levels (Castro-Magana et al 1988, Mendis-Handagama et al 2005), which suggests normal Leydig cell function. However, adult men with hypothyroidism display reduced serum testosterone concentrations and a reduced number of adult Leydig cells (Wortsman et al 1987, Mendis-Handagama et al 2005), which is a similar phenotype to hypothyroid rats, as described above.

1.7.7 Platelet Derived Growth Factor Receptor A

Platelet derived growth factors comprise of four ligands, PDGF- α , β , C and Δ (Ding et al 2000, Basciani et al 2010) and their receptors display conserved signalling, with the PDGF-C ligand capable of binding to PDGF α receptors (Li et al 2000). In relation to humans, fetal Leydig cells express significant levels of platelet-derived growth factor (PDGF) isoforms α and β , and their respective receptors (Basciani et al 2002, 2010). PDGF α is expressed in adult Leydig cells (Mariani et al 2002) along with its receptor, PDGFR α in the human (Basciani et al 2002). The importance of platelet derived growth factor receptor α (PDGFR α) in the development of fetal Leydig cells in the rodent was demonstrated in earlier studies in which deletion/disruption of *PDGFR α* impaired fetal Leydig cell differentiation, potentially due to a failure in progenitor cell proliferation (Brennan et al 2003, Schmahl et al 2008, Griswold and Behringer 2009). PDGFR α is expressed in the interstitium and PTM cells of fetal rodent testes at e12.5-e13.5 (Brennan et al 2003, Ricci et al 2004, Basciani et al 2010), interstitial and PTM cells of the neonate (Gnessi et al 1995, Thuillier et al 2003) and in adult Leydig cells (Ge et al 2005). PDGF α has been shown to be expressed in 3 β -HSD⁺ adult Leydig progenitor cells in the rat at pnd 10 (Fecteau et al 2006). It has been suggested to play in role in adult Leydig cell differentiation, as adult Leydig cells are absent in PDGF α -deficient mice (Gnessi et al 2000). Isolated putative non-steroidogenic Leydig stem cells, from rat testes at pnd 7

which expressed PDGFR α , LIFR and c-kit were shown to proliferate in vitro and express Leydig cell specific genes once stimulated by PDGF α and other factors in differentiation-inducing medium (Ge et al 2006). In line with this, mRNA levels of *pdgfra* increases dramatically and transiently one day post-EDS i.e. adult Leydig cell ablation (O'Shaughnessy et al 2008), which may suggest a role for PDGFR α in adult Leydig stem cell differentiation. A recent study demonstrated that PDGFR α ⁺ cells isolated from pnd 8 rat testis belong to the peritubular myoid cell (PTC) lineage, evident from their expression of PTC-specific genes, pluripotency markers and the expression of genes encoding steroidogenic enzymes (Landreh et al 2013). This suggests neonatal PDGFR α ⁺ PTM cells might give rise to adult Leydig cells, in agreement with the hypothesis of a multifocal adult Leydig cell origin (Habert et al 2001, Haider, 2004).

Conditional deletion of *pdgfra* in steroidogenic cells driven by the *Sf1*-Cre promoter (*Sf1-Cre;PDGFR α ^{-/-}*) in early adulthood resulted in significantly smaller testes, reduced sperm count, reduced adult Leydig cell number which coincided with reduced *Cyp11a1* expression, in comparison to wild-type mice (Schmahl et al 2008, Basciani et al 2010). Interestingly, the mutant mice become fertile between 3-6 months of age, which is coincident with increased expression of PDGFR β in the *Sf1-Cre;PDGFR α ^{-/-}* mouse line (Schmahl et al 2008, Basciani et al 2010). This could suggest a compensatory mechanism by PDGFR β as PDGFRs maintain conserved cell signalling, despite different functions (Li et al 2000, Klinghoffer et al 2001). An earlier study that investigated adult Leydig cell development in an adult Leydig cell ablation/regeneration model (EDS) showed the adult Leydig population arises from vasculature pericytes which expressed PDGFR β (Davidoff et al 2004). Overall, the involvement of PDGFs in adult Leydig cell development is complex and further investigations are necessary to fully elucidate their role in this process.

1.7.8 Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) is a highly pleiotropic cytokine (Hilton, 1992, Ge et al 2005). LIF has previously been shown to promote proliferation of murine embryonic stem cells whilst maintaining these cells in an undifferentiated totipotent state (Smith et al 1988, Ge et al 2005). It is a member of the IL-6 cytokine family and is required for neural stem cell self renewal, as shown in vitro (Wright et al 2003), and is also expressed in PTM cells in rat testes from e13.5 onwards (Piquet-Pellorce et al 2000). In a recent study that investigated putative adult Leydig 'stem cells', isolated interstitial cells from rat testes at pnd 7, which expressed LIFR (and PDGFR α), were shown to proliferate upon stimulation with differentiation-inducing medium containing LIF in vitro (Ge et al 2006). These cells were capable of differentiating into 3 β -HSD⁺ adult Leydig cells which could produce testosterone (Ge et al 2006), thus suggesting a key role for LIF in adult Leydig cell development. Withdrawal of LIF has also been shown to stimulate differentiation in mouse embryonic stem cells (Ward et al 2004). This is also evident in a more recent study that investigated adult Leydig cell regeneration, post-EDS treatment. In this study, mRNA levels of *Lif* increased significantly following adult Leydig cell ablation (24 hours post-EDS) and decreased to basal levels between 3-5 days at the point where newly developing adult Leydig cells are differentiating (O'Shaughnessy et al 2008). Overall, this suggests a role for LIF in adult Leydig stem cell proliferation and differentiation.

1.7.9 Desert hedgehog

Desert hedgehog (Dhh) is a member of the hedgehog family of genes and apart from the gonads, Dhh can also play a role in other organs including pancreas and adipocytes (King et al 2008). In the testis, it is secreted by Sertoli cells and its receptor, Patched 1 (PTCH1), is located on Leydig and peritubular myoid cells in mice (Clark et al 2000, Yao et al 2002). Dhh is known to play a role in Leydig and peritubular myoid cell differentiation (Yao et al 2002), as evident from mice in which the absence of a functional Dhh in male gonads, results in

undescended small testes, failure of Leydig cells to complete differentiation and abnormal seminiferous cord formation (Clark et al 2000, Yao et al 2002, Park et al 2007). The majority of *Dhh* null mice develop into pseudohermaphrodites (Clark et al 2000). In human, mutations in *Dhh* are associated with 46, XY individuals who present with partial/complete gonadal dysgenesis (Umehara et al 2000, Canto et al 2004). The role of *Dhh* in fetal Leydig cell differentiation is highlighted by studies in which *Dhh* was induced in fetal mouse ovaries, resulting in fetal Leydig cell differentiation from SF1⁺ cells, as evident from their production of androgens and *Insl3* (Barsoum et al 2009).

1.7.10 GATA4

GATA4 is the only one of six family members to be expressed in somatic cells, as opposed to germ cells, in the bipotential gonad (Eggers et al 2012). Expression of the transcription factor (GATA4) becomes sexually dimorphic at e13.5 (Eggers et al 2012), and in human fetal Leydig cells, this expression peaks around 15 weeks of gestation, coincident with a peak in testosterone levels and the exponential rise in fetal Leydig cell number (O'Shaughnessy et al 2007, Ketola et al 2000, Svechnikov et al 2010). *Gata4* is evident in fetal Leydig cells, interstitial cells and PTM cells at e13.5 and e17.5 (Bielinska et al 2007) and is expressed in Sertoli and adult Leydig cells of the mouse testis (Ketola et al 2000, Bielinska et al 2007). Isolated putative 'adult Leydig stem' cells from rat testes at pnd 7 which were PDGFR α ⁺, expressed c-Kit, LIF and GATA4, were shown to be capable of differentiating into adult Leydig cells in vitro (Ge et al 2006). Furthermore, in an adult Leydig cell ablation/regeneration model via EDS, the isolated GATA4⁺ Leydig 'stem cells' were also capable of developing into adult Leydig cells (Ge et al 2006). This highlights a role for GATA4 in adult Leydig stem/progenitor cell development. GATA4 is present in Sertoli and adult Leydig cells throughout postnatal mouse and human testis development (Ketola et al 1999, 2000). During puberty in the human testis, GATA4 expression was more intense in developing Leydig cells, which decreased with age (Ketola et al 2000).

Taken together, this suggests GATA4 could provide a useful marker for newly differentiating adult Leydig progenitor cells.

1.7.11 COUP-TFII

Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) derived its title from its ability to regulate expression of the chicken ovalbumin gene (Wang et al 1989). There are two COUP-TF nuclear receptors (I and II), which are ligand-inducible, highly conserved transcription factors, containing identical ligand-binding-domains (LBD) across species (Tsai and Tsai 1997, Kruse et al 2008). COUP-TFs are termed 'orphan' nuclear receptors as their ligand has not yet been identified (Martin and Tremblay 2010). COUP-TFI is expressed in the neural system (Tang et al 2006) whereas COUP-TFII is expressed in the mesenchyme of developing organs. This review will focus on COUP-TFII, as it is involved in male (Qin et al 2008) and female reproduction (Takamoto et al 2005). COUP-TFII has also been reported to play a role in venous identity (You et al 2005), cell fate determination (endothelial lymphatic cells; Yamazaki et al 2009, neural progenitor cells during eye development; Tang et al 2010), organogenesis, angiogenesis (Qin et al 2010), cerebellum development (Kim et al 2009) and metabolic homeostasis (Kruse et al 2008, Li et al 2009, Lin et al 2011).

COUP-TFII is first detected in the visceral mesoderm and myocardium and umbilical veins at e8.5 in the mouse (Pereira et al 1999). COUP-TFII^{-/-} mutant mice were embryonic lethal, due to angiogenesis and cardiovascular defects (Pereira et al 1999). This was confirmed by specific deletion of COUP-TFII in the endothelium via Tie2-Cre recombinase, which resulted in death due to vascular defects. Ectopic expression of COUP-TFII in the endothelium of transgenic mice resulted in down regulation of arterial markers e.g. Jag1, and up-regulation of venous markers, EphB4. This demonstrates a key role for COUP-TFII in the regulation of vein identity (You et al 2005).

Regarding the female reproductive system, COUP-TFII is predominantly expressed in steroidogenic theca cells surrounding ovarian follicles (Takamoto

et al 2005), suggesting potential involvement in the steroidogenic pathway. Following LH stimulation in wild-type female mice, theca cells synthesize precursor C19 steroids in the pre-ovulatory follicle (Chaffin and Vandervoort 2013). The ablation of one allele (haploinsufficiency) of COUP-TFII^{+/-} in female mice, resulted in reduced responsiveness to gonadotrophins and therefore reduced progesterone levels. COUP-TFII^{+/-} mice had a 70% reduction in steroidogenic enzyme expression of CYP11A1, StAR and 3 β -HSD1 which could explain the reduced ability to synthesise progesterone (Takamoto et al 2005).

In relation to the male reproductive system, COUP-TFII has been shown to be involved in adult Leydig cell development, specifically the differentiation but not maintenance of adult Leydig cells (Qin et al 2008). In this study, the embryonic lethality of COUP-TFII null mice was circumvented using a tamoxifen inducible Cre to inactivate COUP-TFII in a time specific manner in a mouse model. Postnatal inactivation of COUP-TFII pre-puberty resulted in infertility, spermatogenic arrest and hypogonadism, presumably as a result of the arrested differentiation of adult Leydig progenitor cells (Qin et al 2008). COUP-TFII does not seem to be required for the maintenance of adult Leydig cells as post-pubertal deletion of COUP-TFII resulted in normal male reproduction and Leydig cell function.

The precise structure or ligand regulation of COUP-TFII has not yet been elucidated. A previous study investigated the conformational status of COUP-TFII and described it as having an 'autorepressed conformation', as the activation binding sites are inaccessible due to its folded conformation (Kruse et al 2008). Retinoic acid, a vitamin A metabolite, can alter this conformational state thus promoting COUP-TFII to recruit co-activators and increase its transcriptional activity (Kruse et al 2008). An earlier study reported that retinoic acid can regulate steroidogenesis in rodents, as evident by reduced testosterone production in male fetuses of pregnant dams who were fed a vitamin A deficient diet (Livera et al 2004). During fetal Leydig cell differentiation, retinoic acid is reported to negatively regulate steroidogenesis,

potentially via down regulation of *Cyp17a1*, as demonstrated in cultured cells from rat testes (Livera et al 2004). There are six receptors via which retinoic acid mediates its effects (NR1B;1,2,3 and NR2B;1,2,3), with NR2B1 in particular expressed in adult Leydig cells (Boulogne et al 1999). Interestingly, *Nr2b1*-deficient mice die during fetal life due to cardiac defects (Sucov et al 1994) thus its role in adult Leydig cell development remains to be elucidated. The role for retinoic acid in negatively regulating steroidogenesis during fetal Leydig cell differentiation (Livera et al 2004) appears somewhat similar to COUP-TFII, which has also been proposed to negatively regulate steroidogenesis in differentiating fetal Leydig cells (van den Driesche et al 2012). However, the precise role and interplay between these factors postnatally, in the potential development/regulation of adult Leydig cells, remains to be elucidated.

1.7.12 Macrophages

Macrophages lie in close proximity to Leydig cells and are believed to play a role in adult Leydig cell function. They constitute 25% of the total interstitial cell population in adult rodents (Giannessi et al 2005) but have been shown to first appear in the fetal testis in rats at e16 (Livera et al 2006). In the absence of macrophages, adult Leydig cells demonstrate abnormal development (Hales, 2002). Macrophages produce several factors which can influence Leydig cell steroidogenesis including macrophage derived factor (MDF) (Lukyanenko et al 2000), interleukin-1 (IL-1) (Verhoeven et al 1988), tumor necrosis factor alpha (TNF α), and reactive oxygen species (ROS). Cultured immature Leydig cells from rats treated with IL-1 resulted in stimulation of steroid production (Verhoeven et al 1988). MDF, or its more recent identification as 25-hydroxycholesterol (HC), is secreted by macrophages and was shown to increase 3 β -HSD activity in developing Leydig cells and to stimulate testosterone production (Nes et al 2000, Chen et al 2002). Testosterone can also regulate 25-HC production in a paracrine fashion via a negative-feedback loop (Lukyanenko et al 2002). The importance of macrophages in Leydig cell

development is evident from adult Leydig cell ablation/regeneration studies, in which macrophages were depleted from one testis prior to EDS-induced adult Leydig cell ablation/regeneration. Adult Leydig cells failed to regenerate in the absence of testicular macrophages, whereas in the contralateral testis (control vehicle only), adult Leydig cells regenerated normally (Gaytan et al 1994). Overall, this suggests macrophages play a key role in adult Leydig cell development.

1.8 Steroidogenesis

Steroidogenesis refers to the synthesis of steroid hormones, which occurs in steroidogenic cells of the testis, ovary, adrenal, placenta and brain (Baulieu 1997, King et al 2002, Papadopoulos and Miller 2012). Steroid hormones are essential for reproductive function and homeostasis. As 95% of testosterone in human males is synthesised within the testis, intratesticular testosterone levels are ~250 times higher than in blood serum with approximately 6-7mg testosterone produced per day (Coffey 1988, Maddocks et al 1993). Steroid concentration in target tissues and body fluids is dependent on binding proteins e.g. sex hormone binding globulin (SHBG) and albumin. Approximately 98% of circulating testosterone is present in bound form, with 2% free, which eventually is metabolised in the liver or converted into dihydrotestosterone (Miller and Bose 2011). The rate of androgen production in adult Leydig cells must be maintained to produce the high levels of testosterone necessary for spermatogenesis and male health. The biosynthesis of testosterone is discussed in detail below, beginning with its substrate, cholesterol.

1.8.1 Cholesterol transport

The transfer of cholesterol to the outer mitochondrial membrane involves high-affinity cholesterol binding proteins. Sterol carrier protein-2 (SCP2) was originally believed to be involved in this trafficking (Vahouny et al 1983, van Noort et al 1988), however a later study that investigated SCP2 expression in

Leydig cells described it as membrane-bound (van Haren et al 1992) and not abundantly present in the cytosol as previously thought. Furthermore, SCP2 was recently described as having a low affinity for cholesterol (Gallegos et al 2001) so its role in cholesterol trafficking may not be important. The most important event in the transfer of cholesterol occurs between the outer and inner mitochondrial membranes in order to initiate steroidogenesis. A crucial player in this critical step is steroid acute regulatory protein, StAR (Payne and Hales 2004, Manna et al 2009, Papadopoulos and Miller 2012) and will be described in the next section. Several other factors have also been described for cholesterol trafficking, which facilitates steroidogenesis, including peripheral benzodiazepine receptor (PBR) or the mitochondrial transporter protein (TSPO) (Fan and Papadopoulos 2013). PBR/TSPO is an 18kDA protein which acts on the outer mitochondrial membrane downstream from StAR, to transfer cholesterol into the inner mitochondrial membrane (Papadopoulos 1993, Liu et al 2006). Targeted deletion of PBR/TSPO in a Leydig cell mouse line resulted in the blockage of cholesterol transport and reduced/disrupted steroidogenesis, which was restored following PBR/TSPO replacement (Li et al 2001).

1.8.2 Steroid Acute Regulatory protein (StAR)

StAR plays a crucial step for the regulated delivery of cholesterol to the inner mitochondrial membrane for steroidogenesis (Clark et al 1994, Bose et al 1996, Miller et al 1999, Stocco 2001, Bose et al 2002, Stocco and Clark 2006,). During embryonic development, StAR transcription in fetal Leydig cells is regulated by SF-1 (Clark et al 1995). The proximal promoter of *StAR* is important for regulation of *StAR* transcription and this region is highly conserved across species (Manna et al 2009). The rate of transcription of *StAR* in adult Leydig cells is regulated by LH, which under normal conditions stimulates the production of a 37kDA protein, StAR (Stocco and Sodeman 1991, Clark et al 1994, 1995, Manna et al 2009, Lee et al 2013). More recently, histone modifications in the proximal promoter region of *StAR* have been shown to play

a key role in StAR transcription (Silverman et al 1999, LaVoie et al 2005, Lee et al 2013) and will be discussed later in this review.

StAR creates a link bridge or a hydrophobic tunnel on the outer mitochondrial membrane allowing for cholesterol transfer from its sterol-binding pocket into the inner mitochondrial membrane (Tsujiyama and Hurley 2000). Continuous StAR synthesis is required to maintain the high levels of steroid production in the testis. LH stimulation induces the phosphorylation of StAR on Ser 195 by protein kinase A, which doubles its activity and is essential for optimal steroidogenesis (Arakane et al 1997, Papadopoulos and Miller 2012). The activity of StAR in promoting steroidogenesis is proportional to the length of time spent on the outer mitochondrial membrane (Bose et al 2002).

The indispensable role for StAR is clearly evident from earlier clinical and rodent studies (Lin et al 1995, Bose et al 1996, Caron et al 1997, Hasegawa et al 2000). In mice with mutated/deleted *StAR*, an accumulation of cholesterol in steroidogenic Leydig and adrenal cells, combined with a failure of steroid production was evident, which is identical to the human condition of lipoid congenital adrenal hyperplasia (CAH). Thus, lipoid CAH was shown to be caused by mutation/inactivation of the *StAR* gene (Lin et al 1995, Miller, 1997, Caron et al 1997, Stocco 2002). As previously mentioned, other proteins apart from StAR are also involved in cholesterol transfer e.g. those encompassing a "transduceosome" (Papadopoulos and Miller 2012, Fan and Papadopoulos 2013). PBR/TSP0 is suggested to interact with StAR for normal cholesterol transfer (Hauet et al 2005), however the significance of the interplay between this protein and others to StAR, is not yet conclusive. Although PBR/TSP0 is present and functions normally in *StAR* mutant mice, its function appears insufficient to support the rapid level of cholesterol transport needed for androgen production. Thus, the external genitalia were not virilised in *Star* mutant mice and adrenal steroidogenesis was absent, which resulted in death (Lin et al 1995, Caron et al 1997). To circumvent the previous lethality, *StAR* knockout mice were kept alive by corticosteroid replacement and analysis of the

Leydig cells revealed limited androgen production (Hasegawa et al 2000). Although StAR is crucial for the acute steroidogenic responses, in its absence, steroidogenesis continues at about 14% of its previous rate (Lin et al 1995). Overall, it highlights that StAR is crucial for the "rapid" transport of cholesterol which is essential for the acute regulation of steroid synthesis, as described in the next section.

1.8.3 Testicular testosterone biosynthesis

Following hormonal stimulation by LH in adult Leydig cells (Section 1.7.1), steroidogenesis is regulated by either a chronic or acute response. The chronic or long term response refers to an increase in transcription of steroidogenic genes encoding enzymes involved in the steroidogenic pathway, mainly CYP11a1/P450_{scc} (Simpson and Waterman 1988). The acute response is an essential one, due to the limited steroid storage by steroidogenic cells (Miller and Auchus 2011). Acute or short term steroid production requires the rapidly synthesized protein StAR, to perform a vital step in steroidogenesis, namely to transfer cholesterol from the outer to the inner mitochondrial membrane, as previously described. Steroidogenesis commences with mobilisation and delivery of the steroid hormone substrate, cholesterol (C₂₇). Cholesterol is either 1) synthesised *de novo* from acetate in the endoplasmic reticulum (Ferguson 1963) which is coordinated by sterol regulatory element binding proteins (SREBPs), 2) taken up from plasma lipoprotein stores (high density lipoprotein (HDL) or low (L)DL) or 3) taken up from intracellular lipid droplets containing cholesterol esters (Miller and Bose 2011). Rodents use cholesterol from circulating HDL, while humans use LDL, as their principal source of cholesterol. Active or free cholesterol is able to move from the plasma membrane to the intracellular membranes (Miller and Auchus 2011), as cholesterol is insoluble in water (Haberland and Reynolds 1973).

Steroidogenic enzymes can be subdivided into two groups; cytochrome P450 enzymes, a term encompassing the oxidative enzymes (CYP11A1/P450_{scc},

CYP17A1/P450c17, CYP19A1/P450arom) and hydroxysteroid dehydrogenase enzymes (3β -HSD and 17β -HSD). The P450 enzymes were named from their yellow pigmentation in solution due to maximal light absorption at 450nm (Miller and Bose 2011). P450 genes are now formally termed CYP genes (Miller and Auchus, 2011). P450 and the HSD enzymes receive electrons from nicotinamide adenine dinucleotide phosphate (NADPH) to catalyze hydroxylation and cleavage of their substrate (Sherbet et al 2007).

Once StAR has promoted the movement of the insoluble steroid substrate cholesterol into the inner mitochondrial membrane, steroidogenesis begins when CYP11A1 cleaves the side chain of cholesterol, forming soluble pregnenolone (C_{21}) (Koritz, 1970, Simpson, 1979). Soluble pregnenolone can then either be converted to progesterone via 3β -hsd, or converted to 17α -hydroxypregnenalone by CYP17A1 (Zuber et al 1986). The formation of androstenedione by CPY17A1 occurs within the endoplasmic reticulum (Zirkin and Chen 2000). The enzyme 3β -HSD can convert pregnenolone into progesterone and dehydroepiandrosterone to androstenedione, and the preference of either of these pathways ($\Delta 5$ to $\Delta 4$) is species-dependent.

The preferred route of testosterone synthesis differs between species, i.e. CYP17A1 will use the $\Delta 5$ pathway for 17α -hydroxypregnenolone as a substrate in human and bovine (Fluck et al 2003, Rey and Grinspon 2011), whereas in rodents, CYP17A1 will use the $\Delta 4$ pathway for 17α -hydroxyprogesterone as a substrate (Brock and Waterman 1999). There are several isoforms of 17β -HSD with type 3 (17β -HSD) found in adult Leydig cells (Miller and Bose et al 2011) but located within the seminiferous cords in fetal mouse testis (O'Shaughnessy et al 2000). The expression of 17β -HSD3 within fetal seminiferous cords, has so far, not been shown in any other species, so its wider significance is unknown. In adulthood, the enzyme 17β -HSD3 marks the final step in steroidogenesis by which androstenedione is converted to testosterone. Deficiency in 17β -HSD3 represents one cause of 46,XY disorders of sex development (DSD) in humans (Werner et al 2012). Presently, there are 27 known mutations in the 17β -HSD3

gene, which result in severe under-masculinisation (Galdiero et al 2013). Defects in 17β -HSD3 in humans is the most common androgen synthesis disorder and cases are reported world-wide (Mendonca et al 2010). Patients present with ambiguous or female-like genitalia and intra abdominal/inguinal testes (Mendonca et al 2010).

Testosterone can be metabolised into either dihydrotestosterone (DHT) by 5α -reductase or oestradiol by CYP19A1 (Normington and Russell 1992, Papadopoulos and Miller 2012). A schematic of testosterone biosynthesis is shown in Figure 1.4 below.

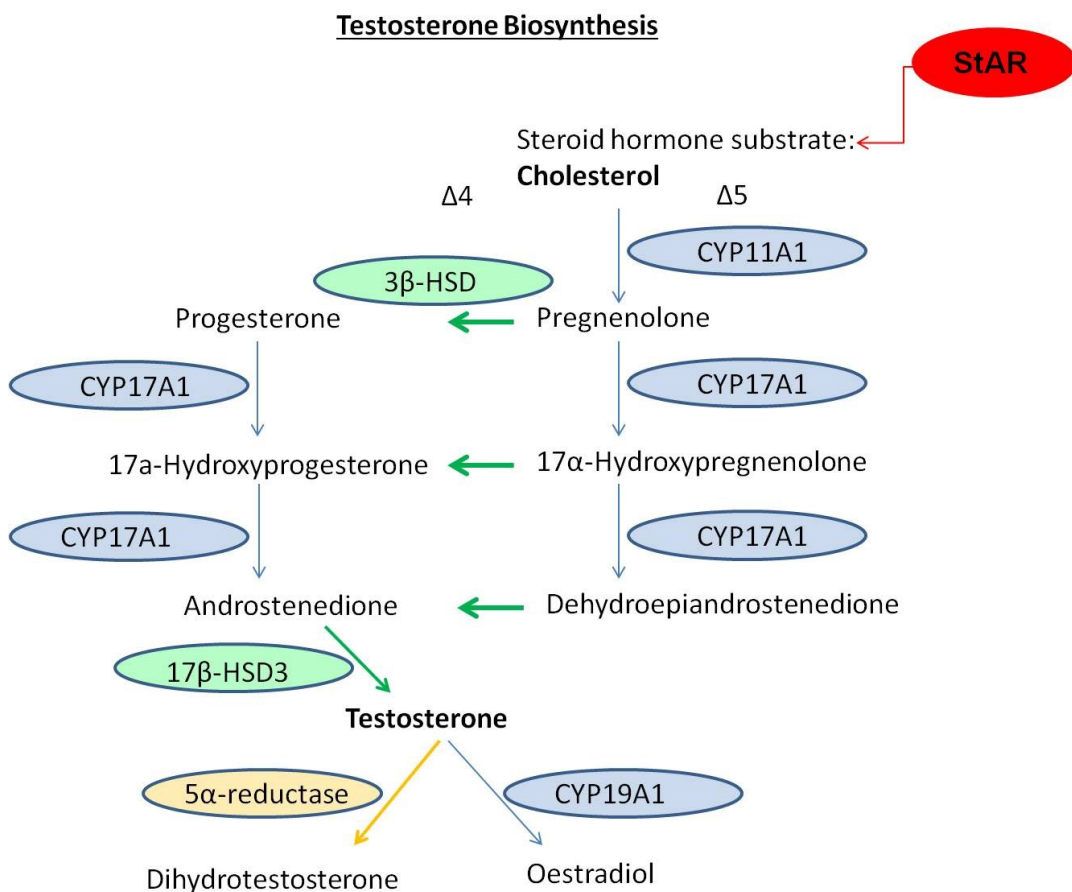


Figure 1.4 Testosterone biosynthesis in the Leydig cell. Following the transport of cholesterol by StAR, CYP11A1 metabolises cholesterol into pregnenolone which is then subjected to degradation by either of two categories of enzymes; Cytochrome 450 (depicted in blue) and the hydroxydehydrogenase enzymes (depicted in green). The action by 17β -HSD3 is the final step in steroidogenesis, forming the end product testosterone. Testosterone can be metabolised into DHT or oestradiol.

1.9 Regulation of steroidogenesis

Steroidogenesis in Leydig cells occurs if the following criteria are met; 1) integrity of the LHR in order to initiate the signalling pathway 2) availability of the steroid hormone precursor cholesterol 3) integrity of transporters of cholesterol from stores to mitochondria 4) integrity of mitochondrion 5) steroidogenic enzymes present at normal levels. Testosterone biosynthesis is regulated by the hypothalamic pituitary gonadal (HPG) axis, as described below.

1.9.1 Hypothalamic Pituitary Gonadal axis

The hypothalamic-pituitary-gonadal axis refers to a significant pathway for steroidogenesis and spermatogenesis. It is composed of three components; gonadotrophin releasing hormone (GnRH) neurons which project from the hypothalamus, the gonadotroph cells in the anterior pituitary and the testicular Sertoli and Leydig cells (Handa and Weiser 2013). The HPG axis controls the release of FSH, required for Sertoli cell proliferation and spermatogenesis and LH, which regulates steroidogenesis in adult Leydig cells. Gonadotrophin releasing hormone (GnRH) is transported from the hypothalamus to the pituitary via the hypothalamic-hypophyseal portal blood system and causes the release of FSH and LH from the gonadotroph cells (Matsumoto and Bremner 1984). In terms of steroidogenesis, a negative feedback system regulates testosterone production either directly via testosterone or indirectly via its metabolites dihydrotestosterone and oestradiol, which act to inhibit the secretion of GnRH and thus the release of LH. In the case of high levels of testosterone, the normal response is low LH. Conversely, where low levels of testosterone occur, LH is usually elevated, a condition termed “primary hypogonadism” (Handa et al 1985, Rey et al 2013).

1.9.2 Other mechanisms controlling Leydig cell function

Several studies have described a pathway regulating steroidogenesis in Leydig cells, which is independent of the pituitary and occurs between the

paraventricular nucleus and the testis. These studies identify a neural based regulatory pathway, which affects steroidogenesis via β -adrenergic receptor activation in the brain (Gerendai et al 1995, Turnbull and Rivier 1997, Ogilvie and Rivier 1998). Leydig cell responsiveness to gonadotrophins was rapidly inhibited following intracerebro-ventricular injection of IL-1 β or corticotrophin releasing factor (CRF), which also occurred when LH release had been blocked, thus suggesting a pituitary-independent pathway (Ogilvie et al 1998, Selvage and Rivier 2003) in rats. This resulted in a blunted response of testosterone production to hCG stimulation. Furthermore, StAR protein expression was reduced in Leydig cells which might have contributed to the reduced steroidogenesis reported. This defect could be partially restored via administration of a water permeable cholesterol to bypass the requirement of StAR (Ogilvie et al 1999).

A factor which has been shown to regulate the HPG axis and thus regulate adult Leydig cell function is 'kisspeptin', which itself is regulated by a steroid negative feedback pathway (Smith 2013). Kisspeptin acts via its receptor (Gpr54) in the arcuate nucleus and the anteroventral periventricular nucleus of the hypothalamus and stimulates GnRH release (Kirilov et al 2013). This has previously been shown to increase levels of LH and testosterone in rats (Patterson et al 2006) and rhesus monkeys (Seminara et al 2006). Mutant mice with a neuron-specific deletion of Gpr54 are infertile with significantly smaller testes, as compared to wild-type (Kirilov et al 2013). Targeted insertion of Gpr54 into GnRH neurons of the mutant mice rescued the normal kisspeptin stimulatory action upon GnRH neurons (Kirilov et al 2013).

Nitric oxide (NO) has also been reported to modulate testosterone production via paracrine signalling between adult Leydig cells and macrophages. NO is formed via the oxidation of L-arginine by nitric oxide synthase (NOS) (Laubach et al 1995). Isoforms of NOS are present in the testes of human (Davidoff et al 1995), horse (Ha et al 2004), pig (Kim et al 2007) and rodents (O'Bryan et al 2000, Jarazo-Dietrich et al 2012). One of the NOS isoforms, inducible nitric oxide

synthase (iNOS), is expressed in testicular cells including adult Leydig cells under normal conditions (Davidoff et al 1995, O'Bryan et al 2000, Kon et al 2002, Lee and Cheng 2004). It has been proposed that NO produced from iNOS activity, can modulate testosterone production in adult Leydig cells, by inhibiting steroidogenesis in the mouse (Adams et al 1994, Pomerantz & Pitelka 1998, Weissman et al 2005). The latter study showed that the generation of NO specifically occurs in testicular macrophages, which then inhibits steroidogenesis in Leydig cells via a paracrine action (Weissman et al 2005).

1.10 Androgen receptor expression in the testis

In adult human males, intratesticular androgen levels range between 340-2000nM compared to 8.75-35nM in serum (Walker 2011) with a similar elevation between intratesticular levels and serum evident in rodents (Maddocks et al 1993). Androgen receptor (AR; also called NR3CA) expression in fetal rat testis is evident in interstitial cells at e17 (Majdic et al 1995), although the identity of these AR⁺ non-steroidogenic interstitial cells is still unclear. At e19, e21.5 and pnd 5, AR expression is localised to PTM and non-steroidogenic interstitial cells (Majdic et al 1995). The authors suggest these cells may represent adult Leydig progenitor cells, although extensive morphological and marker analysis is needed to investigate this further (Eacker and Braun 2007).

Androgen signalling is mediated via the AR, which is present in specific testicular cells, including adult Leydig cells throughout their maturation, as previously described. Testosterone signalling via germ cells is not required for spermatogenesis, as a functional AR is absent from germ cells (Tsai et al 2006, Smith and Walker 2014). Testosterone supports spermatogenesis therefore via ARs present in other testicular cells. AR expression is localised to Sertoli cells (DeGendt et al 2004, Rey et al 2009, Willems et al 2010, O'Shaughnessy et al 2010), peritubular myoid (PTM) cells, (Welsh et al 2009, 2012), arteriole smooth muscle (Bremner et al 1994, Welsh et al 2010), vascular endothelial cells (O'Hara and Smith 2012) and adult Leydig cells (Bremner et al 1994, You

and Sar 1998, DeGendt et al 2005). The role of AR in adult Leydig cells will be discussed in the following sections.

In postnatal life, androgens exert their largest effect on adult Leydig cell development through direct action on Leydig cells themselves (De Gendt et al 2005). Isolated progenitor Leydig cells from rats at pnd 21, express mRNA for *AR*, and acquire increasing *AR* mRNA and protein expression during their differentiation into immature adult Leydig cells at pnd 35 (Shan et al 1992, 1995). Once immature adult Leydig cells undergo a single division into mature adult Leydig cells, *AR* levels decline and both gene and protein expression levels in mature adult Leydig cells are lower than in progenitor or immature adult Leydig cells (Eacker and Braun 2007). To investigate the hormonal regulation of developing adult Leydig cells, isolated progenitor and immature adult Leydig cells were treated with a GnRH antagonist (NalGlu) which resulted in a reduction in mRNA levels and protein expression of AR. Administration of exogenous LH or testosterone following GnRH inhibition, caused an increase in AR protein expression in progenitor and immature, but not mature, adult Leydig cells (Shan et al 1995, 1997). Thus, the postnatal maturation of adult Leydig cells requires hormonal stimulation by testosterone. In order to alter gene expression in specific target cells, androgens bind to their receptors and elicit a signalling response, as described below.

1.10.1 Androgen receptor gene

The AR is encoded by a single gene on the X chromosome (Brown et al 1989), thus any mutation in AR affects males, whereas females transmit the AR mutation to the next generation. The AR gene is conserved across species (Tilley et al 1990, Lindzey et al 1993) and consists of four structurally and functionally distinct domains. These include; a highly conserved central DNA binding domain (DBD) encoded by exons 2 and 3, a hinge region which connects the DBD to a C-terminal ligand-binding domain (LBD) encoded by exons 4-8, and an N-terminal domain (NTD) encoded by exon 1 (Brinkmann et al 1989, McLachlan

et al 2002, Smith and Walker 2014). There are two transcriptional activation domains (AF-1 and 2) (Matsumoto et al 2008). The interaction between the amino/carboxyl termini of the AR (N/C interaction) is essential for its stabilisation, as induced by testosterone. Disruption of the N/C interaction due to naturally occurring AR mutations, have been identified in patients with androgen insensitivity syndrome (AIS), highlighting its crucial role in normal androgen signalling (Thompson et al 2001, Ghali et al 2003, Jaaskelainen et al 2006). Previous studies have shown that adult humans with a mutation in the *AR* gene have generally normal-high testosterone and elevated LH levels (Madden et al 1975, Eacker and Braun 2007).

1.10.2 Androgen signalling pathways

Steroids circulate in blood either freely or bound to steroid hormone binding globulin (SHBG) (or also called androgen binding protein (ABP)) or albumin. As androgens are small lipid soluble steroid hormones, they enter directly through the plasma membrane of target cells to reach the AR. Testosterone can be converted by 5 α -reductase into DHT, as previously described, and its actions are also mediated by AR. Unliganded AR is located in the cytoplasm of cells bound to heat-shock chaperone proteins (HSP) e.g. HSP 70 and 90 (Pratt and Toft 1997, Tyagi et al 2000). These chaperones stabilise the conformation of the LBD and assist in the maintenance of a high affinity AR structure (Fang et al 1996), essential for an efficient response to androgens.

1.10.3 Genomic androgen signalling pathway

Once the androgen ligand has bound to AR, the complex undergoes a conformational change and releases itself from the attached heat-shock proteins. This change is essential to reveal the DBD region, necessary for interaction with AREs on target genes. AR translocates to the nucleus where it binds as a dimer (homodimerisation) to specific regions of the DNA promoter region (DBD) of AR target genes, termed androgen response elements (ARE)

(Chang et al 1995, Kimura et al 2007). Thus, AR homodimerisation and binding to AREs promotes transcriptional activation or repression of specific target genes. The highly conserved DBD region contains two zinc finger domains (Schoenmakers et al 2000), of which the second zinc finger domain and the N/C interaction mediate homodimerisation of the AR. Androgen-dependent homodimerisation then recruits co-activator/repressor proteins and protein complexes which alter gene expression (Nemoto et al 1994, Smith and Walker 2014). This pathway of action has been termed the "genomic or classical" pathway (Figure 1.5) and can take up to 60 minutes to modulate gene expression and produce proteins (Shang et al 2002).

1.10.4 Non-genomic androgen signalling pathways

Recently identified alternative "non-genomic" pathways of testosterone action have been identified. In Sertoli cells, this pathway involves testosterone binding to AR at the plasma membrane which results in rapid activation of Src tyrosine kinase (Cheng et al 2007), as shown in Figure 1.5. This then phosphorylates and stimulates the epidermal growth factor receptor (EGFR), followed by stimulation of the MAP kinase cascade, inducing cAMP response element-binding protein (CREB) phosphorylation and transcription within one minute (Cheng et al 2007, Walker 2010). Furthermore, cells expressing AR and ER which are stimulated by either androgen or oestradiol can activate Src (via induction of an ER α / β -AR-Src ternary complex) which stimulates the Src/Raf-1/Erks signalling pathway (Figure 1.5) and can result in cell proliferation, as shown in a prostate cancer cell line (Migliaccio et al 2000). Antagonists to either steroid hormone can inhibit this pathway. This *in vitro* study used co-transfected cells with AR and ER, and showed that stimulation by either androgen or oestradiol resulted in more efficient/stronger Src activation, in comparison with cells transfected with only AR or ER alone. Src interacts with ER α / β through its SH2 domain, and AR through its SH3 domain (Migliaccio et al 2000). Thus either steroid (androgen or oestradiol), in the presence of both AR

and ER α/β , can enhance Src kinase activity via binding of AR to SH3 and ER α/β to SH2, of the 'inactive' Src. This induces a conformational change, resulting in an 'active' form of the Src complex which then stimulates the Src/Raf-1/Erks pathway, as mentioned above. This suggests that an important balance and crosstalk exists between AR and ER for non-genomic signalling. Whether this pathway is utilised in the fetal or adult testis has not been investigated.

The second pathway of non-genomic testosterone action has been demonstrated in cultured Sertoli cells from pre-pubertal rats (Lyng et al 2000, Loss et al 2011). Testosterone can activate the hydrolysis of PIP2 by phospholipase C (PLC), which causes cell depolarisation and results in an influx of Ca²⁺ (Loss et al 2011), as depicted in Figure 1.5. It is not yet known whether AR or another receptor is required to activate these pathways which, unlike genomic androgen signalling, can occur within seconds (Smith and Walker 2014).

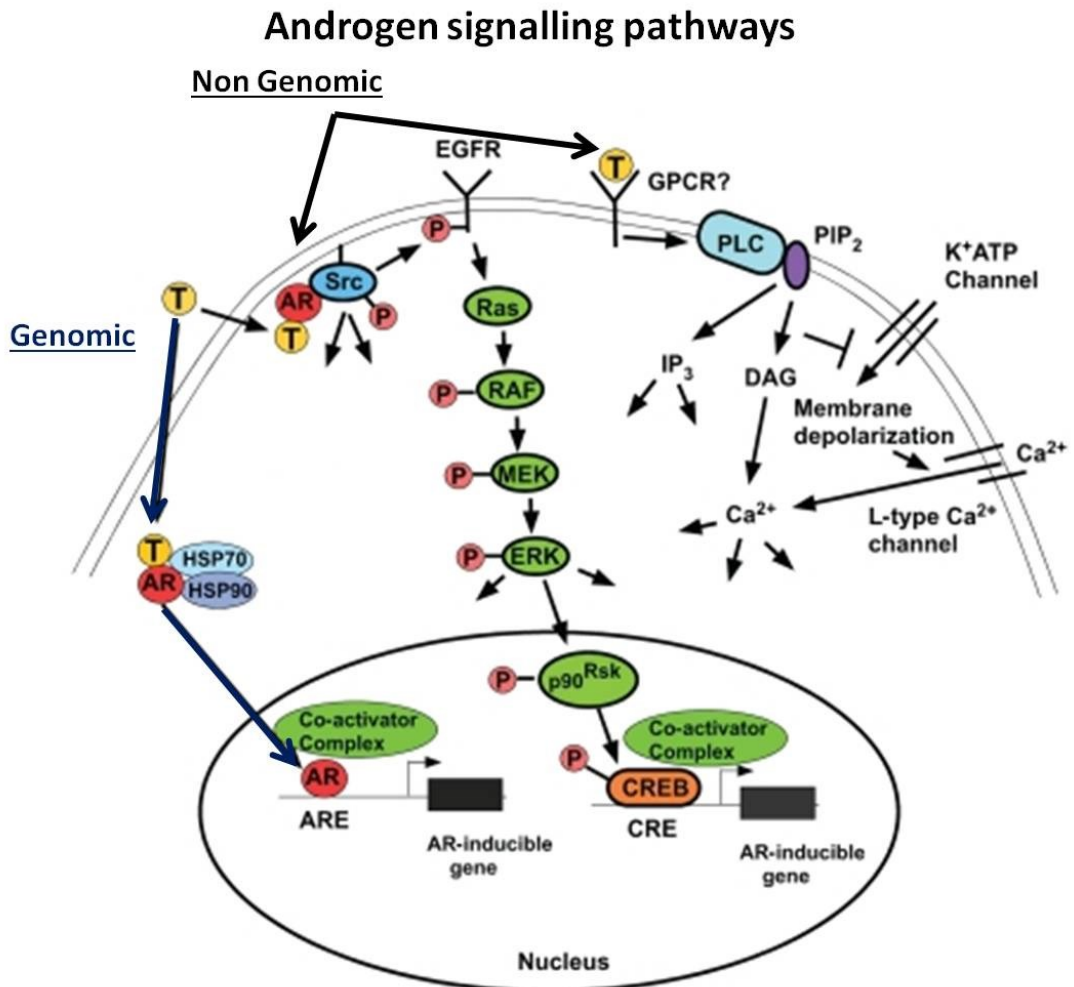


Figure 1.5 Androgen signalling pathways. Genomic androgen signalling is denoted in the blue arrows. Non genomic androgen signalling is shown in black arrows (Adapted from Walker 2011).

1.11 Androgen deficiency

Androgen production commences in the human fetal male between 7-8 weeks gestation, peaks between 12-14 weeks and then slowly declines (Prince 2001). In the rat, testosterone is first detected at e15.5 and peaks before birth at e18.5 (MacLeod et al 2010). Androgen signalling in the MPW is pivotal for normal phenotypical male development (Welsh et al 2008). Insufficient fetal androgen production or exposure interferes with masculinisation and results in disorders of sex development (DSD), which at birth manifests as ambiguous genitalia (Rey

and Grinspon, 2011, Rey et al 2012). Early fetal-onset hypogonadism results in 'dysgenetic DSD' and affects both Leydig and Sertoli cells whereas 'non-dysgenetic DSD' affects either Sertoli or Leydig cells (Josso et al 2012). In humans, insufficient androgen production as a result of fetal Leydig cell dysfunction (LH/hCG receptor mutations or defects in steroidogenic enzymes) results in undervirilization and cryptorchidism. Such patients have normal regression of Müllerian ducts due to normal functioning of Sertoli cells secreting AMH. During puberty, these patients display low-non detectable testosterone levels but LH is elevated (Mendonca et al 2010, Rey et al 2012).

1.11.1 Androgen receptor mutations

The term 'testicular feminisation syndrome' was put forward in the early 1950's to describe 46,XY patients who presented as females with bilaterally cryptorchid testes producing "oestrogen-like hormones" (Morris, 1953). This is now known as complete androgen insensitivity syndrome (cAIS) (Quigley et al 1995, Hughes et al 2012). Mutations in the AR gene can prevent AR-mediated signalling which results in 'androgen insensitivity syndrome' (AIS). In humans, 46,XY individuals with AIS present with female genitalia but incomplete testes descent and with normal and/or high testosterone production (MacLean et al 1995, Quigley et al 1995, Hughes et al 2012). Complete (c)AIS individuals present with complete undermasculinisation of the external genitalia at birth, abnormal pubertal sexual development and infertility during adulthood (Gottlieb et al 2011). Analysis of hormone levels showed normal-elevated testicular testosterone and increased LH levels (Brinkmann, 2001). Partial (p)AIS results in varying degrees of masculinisation of the external genitalia e.g. micropenis, hypospadias or ambiguous genitalia, whereas mild (m)AIS individuals present with typical male external genitalia but gynaecomastia at puberty or infertility later in life (Gottlieb et al 2011, Hughes et al 2012). There are more than 800 X-linked AR gene mutations reported in AIS patients, with the majority affecting the ligand binding domain of the AR gene (Hughes et al

2012). A recent study found a higher number of CAG repeat sequences in the *AR* gene was associated with increased risk of hypospadias (Adamovic et al 2012). High number of CAG repeat sequences is also indicative of Kennedy's disease in humans in which AIS is a common additional disorder (Kerkhofs et al 2009).

1.11.2 Tfm mouse model

The first mouse model for human androgen insensitivity was described in 1970 and was termed the 'testicular feminisation' (Tfm) mouse model (Lyon and Hawkes, 1970). Genetically XY male 'Tfm mice' are infertile, have smaller cryptorchid testes (in the inguinal region), and are phenotypically female as evident by the external genitalia, which depicts a similar phenotype to cAIS humans (Kerkhofs et al 2008). As cytosolic AR protein expression was absent, but DHT and testosterone concentrations could still be detected, this suggested that the resulting phenotype arose from a defect in AR, instead of a lack of androgen production (Gehring and Tomkins, 1974). More specifically, Tfm mice are characterised by a frameshift mutation in the AR gene resulting in non-functioning AR. Results from Tfm mice show that, of the two generations of Leydig cells, (fetal and adult), it is the adult Leydig cell population which fails to differentiate (Murphy et al 1994, O'Shaughnessy et al 2002, Merlet et al 2007).

1.11.3 Androgen Receptor Knock Out

To investigate the role of androgens in testicular cells, the Cre-loxP system has been invaluable for assessing cell-specific function by targeting AR deletion in specific testicular cells via a specific promoter-driven Cre. This provides an excellent in vivo model to investigate human cAIS as both have an inactive AR, and present as phenotypically female but with cryptorchid testes (Verhoeven et al 2010). An advantage of using this over Tfm mice is that Cre/loxP technology inactivates the AR but keeps the reading frame intact so that the mutant AR can bind its ligand but not DNA, thus target gene transcription is not regulated. To date, there have been five transgenic mouse models with ubiquitous knockout

of AR (ARKO) generated, which differ by which exon is targeted or promoter utilised (Kerkhofs et al 2009, Verhoeven et al 2010, Smith et al 2014). The phenotype of the Tfm and ARKO mouse models are comparable, both having greatly reduced testis size, cryptorchidism, absence of Wolffian duct derived structures, a vaginal opening, and increased LH levels with normal/reduced testosterone levels (Kerkhofs et al 2009). Exon 3 encodes the second zinc finger of the DNA binding domain of the AR protein (Berg et al 1989). Using a murine model with deletion of exon 3 of the AR gene (Notini et al 2005) models a population of human CAIS patients who present with a deletion of the third exon of *AR* (Quigley et al 2002). Investigation of adult Leydig cell development and function in ARKO mice revealed a reduction in testosterone levels (Yeh et al 2002) and increased LH (DeGendt et al 2005), as compared to wild-type. The number of adult Leydig cells was severely reduced (DeGendt et al 2005, O'Shaughnessy et al 2012), again implicating a role for androgen signalling in adult Leydig cell development.

1.12 Adult Leydig cell specific ARKO

Androgen signalling via the AR in adult Leydig cells regulates their development and maturation, final population number, and controls steroidogenic gene expression (De Gendt et al 2005, Eacker and Braun 2007). While the role of AR in Leydig cells has been derived from ARKO mouse models, this does not elucidate the specific role of AR signalling within Leydig cells, as AR is ablated in all cell types and results in potentially confounding changes such as cryptorchidism (Smith and Walker 2014). One relatively recent attempt to target AR in Leydig cells, used an anti-Müllerian hormone receptor 2 (AMHR2)-Cre. Although spermatogenesis was blocked in these animals and Leydig cells had a reduced steroidogenic capacity and decreased testosterone production (Xu et al 2007), this model cannot be deemed 'specific to adult Leydig cells' due to the Cre-promoter used, which is also expressed in Sertoli cells (Verhoeven et al 2010, Smith and Walker 2014). Thus, the precise role for AR signalling in adult Leydig cells remains to be fully elucidated, but use of other AR knockout

models (SCARKO, ARKO, PTM-ARKO) are useful tools to investigate adult Leydig cell development, and are described below.

1.12.1 Sertoli cell specific androgen receptor knock out

Sertoli cell-specific ARKO (SC-ARKO) male mice are generated by crossing exon 2-floxed AR mice with an AMH/Mis promoter-driven Cre (specific for Sertoli cells) (De Gendt et al 2004). This method allows investigation of the cell specific role of AR in Sertoli cells and how this might affect adult Leydig cell development. Compared to ARKO and Tfm mice, SCARKO males have a normal external male phenotype, and internal male accessory sex organs (epididymis, vas deferens, seminal vesicles and the prostate). Unlike the undescended and smaller testes in cryptorchid ARKO and Tfm mice, testes from SCARKO mice are normally descended, but reduced in size. SCARKO males are infertile with arrested spermatogenesis at the pre-meiotic stage (De Gendt et al 2004, Tan et al 2005). This suggests a role for AR signalling in germ cell meiosis and spermatogenesis, and also maintenance of cell-cell interactions between Sertoli and germ cells (Denolet et al 2006). As the number of Sertoli cells is significantly decreased in Tfm and ARKO models (De Gendt et al 2004), this might suggest that AR signalling via Sertoli cells is important for determination of Sertoli cell number. However quantification of Sertoli cells in SCARKO mice, demonstrated AR signalling via Sertoli cells was dispensable for final Sertoli cell number and maturation (De Gendt et al 2004, Tan et al 2005, Lim et al 2009, O'Shaughnessy et al 2012). Thus, AR must signal via another testicular cell type to regulate the final number of Sertoli cells.

In relation to adult Leydig cells, SCARKO mice have an approximate 40% reduction in adult Leydig cell number, evident from pnd 20 onwards, although their function was not impaired (De Gendt et al 2005). This suggests that adult Leydig cell number is regulated to some degree by AR signalling via Sertoli cells, but that androgens regulate Leydig cell function via cells other than Sertoli cells. In fact, steroidogenic gene expression in adult Leydig cells of SCARKOs was

upregulated (e.g. *Cyp11a1*, *3 β -HSD1* and *Cyp17a1*) which may suggest a compensatory mechanism to counteract for the 40% reduction in adult Leydig cells (De Gendt et al 2005). However, a more recent study that quantified adult Leydig cells in a SCARKO mouse model using the optical disector stereological method, revealed comparable numbers of adult Leydig cells between wild-type and SCARKO (O'Shaughnessy et al 2012). This difference may be a reflection of different stereological methods or different mouse strains, utilised between studies. In a transgenic mouse model of prematurely induced AR activation in Sertoli cells, the number of both fetal and adult Leydig cells was found to be reduced (Hazra et al 2013). Furthermore, adult Leydig cells displayed advanced maturation and increased androgen production per Leydig cell in order to maintain normal testosterone levels, while LH remained unchanged (Hazra et al 2013). Thus, AR signalling via Sertoli cells appears to play an important role in adult Leydig cell development.

1.12.2 Peritubular myoid cell androgen receptor knock out

The peritubular myoid (PTM) cells lie in close proximity to Sertoli cells and are involved in AR signalling via Sertoli cells to mediate spermatogenesis. Knockout of AR in 40% of PTM cells (PTM-ARKO model) demonstrated that males were infertile and azoospermic with reduced Sertoli cell function (Welsh et al 2009). In relation to fetal Leydig cells, there was no evidence for abnormal steroidogenesis or protein expression, indicating that development of the fetal Leydig cell generation, occurs independent of androgen signalling via AR in PTM cells. Although the number of adult Leydig cells in PTMARKO mice was comparable to wild-type (Welsh et al 2009), Leydig cells displayed reduced function, evident by decreased mRNA for *3 β -hsd6*, and an increase in *CYP17a1* and evidence for compensated adult Leydig cell failure, evident by increased LH with normal intratesticular testosterone levels (Welsh et al 2009, 2012).

1.13 Adult Leydig cell ablation/regeneration model

Ethane dimethane sulphonate (EDS) is an alkylating sulphonic acid ester used to specifically ablate mature adult Leydig cells in rats, which results in a short period of sterility for 2-8 weeks while having few other toxicological or adverse effects (Bartlett et al 1986, Sharpe et al 1990, Teerds et al 1999). Following a single injection of 75mg/kg EDS, mature adult Leydig cells are ablated which results in the absence of testosterone production post-EDS, which returns to pre-injection levels after 3-7 weeks, coincident with redeveloping adult Leydig cells (Teerds et al 1999). Weight of the testes, seminal vesicles and prostate are reduced as a consequence of reduced testosterone production. Numbers of regenerating adult Leydig cells are restored to their pre-injection levels, after approximately 5-6 weeks post-EDS injection (Teerds and Rijintjes, 2007).

EDS can exert its cytotoxic effect on other cell types e.g. the parenchymal cells of the adrenal cortex (Plecas et al 1997), but its main cytotoxic targets are mature adult Leydig cells. The counterpart in females to adult Leydig cells in males, are steroidogenic ovarian theca cells, however EDS did not affect the thecal cells (Smart et al 1990). Interestingly, EDS does not ablate progenitor/immature Leydig cells, as shown in studies in which EDS was administered to prepubertal rats (Kelce et al 1991). It is still unknown precisely how EDS specifically induces adult Leydig cell death but the biosynthesis of testosterone is inhibited as steroidogenic activity is severely reduced (Stocco and Clark 1996), which coincides with an increase in intracellular glutathione levels (Kelce and Zirkin 1993). The majority of mature adult Leydig cells become apoptotic 24 hours post-EDS, evident by chromatin condensation and nuclear fragmentation, along with the presence of apoptotic proteins Fas and Fas-ligand, which cleave pro-caspase3 into active-caspase 3, a known apoptotic marker (Taylor et al 1999, Kim et al 2000). EDS is an invaluable tool to investigate adult Leydig cell regeneration, as it recapitulates normal adult Leydig cell development. Furthermore, progenitor adult Leydig cells can be investigated in this model, prior to their differentiation into newly developing adult Leydig cells.

1.14 Di (*n*-butyl) phthalate

Di (*n*-butyl) phthalate (DBP) is a phthalate ester which has wide applications in plastics but is also used in some cosmetics and medications (Hu et al 2009, Martinez-Arguelles et al 2013). An earlier study that investigated the effects on the F1 generation of dams exposed to DBP in their diet, highlighted the significance of exposure during gestation, as opposed to adulthood. DBP-exposed F1 male offspring exhibited abnormal testes and reproductive tract development along with decreased sperm production in adulthood (Wine et al 1997). Thus, DBP disrupts fetal testis development and results in a variety of reproductive disorders (Albert and Jegou 2013), which can include; malformations of the epididymis, vas deferens, seminal vesicles or prostate (Mylchreest et al 1998), retained nipples (Foster, 2006), decreased AGD (Mylchreest et al 1998, van den Driesche et al 2012), hypospadias (Mylchreest et al 1998), cryptorchidism (McKinnell et al 2005). These hallmarks are a result of DBP-induced inhibition of fetal Leydig cell function e.g. testosterone and *Insl3* production.

The primary mechanism via which DBP exerts its effects on fetal Leydig cells is still unclear but DBP exposure clearly inhibits a key player in the transport of cholesterol to initiate steroidogenesis, *StAR* (Clark et al 1995, Shultz et al 2001, Manna et al 2009, Papadopoulos and Miller 2012, van den Driesche et al 2012), as well as repression of steroidogenic genes encoding enzymes involved in the steroidogenic pathway e.g. *Cyp11a1*, *3 β -hsd1*, *Cyp17a1* (Barlow et al 2003, Lehmann et al 2004, Thompson et al 2004, Plummer et al 2007, van den Driesche et al 2012, Desdoits-Lethimonier et al 2012). A large gene analysis study which investigated the effects of fetal rat exposure to different phthalates between e12-e19, showed that the most marked disruption involved DBP disruption of steroidogenic pathways and cholesterol transport (Liu et al 2005). As a consequence, fetal exposure to DBP significantly lowers intratesticular testosterone levels in the rat (Mylchreest et al 1999, Scott et al 2008, van den Driesche et al 2012). The number of fetal Leydig cells is unaltered by fetal DBP-exposure, rather their cytoplasmic volume is reduced (van den Driesche et al

2012). A study that compared steroidogenesis in the fetal testis and adrenal gland in the rat following fetal DBP exposure, showed that steroidogenic gene expression in the adrenal gland was unaltered whereas there was a rapid change in the fetal testis e.g. repressed transcription of *StAR*, *Cyp11a1*, *Cyp17a1* (Thompson et al 2005), thus demonstrating DBP-induced fetal Leydig cell dysfunction. An obvious hallmark of fetal DBP-exposure is the occurrence of large focal areas of fetal Leydig cell aggregates (Mahood et al 2005, Lin et al 2008, van den Driesche et al 2012). Moreover, ectopic Sertoli cells form within these Leydig cell aggregates and manifest postnatally, as focal areas of dysgenesis containing malformed seminiferous tubules, Sertoli cell only tubules or tubules containing entrapped Leydig cells (Hutchinson et al 2008).

Long term effects of fetal DBP exposure in rats were observed at 6, 12 and 18 months of age, which included male reproductive tract abnormalities and Leydig cell dysgenesis (Barlow et al 2004). A recent study which investigated the effects of fetal DBP-exposure on adult rats, showed a reduction in testicular 3β -HSD and 17β -HSD enzymatic activity, associated with compensated adult Leydig cell failure (Giribabu et al 2012), indicating an altered/abnormal steroidogenic pathway throughout postnatal life as a result of fetal exposure to DBP.

The purpose of exposing fetal rats to DBP during development and thus altering androgen production is to induce a TDS-like syndrome as evident in man, which similarly results in cryptorchidism, hypospadias, abnormal serum hormone levels and dysfunctional Leydig cells (Mylchreest et al 2002, Fisher et al 2003, Barlow and Foster 2003, Sharpe and Skakkebaek 2008). In cryptorchid 3-month old boys, FSH and LH levels were significantly increased compared to control infants (Suomi et al 2006), an abnormality also found in boys with hypospadias (Hsieh et al 2008, Wohlfahrt-Veje et al 2009). In a large clinical study investigating hormone levels in 357 infertile men, 18% were shown to have increased serum LH and 19% had lower testosterone levels, in comparison to 318 fertile men, which is indicative of compensated adult Leydig cell failure

(Andersson et al 2004). Thus, the use of DBP-induced testicular dysgenesis in rats provides an experimental model to investigate human TDS.

1.15 Flutamide

Flutamide is a synthetic non steroidal androgen receptor antagonist, specifically designed by pharmaceutical companies to competitively bind to AR and thereby block the action of testosterone and DHT (Peets et al 1974, Virtanen and Adamsson 2012). More specifically, flutamide can disrupt the N/C interaction of AR which prevents transcriptional activation of AR in target tissues. Flutamide is a useful tool to study androgen-dependent male reproductive development. Fetal exposure of flutamide in rats (e12-e21) resulted in reduced reproductive organ weights, reduced AGD, cryptorchidism, hypospadias and increased nipple retention, evident during postnatal life (McIntyre et al 2001). Perinatal exposure to flutamide in rats (e14-pnd 3) results in reduced reproductive organ weights, reduced AGD, cryptorchidism, hypospadias, penis malformation, and retained nipples (Miyata et al 2002). Furthermore, a long term effect of perinatal exposure to flutamide was evident during adulthood (pnd 60) from abnormal adult Leydig cell function i.e. significantly increased LH levels with normal testosterone levels (Miyata et al 2002). Administration of flutamide during gestational development in a porcine model demonstrated a long-term effect in adult boars with impaired Leydig cell responsiveness to LH and reduced testosterone (Kotula-Balak et al 2012). Fetal exposure to flutamide, at a dose of 100 mg/kg/day between e15-18 in the rat, inhibited masculinisation of the reproductive tract by blocking peripheral androgen action (Welsh et al 2008, 2009). Postnatal exposure (pnd1-15) to flutamide reduced the number of Sertoli cells by 18% at pnd 25 and this reduction was exacerbated by prenatal DBP-exposure (Auharek et al 2010). Overall, while flutamide is a potent AR antagonist, it may have limited ability to antagonise the high intratesticular testosterone levels present in the fetal testis (Welsh et al 2008).

1.16 Testosterone Propionate

Testosterone propionate (TP) is an androgenic steroid which has previously been used to manipulate testosterone levels, as described below. Excess androgen exposure via TP during fetal development of rats, results in masculinised females, as evident from an increased AGD, absence of a vaginal opening, lack of nipples, masculinised external genitalia, and the presence of a prostate and seminal vesicles (Wolf et al 2002, Welsh et al 2008, 2010). In postnatal life, this resulted in anovulation and infertility (Wolf et al 2002). Increased masculinisation of the external genitalia in females was most likely due to increased circulating TP being converted to DHT. Increased exposure to TP during fetal life can also result in intrauterine growth retardation (IUGR) in rats (Wolf et al 2002, 2004) and in humans (Carlsen et al 2006). The effect of fetal TP-exposure on male offspring was assessed in postnatal life in rats, and demonstrated a delayed onset of puberty and reduced testosterone plasma levels in adulthood (Dela Cruz and Pereira, 2012).

1.17 Dihydrotestosterone

Dihydrotestosterone (DHT), is a testosterone metabolite which is produced following testosterone conversion by 5 α -reductase (type II). 5 α -reductase (type I) is found in the skin and liver whereas type II is predominantly found in male reproductive tissues (Miller and Auchus, 2011). It is responsible for development of the prostate (Schultz and Wilson 1974, Imperato-McGinley et al 1992), prevention of areola and nipple formation (Goldman et al 1976, Topper and Freeman 1980, Imperato-McGinley et al 1992), as well as determining the length of AGD (Imperato-McGinley et al 1985, 1986, Clark et al 1990, Bowman et al 2003, Welsh et al 2008). DHT binds to AR with a higher affinity than testosterone (Brinkmann, 2011). In humans, the vital role of DHT in masculinising external genitalia and the prostate was first highlighted in 1974 (Imperato-McGinley et al 1974). Patients with a mutation in the gene coding for 5 α -reductase type II enzyme have a form of pseudohermaphroditism (normal differentiation of the Wolffian duct but defective external virilisation) (Wilson et

al 1993, Verhoeven et al 2010). Lowering fetal DHT exposure levels in rats via finasteride, a 5 α -reductase (type II) inhibitor (i.e. blocks the conversion of testosterone to DHT) results in permanent changes to AGD and nipple retention in males. This study also highlighted that DHT is not essential for differentiation of the Wolffian duct (Bowman et al 2003). A recent study that investigated the effect of exogenous fetal DHT-exposure in rats, demonstrated that 10mg/kg of DHT could masculinise females but could not advance or enhance male reproductive development, although a potential effect on Leydig cells was not examined (Dean et al 2012).

1.18 Fetal/developmental programming

The importance of the intrauterine environment in promoting lifelong health and/or disease risk is a relatively recent phenomenon, which began with the association between low birth weight and hypertension in adulthood (Barker et al 1993). Most human data of early life programming utilised fetal birth weight as an indication of intrauterine growth and development (Waterland and Michels 2007). It is becoming increasingly apparent that in terms of androgen exposure during fetal life, this can be measured by AGD throughout postnatal life (Eisenberg et al 2011, 2012). Furthermore, reduced AGD i.e. reduced fetal androgen action, is associated with increased risk of male reproductive disorders (Welsh et al 2008, Dean and Sharpe, 2013).

1.19 Epigenetic changes

The term 'epigenetics' was first coined by Conrad Waddington to describe the study of "interactions between genes and their products which bring phenotype into being" (Waddington, 1968). The term epigenetics refers to a mitotically and trans-generationally heritable alteration to the genetic make-up by altering gene expression without altering the DNA sequence (Berger et al 2009). Thus, the phenotype of daughter cells is altered without any change to the genotype, although this alteration is potentially reversible (Weaver et al 2005). Epigenetic factors that can alter gene transcription include histone modifications,

methylation of DNA and microRNAs (Bibikova et al 2008), which will be discussed below. These mechanisms interact with one another and abnormal activity of one or all of these mechanisms, can result in epigenetic diseases e.g. cancer. Together, these processes can determine when and where, various sets of genes are expressed in a tissue or cell (Zhang and Ho, 2011).

Epigenetic modifications can occur through micro RNAs (miRNAs), which are a class of small non-coding RNAs which can bind to target mRNAs and suppress protein translation (Kong et al 2008). One miRNA can target several mRNAs, with each mRNA susceptible to negative influence by a variety of miRNAs. Thus extensive miRNA profiling in the future will open up the field of miRNAs in gene regulation. Epigenetic modifications can also occur via both methylation of DNA CpG dinucleotides and through histone modifications, in which the latter acts by remodelling chromatin to control gene expression (Warner and Ozanne, 2010). Thus, the organism can respond to its environment and change its gene expression accordingly, which has implications for fetal development in a perturbed intrauterine environment e.g. deficient androgen action.

1.19.1 DNA methylation

DNA methylation occurs at cytosines within cytosine-guanine dinucleotides (CpG) (the 'p' denotes a phosphate group) islands and converts cytosine to 5-methylcytosine, and is generally associated with transcriptional silencing (Waterland and Michels 2007). A CpG island on one DNA strand always pairs with a CpG island on the complementary strand. To transfer epigenetic information during DNA replication, the newly synthesised DNA strands are unmethylated (hemimethylation). DNA methyltransferase 1 (DNMT1) maintenance methylase restores the pattern on the daughter DNA strand to that matching the parent DNA molecule (Waterland and Michels, 2007). This provides a mechanism for transferring epigenetic information in proliferating cells. DNMT3a and 3b generate new methylation patterns in quiescent cells during embryonic development (Siedlecki and Zielenkiewicz, 2006). CpG

methylation in promoter regions of genes is associated with transcriptional silencing, potentially by affecting the binding of methylation-sensitive DNA-binding proteins and interacting with histone modifications (Jaenisch and Bird 2003). Site specific changes in DNA methylation are highly correlated with gene expression (Ehrlich 2003, Waterland and Michels 2007), thus CpG methylation studies can provide a read-out of epigenetic regulation.

1.19.2 Histone modifications

Transcriptional silencing is associated with methylated promoters, as described above; a process also associated with repressive histone markers (Tiwari et al 2008), as discussed below. Chromatin is mainly composed of DNA wrapped around histones (H1, H2A, H3 and H4) in the nucleosome, which can be modified by acetylation, methylation, phosphorylation, sumoylation or ubiquitination (Matinez-Arguelles and Papadopoulos, 2010). 'Histone modifications' refer to modification/remodelling of the chromatin structure via histones, a process which can regulate gene transcription (Shen et al 2009) via transient or permanent reorganisation of chromatin (Kadonaga 1998, Kondo 2009). Acetylation and methylation of histones are the best characterised of histone modifications. When histone lysine residues are acetylated, the charge attraction between DNA and histones weakens, thus decondensing the chromatin and in such manner, promotes gene transcription. Methylation of histone lysine residues can be activating or repressive e.g. methylation of H3K4me promotes an active chromatin structure leading to transcriptional activation. Conversely methylation of H3K27me for example, is known to be a repressive transcriptional mark (Zhang and Ho 2011). Thus transcriptional activation can occur via histone methylation e.g. of lysine 4 at histone 3 (H3K4) or transcriptional repression by methylation of lysine 27 at histone 3 (H3K27) (Lee et al 2005). Tri-methylation at lysine 27 of histone 3 (H3K27me₃) is one such histone modification, the expression of which commonly acts as a repressive mark (Kirmizis et al 2004, Barski et al 2007, Sui et al 2012) by

condensing the chromatin, thus impeding the binding of transcription factors to response elements in the promoter and thus preventing transcription (Li et al 2007).

Epigenetic changes can be meiotically and/or mitotically heritable, altering gene expression potential without altering the gene sequence (Wu and Morris, 2001), and can occur early during cell proliferation and development (Klein et al 2005). Of the histone modification marks, H3K27me3 and H3K9me3 demonstrate mitotic inheritance (Bernstein et al 2007, Margueron et al 2009). Epigenetic changes can also occur within the proximal promoter region of a gene (Bernstein et al 2006). This region in the promoter of *Star* was found to have reduced H3K27me3 upon *StAR* transcription, as induced by hCG injection in granulosa cells (Lee et al 2013). The proximal promoter region has also been shown to be important for *StAR* transcription in MA-10 cells (Hiroi et al 2004) and granulosa cells (Christenson et al 2001). In other recent studies investigating *StAR* regulation, its transcription was induced by hCG injection in granulosa cells (Lee et al 2013) or by 8-Br-cAMP in MA-10 cells (Hiroi et al 2004) and in both cases, increased *StAR* transcription was associated with a reduction in H3K27me3 in the proximal promoter region of *StAR*.

Histone methyltransferase (HMT) enzymes can modify specific lysine residues on core histones, in relation to H3, it can catalyse trimethylation of lysine residues on H3 (Zhang and Reinberg 2001, Lachner et al 2003). Lysine-specific demethylase 1 (LSD1) functions to remove transcriptionally repressive methylation marks from histone in favor of gene expression (Martinez-Arguelles 2010). Knockdown of LSD1 results in decreased transcriptional activation of AR-driven genes (Metzger et al 2005). Thus, by relieving repressive histone marks, demethylases can control specific gene expression.

Several studies have pointed to a role for polycomb-repressive complex 2 (PRC2), which is comprised of histone methyltransferases e.g. enhancer of zeste (Ezh1/2), suppressor of zeste-12 (Suz12), and embryonic ectodermic development (Eed) (Cao et al 2002, Muller et al 2002, Shen et al 2008,

Margueron et al 2008, 2009) and more recently a coregulator, 'Jarid2' (Shen et al 2009, Li et al 2010) as the main mediators of this process. More specifically, EZH2 catalyses the methylation of lysine 27 at histone 3 (H3K27me3) (Cao and Zhang 2004, Schuettengruber et al 2007). Overall, histone modifications in controlling gene regulation in postnatal life, warrants further investigation of 'pre-programmed' progenitor cells during fetal development.

1.20 Hypotheses

The 'window of epigenetic reprogramming' is considered to occur *in utero* (Prins, 2008, Gluckman et al 2009, Thompson & Einstein 2010), and altering levels of androgen exposure during the MPW (Welsh et al 2008) can result in reproductive disorders manifesting in newborns and young men (Sharpe and Skakkebaek 2008). This may suggest that somatic testicular cells, altered by deficient androgen action, are epigenetically programmed during fetal development. Thus, the hypothesis underpinning this thesis, was that adult Leydig progenitor cells are present in the fetal testis and are susceptible to effects of altered androgen action/production, thus affecting their differentiation and functional development at puberty into normal functioning adult Leydig cells. As a consequence, adult Leydig cell function is altered, for example resulting in reduced testosterone production during adulthood. Lower testosterone levels in adulthood are associated with 'Western disorders', morbidity and mortality (Tivesten et al 2009, Traish et al 2011). The experimental work in this thesis aimed to investigate how fetal events, i.e. androgen exposure, can programme adult testosterone levels.

To achieve this, adult Leydig cell development was first recapitulated via the EDS model, to characterise putative adult Leydig progenitor cells and assess whether COUP-TFII-expressing interstitial cells could differentiate into adult Leydig cells (Chapter 3). The next aim was to establish whether COUP-TFII+ interstitial cells might be fetal androgen targets i.e. express the AR (Chapter 3). To investigate whether the putative adult Leydig progenitor cells are androgen-modulated, various AR knockout models including ubiquitous ARKO, Sertoli-cell

specific ARKO and PTM-ARKO were analysed, in order to investigate whether this affected the number of adult Leydig progenitor cells and/or the function of adult Leydig cells (Chapter 4). This was also carried out in a DBP model to investigate whether lowered fetal testosterone levels affected the number of adult Leydig progenitor cells and/or the function of adult Leydig cells (Chapter 4). The mRNA expression of adult Leydig cell specific genes in adulthood, after fetal exposure to DBP was also investigated (Chapter 4). Pregnant rats were exposed to various treatments to manipulate intratesticular androgen levels during fetal/postnatal development (Chapter 4). Finally, to investigate a mechanism of fetal 'programming' in DBP-exposed animals, ChIP analysis was undertaken to explore the hypothesis of altered *StAR* regulation via histone modifications i.e. increased histone repressor, H3K27me3 (Chapter 5).

1.21 Aims

To characterise adult Leydig progenitor cells in an adult Leydig cell ablation/regeneration model and throughout normal pubertal and adult Leydig cell development.

To determine if adult Leydig progenitor cells are conserved across species in the fetal testis.

To establish whether adult Leydig progenitor cells are androgen-modulated via several androgen receptor knockout models and altered testosterone production/action rodent models.

To investigate potential 'gain of adult Leydig cell' function models.

To investigate a mechanism of fetal 'programming' of adult Leydig progenitor cells, which may explain how fetal events programme adult testosterone levels.

Chapter 2 General Materials and Methods

2.1 Human fetal tissue collection

Human fetal testes were obtained following termination of pregnancy. Women gave written consent according with UK national guidelines (Polkinghorne, 1989) and the study was approved from the Local Research Ethics Committee. Medical/surgical termination was induced by treatment with mifepristone (200mg orally), followed by either misoprostol (Pharmacia, Surrey UK 200 mg every 3 h, per vaginam) or depending on weeks of gestation, vacuum aspiration under general anaesthetic. None of the terminations were for reasons of fetal abnormality. Gestational age was determined using ultrasound and confirmed by subsequent measurement of foot length in 2nd trimester samples examined. Micro-dissection of the testes from the fetal abdominal cavity was performed and samples were fixed in Bouin's for immunohistochemistry, as described below.

2.2 Animal work

All animal work was completed in the Biomedical Research Facility (BRF) Little France animal facility in accordance with the UK Home Office Animal Experimentation Scientific Procedures Act 1986, under project licences 60/4564 and 60/4200. Wistar rats were purchased from Harlam UK and bred to generate stock within the BRF animal facility. William Mungal performed licensed procedures including treatments and day-to-day animal husbandry, while I performed licensed procedures under personal animal licence 60/12997.

2.2.1 Welfare conditions

Animals were housed in the BRF animal facility in a room with fixed lighting for 12h per day from 07:00 to 19:00. Humidity levels were maintained at approximately 55% while room temperature was kept between 20-25°C. Food

and water were available *ad libitum*. Rats were housed in clear-sided cages, which contained solid bottoms covered in wood shavings for bedding.

2.2.2 Time-matings

Time-matings were used to allow calculation of gestational stages. For mating purposes, male and female rats were paired together in a grid-bottomed cage overnight, which was subsequently inspected for evidence of copulatory plugs which had fallen through the grid overnight. This indicated successful mating and the gestational stage was immediately recorded as embryonic day (e), 0.5. The male was removed from the cage and pregnant female rats housed separately. Rats become sexually mature at approximately six weeks of age. Therefore, to ensure successful pregnancies, proven fertile male rats were used aged between 10 and 14 weeks of age.

2.3 Treatments during pregnancy

Androgens are required during fetal life for masculinisation and normal somatic cell development. Therefore, the purpose of the experiments described below was to manipulate fetal androgen exposure during development in order to investigate the effect on adult Leydig cell development. To address this, pregnant female rats were administered treatments during defined stages of pregnancy. In this manner, offspring could be assessed for abnormalities after indirect exposures during fetal development. The treatments used were: di-*n*-butyl phthalate (DBP), testosterone propionate (TP) or dihydrotestosterone (DHT). DBP was administered via oral gavage using a 15-16G blunt ended steel gavage cannula, 10-12cm in length (Medicut, Sherwood Medical Industries Ltd, UK) attached to a disposable plastic 1ml syringe. TP and DHT were administered by subcutaneous injection using a 25G, 0.5 x 16mm needle (BD Microlance) attached to a 1ml plastic syringe. To ensure correct dosing of the relevant chemical, rats were weighed directly prior to dosing. Treatments were administered between 09:00 and 11:00 for both consistency and to allow time

to check the animals regularly for any sign of toxicity or discomfort during the rest of the day.

2.3.1 Di (n-Butyl) phthalate

Di (*n*-butyl) phthalate (DBP; Sigma) was administered via gavage to pregnant dams from e13.5-21.5, which encompasses the masculinisation programming window (MPW) (Welsh et al 2008, Macleod et al 2010), a period in which testosterone production is crucial for masculinisation. To investigate the effect that lowered fetal testosterone production has on adult Leydig cells and their progenitors, pregnant dams were gavaged daily with DBP at 500mg/kg maternal bodyweight. This dose has previously been shown to lower fetal testosterone by >70%, resulting in areas of focal dysgenesis at birth and adulthood, along with other reproductive tract abnormalities (Mylchreest et al 1998, Foster et al 2001, Mahood et al 2005, Mahood et al 2006, Hutchison et al 2008, van den Driesche et al 2012). DBP treatment did not cause any obvious adverse toxicological effects to the dams nor was there any increase in fetal mortality. DBP was diluted in corn oil (purchased from a supermarket) as per previous studies from our laboratory, e.g. to make up a dose of 500mg/kg dam bodyweight; 5ml DBP was made up to 10ml with corn oil and administered at 1ml/kg maternal bodyweight. Treatments were freshly prepared and stored at room temperature in an airtight container. DBP-exposed fetuses were studied at the end of gestation (e21.5) or on postnatal days 6, 10, 25 or 90.

2.3.2 Flutamide

Flutamide (Sigma, Poole, UK) is a potent synthetic anti-androgenic compound which blocks the action of both testosterone and DHT by competitively binding to the androgen receptor. The purpose of exposing pups to flutamide postnatally was to investigate the modifying effects of androgen deprivation to adult Leydig progenitor cells during the postnatal period, prior to adult Leydig cell differentiation (~Pnd 15). To expose the pups via breast milk, flutamide was

administered to nursing dams by oral gavage (100mg/kg maternal bodyweight) in 2.5% dimethylsulfoxide (DMSO; Sigma) in 1ml/kg corn oil (purchased from a supermarket) from pnd 1-15. In a separate series of studies, the postnatal flutamide treatment as described was combined with fetal DBP-exposure (Section 2.3.1) to investigate the cumulative effects of altered androgen exposure during pre- and postnatal life on adult Leydig progenitor cell development. Treatments were freshly prepared and stored at room temperature in an airtight container.

2.3.3 Testosterone Propionate

As the purpose of administering the previous treatments was to decrease fetal androgen exposure, the aim of the next experiments were to increase the level of fetal or postnatal androgen exposure. The purpose of which, was to investigate potential effects to adult Leydig and/or their progenitor cells, potentially resulting in a "gain of adult Leydig cell function" model. Testosterone propionate (TP; Sigma) is an androgenic steroid which has previously been used to manipulate testosterone levels (Wolf et al 2002, 2004, Welsh et al 2008, 2010). TP was administered daily by subcutaneous injection to pregnant rats on e14.5-e21.5 at 20mg/kg maternal body weight, as in previous studies (Welsh et al 2008, 2010). In a separate series of experiments, newborn male rats were administered TP daily by subcutaneous injection from postnatal day 1-24, to investigate if increased postnatal TP affects the development of adult Leydig cells and/or their progenitor cells. TP was dissolved in corn oil (purchased from a supermarket) with treatments freshly prepared and stored at room temperature in an airtight container.

2.3.4 Dihydrotestosterone

As TP administration resulted in confounding factors, including fetal growth restriction and the potential aromatisation of TP to estradiol, this prompted the use of another model to potentially and effectively elevate intratesticular

testosterone exposure. Dihydrotestosterone (DHT) has a higher affinity for the androgen receptor compared to testosterone and unlike the aforementioned, DHT cannot be aromatised into estradiol. To investigate whether increasing androgens during fetal development would affect adult Leydig cells and/or their progenitor cells, DHT was administered daily by subcutaneous injection to pregnant rats on e15.5-e21.5 at 10mg/kg maternal body weights. This dose was chosen as previous studies have shown administration of 10mg/kg induced a degree of female masculinisation (Dean et al 2012). DHT was dissolved in corn oil (purchased from a supermarket) with treatments freshly prepared and stored at room temperature in an airtight container.

2.4 Inducible nitric oxide synthase knock out mouse model

To investigate the effect that potentially increased fetal androgen exposure might have on adult Leydig progenitor cell development, the iNOS^{-/-} mouse model was used. This model has previously been shown to have increased testis weight, increased Sertoli and adult Leydig cell number and increased AGD at all postnatal ages examined (Auharek et al 2011, 2012). As the administration of TP or DHT in the previous models used, as above, may not definitively increase intratesticular testosterone (ITT), the iNOS^{-/-} model was chosen for its potentially increased endogenous ITT. Slides containing sections of wild-type control and iNOS^{-/-} (from Prof. Luiz De Renato) were analysed. Briefly, wild type (C57BL/6) and iNOS-deficient mice (B6.129P2-Nos2tm1Lau/J) were purchased from the Jackson Laboratory. All animals were treated according to the Ethics Committee in animal experimentation from the Federal University of Minas Gerais, as described previously (Auharek et al 2011, 2012).

2.5 Adult Leydig cell ablation/regeneration via ethane dimethane sulphonate

Ethane dimethane sulphonate (EDS) is a toxic chemical used to specifically ablate mature adult Leydig cells in rats while having few other toxicological or adverse effects (Bartlett et al 1986, Sharpe et al 1990, Teerds et al 1999). One

week after a single injection of 75mg/kg EDS, new adult Leydig cells begin to regenerate, with normal numbers restored after approximately 5-6 weeks post EDS injection. EDS (gift from Prof. Ian Morris) was used in this study as an ablation/regeneration model to recapitulate normal ALC development and to investigate the phenotype of the progenitor cells from which the 'new' adult Leydig cells differentiate. EDS was administered by a single i.p. injection to adult male Sprague Dawley rats at a dose of 75mg/kg/ml. Control animals received vehicle only (DMSO; Sigma). To dissolve the EDS in DMSO, the solution was heated for ten minutes. It was prepared fresh on the morning of injection and kept in an airtight container for use.

2.6 Lineage-tracing of adult Leydig cells

In order to demonstrate whether COUP-TFII⁺ adult Leydig progenitor cells could develop into adult Leydig cells, a model for adult Leydig lineage tracing was utilised, generated by Dr. Laura O'Hara and Prof. Lee Smith. Briefly, mice with Cre recombinase driven by the adipocyte protein (aP2) promoter were bred with a YFP reporter line which showed aP2 was restricted to Leydig cells (O'Hara et al, unpublished). The use of a YFP/GFP antibody allowed for visualisation of aP2 expression in adult Leydig cells and their progenitors in fetal life. More specifically, male congenic C57BL/6J mice hemizygous for an aP2-Cre transgene (He et al 2003) were mated to homozygous R26R-EYFP females (Srinivas et al 2001). An aP2-YFP (without Cre) was utilised as an appropriate control.

2.7 Androgen receptor knockout mouse models

Both the Sertoli cell androgen receptor knockout (SCARKO) and the androgen receptor knockout (ARKO) mouse model were generated by Karel de Gendt in Belgium, using cyclisation recombination (Cre) recombinase / loxP technology, as previously described (De Gendt et al 2005). Briefly, to generate Sertoli cell-selective Androgen Receptor Knockout (SCARKO) mice, ARflox^{+/+} female animals were crossed with anti-Müllerian hormone (AMH)-Cre^{+/+} male mice

expressing Cre recombinase selectively in Sertoli cells. The complete Androgen Receptor Knockout (ARKO) mouse line was generated by crossing AR^{flox}/AR⁺ female animals (129/Swiss) with phosphoglycerate kinase-1 (PGK)- Cre^{+/+} male animals, (C57BL/6) in which the Cre is expressed ubiquitously in cells. Peritubular myoid cell-specific AR knockout (PTM-ARKOs), were generated by mating male mice heterozygous for Cre recombinase under the control of a smooth muscle myosin heavy chain (MH; Xin et al 2002) promoter with female mice homozygous for a floxed AR (De Gendt et al 2004, Welsh et al 2009). P_{gk1}-Cre without a floxed AR gene was used as an appropriate control in these mouse lines. A schematic representation is shown below (Figure 2.1). All animals were generated and maintained according to the UK Home Office guidelines for the Care and Use of Laboratory Animals and all experiments were approved by the Local Ethics Committees.

Androgen receptor knockout models via Cre/loxP system

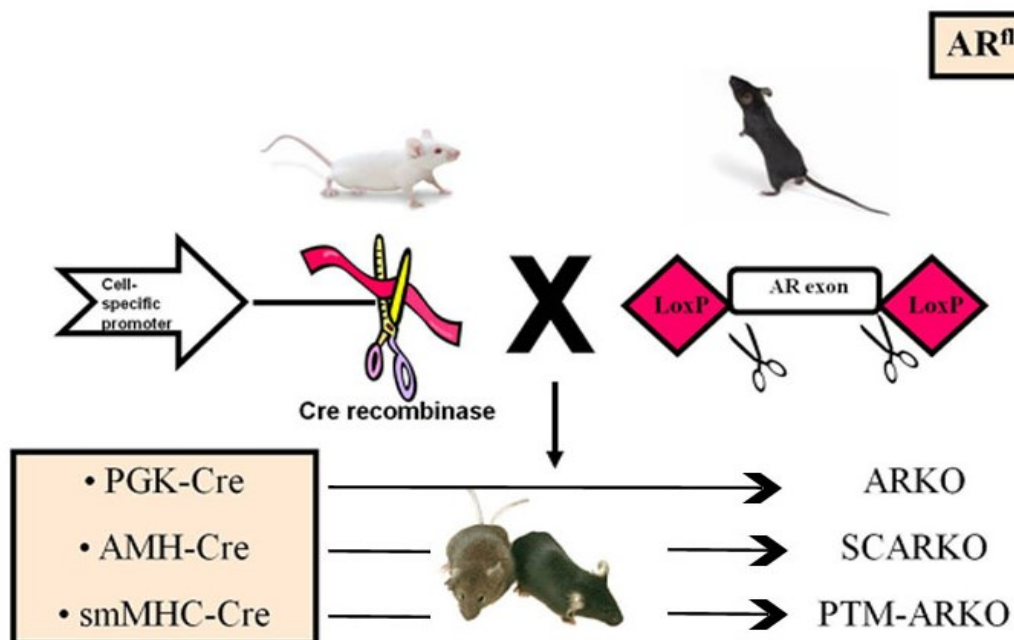


Figure 2.1 Androgen Receptor knockout mouse models via Cre/loxP system. A specific promoter-driven Cre recombinase (scissors), targets loxP sites which flank the gene of interest, namely AR, in these studies.

2.8 Necropsy

Postnatal animals were killed by inhalation of carbon dioxide (CO₂) and subsequent cervical dislocation under schedule 1 of the animal (Scientific Procedures) Act 1986. Neonates from pregnant dams were killed by decapitation and placed in ice-cold 0.01M phosphate buffered saline (PBS; Sigma; section 2.16.3).

2.8.1 Gross dissection

Fetuses were removed from the deceased pregnant dam via standard gross dissection techniques. The dam was placed on her back and an incision to the abdomen allowed the uterus to be removed intact. Once each amniotic sac and umbilical cord was cut, measurements for each fetus were taken immediately prior to placing them in ice-cold PBS. This minimised tissue degradation occurring during transportation, prior to carrying out fine dissections (Section 2.8.3).

2.8.2 Anogenital distance

The anogenital distance (AGD), refers to the measurement of the distance from the middle of the anus to the base of the genital tubercle. AGD is increased (nearly two-fold) in normal males compared to normal females in response to masculinisation and thus provides a read-out of androgen exposure during fetal life (Welsh et al 2008, Macleod et al 2010, Dean & Sharpe 2013). Prior to any incision being made for tissue dissection, AGD was measured using electronic digital callipers with a resolution of 0.02mm (Faithfull Tools, Kent) which provided a measure of any increase/decrease in androgen exposure during fetal development.

2.8.3 Fine dissection

Bodyweight for each animal was measured using an electronic analytic balance (Handy H110, Sartorius) prior to decapitation. Each fetus was placed in a petri dish (Corning, UK) containing tissue paper soaked in PBS and placed under a binocular dissecting microscope (Leica, MZ6) which contained a trans-illuminated stage. Extra lighting, when needed, was provided by external cold lights (Leica CLS 150x) to minimise any heat damage caused by the lights. Testes were trimmed using the bevelled edge of 27G needles (Monoject, sterile needles, 0.4mm x 12mm) attached to disposable plastic 1ml syringes. This allowed accurate removal of the fetal epididymis and efferent ducts, prior to weighing the testes for fixing or freezing.

2.8.4 Postnatal tissue collection

Postnatal tissue was collected in male rats on postnatal day 6, 10, 25, 75 and 90. Male rats were placed in a supine position for an incision to be made in their abdomen, to enable removal of the prostate and testes. The latter were obtained by pulling on the fat pad attached to the scrotal sac. Following tissue excision, the testes and prostate were weighed using an electronic analytic balance (Handy H110, Sartorius). Images of the dissected tissue were photographed using a D70 camera (Nikon, Surrey, UK) fitted with a Nikon AF Nikkor 24-120 mm lens.

2.9 Tissue preservation

Following retrieval of tissue and their relevant measurements, samples were immediately either snap-frozen or stored in Bouin's solution to preserve the tissue for future use.

2.9.1 Frozen tissue

One testis per animal was cut in half and both halves were separately placed in a 2ml eppendorf on dry-ice prior to being stored in the freezer at -80°C until required.

2.9.2 Fixed tissue

The isolated tissue was fixed in Bouin's solution (see section 2.16.1) in an airtight container. For adult male rats, testes were left in Bouin's solution for a total of 6 hours. Following the initial three hours, the partially fixed testis was halved using a razorblade and re-submerged in Bouin's for a further three hours, to allow the solution to fully diffuse through the whole testis. Fetal testes were submerged in Bouin's for two hours in an airtight container. Bouin's fixed tissue was then placed into 70% EtOH (ethanol) and processed by the histology team (SuRF, CRH, Edinburgh). Briefly, the tissue was processed through a series of graded alcohols for 17.5 hours in an automated Leica TP1050 tissue processor (Leica Microsystems, Milton Keynes, UK) and embedded into molten paraffin wax. Once the wax blocks containing the sample of interest had cooled and set, all samples were stored at room temperature for future use.

2.10 Protein Expression

2.10.1 Immunohistochemistry

To detect protein expression in cells, antibodies against a specific protein of interest were detected by immunohistochemistry on Bouin's fixed tissue, as outlined below. For immunohistochemistry undertaken on sections of animals exposed to treatments or otherwise, an appropriate age-matched control was also included in each experiment. Where possible, tissue sections from both control and treated animals were analysed on the same slide to allow for direct comparison. A sample size of at least five animals per experimental group, with at least two sections from the same animal, was included in each experiment, which ensured reproducibility of the results found. The protocols from

sectioning to imaging for immunohistochemistry are outlined briefly, full details of which are given in sections 2.10.2-2.10.11.

- Samples embedded in paraffin blocks were cut into 5µm sections using a microtome.
- Sections were mounted onto labelled slides.
- Slides containing sections of tissue were dewaxed and re-hydrated.
- Tissue underwent antigen retrieval in a decloaking chamber.
- Non-specific antigens were blocked.
- Specific primary antibody was added and detected using an amplification system.
- Protein of interest was visualised using a colour reaction (DAB).
- Sections of tissue were covered with a glass coverslip using permafluor.

2.10.2 Sectioning

Paraffin wax blocks containing the tissue of interest were placed on ice for 10 minutes to ensure that the wax was rigid to allow ease of sectioning at 5µm thickness using a microtome (Leica, model RM 212 5RT). The sections were then floated onto 30% industrial methylated spirits (IMS; Fischer Scientific) prior to being floated in a heated water bath (Lamb RA, model E/65) at 45-55°C to eliminate any creases in the section. Sections were teased apart using a thin brush and mounted onto a pre-labelled (slide labeller, Leica IPS) electrostatically charged glass slide (Leica Biosystems, Peterborough Limited). Slides were placed in a metal rack and left overnight in an incubator (Lamb RA, model E28.5) at 55°C, to allow the sections to adhere to the glass slide. Slides were then stored at room temperature until required.

2.10.3 Dewaxing and rehydrating

In order to remove paraffin wax from the section (dewaxing), slides were left in xylene for 5 minutes x2. Tissue was then rehydrated by immersion in decreasing concentrations of ethanol for 30 seconds each i.e. 100% ethanol (x2), 95%, 80%, 70% and then rinsed in dH₂O.

2.10.4 Antigen retrieval

Bouin's solution induces protein cross-linking to preserve the tissue integrity, which can often result in masking the protein/antigenic sites; thus cross-link removal was required for successful immuno detection. This was achieved by heat-induced antigen retrieval using a decloaking chamber/pressure cooker (Biocare Medical; CA, USA). Following rehydration, slides were immersed in a plastic holder which contained retrieval solution (25mls Citrate buffer pH6; section 1.14.6 and 225mls dH₂O). The holder was placed into the decloaking chamber (containing 500mls dH₂O) and the programme was run for 40 mins, which involved heating to 125°C followed by cooling to 90°C. Slides were rinsed in tris buffered saline (TBS; section 2.16.2) solution for 5 minutes.

2.10.5 Blocking

To prevent any non-specific binding of primary/secondary antibodies or detection agents to any molecules other than the protein of interest, slides were submerged in hydrogen peroxide (30mls; BDH) and methanol (270mls; Fischer Chemical) for 30 minutes on a rocker at room temperature. Slides were then washed with TBS for 5 minutes x2. Excess TBS was carefully dried from around the section with tissue paper. Blocking serum (Diagnostic; Scotland) was then added to the tissue section to block any potential remaining non-specific binding sites. Blocking serum was chosen from the species in which the secondary antibody was raised and was diluted 1:4 in TBS containing 5% bovine serum albumin (BSA; Sigma). Slides were incubated in a humidity chamber at room temperature for 30 mins. To further prevent any non-specific

background staining, slides were washed for 5 minutes x2 between each step with TBS, on a rocker at 40-50 rpm at room temperature.

2.10.6 Primary antibody

Each primary antibody was initially optimised for all animals and ages used in this thesis. The primary antibody was diluted in blocking serum at its optimal concentration, carefully placed on the section and evenly distributed. For a negative control, sections were incubated with blocking serum only, in place of a primary antibody. The general conditions used for a sample of primary antibodies in this thesis are listed below (Table 2.1). All primary antibodies used for protein detection on sections were incubated overnight in a humidity chamber at 4°C.

Table 2.1 Antibodies and Dilutions for Immunohistochemistry

Primary Antibody	Species Raised	Dilution	Manufacturer
SMA	Mouse	1:3,000	Santa Cruz
COUP-TFII	Mouse	1:100	Sigma
3B-HSD	Goat	1:500	Santa Cruz

2.10.7 Secondary antibodies

Slides were washed in TBS (5 minutes x2) to remove any unbound primary antibody. A biotin labelled (biotinylated) secondary antibody was then added to amplify the localisation of the primary antibody. Secondary antibodies were raised against a species-specific sequence on the primary antibody. Also, the secondary antibodies were raised in the same species as the blocking serum used. The biotinylated secondary antibody was diluted 1:500 in blocking serum and incubated on slides in a humidity chamber at room temperature for 30 minutes.

Table 2.2 Secondary antibodies used for Immunohistochemistry

Target Antigen Species	Host Species	Supplier
Rabbit	Mouse	Dako
Rabbit	Goat	Dako







2.10.8 Antigen Detection

In order to amplify the biotinylated secondary antibody signal, slides were incubated with streptavidin/horseradish peroxidase (Strep HRP; Vector) diluted 1:500 in TBS for 30 minutes in a humidity chamber at room temperature. To localise the primary antibody, slides were incubated with 3,3'-diaminobenzidine (liquid DAB⁺; DAKO) which was diluted in its own buffer according to manufacturer's instructions: 1 drop/ml buffer, immediately prior to use. A brown colour ensued following successful reaction to the specific antigenic sites of interest, as monitored under a microscope (Axiostar plus; Zeiss) after approximately 5 minutes. The reaction was quenched by immersing the slides in water, followed by TBS for 5 minutes to wash off any excess unbound DAB. A schematic representation of the reaction is shown in Figure 2.2.

2.10.9 Double/Triple immunohistochemistry

For detection of more than one antigen at a time, instead of using Strep-HRP to amplify the signal, Streptavidin Alkaline phosphatase (Strep-AP; Sigma) was used. Following TBS washes, the chromagen used to detect the primary antibody was commercial fast blue/red (Diagnostics Biosystems, Pleasanton, CA) diluted in its own buffer as per manufacturers' instructions. Slides were washed in TBS to remove any excess chromagen and underwent heat-induced antigen retrieval for a second time. This involved bringing retrieval solution (450mls water and 50 mls citrate pH6.0) to boiling point (4 minutes) and then

immersing the slides in a plastic rack within a plastic container. A lid was placed on loosely and slides were left in the boiling retrieval solution for 2.5 minutes and left to cool for a further 20 minutes. Blocking serum was then placed on the sections prior to the subsequent primary antibody. The process was repeated until the final primary antibody detection stage where Strep-HRP was added for 30 minutes at room temperature followed by a TBS wash (5 minutes x 2) and finally DAB, as the last chromagen.

	Antigen		Primary Antibody
	Secondary Antibody		Biotinylation
	Streptavidin HRP		DAB/Fast Red/Blue Enzyme Reporter

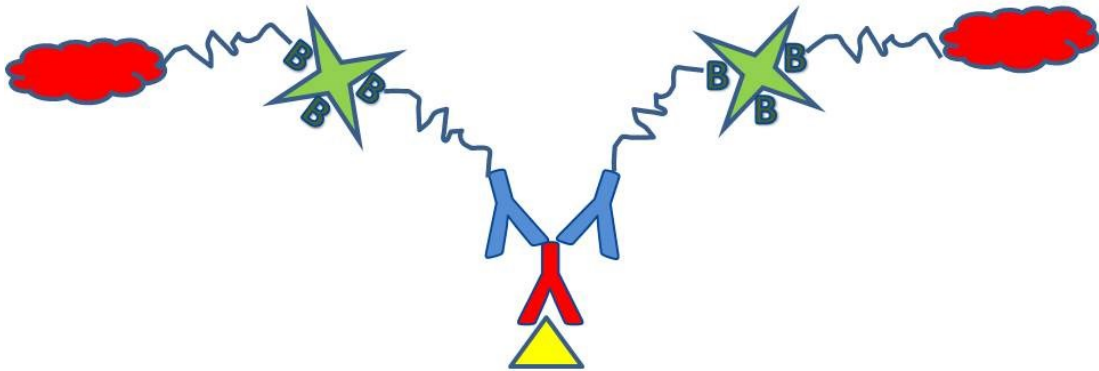


Figure 2.2 Schematic representation of localising the protein of interest. Proteins/antigens are expressed in cells which specific primary antibodies can bind to. A biotin-labelled secondary antibody specifically binds to the primary antibody and streptavidin-HRP then binds to the biotin label forming a complex. Addition of a chromagen substrate (Fast Red/Blue/DAB) reacts with the complex and releases a colour, allowing the protein of interest to be localised.

2.10.10 Dehydration and mounting

As 'fast blue/red' is soluble in alcohol, placing the slides through alcohol dehydration would remove the staining from the sections. For this reason following immunostaining, slides were immediately mounted using glass coverslips (Surgipath; Leica Biosystems) with an aqueous mounting medium, Permafluor (Thermo Scientific). Slides were left to dry and stored at room temperature.

2.10.11 Imaging

Localised target proteins of interest in tissue sections were examined using a Provis microscope (Olympus Optical, London, UK) and images were captured using an attached digital camera (Canon DS126161). Captured images were compiled using Adobe Photoshop 5.0 (Adobe Systems Inc, Mountain View, CA).

2.11 Image analysis

To analyse immunostained sections, slides were visualised using a Zeiss Axio-Imager microscope (CarlZeiss Ltd., Welwyn Garden City, UK) fitted with a Hitachi HVC20 camera (Hitachi Denshi Europe, Leeds, UK) and a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK) and utilised Image-Pro Plus 7.0 with Stereologer Analyser Pro (Media Cybernetics, UK).

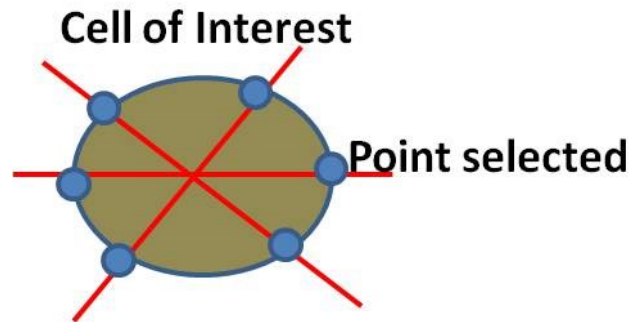
2.11.1 Determination of numbers of adult Leydig cells and their progenitor cells

Adult Leydig progenitor cells, stained with DAB⁺ (Section 2.10.8) were counted using standard stereological techniques in order to determine whether specific treatments, or otherwise, altered the number of these cells per testis. Oil immersion was used to examine slides under the microscope using a Leitz x100 objective fitted to a Zeiss Axio-Imager microscope (CarlZeiss Ltd; Welwyn Garden City, UK) fitted with a HVC20 camera (Hitachi Denshi Europe, Leeds, UK). This allowed the immunostaining to be visualised on screen using Image-

Pro 7.0 with Stereologer plug-in software (MagWorldwide; Wokingham, UK). The software generated a grid which was placed over the testis section and produced, at least 150, random fields of testicular cross-sections for each animal to be analysed. Points (crosses) falling over cell nuclei of interest were scored i.e. when the centre of a cross fell over a COUP-TFII⁺ interstitial cell. Cells which fell under the edge of the cross were excluded, ensuring consistency of the results. This data was expressed as a percentage of the total points counted and values were converted to absolute nuclear volumes per testis by multiplying by testis weight, which is equivalent to volume, as shrinkage is minimal.

2.11.2 Determination of the mean nuclear volume for counted cells

For each cell type quantified, the mean nuclear volume was determined, which allowed the conversion of nuclear cell volumes per testis to be expressed as the absolute number of cells per testis. Oil immersion was used to examine slides under the microscope and image analysis was carried out, as described above (Section 2.11.1), in which random fields were generated in the testis section. Fields containing cells of interest for each sample, group and age were measured. To measure the nuclear cell volume, the centre of each cell was selected which automatically generated three diameter measurements (μm) to trisect the cell. At each point on the cell of interest, where the line met the cell edge, a score was made and the nuclear volume was calculated by the software (Figure 2.3). The nuclear volume for at least 150 cell nuclei (e.g. in adult testes) was taken in order to have an accurate representation of the mean nuclear volume of each cell type. As I performed the scoring for cell counting and nuclear volume measurements for all studies in this thesis, it ensured that measurement errors were consistent.



$$\text{Number of cells (millions)} = \frac{\text{Absolute nuclear volume/testis (mg)} \times 1000}{\text{mean nuclear volume } (\mu\text{m}^3)}$$

Note: The fraction is multiplied by 10^9 to convert mg to μm^3 (assuming specific gravity = 1) and divided by 10^6 because the result is expressed in terms of millions of cells.

Figure 2.3 Schematic representation of the method for measuring nuclear cell volume and the formula used to calculate the absolute number of cells/testis.

2.12 Immunofluorescence

Fluorescent immunohistochemistry (immunofluorescence) is often more sensitive than colorimetric immunohistochemistry and thus proteins of interest are better visualised. To investigate whether different proteins of interest (each with a different colour of fluorophore attached) are present within the same cell, immunofluorescence was carried out to clearly visualise when fluorescent colours combine, i.e. co-localisation of proteins within a cell type. For the purpose of this thesis, any co-localisation is shown in yellow, as a result of green and red fluorophores combining. The protocols from sectioning to addition of primary antibodies, were similar to that described above for colorimetric immunohistochemistry (Section 2.10), and are outlined below.

- Samples embedded in paraffin blocks were cut into $5\mu\text{m}$ sections using a microtome.
- Sections were mounted onto labelled slides.
- Slides containing sections of tissue were dewaxed and re-hydrated.
- Tissue underwent antigen retrieval in a decloaking chamber.

- Non-specific antigens were blocked via hydrogen peroxide in methanol.
- Primary antibody one was added followed by peroxidase-conjugated secondary antibody.
- Specific protein of interest was detected using a fluorescent tyramide amplification system.
- Tissue underwent antigen retrieval in microwave.
- Non-specific antigens were blocked via hydrogen peroxide in TBS-Tween
- Primary antibody two was added followed by peroxidase-conjugated secondary antibody
- Specific protein of interest was detected via fluorescent tyramide amplification system.
- A nuclear counterstain, DAPI was added to section.
- Sections of tissue were covered with a glass coverslip using permafluor.

2.12.1 Blocking

The conditions for blocking are identical to those outlined in section 2.10.5. For the majority of fluorescent immunohistochemistry used in this thesis, normal chicken serum was used as blocking serum to ensure cross-reactivity did not occur.

2.12.2 Primary antibody

Each primary antibody was initially optimised for all animals and ages used in this thesis. The primary antibody was diluted in blocking serum at its optimal concentration, carefully placed on the section and evenly distributed. For a negative control, sections were incubated with blocking peptide where available or blocking serum only, in place of a primary antibody. General conditions used

for primary antibodies in this thesis are listed below (Table 2.4). All primary antibodies used for protein detection on sections were incubated overnight in a humidity chamber at 4°C.

2.12.3 Secondary antibody

The secondary antibodies used are summarised below (Table 2.3). Instead of using a biotin labelled secondary as in colorimetric immunohistochemistry, peroxidase (HRP) labelled secondary antibodies were used for fluorescence immunohistochemistry. The peroxidase labelled secondary antibody was diluted 1:200 in blocking serum and incubated on the tissue section in a humidity chamber at room temperature for 30 minutes.

Table 2.3 Secondary antibodies for Immunofluorescence

Target Antigen Species	Host Species	Supplier
Chicken	Rabbit	Santa Cruz
Chicken	Goat	Santa Cruz
Chicken	Mouse	Santa Cruz

2.12.4 Antigen detection

In order to visualise the peroxidase labelled secondary antibody signal, slides were incubated with green, red or blue fluorescent dye using the ‘Tyramide Signal Amplification’ (TSA; PerkinElmer, MA, USA) system. This was diluted in its own buffer according to manufacturers’ instructions: 1:50, immediately prior to use and left to incubate on sections for 10 minutes in a humidity chamber at room temperature. The reaction was quenched by immersing the slides in TBS (5 minutes x2) in a covered dark container to prevent any photo bleaching occurring.

2.12.5 Counterstaining and mounting

Following TBS washes, the sections were counterstained with 4',6- Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Sigma) which is a nuclear-specific dye. This was useful both to check the integrity of the section and also for orientation when visualising the section with the confocal microscope. DAPI was diluted 1:500 in TBS and left to incubate for 10 minutes. Unbound/excess DAPI was washed off with TBS for 5 minutes and slides were mounted using glass coverslips (VWR, international, UK) with an aqueous mounting medium, Permafluor (Thermo scientific). Slides were covered in tinfoil to prevent photo-bleaching occurring and stored at 4°C for subsequent imaging.

2.12.6 Double/triple/quadruple immunofluorescence

For co-localisation studies i.e. detection of more than one antigen, following initial tyramide detection, slides were washed in TBS (5 minutes x 2) and citrate retrieved (see section (2.10.4) to remove the previous tyramide dye. Slides were then blocked with hydrogen peroxide (30mls) in TBS-Tween (270mls) to block any endogenous peroxide. Slides were further blocked in serum for 30 minutes in a humidity chamber at room temperature, followed by incubation with the subsequent primary antibody and left overnight at 4°C. The protocol was continued as above for peroxidase labelled secondary antibodies and washed with TBS between steps. Each primary antibody was visualised with a separate fluorescent colour of tyramide. Slides were counterstained and mounted (Section 2.12.5).

2.12.7 Fluorescent microscopy and imaging

Localised/co-localised protein(s) of interest were visualised and imaged using a Zeiss LSM 710 Confocal Microscope (Carl Zeiss Ltd, Hertfordshire, UK) and Zen software (Carl Zeiss Ltd, 2009). Captured images were compiled using Adobe Photoshop 5.0 (Adobe Systems Inc, Mountain View, CA).

Table 2.4 Antibodies and dilutions used for Immunofluorescence

Primary	Species	Dilution	Manufacturer	Secondary	Detection
SMA	Mouse	1:10,000	Santa Cruz	C ¹ α M ²	Tyr-Cy5
COUP-TFII	Mouse	1:1,000	Perseus Proteomics	C α M	Fluorescein
3B-HSD	Rabbit	1:8,000	Santa Cruz	C α G ³	Tyr-Cy3
INSI3	Rabbit	1:500	Santa Cruz	C α R ⁴	Tyr-Cy3
GATA4	Mouse	1:500	Santa Cruz	C α M	Tyr-Cy3
CD31	Goat	1:500	Santa Cruz	R α G	Tyr-Cy3
H3	Rabbit	1:5,000	Abcam	C α R	Tyr-Cy3
H3K27me3	Rabbit	1:5,000	Active Motif	C α R	Tyr-Cy3
H3K4me3	Rabbit	1:5,000	Active Motif	C α R	Tyr-Cy3
PCNA	Rabbit	1:500	Santa Cruz	C α R	Tyr-Cy3
CD146	Rabbit	1:500	Abcam	C α R	Tyr-Cy3
CD68	Mouse	1:1000	AbD Serotec	R α M	Tyr-Cy3
AR	Rabbit	1:200	Abcam	G α R	Tyr-Cy3
SF1	Rabbit	1:200	Santa Cruz	G α R	Tyr-Cy3
CD34	Goat	1:500	Santa Cruz	R α G	Tyr-Cy3
S100A4	Rabbit	1:100	Dako	C α R	Tyr-Cy3
LHR	Rabbit	1:200	Santa Cruz	G α R	Tyr-Cy3
MafB	Rabbit	1:400	Abcam	C α R	Tyr-Cy3
StAR	Rabbit	1:500	Affinity BioReagents	C α R	Tyr-Cy3
GFP	Rabbit	1:1,500	Abcam	C α R	Fluorescein

C¹: Chicken; M²: Mouse; G³: Goat; R⁴: Rabbit sera.

2.13 RNA analysis

To investigate gene expression in tissues of interest, firstly RNA was extracted, followed by cDNA synthesis (PCR) and lastly qRT-PCR to investigate mRNA expression levels of any gene of interest. These procedures are detailed below.

2.13.1 RNA extraction

Tissue was retrieved from storage in the -80°C freezer and left to thaw for 10 minutes on ice. RNA was isolated from the tissue of interest (adult rat testis) using the RNeasy Mini extraction kit (Qiagen, Crawley, UK), according to manufacturers' instructions. This entailed excising 30mg of frozen testis tissue from the centre of the testis and submerging in 600µl lysis buffer (β -mercaptoethanol (Sigma) in RLT buffer (Qiagen; 1:100) in a 2ml eppendorf containing a 5mm diameter stainless steel bead for homogenisation. Homogenisation was carried out using a tissue lyser (Qiagen) for 5 minutes in total (2.5 minutes in opposite positions) at 25 Hz, which served to disrupt the tissue and thus release RNA. Samples were centrifuged ('eppendorf' centrifuge 5424) for 3 minutes, 70% ethanol (600µl) was added to the supernatant and manually mixed by pipetting to promote binding of RNA to the RNeasy membrane. This mixture was transferred into supplied RNeasy mini columns and centrifuged for 15 secs at 10,000 rpm. Flow-through was discarded and RW1 buffer (350µl) was added and samples centrifuged for 15 seconds at 10,000rpm to wash the spin column. Any genomic DNA contamination was removed using the supplied RNase-free DNase on-column digestion kit (10µl DNase in 70µl RDD buffer), incubated for 15 minutes at room temperature. RW1 buffer (350µl) was added before samples were centrifuged for 15 seconds at 10,000rpm. RPE buffer (500µl) was added and samples centrifuged for 15 seconds at 10,000 rpm and flow-through discarded. A second 500µl of RPE buffer was added and samples centrifuged for longer (2 minutes at 10,000rpm) in order to dry the membrane to eliminate ethanol carry-over when eluting RNA. Using a new 2ml collection tube (Qiagen) to further eliminate ethanol and RPE buffer carry-over, samples were centrifuged at full speed (14,000 rpm) for

1 minute. Lastly, to elute the RNA, the column was gently placed into a 1.5ml collection tube and 30µl of RNase-free water was pipetted directly onto the column membrane and incubated for 5 minutes at room temperature. Samples were then centrifuged for 1 minute at 10,000 rpm to collect the extracted RNA which was then placed on ice for immediate RNA quantification and quality check.

2.13.2 RNA quantification

To correct for varying amounts of RNA collected between samples, the concentration and quality of RNA was measured using a Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA). The ratio of absorbance at 260 and 280 nm is used as a measure of purity in RNA extractions. A ratio of ~2.0 is generally accepted as “pure” for RNA, a ratio <2.0 may indicate a contamination in the sample e.g. the presence of proteins. Thus for the studies herein, only samples with an absorbance ratio of ~2.0 were used.

2.13.3 Reverse transcription

RNA was converted to cDNA via reverse transcriptase polymerase chain reaction (RT-PCR) using a ROX vilo kit (Invitrogen). The master mix for each reaction is listed below (Table 2.5). Control tubes were also included (master mix + water and another tube: water + sample). Once the reagents were added together into a labelled 0.2ml sterile thin walled PCR tube (Continental Lab Products, Oxford, UK), these were placed in a thermocycler (DNA Engine Thermal Cycler) for cycling on the following VILO programme (Table 2.5). Samples were stored at -20°C for future use.

Table 2.5 Reagents and thermocycling programme for cDNA

Stock	1 reaction (20µl tube)
5x Vilo Reaction Mix	4µl
10x Superscript enzyme Mix	0.25µl
RNA 100ng/µl	1µl
RNase free water	14.75µl
Temperature (°C)	Time (mins)
25	10
42	60
85	5
4	Holding

2.13.4 Primer and probe design

In order to design primers and probes for Taqman PCR, the Universal Probe Library assay design centre was utilised (www.universalprobelibrary.com; Roche). This system designs specific primers for any gene of interest and assigns the best matched probe. Forward and reverse primers (MWG Biotech, London, UK) were reconstituted in nuclease free H₂O and diluted to 20µM (20µl primer in 80µl H₂O). Aliquoting the primers reduced the likelihood of stock contamination. The primer sequences and the assigned probe number for each set of primers, used for studies in this thesis, are listed below (Table 2.6).

Table 2.6 Primer sequences and assigned probe used for taqman PCR

Gene	Forward Primer	Reverse Primer (Probe #)
<i>Sox9</i>	<i>CTGAAGGGCTACGACTGGAC</i>	<i>TCTTGATGTGCGTTCTCTGG (63)</i>
<i>Lhr</i>	<i>CTGGAGAAGATGCACAGTGG</i>	<i>CTGCAATTTGGTGGGAAGAAATA (107)</i>
<i>StAR</i>	<i>TCACGTGGCTGCTCAGTATT</i>	<i>GGGTCTGTGATAAGACTTGGTTG (83)</i>
<i>CYP11a1</i>	<i>TCACATGCAGAATTTCCAGAAG</i>	<i>AGGATGTAAACTGACTCCATGTTG (7)</i>
<i>3β-hsd</i>	<i>TCATCTGATTTTGAACAATTTAGC</i>	<i>CTCCTGCTCCTGTACCAG (105)</i>
<i>CYP17a1</i>	<i>CATCCCCACAAGGCTAAC</i>	<i>TGTGTCCTTGGGGACAGTAAA (67)</i>
<i>17β-hsd3</i>	<i>AATATGTCACGATTGGAGCTGA</i>	<i>AAGGAATCAGGTTTCAGAATTATCG (5)</i>

2.13.5 Taqman PCR

Quantitative real-time/reverse transcriptase polymerase chain reaction (RT-qPCR) was carried out using the ABI Prism 7900 Sequence detection system (Applied Biosystems). It operates by amplifying the target DNA sequence while quantifying the number of cycles it takes to do so. Taqman uses a fluorescent reporter molecule to detect the PCR product as it accumulates during cycles i.e. as the product increases, so too does the fluorescence emitted. Both forward and reverse primers were designed along with an assigned probe from the Universal Probe library (Roche). For studies utilising Taqman PCR in this thesis, the probe contained a reporter dye (6-carboxyfluorexsein: FAM) at the 5' end and a non-fluorescent quencher (6-carboxy-tetramethyl-rhodamine: TAMRA) at the 3' end, which held the probe in a non-fluorescent state.

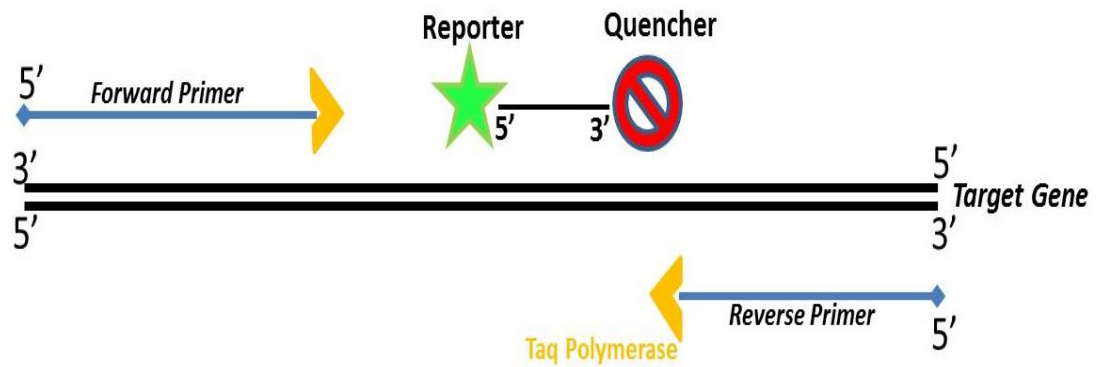


Figure 2.4 Fluorescent Reporter is suppressed by quencher present on probe

During Taqman PCR, the probe anneals to the target gene cDNA of interest (if present in the sample), between the forward and reverse primers. Taq polymerase enzyme binds to the 5' of the primer until it reaches the probe, which is then cleaved and releases the fluorescent FAM dye. With every cycle and PCR product formed, the reporter is thus cleaved from the probe and fluoresces. In this manner, the amount of PCR product is measured quantitatively by the level of fluorescence produced.

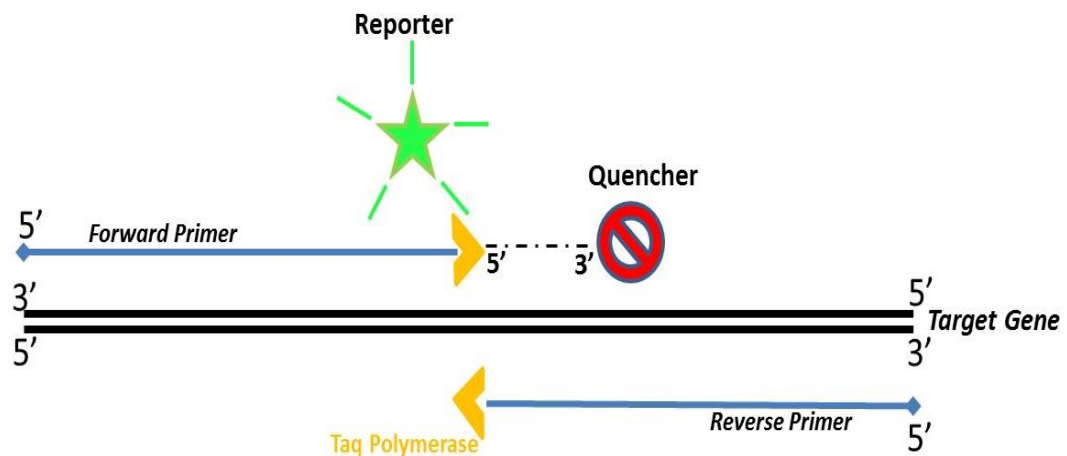


Figure 2.5 Taq Polymerase cleaves probe to release fluorescent reporter

To ensure accuracy of results, samples were run in triplicate on a 96 well MicroAmp optical reaction plate (Applied Biosystems). The expression level of the gene of interest was related to an internal control 18s, a ribosomal RNA, which was used as a reference for RNA variation between samples. Both 18s RNA and sample RNA were analysed in the same wells but with different fluorescent endpoints. Positive controls (e.g. adult testes; Ambion) along with negative controls (water only/master mix only) were included in all runs. All samples and reagents were kept on ice while preparing the Taqman plate. Master mix, primers and probes were vortexed prior to use, while 18s was gently inverted. For each sample, the following reaction was prepared (Table 2.7) with 13.5ul pipetted into each well, along with 1.5ul cDNA. To cover the plate, an optical adhesive cover (Applied Biosystems) was evenly applied and the plate was loaded onto an ABI 7900 sequence detection system. To minimise any potential variations in gene analysis, potentially due to different ABI machines, plates were consistently loaded onto ABI 7900 machine no.3 for every Taqman run.

Table 2.7 Reagents used for Taqman PCR

Reaction	Volume per well (15µl)
Nuclease free H ₂ O	5.325µl
Taqman Master mix	7.5µl
Forward Primer (5' - 3')	0.15µl
Reverse Primer (3' - 5')	0.15µl
Universal Probe	0.15µl
18s	0.225µl
cDNA	1.5µl

2.13.6 Analysis of results

Taqman PCR results are represented on an amplification plot. This demonstrates the amount of fluorescence produced as a result of the PCR product formed (Section 2.13.5) and thus the amount of target gene expressed in the tissue of interest. The baseline (d) refers to basal fluorescence present in the reaction plate while background (c) refers to any background fluorescence. As the level of fluorescence increases, it is associated with an exponential increase of PCR product, referred to as the threshold. Therefore, the threshold line must be set in the exponential phase, rather than in the linear (b), plateau (a) or background (c). Once the fluorescence signal is detected above the threshold, this is termed the threshold cycle (Ct value). It is this 'Ct value' which demonstrates the required number of cycles for amplification of the PCR product, which is significantly above the background level (Figure 2.6).

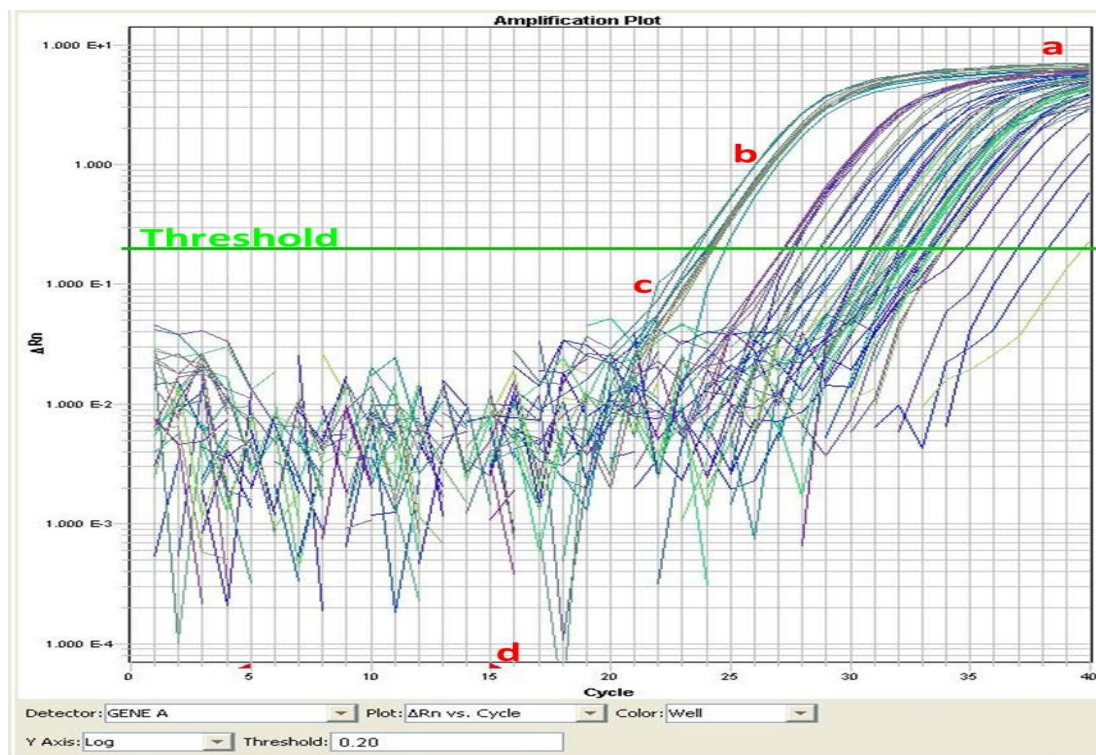


Figure 2.6 A Taqman real time quantitative PCR plot. a=plateau phase; b=linear phase; c=background; d=baseline. Threshold is indicated in green. Amplification plot is representative of a successful taqman run from studies in this thesis.

In order to analyse any expression changes in a gene between control and treated samples, this was achieved using the comparative Ct method. To calculate the ΔCt for each sample, the Ct value of the internal positive control gene was subtracted from the Ct of the target gene (e.g. StAR Ct-18S Ct= ΔCt). As the sample reactions were prepared in triplicates (to account for pipetting errors), this result was averaged and compared to a reference sample (e.g. control adult rat testes total RNA) to give the $\Delta\Delta Ct$. This was achieved by simply subtracting the ΔCt of the reference sample from the ΔCt of the target gene (e.g. ΔCt control- ΔCt StAR= $\Delta\Delta Ct$). To calculate the amplified target, the formula $2^{-\Delta\Delta Ct}$ was applied. This is derived from an equation describing the exponential amplification of PCR; ($X_n=X_o \times (1+E_x)^n$) (Livak and Schmittgen, 2001) where X_n =number of target molecules at threshold level, n =cycle number, X_o =number of target molecules at the start, $1+E_x$ =efficiency of target amplification (should be equal between target and internal control). Using the formula $2^{-\Delta\Delta Ct}$ provides a measure of relative quantification i.e. an increase or decrease in mRNA expression between samples and controls.

2.14 CHIP assay

Chromatin immunoprecipitation (ChIP) assay allows the analysis of epigenetic modifications of a selected region on a gene, which in these studies, referred to histone modifications. The expression levels of a methylated histone at a specific location on a gene of interest were examined to determine whether there was a specific histone modification which would affect transcription. The protocol is outlined below.

- Histones are cross-linked to DNA to isolate chromatin.
- Chromatin is sonicated.
- Chromatin fragments are immunoprecipitated with a specific antibody.
- PCR is carried out to quantify the level of histone modification to the gene of interest.

2.14.1 Cross-linking

Testis samples were retrieved from the -80°C freezer and left to thaw on ice for 10 mins. A small segment (150mg) was excised, weighed (Sartorius, Northern Balance) and placed in a petri dish (Corning) on ice. The segment was immediately sliced with a sharp blade into 1mm fragments and re-suspended in 10mls PBS containing protease inhibitor (PI; Roche Complete), diluted 1:50 e.g. 200µl of PI was added to 9.8mls of PBS to re-suspend the sample. In order to cross-link histones to DNA, 270µl of 37% formaldehyde (Sigma) was added and left to incubate for 10 minutes rotating on a rocker at room temperature. Formaldehyde was used as it is a reversible cross-linker. To quench cross-linking, 125µl of glycine (Sigma) was added, the sample was centrifuged ('epENDORF' centrifuge 5415) at 5,000rpm for 10 minutes and the supernatant discarded. The pellet was washed twice using ice-cold PBS containing PI and centrifuged. Finally the pellet was re-suspended in 200µl ice-cold PBS in a 2ml eppendorf tube.

2.14.2 Sonication

Tissue was homogenised with 20 strokes of a probe homogeniser (T10 Basic Ultra-Turrax, IKA, Staufen, Germany). Lysis buffer (200µl; section 2.16.7) was added and the sample incubated for 20 minutes on ice. The sonicator (Bioruptor; Diagenode, Belgium) was cooled for 10 minutes by addition of ice and the temperature was carefully monitored using a thermometer. Once the ice melted, dropping the temperature to 1°C, the samples were sonicated on 'high' using alternating pulses at 30 seconds on/off. Samples were stored in a -20°C freezer before immunoprecipitation. To check whether the sonication successfully sheared the DNA into fragments of 200-1000bp, 200µl of sonicated supernatant was removed from a sample, 8µl of NaCl was added and the sample was incubated for 4 hours at 65°C to reverse cross-links. This was followed by purification of the DNA (Section 2.14.4), which then underwent electrophoresis (Section 2.14.6) to visualise the size of DNA fragments after sonication.

2.14.3 Immunoprecipitation

The sonicated supernatant was diluted ten-fold with ChIP dilution buffer (Section 2.16.9) and a portion of this (1%) was kept as the "input control", used to quantitate the amount of DNA present. The solution was then divided into three aliquots and pre-cleaned with 75ul of 'salmon sperm DNA/protein A agarose-50% slurry' (Millipore) for 30 minutes at 4°C to reduce any non-specific background. To pellet the agarose, samples were then centrifuged for 1 minute at 10,000rpm at 4°C ('eppendorf' centrifuge 5415R). The three aliquots of supernatant fraction were appropriately labelled and incubated overnight with either 1) 10µl of a specific primary antibody (to attach to modified histone of interest), 2) negative control (IgG; Abcam) or 3) positive control (unmodified H3; Abcam) and rotated at 4°C. To collect the antibody/histone complex, 60µl sperm DNA/protein A agarose-50% slurry was added to the samples and incubated for 4 hours at 4°C. Samples were centrifuged at 4°C for 2 minutes at 1,000 rpm and the supernatant containing unbound, non-specific DNA was discarded. Samples were then washed on a rotating platform with 1ml of buffers in the following order: low salt, high salt, LiCl, TE and a final TE buffer wash (see section 2.16.10-2.16.13). Between washes, the samples were centrifuged at 4°C for 1 minute at 10,000 rpm. To elute the histone complex from the antibody, 100µl elution buffer (section 2.16.14) was added to the pelleted protein A agarose/antibody/histone complex and incubated on rotation for 15 minutes at room temperature. Following 2 minutes of centrifugation, the supernatant fraction (eluate) was carefully transferred to another labelled tube and the elution step was repeated with the subsequent eluates combined. To reverse the histone-DNA crosslinks, 8ul 5M NaCl was added to the sample and input control and heated at 65°C overnight, followed by 10µl 0.5M EDTA, 20µl 1M Tris HCl and 1µl 20 mg/ml proteinase K, with incubation at 45°C for 60 minutes.

2.14.4 DNA purification

DNA was purified from the samples using the QIAquick PCR purification kit (Qiagen). To bind DNA, the sample was placed in a QIAquick column and centrifuged for 30 seconds with the flow-through discarded. To wash the sample, 750µl buffer PE (containing ethanol) was added to the column, and the sample centrifuged for 30 seconds with the flow-through subsequently discarded. To remove any residual wash buffer, the column was centrifuged in a new 2ml collection tube (Qiagen). To elute DNA, the column was placed in a new 1.5ml eppendorf containing 50µl elution buffer (containing 10mM Tris HCl) which was carefully added to the centre of the column, and left for 5 minutes before being centrifuged for 1 minute at 5,000 rpm. The purified DNA was stored in a -20°C freezer for future use.

2.14.5 PCR using SYBR green

In order to amplify purified DNA bound to the immunoprecipitated histone, PCR was carried out using SYBR green (Brilliant III Ultra-Fast SYBR; Agilent Technologies). Fluorescent SYBR green dye binds to double-stranded DNA and fluoresces, i.e. at the end of the PCR cycle when the double-stranded DNA product is produced. As the temperature increases, the DNA product melts and releases the SYBR green fluorescent dye and this melting/dissociation can be visualised at the end of the PCR cycle as a 'melting curve'. As SYBR green has the disadvantage of non-specifically binding to any double stranded DNA, analysing a melt curve (Figure 2.7) is essential to check that only a single gene-specific amplicon (visualised as a single peak in the melt curve) was generated. This is advantageous for primer design as any non-specific amplification (visualised as multiple peaks) identified in the melt curve may indicate primer dimer or genomic DNA contamination. Using a reference dye provides a stable baseline to normalise the samples, as the fluorescence in the reference dye remains unchanged throughout the programme.

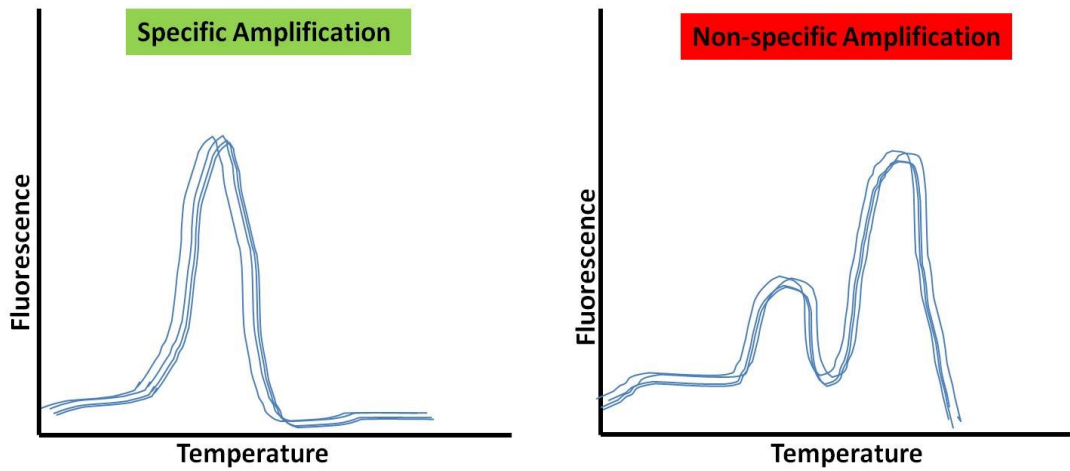


Figure 2.7 Melt Curve Analysis. The left plot shows a unique melt peak, indicative of specific amplification while the right plot shows two clearly distinct melt peaks, indicative of non-specific amplification.

To ensure accuracy of results, samples were run in triplicate on a 96 well MicroAmp optical reaction plate (Applied Biosystems). All samples and reagents were kept on ice while preparing the PCR plate. For each sample, the following reaction was prepared (Table 2.8) with 8 μ l reaction mix pipetted into each well, followed by 2 μ l purified DNA. To cover the plate, an optical adhesive cover (Applied Biosystems) was evenly applied and the plate was loaded onto an ABI 7900 sequence detection system and run on the programme (Table 2.9). To minimise any potential variations in gene analysis due to different ABI machines, plates were loaded onto ABI 7900 machine no.3 for every run.

Table 2.8 Reagents used for SYBR green PCR

Reaction	Volume per well (10µl)
2x Brilliant III SYBR Mix	5 µl
25mM Forward Primer	0.2 µl
25mM Reverse Primer	0.2 µl
Diluted (1:50) Reference Dye	0.15 µl
Nuclease free water	2.45 µl
DNA	2 µl

Table 2.9 PCR programme used with SYBR green

Cycle	Duration	Temperature
1	3 mins	95° C
40	5 secs	95° C
	12 secs	60° C

2.14.6 Electrophoresis

To make a 2% agarose gel for electrophoresis, 4g agarose (Sigma) was added to 200mls TBE buffer (Section 2.16.5) in a conical flask (Corning). To fully dissolve the agarose, the solution was heated in a microwave for 2 minutes. As DNA cannot be visualised in natural light, 7µl of coloured dye (GelRed; Biotium), which binds to DNA and fluoresces under UV light, was added to the solution. This was then gently poured into the tray and all bubbles promptly removed. A comb was placed into the solution to create a well, to load the samples into once the gel was set after 1 hour. TBE buffer (500mls) was poured into the tank in

preparation for electrophoresis. To determine the molecular weight of the DNA fragments, 5µl of 500bp DNA ladder (Bioline) was pipetted into the first well in the gel. Prior to loading samples into the wells, 1 volume (5µl) of loading buffer (Bioline) was mixed with 5 volumes (30µl) purified DNA. The loading buffer contains glycerol, a dense reagent, which allowed the DNA sample to sink to the bottom of the well. The electrophoresis was run at 90V (Bio-Rad Powerpac 300) for 90 minutes. This was monitored regularly so that the DNA did not run off the bottom of the gel.

2.14.7 Densitometry

DNA was visualised on the gel using UV light (GeneFlash Transilluminator Syngene, Cambridge, UK) and an image was generated (model P91 Mitsubishi, Hertz, UK) for analysis. ImageJ (version 1.46h, National Institutes of Health, USA) software was used to analyse the density (intensity) of the DNA bands.

2.15 Statistical analyses

Values are expressed as means \pm SEM, where appropriate. Differences between means were analysed using an unpaired two-tailed t-test for two groups or one way Analysis of Variance (ANOVA) (for 3 or more groups) followed by the Bonferroni post-hoc test. Where unequal variances occurred, data were log transformed prior to statistical analysis. The criteria for significance, as indicated by asterisks, was set at $p \leq 0.05 = *$; $p \leq 0.01 = **$; $p \leq 0.001 = ***$. All statistical analyses in this thesis were carried out using GraphPad Prism 5.0 (version 5, GraphPad software Inc, San Diego, CA).

2.16 Common Solutions

2.16.1 Bouin's

Bouin's Solution is a well-established formaldehyde based fixative, which appears bright yellow in colour (purchased from Triangle Biomedical Sciences Ltd, Lancashire, UK) and works by forming cross links between protein and aldehydes to produce a stable structure and avoids damaging antigenic sites.

2.16.2 Tris-Buffered Saline

To make 0.5M of 10 l solution of TBS, the following were added together:

Tris (Sigma) 60.5 g

NaCl (Sigma) 87.6 g

HCl (BDH) 300 mls

dH₂O Up to 10 l

TBS solution was very carefully adjusted to pH 7.4 using concentrated HCl and stored as a stock solution at 4°C. When needed, 1x TBS was made by diluting 1 l of 10x TBS with 9 l of dH₂O and stored at room temperature.

2.16.3 Phosphate Buffered Saline (PBS) Solution

One PBS tablet (Sigma) was dissolved in 1 l of dH₂O

2.16.4 EDTA Buffer 0.5M

EDTA (Sigma) 186.1g

dH₂O 800ml

The pH was adjusted as required, e.g. for pH 8.0, NaOH was added and made up to 1 l with dH₂O

2.16.5 Tris-Borate-Edta (TBE) Buffer

For 1 l of 5x stock solution, add:

Tris 54g

Boric Acid (Sigma) 27.5g

0.5M EDTA pH 8 20mls

Add dH₂O to make 1L

Dilute to 1x with dH₂O prior to use

2.16.6 Citrate Buffer

For 2 l of Citrate buffer, add:

Citric acid monohydrate (Sigma) 42.02 g

1900 mls dH₂O

The pH of citrate buffer was adjusted to pH6.0 using NaOH and stored as a stock solution at 4°C or used at 0.01M by dilution (1:10) in dH₂O.

2.16.7 Lysis Buffer

For 10 mls:

1ml 10% SDS

10mM EDTA (200µl of 0.5M)

50mM Tris HCl pH8 (5ml 0.1M)

2.16.8 Glycine

For 2.5 M of a stock solution:

93.8g Glycine (Sigma)

500mls dH₂O

2.16.9 ChIP Dilution Buffer

1ml of 1% SDS

1.1 ml of Triton X (Sigma)

240ul of 0.5M EDTA (1.2mM)

16.7mls of 0.1m Tris HCl pH 8.1

3.34mls of 5M NaCl

Make up to 100mls with dH₂O

2.16.10 Low Salt Solution

For 100mls, add:

1ml SDS

1ml Triton X

400µl 0.5M EDTA

20mls 0.1M Tris HCl pH 8.1

3mls 5M NaCl

2.16.11 High Salt Buffer

1ml of 1% SDS

1ml Triton-X

400µl of 0.5M EDTA

20mls of 0.1M Tris HCl pH 8.1

10mls of 5M NaCl

2.16.12 LiCl Buffer

25mls of 1M LiCl

1ml Igepal ca-630 (Sigma)

1g deoxycholic acid (Sigma)

200µl of 0.5M EDTA

10mls of 0.1M Tris HCl pH 8.1

2.16.13 TE Buffer for CHIP

200µl of 0.5M EDTA

10mls of 0.1M Tris HCl pH8

2.16.14 Elution Buffer (Make fresh on day of use)

1ml 10% SDS

840µg NaHCO₃

100mls dH₂O

Chapter 3 Characterisation of Adult Leydig Progenitor Cells

3.1 Introduction

The importance of normal functioning adult Leydig cells, which only 'appear' at puberty, is reflected by the significance of normal testosterone levels and/or function for spermatogenesis and overall male health. When puberty commences, residing adult Leydig progenitor cells express the definitive steroidogenic cell-specific marker 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (Baker et al 1999) from as early as postnatal day (Pnd) 12 in the rat (Ariyaratne et al 2000) and at pnd 7 in the mouse (Baker et al 1999). This marks their differentiation into adult-type Leydig cells (Mendis-Handagama and Ariyaratne, 2001, Zirkin, 2010). However, the precise origin of adult Leydig progenitor cells remains a mystery. As all adult testicular cell types, excluding adult Leydig cells, are already present in the fetal testis, it seems a logical assumption that adult Leydig progenitor cells are also present and remain quiescent in the interstitium until their differentiation into Leydig cells at puberty. The vast majority of research on adult Leydig progenitor cells to date, is based on their differentiation into adult Leydig cells during puberty. However the studies described in this chapter aimed to investigate whether adult Leydig progenitor cells might be present from fetal life onwards.

Currently, there is no unifying/defined marker for adult Leydig progenitor cells prior to them differentiating into adult Leydig cells. Regarding this fact, an earlier study which demonstrated that inducible knockout of COUP-TFII in pre-pubertal male mice results in failure of adult Leydig cells to develop (Qin et al 2008) promoted the hypothesis that COUP-TFII might represent a marker for adult Leydig progenitor cells. In order to test this hypothesis, the first aim was to investigate EDS-induced adult Leydig cell ablation/regeneration to characterise the 'progenitor' cells from which the adult Leydig cells regenerated. A second approach was also used via a lineage tracing mouse model, in order to determine whether adult Leydig cells arise from COUP-TFII⁺ cells. The overall purpose of using these experimental models was to investigate if COUP-TFII

could represent a useful marker for adult Leydig progenitor cells, and if so, whether these cells were conserved across species in the fetal testis.

3.2 Materials and Methods

3.2.1 EDS injection

Selective destruction of all mature Leydig cells in adult wistar rats (90-100d) was achieved by a single i.p. injection of ethane dimethane sulphonate (EDS) at a dose of 75mg/kg bodyweight in 2ml/kg DMSO:H₂O 1:3 v:v (Section 2.5). Control rats received vehicle only.

3.2.2 Tissue collection

EDS-injected male rats were randomly assigned into 4 groups (n=5/group) and each group was killed at a specific time point, namely at 1, 2, 3 or 5 wks post-EDS, via inhalation of carbon dioxide followed by cervical dislocation. Prostate and testes were removed from the rats and weighed. One testis was frozen for subsequent RNA analysis (Section 2.13), while the other was fixed in Bouin's (Section 2.16.1) for subsequent protein analysis via immunohistochemistry (Section 2.12). Human fetal tissue was collected by Prof. R Anderson (Section 2.1). Sections of human fetal testis from second trimester (14-18wks gestation) were analysed via immunohistochemistry (Section 2.12).

3.2.3 Lineage-tracing of adult Leydig cells

To investigate if COUP-TFII could be used as a marker of the adult Leydig progenitor cell population in fetal life, a transgenic Leydig cell lineage tracing mouse model, generated by Dr. Laura O'Hara and Prof. Lee Smith, was utilised (Section 2.6). Sections from ap2-Cre/YFP⁺ mouse testes in fetal life underwent double immunohistochemical staining (Section 2.12.6) using an antibody that detects YFP/GFP to localise YFP⁺ cells and a second antibody, COUP-TFII, to mark the adult Leydig progenitor cell population. For the purpose of co-

localisation studies in this chapter, the YFP/GFP antibody localising YFP⁺ cells, is shown in green and labelled GFP.

3.2.4 RNA extraction and preparation of cDNA

Total RNA was isolated from frozen adult testes of control and EDS-injected rats using the RNeasy kit (Qiagen Ltd), (Section 2.13.1). RNA quality and concentration were measured (Section 2.13.2) and cDNA was prepared using the ROX Vilo kit (Invitrogen) (Section 2.13.3).

3.2.5 Taqman qRT-PCR

Quantitative RT-PCR was performed to analyse the expression of the steroidogenic genes *Lhr* and *3 β -HSD* in adult testes of control and EDS-injected rats using the ABI Prism 7900 Sequence Detection System (Applied Biosystems) (Section 2.13.5). Values were normalised to a control adult testis (Ambion). Primers and probes designed by Roche are detailed below (Table 3.1).

Table 3.1 Primers and probes used for mRNA analysis of testis extracts from EDS-injected rats

Gene	Forward Primer	Reverse Primer (# probe)
<i>3β-hsd</i>	TCATCTGATTTTGAACAATTTAGC	CTCCTGCTCCTGTCACCAG (105)
<i>Lhr</i>	CTGGAGAAGATGCACAGTGG	CTGCAATTTGGTGGGAAGAAATA (107)

3.2.6 Immunohistochemistry

Bouins fixed tissue was cut into sections on a microtome, mounted on glass slides, dewaxed and rehydrated (Section 2.10). Triple fluorescence immunohistochemistry was used to distinguish between cell types (Section 2.12.6) and to investigate protein co-localisation. For stereological analysis to be performed, the stereological software required images to be stained

colorimetrically and not fluorescently. Therefore triple colorimetric immunohistochemistry was used, as described below.

3.2.7 Colorimetric Immunohistochemistry

In order to investigate both the expression and to quantify the volume per testis of COUP-TFII⁺ cells, testis sections were firstly stained with specific markers to distinguish between cell types. Staining for colorimetric immunohistochemistry was performed on postnatal mouse testes at day 2, 12, 20, 50 or 140 and also on adult control and EDS treated rats using antibodies listed below (Table 1.2). Leydig cells were identified as cells immunopositive for the steroidogenic enzyme 3-Beta-Hydroxysteroid Dehydrogenase (3 β -HSD). COUP-TFII was used to identify both presumptive adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) and also adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺). Smooth Muscle Actin (SMA) was used to distinguish peritubular myoid (PTM) cells from interstitial cells and also to define the seminiferous cords/tubules.

3.2.8 Triple colorimetric immunohistochemistry

Following rehydration, the slides containing the testis sections underwent heat induced antigen retrieval (Section 2.10.4). To block non-specific binding, sections were placed in 3% (v/v) H₂O₂ in methanol for 30 minutes and washed in tap water. Slides were washed in tris-buffered saline (TBS) (Section 2.16.2) for 5 minutes and incubated with normal rabbit serum (NRS), (Biosera, Ringmer, UK) (4:1 TBS to NRS with 5% bovine serum albumin (BSA) (Sigma, Poole, Dorset)) for 30 minutes. Sections were then incubated with primary antibody diluted in NRS (Table 3.2) overnight at 4°C.

Table 3.2 Primary antibodies used for colorimetric immunohistochemistry

Primary Antibody	Source	Retrieval	Species	Dilution
3 β -HSD	Santa Cruz	Yes	Goat	1:100
SMA	Santa Cruz	Yes	Mouse	1:3000
COUP-TFII	Perseus Proteomics	Yes	Mouse	1:100
MafB	Abcam	Yes	Rabbit	1:400

Sections were washed in TBS (5 minutes x 2) and incubated for 30 minutes with rabbit anti-goat biotinylated secondary antibody, diluted 1:500 in NRS, followed by 5 min TBS washes. Slides were incubated with Streptavidin-AP (alkaline phosphatase) at 1:200 diluted in TBS for 30 minutes followed by a TBS wash. Detection was performed using commercial fast blue buffer. Reactions were stopped in TBS and slides underwent further antigen retrieval in a microwave with 0.01 M citrate (pH 6.0) buffer (Sigma) for 2.5 minutes on medium heat. These were left to cool for 20 minutes and rinsed in TBS. Sections were then blocked in NRS/TBS/BSA as before for 30 minutes before application of the primary antibody. The same protocol was then followed as before, with the exception that rabbit anti-mouse biotinylated secondary antibody, diluted at 1:500 in NRS/TBS/BSA was used. Fast red buffer was used for detection of SMA. COUP-TFII was optimised and applied at 1:100 to testis sections from mice at pnd 2, 12, 20, 50 and 140. Streptavidin-HRP (horseradish peroxidase) at 1:1000 was used to detect RAM-biotinylated secondaries (1:500) in TBS. Detection of COUP-TFII⁺ cells was performed using 3,3-diaminobenzidine tetrahydrochloride (DAB) (Dako, UK) and stopped in TBS. Slides were mounted with Permafluor (Thermo Scientific, Fremont, CA). Two separate slides containing tissue from the same animal were included in each experiment to ensure consistency of the resultant staining.

3.2.9 Quantification of cell types

The volume of adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) and adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) per testis was determined using stereological techniques involving point-counting methods (Section 2.11). This used a Zeiss Axio-Imager microscope (CarlZeiss Ltd; Welwyn Garden City, UK) fitted with a Hitachi HVC20 camera (Hitachi Denshi Europe, Leeds, UK) and a Prior automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK) and utilised Image-Pro Plus version 7.0 with Stereologer Analyser Pro (Media Cybernetics, UK). Although the optical dissector method (Wreford, 1995), which involves the recognition of cell types by their position and shape, is more definitive for cell counting, it could not be used in these studies. This was due to the fact that these studies involved staining for three proteins, via triple colorimetric immunohistochemistry, in order to distinguish between both adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) and adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) for their subsequent quantification.

3.2.10 Triple immunofluorescence

Testis sections from rat, mouse, marmoset and human underwent triple fluorescence immunohistochemistry. Antibody dilutions were optimised for fluorescence prior to experimentation. As above, Leydig cells were identified as cells immunopositive for the steroidogenic enzyme 3 β -HSD, in their cytoplasm. COUP-TFII was used to localise both interstitial adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) and also adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺). Smooth Muscle Actin (SMA) was used to distinguish peritubular myoid (PTM) cells from interstitial cells and also to define the seminiferous cords/tubules. Other interstitial cell types were identified using the following markers; macrophages (CD68), pericytes (CD146), endothelial cells (CD31), fibroblasts (S100A4), and haematopoietic stem cells (CD34). Other factors known to be expressed by adult Leydig cells were also examined e.g. Insl3 and GATA4. Interstitial cells expressing COUP-TFII in their nucleus, but not expressing any of the other aforementioned cell-specific markers, are termed 'adult Leydig

progenitor cells'. Following citrate retrieval, slides were blocked in normal chicken serum (NChS) and incubated overnight at 4°C with the primary antibody of interest (Table 3.3). After TBS washes, slides were incubated at room temperature for 30 minutes with peroxidase labelled secondary antibodies (1:200, section 2.12.3). Tyramide dye (Tyr-Cy5/Cy3) was used as a detector dye to localise the proteins of interest and diamidinophenylindole (DAPI) was used as a nuclear counterstain. For co-localisation studies, NChS was used as a block to prevent cross-reaction with mouse/goat antibodies and the protocol continued as before. Slides were mounted with Permafluor (Thermo Scientific, Fremont, CA) prior to visualisation.

3.2.11 Image capture

Triple colorimetric or single immunohistochemistry images were captured using a Provis BX2 microscope (Olympus America Inc. Center Valley, PA, USA), that was fitted with a Canon DS126131 camera and EOS image capture software (Canon, Woodhatch, Surrey, UK). Fluorescent images were captured using a laser scanning confocal microscope (LSM) 710 Axiovert Observer Z1 (Carl Zeiss, AG, Germany). Images were compiled using Photoshop 5.0 (Adobe Systems Inc., Mountain View, CA).

Table 3.3 Primary antibodies used for immunofluorescence

Primary Antibody	Source	Retrieval	Species	Dilution
3 β -HSD	Santa Cruz	Yes	Goat	1:500
SMA	Santa Cruz	Yes	Mouse	1:6000
COUP-TFII	Perseus Proteomics	Yes	Mouse	1:1000
SF1	Santa Cruz	Yes	Rabbit	1:200
LHR	Santa Cruz	Yes	Rabbit	1:200
S100A4	Dako	Yes	Rabbit	1:1000
CD34	Santa Cruz	Yes	Goat	1:500
CD31	Santa Cruz	Yes	Goat	1:500
CD146	Abcam	Yes	Rabbit	1:500
CD68	AbD Serotec	Yes	Mouse	1:1000
Insl3	Santa Cruz	Yes	Rabbit	1:500
Gata4	Santa Cruz	Yes	Mouse	1:500
GFP/YFP	Abcam	Yes	Rabbit	1:1500
PCNA	Santa Cruz	Yes	Rabbit	1:500

3.3 Results

3.3.1 Adult Leydig cell ablation/regeneration model

Given the recent study illustrating the importance of COUP-TFII for adult Leydig cell differentiation (Qin et al 2008), the initial aim was to determine whether cells expressing COUP-TFII may be putative progenitors for adult Leydig cells. Using ethane dimethane sulphonate (EDS), to recapitulate normal adult Leydig cell differentiation in rats, is a well-established model (Bartlett et al 1986, Sharpe et al 1990, Teerds et al 1999, Ariyaratne et al 2003, O'Shaughnessy et al 2008) in which the adult Leydig cell population is ablated, followed subsequently by regeneration to their initial population size. Firstly, to determine whether EDS had effectively ablated adult Leydig cells; tissue weight, plasma LH and testosterone levels, along with steroidogenic enzyme transcript levels were analysed at 1, 2, 3 or 5 wks post-EDS. These results demonstrated a significant reduction in both prostate and testis weight, (Figure 3.1), elevation of blood LH with a reduction in blood testosterone levels (Figure 3.2), and finally as a measure of Leydig cell function, expression of *3 β -hsd* and *Lhr* were found to be significantly decreased (Figure 3.2) post-EDS. These results are consistent with previous EDS studies (as above), indicating effective ablation of adult Leydig cells in this study.

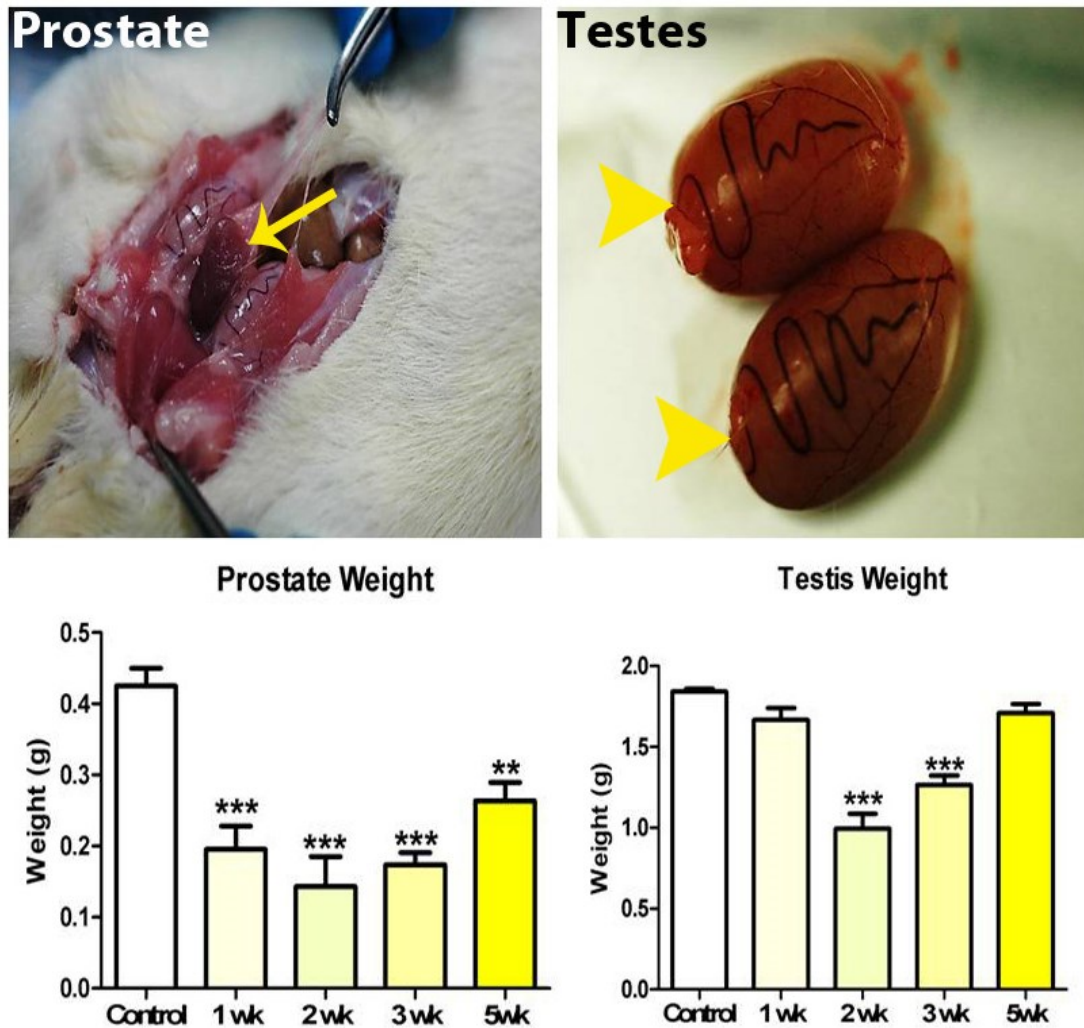


Figure 3.1 Prostate and testes weight post-EDS in rats. Representative image of prostate (top left; arrow) and testes (top right: arrowhead) is shown from a control animal. The weight of the prostate (bottom left) and testes (bottom right) is shown from both control and EDS-injected animals at specific timepoints post-EDS at 1, 2, 3 or 5 weeks. Both prostate and testis weight were reduced following EDS injection. Values are mean \pm SEM for control (n=8) and EDS (n=5). Data were analysed by one-way ANOVA followed by Bonferroni post-hoc test; ***p<0.001, **p<0.01, in comparison with control animals.

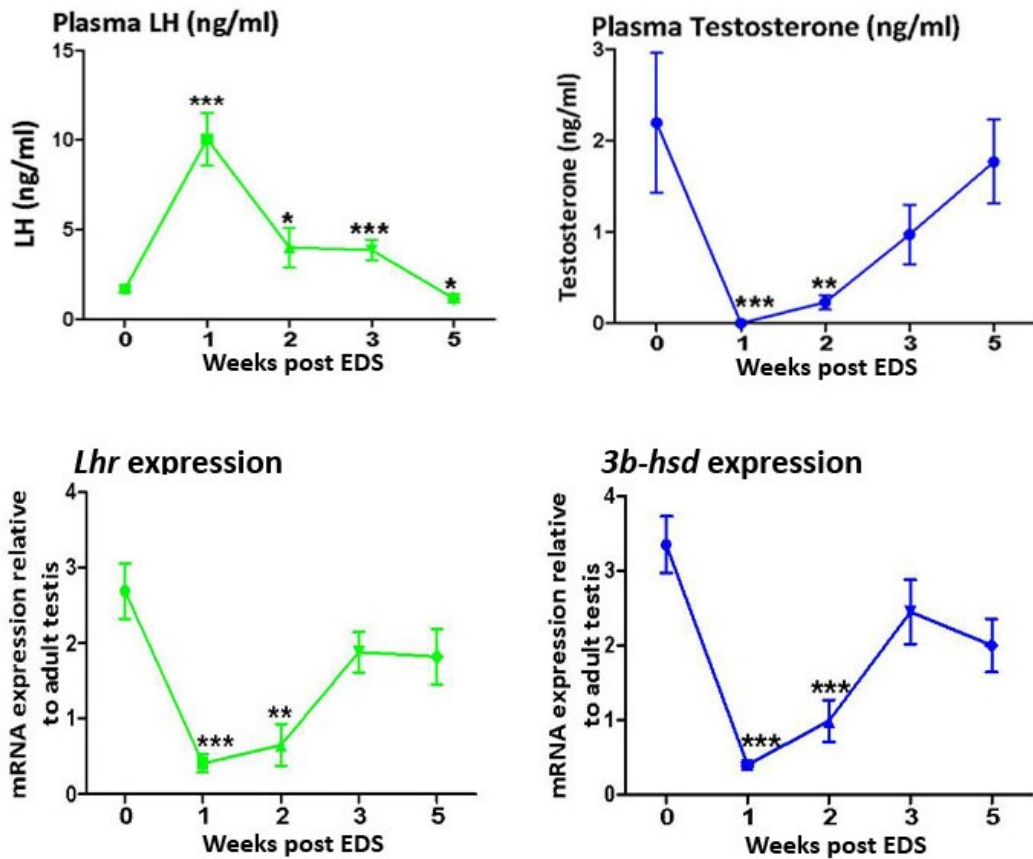


Figure 3.2 Plasma levels and Leydig cell gene expression post-EDS in rats. Change in plasma levels of LH (top left) and testosterone (top right) and in gene expression of *Lhr* (bottom left) and *3b-hsd* (bottom right) in testes from EDS-injected rats after 1, 2, 3 or 5 wks. Values are means \pm SEM for $n=5-7$ per group. Data were analysed using an unpaired t-test; *** $p<0.001$, ** $p<0.01$ in comparison with vehicle control group (time 0).

3.3.2 Adult Leydig cells (3β -HSD⁺) differentiate from COUP-TFII⁺ cells

Having validated the EDS model for successful adult Leydig cell ablation, the following experiments aimed to determine firstly, whether COUP-TFII⁺ cells were sensitive to EDS and secondly, whether the re-population of newly formed adult Leydig cells expressed COUP-TFII. Double immunofluorescence using COUP-TFII (putative adult Leydig progenitor cell marker) and 3β -HSD (to distinguish adult Leydig cells), was carried out on testis sections from control and EDS-injected adult rats. In adult control testes (vehicle injected animals (DMSO+H₂O)), the interstitium contained an abundance of COUP-TFII⁺ spindle-

shaped cells (arrow) while adult Leydig (3β -HSD⁺) cells expressed COUP-TFII in their nuclei (Figure 3.3; arrowhead). One week after EDS-induced adult Leydig cell ablation, there were no identifiable adult Leydig cells present while COUP-TFII⁺ spindle-shaped cells were still present (Figure 3.3; arrow). Two weeks post-EDS, COUP-TFII⁺ interstitial cells acquired 3β -HSD in their cytoplasm, marking the regeneration of identifiable new Leydig (3β -HSD⁺) cells (Figure 3.3; arrowhead). The recovery of adult Leydig cells, close to their previous number pre-EDS, was evident at 3 weeks post-EDS, in which the majority contained a COUP-TFII⁺ nucleus (Figure 3.3; arrowhead). Adult Leydig progenitor cells (COUP-TFII⁺/ 3β -HSD^{neg}) cells remained in the interstitium along with regenerating COUP-TFII⁺ adult Leydig cells (COUP-TFII⁺/ 3β -HSD⁺) throughout the regeneration period.

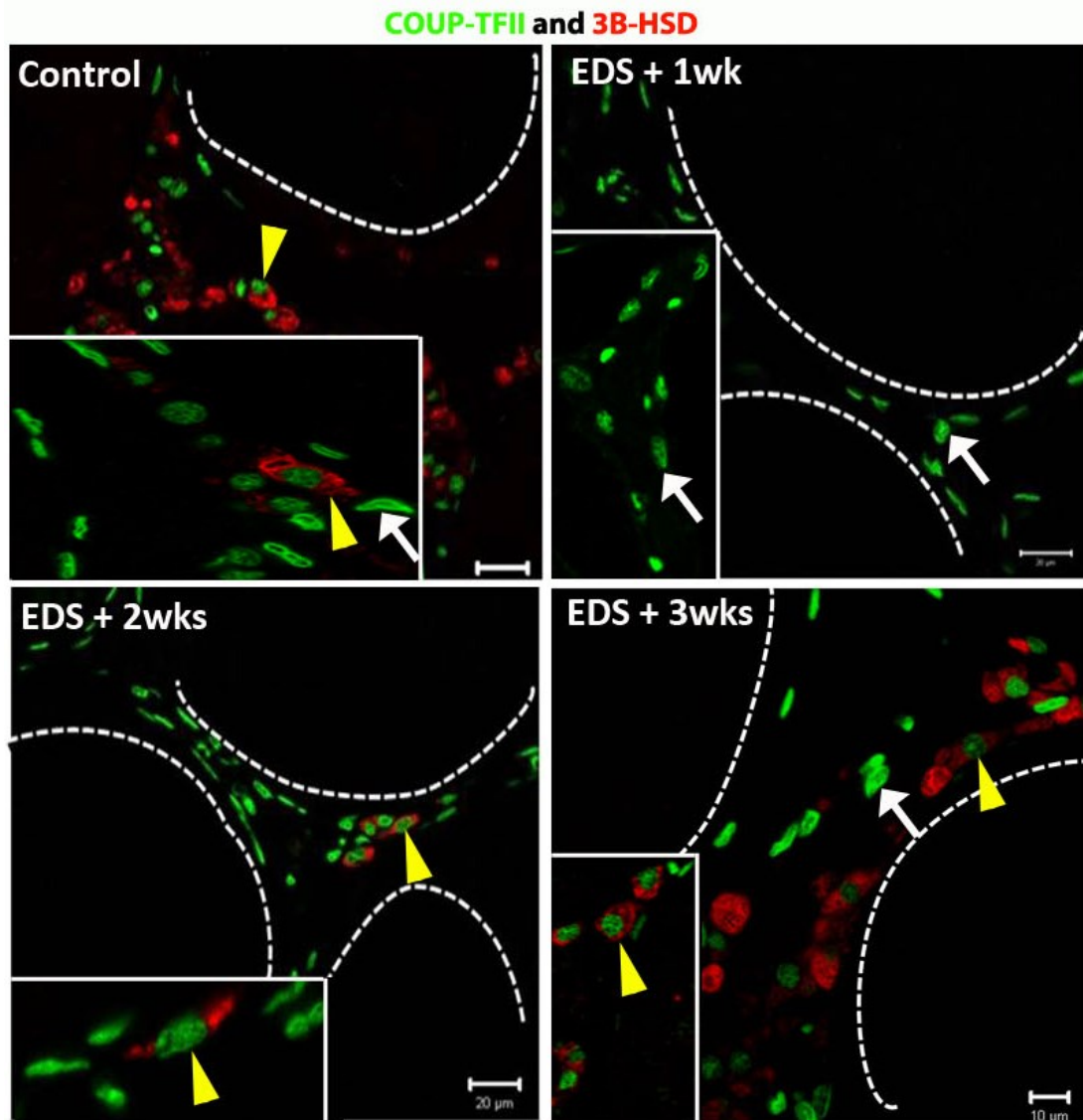


Figure 3.3 Adult Leydig cell regeneration from COUP-TFII⁺ progenitor cells following EDS-induced ablation in the rat. In adult controls (upper left), adult Leydig progenitor cells are identified by their COUP-TFII nuclear staining (green; white arrow) while adult Leydig cells, which also express nuclear COUP-TFII, are stained with the cytoplasmic steroidogenic enzyme marker 3 β -HSD (red; yellow arrowhead). One week post-EDS (upper right), Leydig cells are absent but COUP-TFII⁺ Leydig progenitor cells (green nuclei) remain (white arrow). Two weeks post-EDS (lower left), some spindle shaped COUP-TFII⁺ Leydig progenitor cells (yellow arrowhead) have begun to acquire 3 β -HSD⁺ staining (red; yellow arrowheads), thus marking their onset into the adult LC lineage. Three weeks post-EDS (lower right) numerous adult Leydig cells have regenerated and continue to express COUP-TFII in their nuclei, as in controls. Insets show higher magnification of cells in transition from progenitor to adult Leydig cells. Dashed line depicts the outline of seminiferous tubules. Scale bars = 20 μ m or 10 μ m: bottom right image.

3.3.3 Adult Leydig cells (Insl3⁺/3 β -HSD⁺) differentiate from COUP-TFII⁺ cells

In order to further examine whether COUP-TFII putative adult Leydig progenitor cells differentiated into adult Leydig cells, as indicated by the co-expression of 3 β -HSD two weeks post-EDS (Figure 3.3), a second marker for adult Leydig cells, Insl3 (Ivell et al 2013) was studied. Triple immunofluorescence using COUP-TFII (green) and adult Leydig cell markers; Insl3 (red) and 3 β -HSD (blue), was carried out. Adult Leydig cells are evident in control testes (Figure 3.4; arrowheads). COUP-TFII is evident within the nuclei of adult Leydig cells (arrowhead) and putative adult Leydig progenitor cells (arrows). One week post-EDS, only COUP-TFII⁺ putative adult Leydig progenitor cells are present (arrow), which acquire both adult Leydig cell markers, 3 β -HSD (blue) and Insl3 (red) after two weeks (Figure 3.4; arrowhead). Three weeks post-EDS, putative adult Leydig progenitor cells (COUP-TFII⁺; green) and adult Leydig cells expressing COUP-TFII are evident (Figure 3.4; arrowhead). These results demonstrated that adult Leydig cells (3 β -HSD⁺ and Insl3⁺) express COUP-TFII in their nuclei, which may suggest their differentiation from COUP-TFII⁺ putative adult Leydig progenitor cells. This prompted the question as to whether the number of COUP-TFII⁺ putative adult Leydig progenitor cells changed during adult Leydig cell regeneration post-EDS injection.

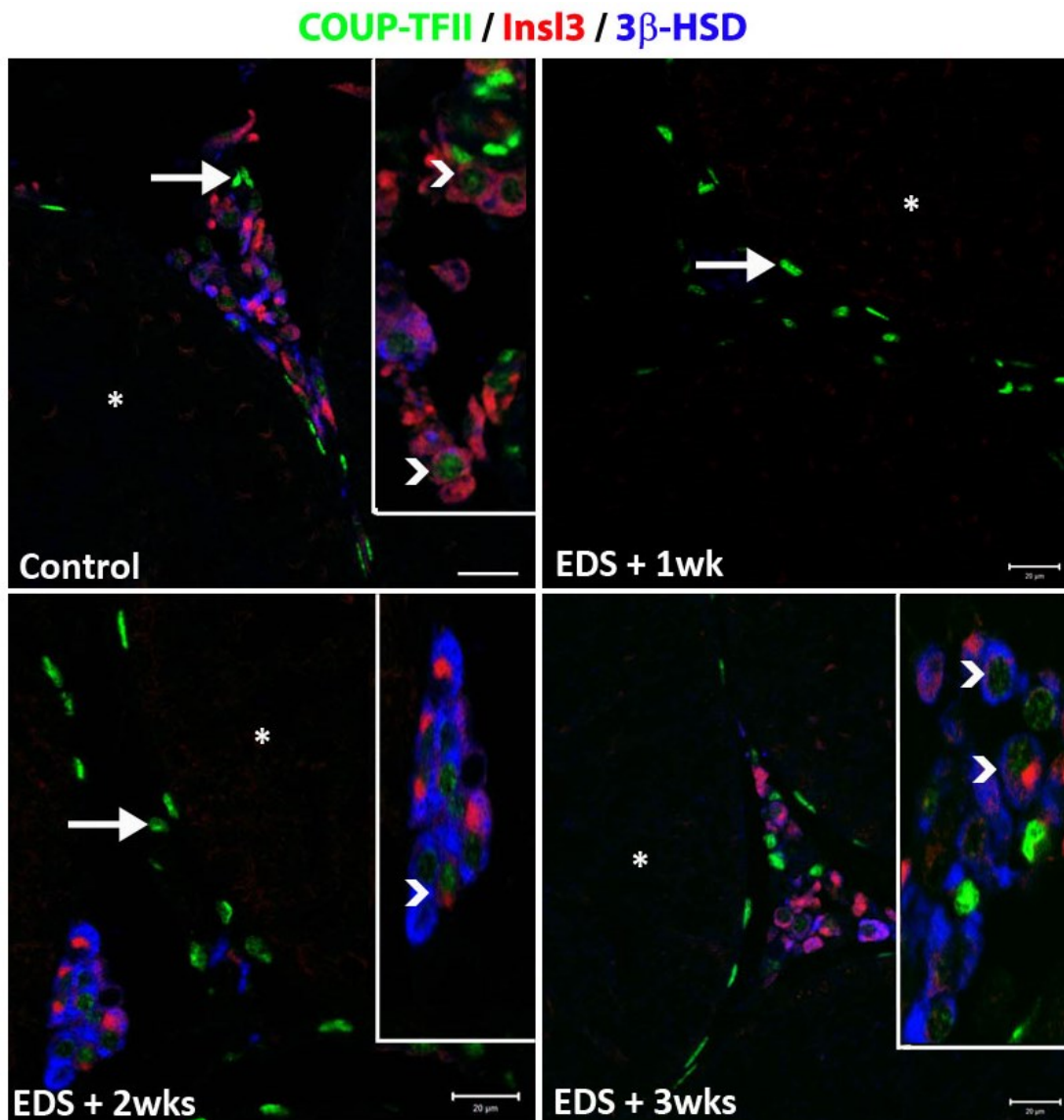


Figure 3.4 Adult Leydig cells ($3\beta\text{-HSD}^+$ and InsI3^+) differentiate from COUP-TFII^+ cells following EDS-induced ablation in the rat. In adult controls (top left), Leydig progenitor cells (COUP-TFII^+ : green; white arrow) are evident alongside adult Leydig cells ($3\beta\text{-HSD}^+$: blue and InsI3^+ : red), which also express COUP-TFII in their nucleus. One week post-EDS (top right), Leydig cells are absent but COUP-TFII^+ Leydig progenitor cells (green nuclei) remain (white arrow). Two weeks post-EDS (bottom left), some COUP-TFII^+ Leydig progenitor cells have acquired $3\beta\text{-HSD}$ and InsI3 staining (arrowhead), which marks the onset of adult Leydig cell regeneration. Three weeks post-EDS (bottom right) numerous adult Leydig cells ($3\beta\text{-HSD}^+$ and InsI3^+ ; arrowhead) have regenerated expressing COUP-TFII in their nuclei, as in controls. Insets show higher magnification views of cells in transition from progenitors to adult Leydig cells. * indicates the seminiferous tubules. Scale bars = $20\mu\text{m}$.

3.3.4 Reduction in COUP-TFII⁺ adult Leydig progenitor cell number after EDS

As adult Leydig cells appear to differentiate from COUP-TFII⁺ putative adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}), it raised the question as to whether their number changed during adult Leydig cell regeneration. Staining with the proliferation marker PCNA (red) showed that a small proportion of COUP-TFII⁺ cells (green nuclei; arrow) were proliferating, as indicated by 'yellow' co-localisation of COUP-TFII⁺ and PCNA⁺ cells, one week post-EDS (Figure 3.5). However, following quantification of the putative adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}), their number declined significantly over the 5-week period of adult Leydig cell regeneration (Figure 3.5), consistent with some of these cells differentiating into adult Leydig cells. The number of adult Leydig cells increased in number during their regeneration, to near normal levels (Figure 3.5).

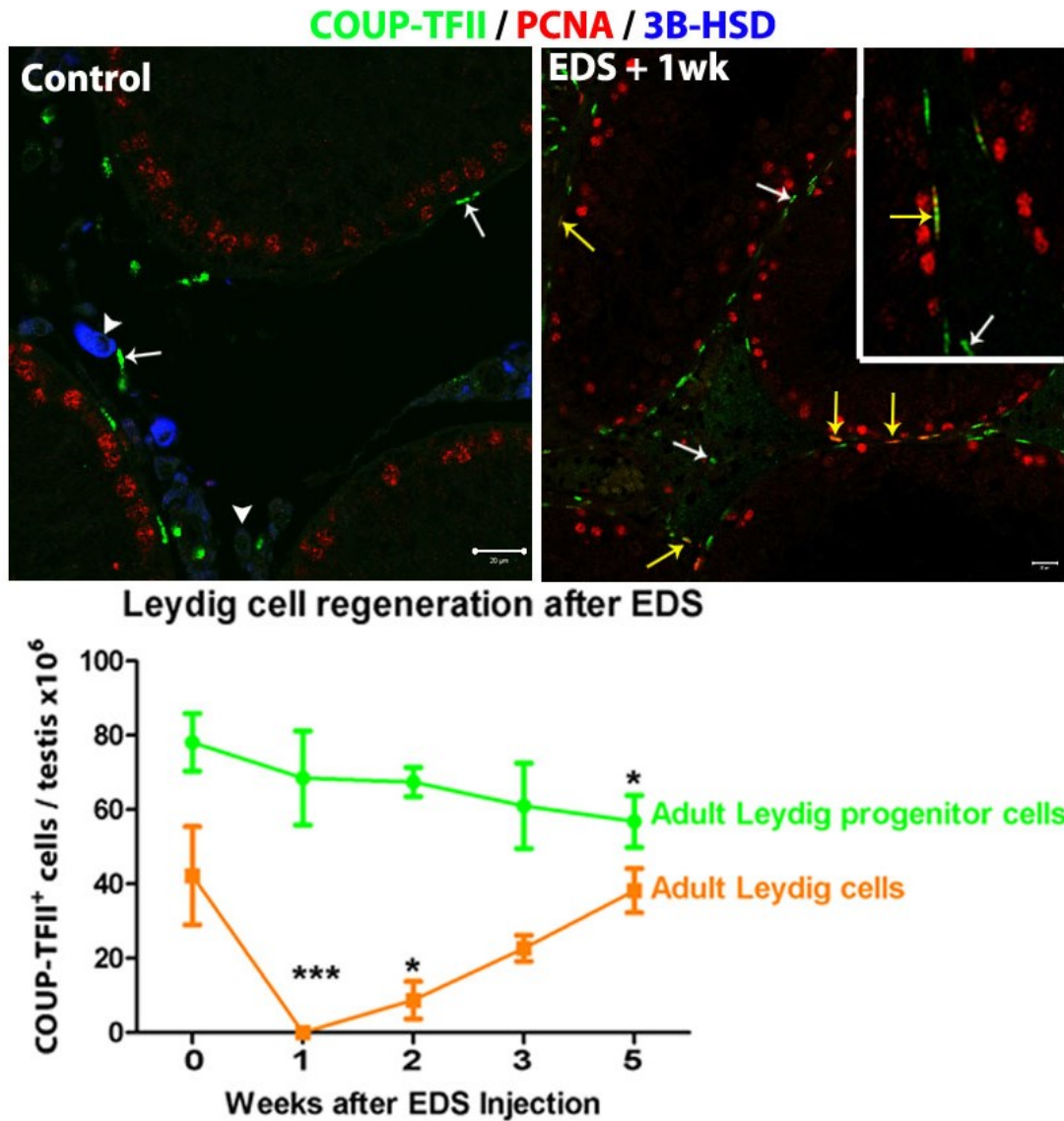


Figure 3.5 Quantification of adult Leydig cells and their putative progenitors. Adult Leydig cells (COUP-TFII⁺/3β-HSD⁺) and putative adult Leydig progenitor cells (COUP-TFII⁺/3β-HSD^{neg}) were quantified at 1, 2 3 or 5 weeks post-EDS in the rat. Adult Leydig cells were ablated and recovered after 5 weeks to near normal levels as in control. Putative adult Leydig progenitor cells significantly declined in numbers at 5 weeks post-EDS. Values are means ± SEM; n=5-7 per group; Data were analysed using an unpaired t-test; ***p<0.001, *p<0.05 in comparison with vehicle control group (time 0). Scale bars = 20µm.

3.3.5 COUP-TFII⁺ adult Leydig progenitor cells do not express key interstitial cell markers

To investigate any alternative interstitial cell source for regenerating adult Leydig cells, other than COUP-TFII⁺ cells, double immunofluorescence was carried out using COUP-TFII (putative adult Leydig progenitor cells; green) and a range of other testicular interstitial cell markers (red). These included macrophages (CD68), pericytes (CD146), endothelial cells (CD31), peritubular myoid cells (SMA), fibroblasts (S100A4), or hematopoietic stem cells (CD34). Other immature/mature Leydig cell markers were also studied including SF1, LHR, Insl3 and Gata4 to investigate their expression profile in the putative adult Leydig progenitor cells. The results showed that COUP-TFII⁺ putative adult Leydig progenitor cells (green) did not express any other interstitial marker as listed above (red), other than COUP-TFII (Figure 3.6). Furthermore these cells did not express the phenotypical adult Leydig cell markers 3 β -HSD (Figure 3.3) or Insl3 (Figure 3.4). MafB was also examined as a potential marker of adult Leydig progenitor cells, as it has been implicated in fetal Leydig cell development (DeFalco et al 2011). However adult Leydig progenitor cells did not express MafB at 1 or 2 weeks post-EDS (Figure 3.7). Lastly, the adult Leydig cell marker GATA4 (red) was examined. This demonstrated that, 1-2 weeks after EDS treatment, a proportion of putative adult Leydig progenitor cells (COUP-TFII⁺; green), located within the interstitium or bordering the seminiferous tubules, co-expressed GATA4 (COUP-TFII⁺/GATA4⁺; yellow arrows) whereas these cells were only rarely evident in control testes (Figure 3.8). Two and three weeks post-EDS, a proportion of COUP-TFII⁺ progenitor cells expressing GATA4, differentiated into adult Leydig cells, as indicated by expression of 3 β -HSD (Figure 3.8; arrowheads). A summary of interstitial cell markers and immature/mature adult Leydig cell markers investigated for expression in COUP-TFII⁺ putative adult Leydig progenitor cells is shown below (Table 3.4). Other factors which are associated with fetal/adult Leydig cell differentiation/development e.g. LHX9 (Birk et al 2000, Mazaud et al 2002, Tang et al 2008), POD1 (Tamura et al 2001, Cui et al 2004, Bhandari et al 2011), LIFR (Ge et al 2006) and GPRC6A (Karsenty, 2012, Oury et al 2013), were also

investigated, but excluded from this thesis due to the non-specificity of the antibodies used.

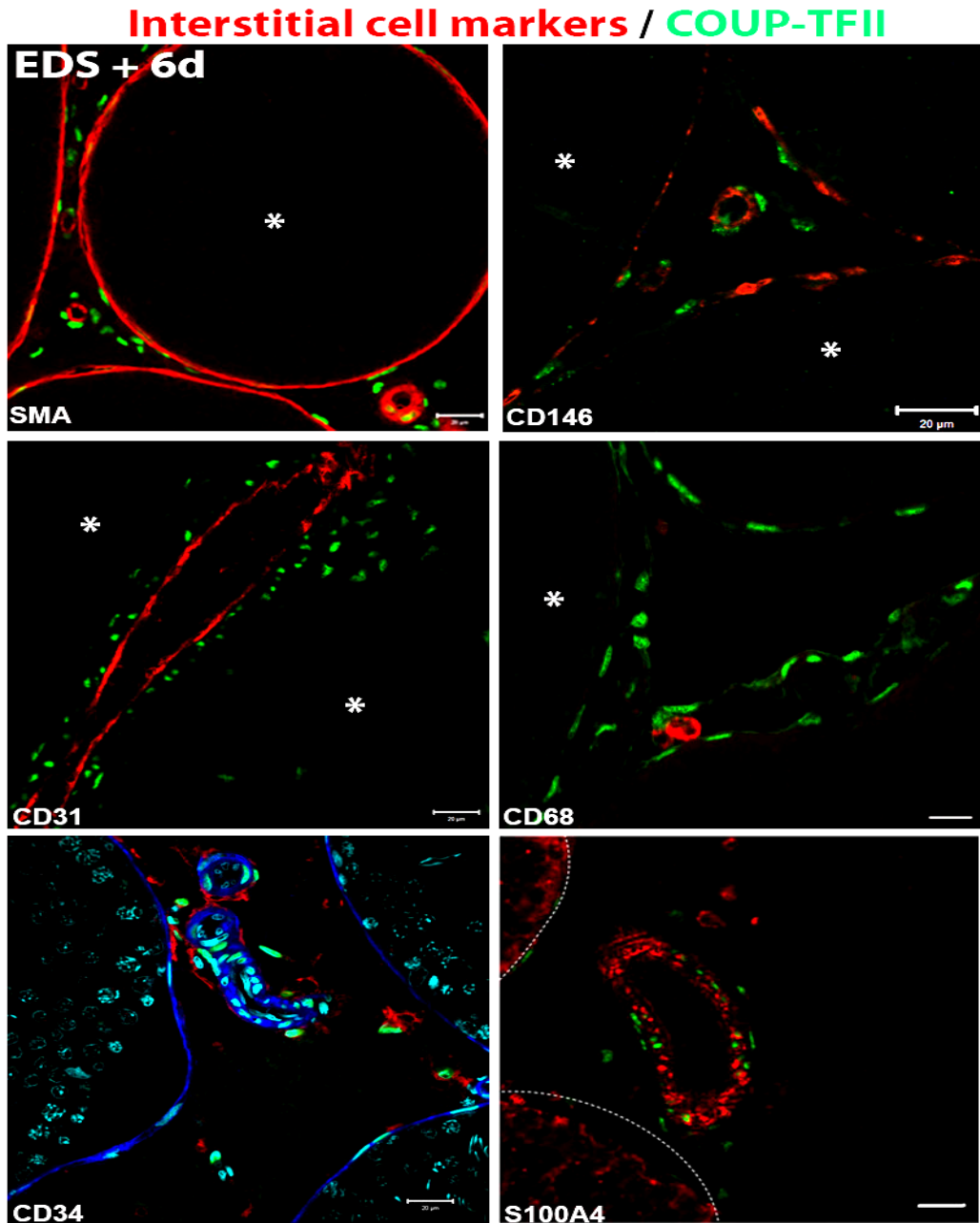


Figure 3.6 Adult Leydig progenitor cells do not express any other interstitial cell markers one week-post EDS in the rat. Adult Leydig progenitor cells (COUP-TFII⁺; green nuclei) are negative for the following: SMA staining, found in PTM cells surrounding the seminiferous tubules and blood vessels (red); pericyte marker, CD146 (red), endothelial cell marker CD31 (red), macrophage marker CD68 (red), haematopoietic stem cell CD34 (red) (SMA:blue) and a fibroblast marker S100A4 (red). Note that in the bottom left image (CD34); nuclear counterstain DAPI is shown in blue. * depicts seminiferous tubules. Scale bars = 20µm and represent n=5.

MafB

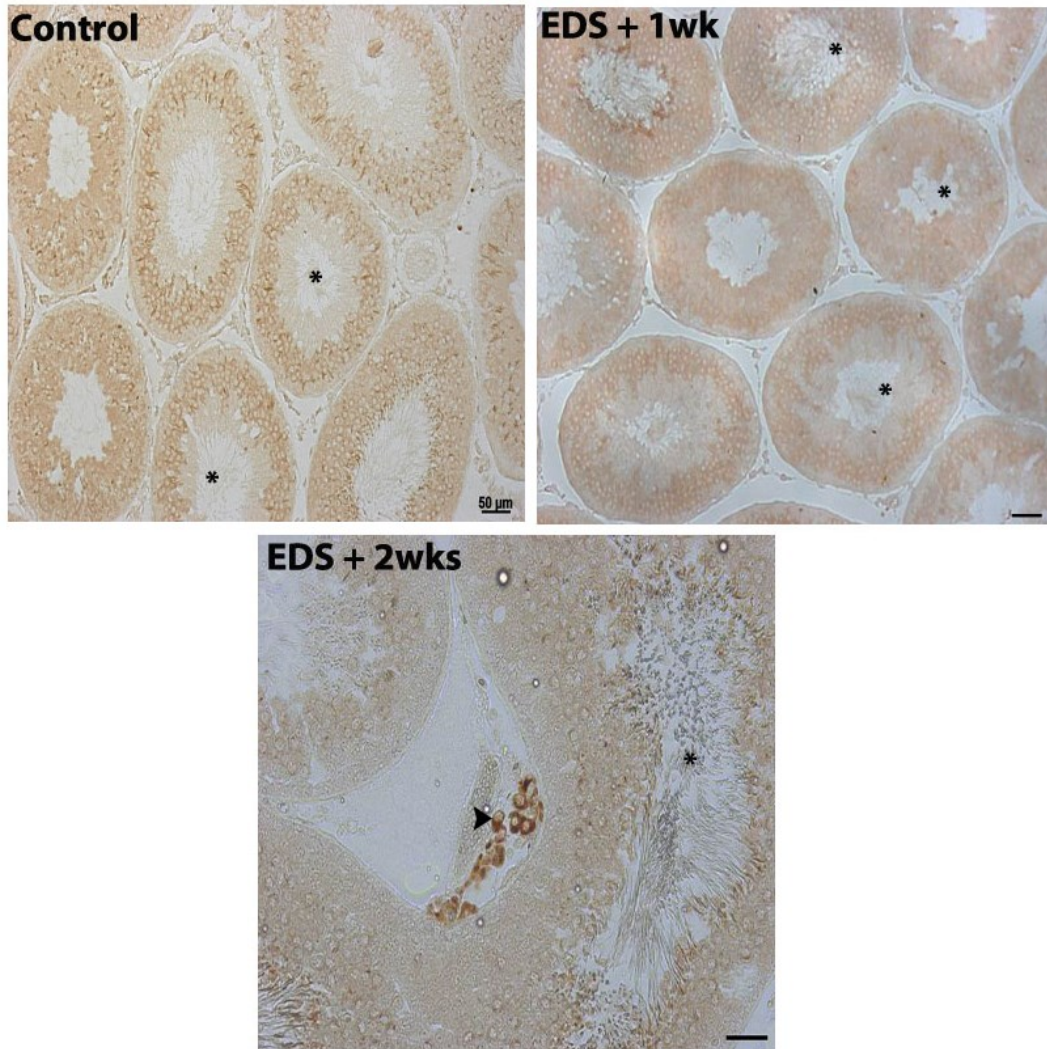


Figure 3.7 MafB expression is restricted to regenerating adult Leydig cells post-EDS in the rat. In control adult rat testis (top left), MafB expression is minimal/absent in the interstitium. One week post-EDS injection, the testis was negative for MafB staining (top right). Two weeks post-EDS, when adult Leydig cells begin to regenerate, MafB (brown) expression was evident in the cytoplasm of Leydig cells, as based on morphology (bottom image). * depicts seminiferous tubules. Scale bar = 50μm.

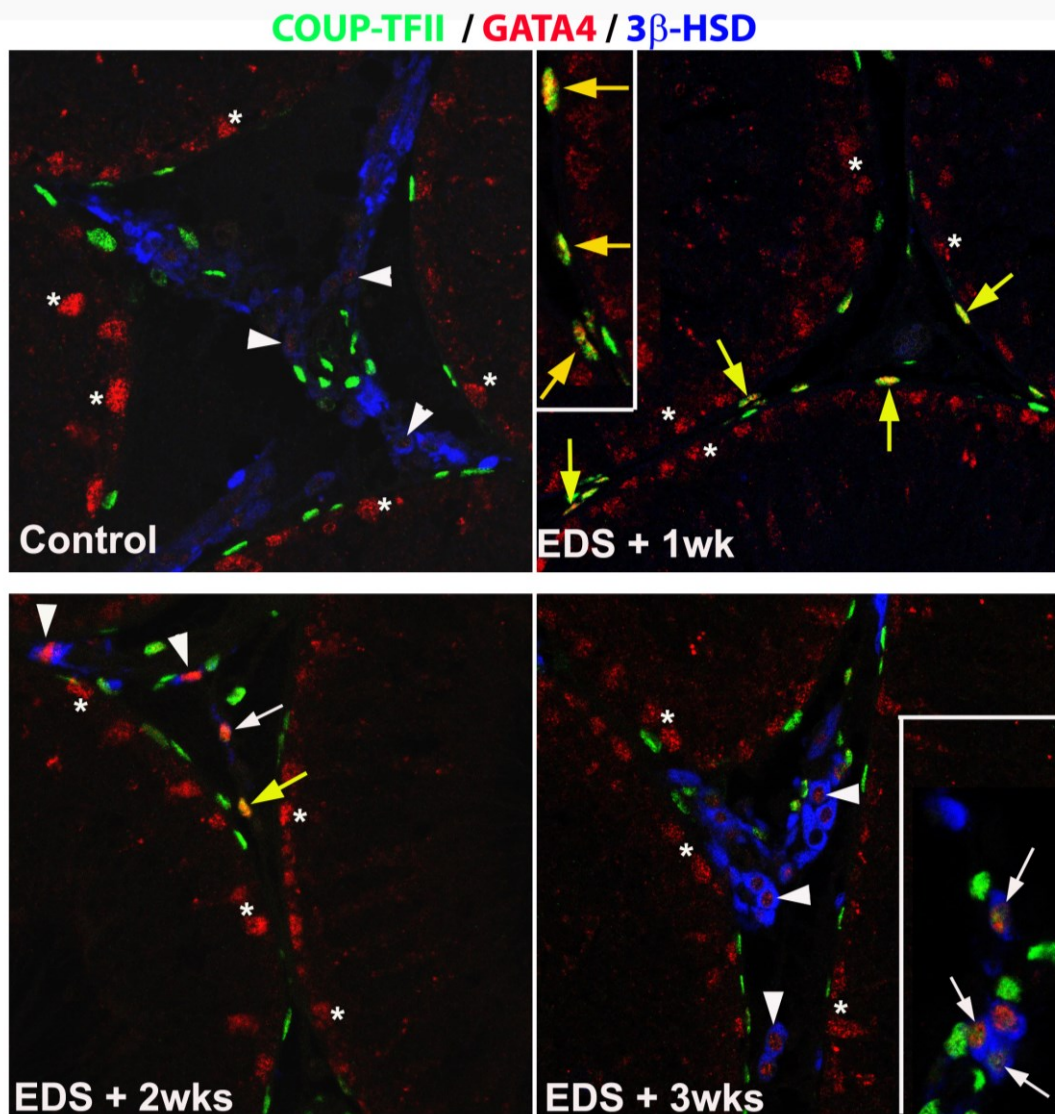


Figure 3.8 Expression of GATA4 in COUP-TFII⁺ putative adult Leydig progenitor cells is an early step in their differentiation into adult Leydig cells (ALC) in the rat. In adult controls (top left), GATA4 expression (red) is confined to the nuclei of Sertoli cells (asterisks) and 3 β -HSD⁺ (blue) Leydig cells (white arrowheads). COUP-TFII-expressing Leydig progenitor cells (green) are negative for GATA4. One week post-EDS (top right), Leydig cells are absent, but GATA4 has been switched on in a proportion of putative adult Leydig progenitor cells (yellow arrows; co-expression of green and red in nuclei). Two weeks post-EDS (bottom left), some of the GATA4-expressing interstitial cells have switched on 3 β -HSD (blue) and by three weeks (bottom right), these cells have completed their differentiation into adult Leydig cells. Cells co-expressing GATA4, 3 β -HSD and COUP-TFII are labelled with white arrows. Insets show higher magnification. For technical reasons with triple immunofluorescence, nuclear COUP-TFII is only evident in a proportion of ALC in these images, whereas it is more clearly evident in ALC of earlier images with double immunofluorescence.

Table 3.4 Summary of interstitial and adult Leydig cell markers investigated post EDS injection in rats, and whether or not they were expressed in COUP-TFII⁺ putative adult Leydig progenitor cells.

Non-Leydig Interstitial cell	Cell Marker	Expression in Leydig Progenitor cell
Macrophage	CD-68	--
Pericyte	CD-146	--
Endothelial	CD-31	--
Peritubular Myoid	SMA	--
Haematopoietic	CD-34	--
Fibroblast	S100A4	--

Immature/adult Leydig cell marker	Expression in Leydig progenitor cell
COUP-TFII	Yes
GATA4	+/-
3B-HSD	--
INSL3	--
LHR	--
SF-1	--
MafB	--

3.3.6 Lineage tracing of adult Leydig cells

To establish whether COUP-TFII could be used as a true marker of the adult Leydig progenitor population, a model for adult Leydig lineage tracing was utilised. Mice with Cre recombinase driven by the adipocyte protein (aP2) promoter were bred with a YFP reporter line which showed aP2 was restricted to Leydig cells, and YFP reporter mice without a Cre transgene did not express YFP (O'Hara et al, unpublished). For the purpose of co-localisation studies in this chapter, the YFP/GFP antibody used to detect YFP⁺ cells is labelled GFP, as indicated in green. During fetal life, aP2⁺/GFP⁺ cells co-localised with COUP-TFII

in Leydig progenitor cells when examined at birth (Figure 3.9; top panel). Follow up analysis demonstrated that this fetally-induced YFP expression was restricted to adult Leydig cells in adulthood (Figure 3.9; bottom panel), confirming that COUP-TFII marks a progenitor population which later develop into adult Leydig cells. Therefore, the COUP-TFII⁺ interstitial cells will be termed 'adult Leydig progenitor cells' for the remainder of this thesis. However, it should be noted that it is unclear if all of the progenitor cells identified have the capacity to develop into adult Leydig cells, or only a sub-population. Moreover, the lineage tracing experiment also showed that some adult Leydig cells did not express GFP (Figure 3.9). This may indicate that some adult Leydig cells may have differentiated from cells other than COUP-TFII⁺ adult Leydig progenitor cells, which were present at birth.

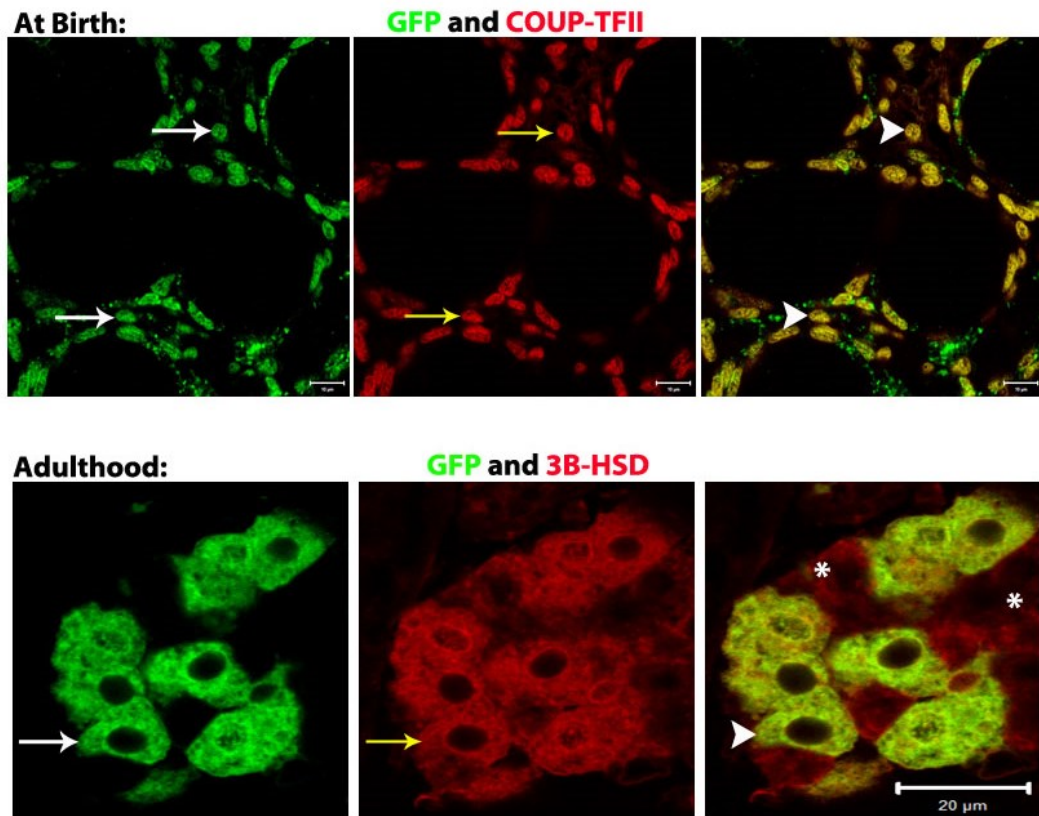


Figure 3.9 COUP-TFII marks adult Leydig progenitor cells as evident from transgenic lineage of adult Leydig cells in mice. At birth (e18.5; top row), the adipocyte protein 2 (aP2) Cre Recombinase induced GFP expression (white arrows) was coincident with COUP-TFII-expressing cells in the testis (yellow arrows). Merged image shows both COUP-TFII and GFP co-localisation (white arrowheads) in adult Leydig progenitor cells. Follow up analysis in adulthood (pnd 50; bottom panel), demonstrated that this fetally-induced GFP expression (white arrow) was restricted to 3β -HSD-expressing (yellow arrow) adult Leydig cells, as indicated by their co-expression (merged image; white arrowhead). Asterisks indicate non-GFP⁺ adult Leydig cells. Scale bars = 20 μ m.

3.3.7 Expression of COUP-TFII during puberty and adulthood

As the results (above) demonstrated that adult Leydig cells regenerate from COUP-TFII⁺ progenitor cells, the next aim was to study Leydig cell differentiation during normal development i.e. puberty. Triple immunofluorescence was used on rat testes to distinguish adult Leydig cells (3β -HSD; red), to identify peritubular myoid (PTM) cells and delineate

seminiferous tubules via expression of smooth muscle actin (SMA; blue) and to investigate adult Leydig progenitor cells (COUP-TFII⁺; green). As with adult Leydig cell regeneration, these results demonstrated that COUP-TFII is expressed in two cell types; 1) spindle shaped interstitial cells in close proximity to the tubules and within the interstitium (green; arrows) and 2) adult Leydig cells (red; arrowheads) expressing nuclear staining of COUP-TFII (COUP-TFII⁺/3 β -HSD⁺) (Figure 3.10). During adulthood, COUP-TFII is expressed in most adult Leydig cells (arrowheads) while COUP-TFII⁺ interstitial cells (arrows) are also present (Figure 3.10).

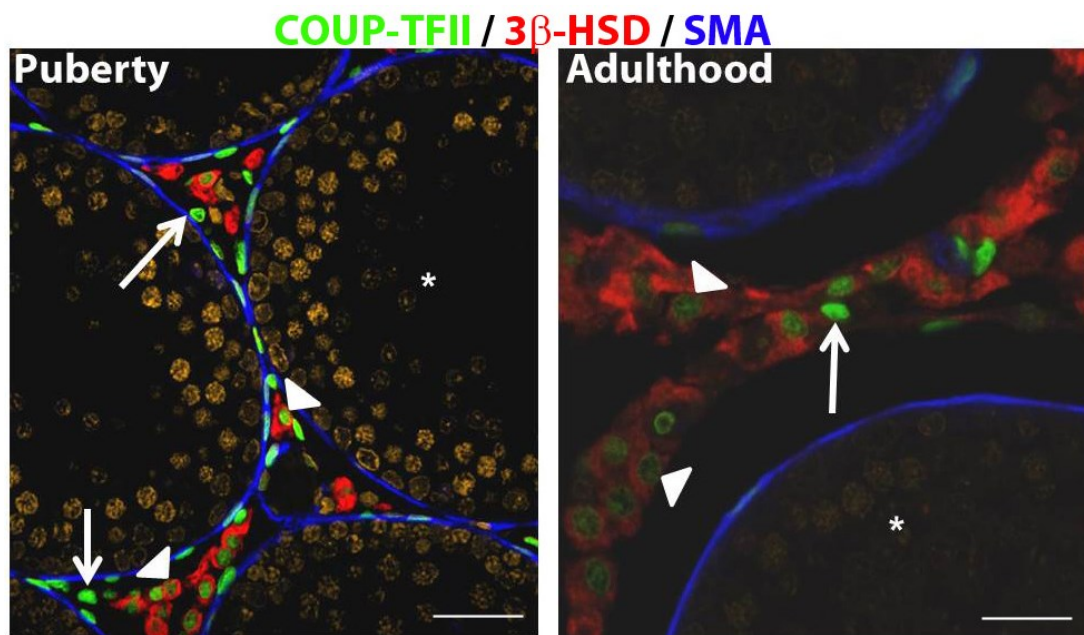


Figure 3.10 COUP-TFII⁺ is expressed in adult Leydig progenitor cells and adult Leydig cells in puberty and adulthood in the rat. At puberty, COUP-TFII⁺ (green; arrows) spindle shaped interstitial cells are present (left), along with COUP-TFII⁺ adult Leydig cells with cytoplasmic 3 β -HSD (red; arrowheads). Similarly, in adulthood (pnd 90), both COUP-TFII⁺ interstitial cells and adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) are present. A counterstain was used to visualise nuclear staining (DAPI; orange). Asterisks depict seminiferous tubules. Scale bar = 20 μ m.

Recapitulating normal adult Leydig cell development via EDS demonstrated that GATA4 was switched on in COUP-TFII⁺ adult Leydig progenitor cells (Figure 3.8) prior to adult Leydig cell differentiation. To investigate whether this occurs during normal adult Leydig cell development, a similar expression study was undertaken using triple immunofluorescence for GATA4 (red), 3 β -HSD (blue) and COUP-TFII (green) in control rats throughout puberty. At the initiation of puberty (day 15), GATA4 expression was evident in a proportion of the COUP-TFII⁺ adult Leydig progenitor cells (yellow arrows), and by pnd 25, these cells had begun to transform into adult Leydig (3 β -HSD⁺) cells (white arrows) (Figure 3.11), which mimics the sequence of protein expression during adult Leydig cell regeneration, after EDS (Figure 3.8).

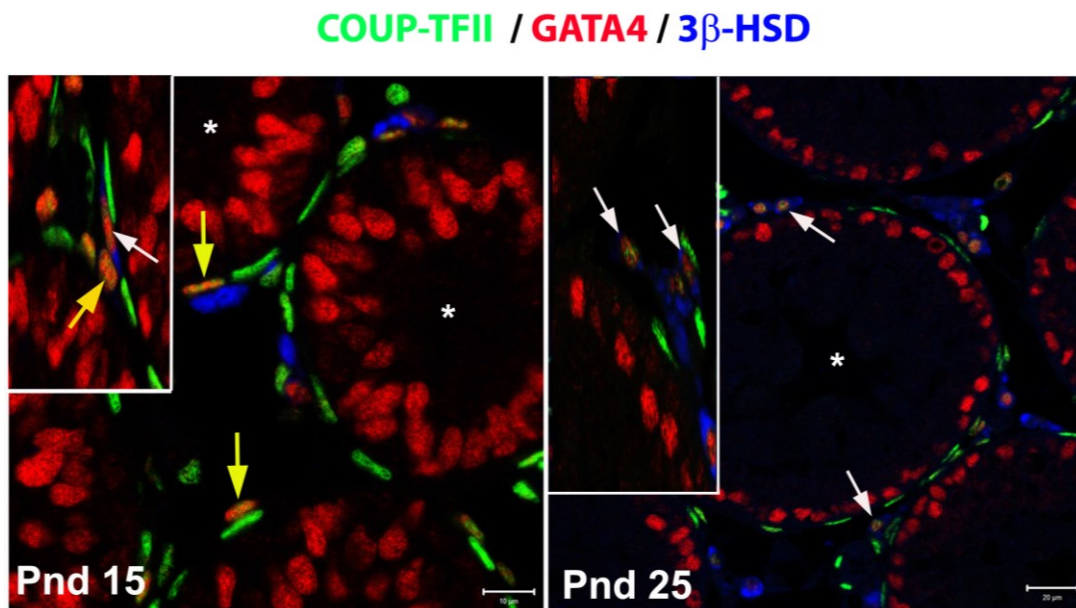


Figure 3.11 GATA4 is expressed in COUP-TFII⁺ adult Leydig progenitor cells during normal pubertal development. At the start of normal puberty (Pnd15) in the rat, co-expression of GATA4 in COUP-TFII⁺ Leydig progenitor cells was common (yellow arrows) and by Pnd25 (bottom right) many of these cells had also begun to switch on 3 β -HSD (white arrows). Insets show higher magnification to emphasise the cascade of protein expression changes in the Leydig progenitor cells during their differentiation into Leydig cells. Asterisks depict seminiferous tubules. Scale bars = 10 μ m (left image) and 20 μ m (right image).

3.3.8 Adult Leydig progenitor cells are present during fetal life

From the earlier lineage tracing experiment, it was evident that COUP-TFII⁺ adult Leydig progenitor cells were present at birth (Figure 3.9). To further investigate the presence of these cells during fetal life, double immunofluorescence was carried out using COUP-TFII (green nuclear staining; adult Leydig progenitor cells) and 3 β -HSD (red cytoplasmic staining; fetal Leydig cells) on control fetal testis sections from rats at e21.5. From both the tiled and high magnification images (Figure 3.12), COUP-TFII⁺ (green) interstitial cells are clearly the most abundant cell type in the fetal testis. Fetal Leydig cells (red) were negative for COUP-TFII (Figure 3.12) at this age. To ensure this was not a species specific finding, triple immunofluorescence with COUP-TFII, 3 β -HSD and SMA (to delineate seminiferous tubules) was carried out on sections from fetal human, marmoset and mice, all of which demonstrated the numerous presence of COUP-TFII⁺ adult Leydig progenitor cells (Figure 3.13).

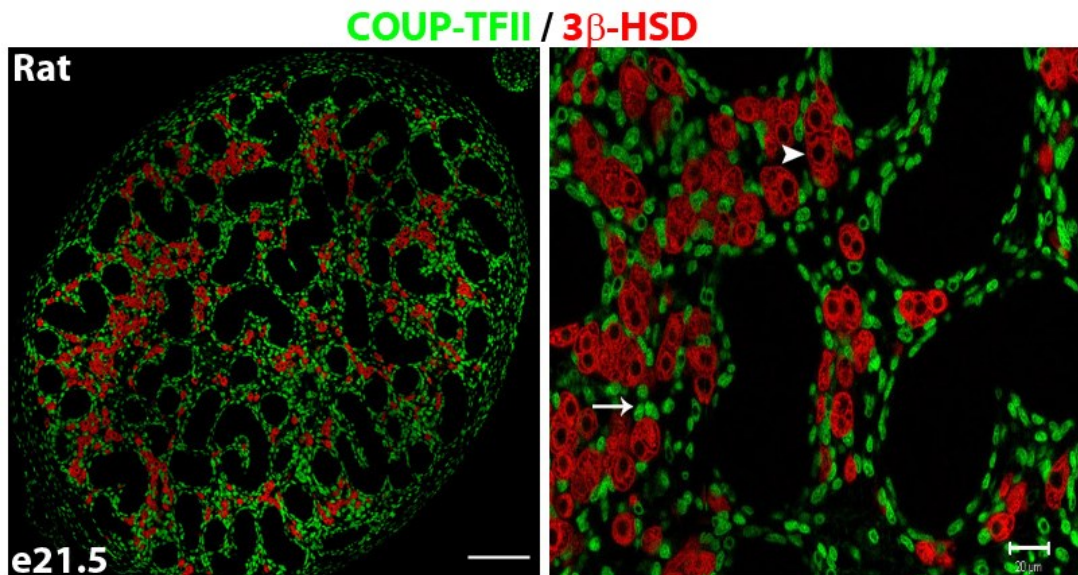


Figure 3.12 COUP-TFII⁺ cells are abundant in the fetal rat testis. Double immunofluorescence for COUP-TFII (green) and 3 β -HSD (red) in fetal rat testis (e21.5). COUP-TFII is expressed in the interstitial cells (green; arrows) while fetal Leydig cells (red; arrowhead), are negative for COUP-TFII staining. Scale bars = 100 μ m (tiled image; left) and 20 μ m (right image). Images are representative of at least 3 control animals.

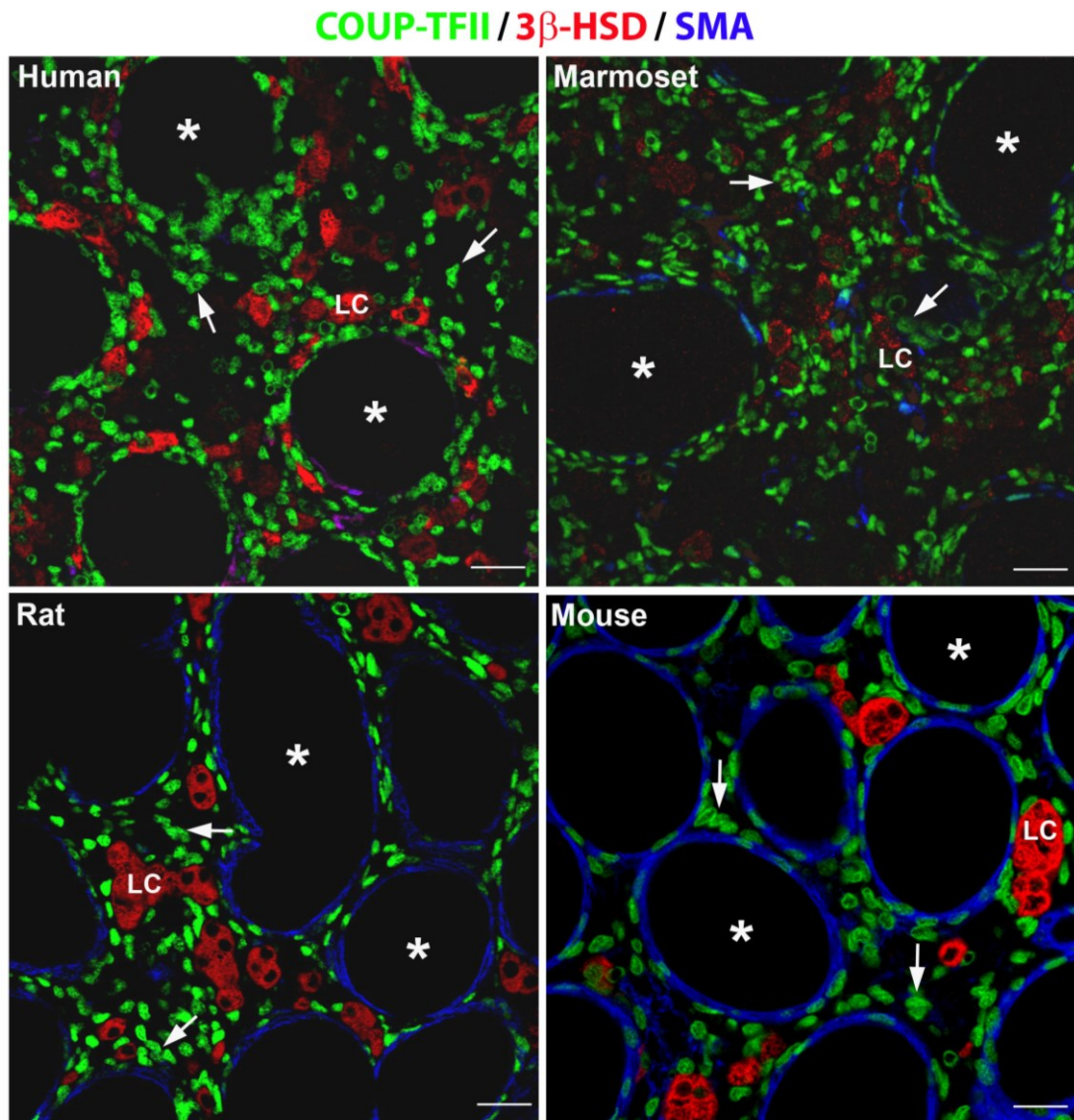


Figure 3.13 Expression of COUP-TFII in fetal testes across species. COUP-TFII⁺ (green) Leydig progenitor cells (white arrow) are the most abundant cell type in the fetal testis of human (top left), marmoset (top right), rat (bottom left) and mouse (bottom right) where red = 3 β -HSD (fetal Leydig cells; LC) and blue = smooth muscle actin, (asterisks indicate seminiferous tubules). The majority of fetal Leydig cells are negative for COUP-TFII. Human sample = 16 weeks gestation; marmoset = 15 weeks gestation; rat = e21.5; mouse = e18.5. Scale bars = 20 μ m.

3.3.9 Steroidogenic Factor-1 and LHR are not expressed in adult Leydig progenitor cells

Steroidogenic factor-1 (SF1), is required during fetal life for two fundamental events 1) normal testis development and 2) differentiation and steroidogenic function of fetal Leydig cells. Given the hypothesis that COUP-TFII⁺ cells present during fetal life are the progenitors for adult Leydig cells, triple immunofluorescence was undertaken to investigate whether SF-1 was expressed in adult Leydig progenitor cells in the fetal testis. This was carried out using COUP-TFII (green; nuclear staining), SF1 (red; nuclear staining) and 3B-HSD (blue; cytoplasmic staining). From the tiled and higher power images (Figure 3.14) in rat (top panel) and mouse (bottom panel), the expression of COUP-TFII (green) is localised to the interstitium while SF1 (red) is clearly localised to Sertoli cells (within the tubules; red) and fetal Leydig cells (blue). It is clearly evident that the COUP-TFII⁺ adult Leydig progenitor cells do not express SF1. Furthermore, to investigate whether COUP-TFII adult Leydig progenitor cells express the luteinising hormone receptor (LHR), double immunofluorescence was carried out using COUP-TFII (green) and LHR (red), which demonstrated that COUP-TFII⁺ cells do not express LHR (Figure 3.15).

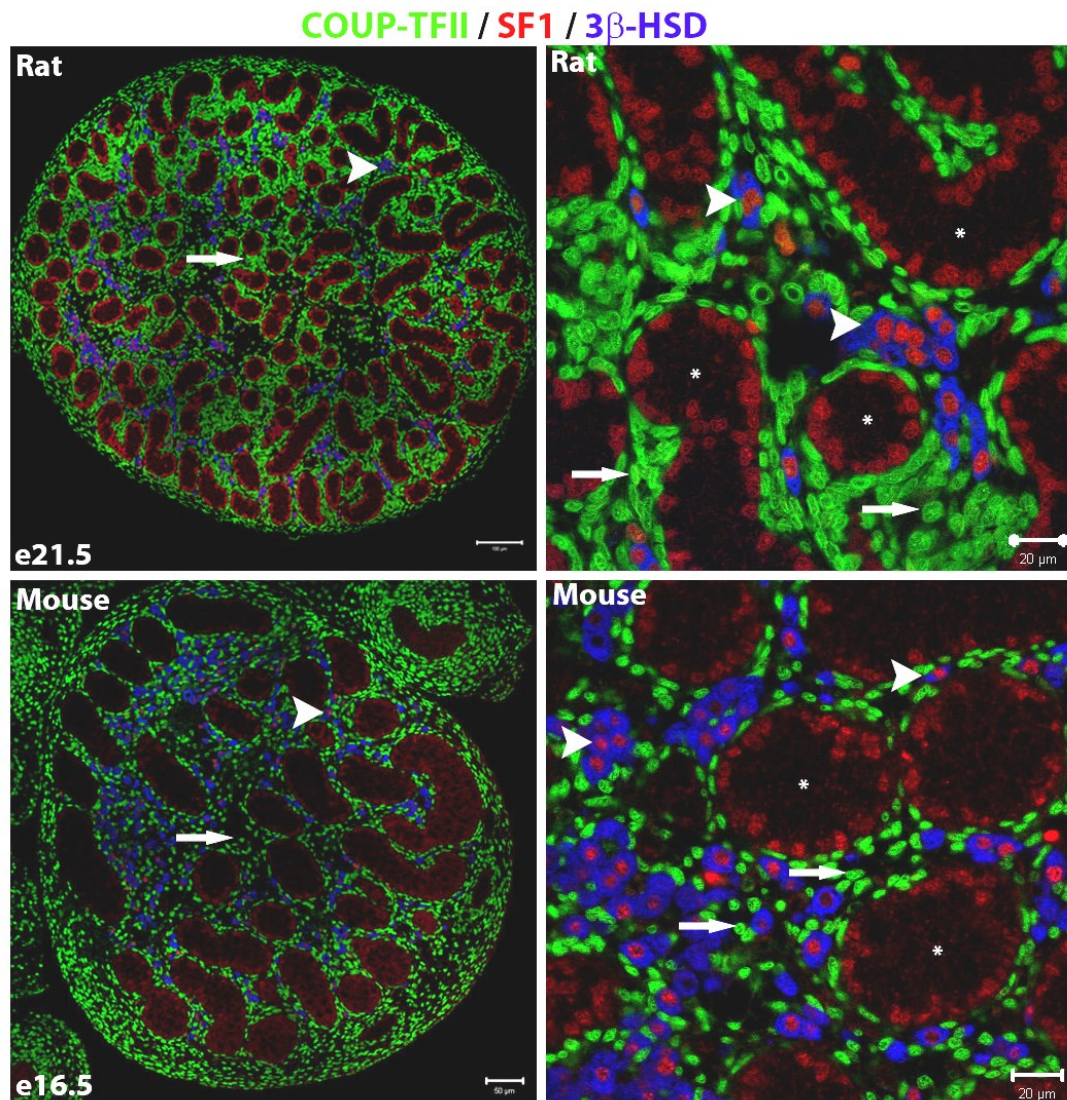


Figure 3.14 COUP-TFII⁺ adult Leydig progenitor cells do not express SF1 in rodents. COUP-TFII⁺ (green) adult Leydig progenitor cells (white arrow) do not co-localise with SF1 (red; Sertoli and fetal Leydig cells), as shown in the fetal rat testis at e21.5 (top; tiled image and zoom) and mouse at e18.5 (bottom; tiled image and zoom). The majority of fetal Leydig cells (3 β -HSD⁺; blue cytoplasmic staining), are negative for COUP-TFII. Asterisks indicate seminiferous cords. Scale bars = 100 μ m (tiled images; left panel) and 20 μ m (right panel).

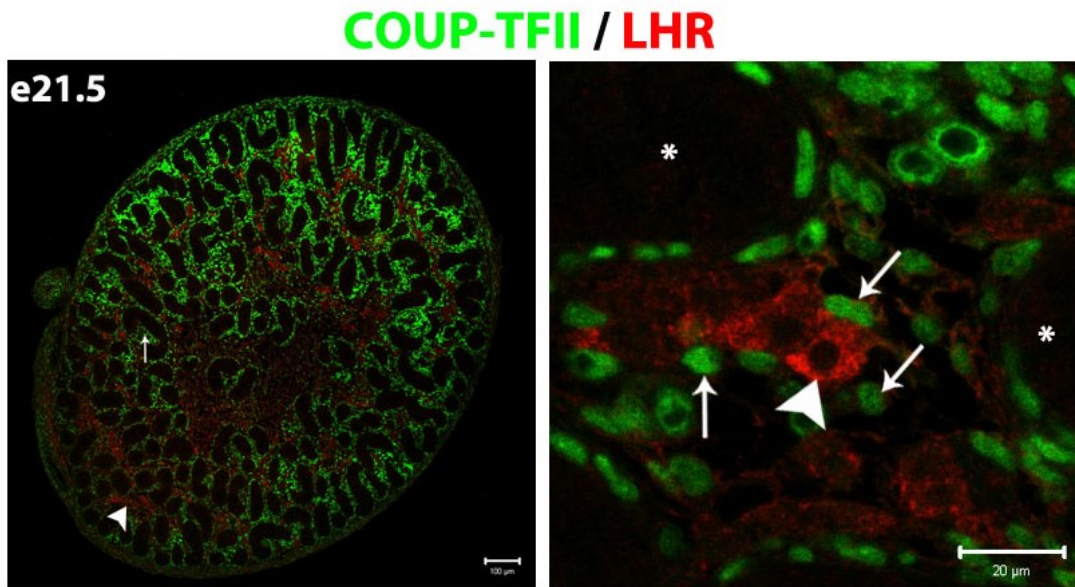


Figure 3.15 COUP-TFII⁺ cells do not express LHR in rat testes at e21.5. COUP-TFII⁺ adult Leydig progenitor cells (green) do not express the cytoplasmic marker LHR (red). Scale bars = 100 μ m (tiled image; left) and 20 μ m (right image).

3.3.10 MafB is not expressed in adult Leydig progenitor cells in the fetal testis

A recent study reported that 'MafB' is involved in the differentiation of fetal Leydig cells (DeFalco et al 2011), however little is known about its involvement in adult Leydig cell differentiation. To investigate whether MafB was potentially expressed in the adult Leydig progenitor cells, single immunofluorescence using DAB to localise Mafb (brown; arrowheads) was carried out on mouse testes at e18.5. Based on morphology, MafB was found to be solely expressed in the cytoplasm of fetal Leydig cells (arrowheads; Figure 3.16), therefore co-localisation studies of Mafb with interstitial COUP-TFII⁺ adult Leydig progenitor cells, was not further investigated.

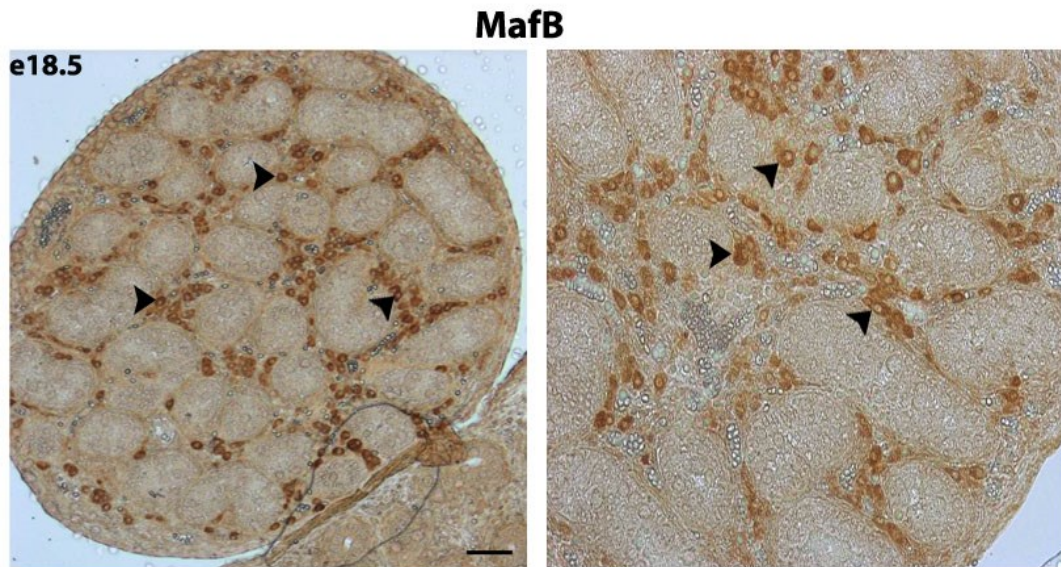


Figure 3.16 Expression of MafB in fetal Leydig cells. MafB (brown) staining is restricted to the cytoplasm of fetal Leydig cells (arrowheads) in the mouse testis at e18.5. Scale bar = 100 μ m (tiled image; left) with a higher magnification image shown on the right. Images are representative of at least 3 control animals.

3.3.11 Volume per testis of adult Leydig cells and their progenitors

As COUP-TFII is expressed in adult Leydig progenitor cells, adult Leydig cells and some peritubular myoid (PTM) cells, the only method of specifically investigating the volume per testis of COUP-TFII⁺ adult Leydig progenitor cells was to use triple immunohistochemistry to distinguish between cell types followed by stereological analysis. COUP-TFII⁺ interstitial cells that did not express SMA (i.e. PTM cells) or 3 β -HSD (i.e. fetal/adult Leydig cells), were quantified using established stereological techniques (Auharek et al 2010, van den Driesche et al 2012) (Section 2.11). The software for stereological analysis in these studies required colorimetric immunohistochemistry and not fluorescence. Thus, sections underwent triple colorimetric immunohistochemistry using 3 β -HSD (blue; Leydig cells) and SMA (red; seminiferous tubule lining) and COUP-TFII (brown; adult Leydig progenitors). This was performed on wild-type mice throughout development e.g. just after birth (Pnd 2), pre-puberty (Pnd 12, 20) and during adulthood (Pnd 50 and 140). As in the previous immunofluorescence images above, the representative triple

colorimetric images revealed an abundance of COUP-TFII⁺ (brown) interstitial cells after birth (Pnd 2) with fetal Leydig cells (blue) negative for COUP-TFII expression (Figure 3.17). Prior to puberty, (Pnd 12) COUP-TFII⁺ (brown; arrows) interstitial cells were present along with differentiating adult Leydig cells expressing cytoplasmic 3 β -HSD (blue) and nuclear COUP-TFII (arrowheads). Both adult Leydig progenitor cells (COUP-TFII⁺) and adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) were also present at adulthood (pnd 90) (Figure 3.17).

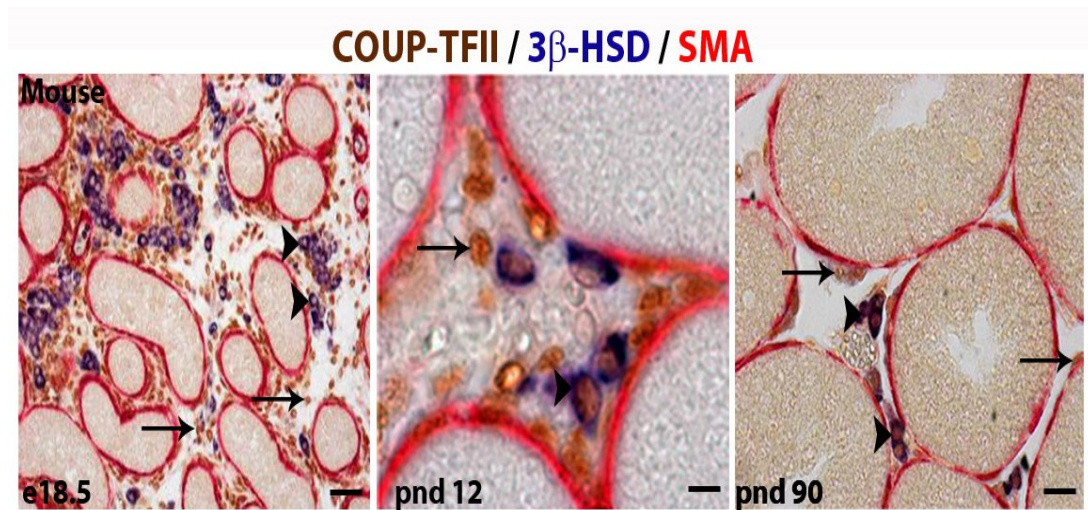


Figure 3.17 COUP-TFII expression in mouse testis across development. Triple immunostaining for adult Leydig progenitor cells (COUP-TFII; brown), Leydig cells (3 β -HSD; blue) and peritubular myoid cells (SMA; red). At e18.5, COUP-TFII⁺ cells are abundant in the interstitium, (arrow). Fetal Leydig cells are negative for COUP-TFII staining. Pre-puberty (Pnd 12), differentiating adult Leydig cells (arrowhead) exhibit nuclear staining for COUP-TFII. Interstitial COUP-TFII⁺ cells are also present (arrow). During adulthood (Pnd 90), both adult Leydig progenitor cells (COUP-TFII⁺) and adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) were also present. e18.5, pnd90 (x 20 magnification; scale bars = 50 μ m), pnd 12 (x 40 magnification; scale bar = 20 μ m). Images are representative of at least 3 control animals per age.

To determine the volume of adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) and adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) per testis, stereological analysis was performed on testis sections in mice after birth (Pnd 2), pre-puberty (Pnd 12, 20) and into adulthood (Pnd 50 and 140). Numbers of adult Leydig progenitor cells increased steadily across development, as did adult

Leydig cells (Figure 3.18). It has been well established that adult Leydig cells (3β -HSD⁺) increase in number following their differentiation at puberty, which these results firstly confirm and secondly add a new dimension to the current understanding, as the developing adult Leydig cells also expressed COUP-TFII⁺ in their nuclei (Figure 3.17, 3.18). The adult Leydig progenitor cells were also quantified at e15.5 and e17.5 in the rat, which demonstrated a significant increase in cell number during fetal development (Figure 3.19).

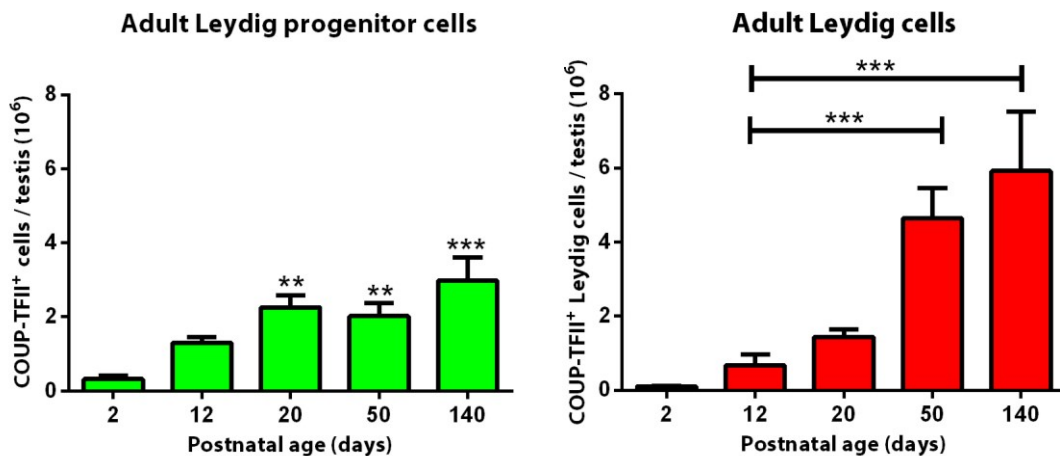


Figure 3.18 Increase in adult Leydig progenitor and adult Leydig cell numbers across development in the mouse. Adult Leydig progenitor cells (COUP-TFII⁺/ 3β -HSD^{neg}) and adult Leydig cells (COUP-TFII⁺/ 3β -HSD⁺) were quantified throughout development in mice; after birth (pnd 2), pre-puberty (pnd 12, 20) and adulthood (pnd 50, and 140). Values are means \pm SEM for n=7-10 wild-type mice at each age. Data were analysed by one-way ANOVA followed by Bonferroni post-hoc test; **p<0.01, ***p<0.001 in comparison with pnd 2 and pnd 12 for progenitor Leydig cells and adult Leydig cells, respectively.

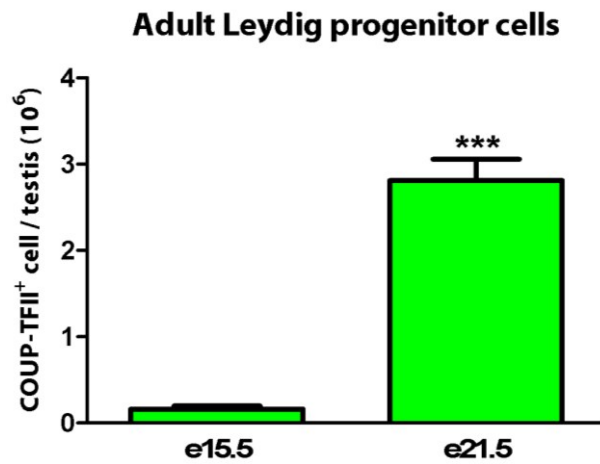


Figure 3.19 Increase in COUP-TFII⁺ adult Leydig progenitor cells during fetal development in the rat. Adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) were quantified during fetal rat development. Values are means \pm SEM for e15.5 (n=4) and e21.5 (n=7). Data were analysed using an unpaired t-test. ***p<0.001 in comparison to e15.5 value.

3.4 Discussion

The principal aim of this chapter was to establish whether COUP-TFII marks the adult Leydig progenitor cell population and whether these cells were present in the fetal testis. The current findings demonstrate adult Leydig cells differentiate from a population of COUP-TFII⁺ interstitial cells evident from 1) an EDS-induced adult Leydig cell ablation/regeneration rat model, 2) lineage tracing in a mouse model and 3) their differentiation into adult Leydig cells during normal puberty. Furthermore, the importance of COUP-TFII⁺ adult Leydig progenitor cells is highlighted by their abundant and conserved presence within the fetal testis.

Using ethane dimethane sulphonate (EDS) to ablate the population of mature adult Leydig cells is a valuable and well-established model to investigate adult Leydig cell development. The present findings support previous studies in showing complete Leydig cell ablation and subsequent regeneration two to three weeks post-EDS. This was evident from 1) the decrease in testis and prostate weight as a result of decreased testosterone, 2) immediate reduction followed by a gradual increase in mRNA transcripts for *3 β -HSD* and *Lhr* as a consequence of regenerating adult Leydig cells, 3) elevation of LH blood levels which corresponded with a reduction in blood testosterone levels between 2 and 3 weeks post-EDS, and 4) definitive *3 β -HSD* and *Insl3* immunoexpression at 2 weeks post EDS, when regenerating adult Leydig cells are first detectable (Morris & Jackson 1978, Kerr et al 1985, Molenaar et al 1985, Bartlett et al 1986, Edwards et al 1988, Sharpe et al 1986, 1990, Teerds et al 1999, Ariyaratne et al 2003, O'Shaughnessy et al 2008). These results demonstrate that unlike the mature adult Leydig cells which are sensitive to EDS and thus ablated COUP-TFII⁺ progenitor cells are resistant to such an insult. Furthermore, COUP-TFII⁺ interstitial cells acquire adult Leydig cell markers (*3 β -HSD* and *Insl3*) two weeks post-EDS, suggesting their differentiation into adult Leydig cells, which is further supported by the significant reduction in the number of adult Leydig progenitor cells five weeks post-EDS.

Previous studies which investigated the differentiation of adult Leydig cells during normal puberty or after EDS-induced ablation/regeneration, have suggested that adult Leydig cells arise from peritubular myoid cells/fibroblast cells (Haider et al 1995, Russell et al 1995, Landreh et al 2013), perivascular smooth muscle cells and pericytes (Davidoff et al 2004, 2009), macrophages (Clegg and Mcmillan 1965) or vascular endothelial cells (Russell et al 1995). Using COUP-TFII as a marker for adult Leydig progenitor cells during adult Leydig cell regeneration (post-EDS) and examining any co-expression of the aforementioned interstitial cells, demonstrated that COUP-TFII⁺ adult Leydig progenitor cells are a distinct population, located within the interstitium and in peritubular locations.

A proportion of COUP-TFII⁺ adult Leydig progenitor cells were located close to the vasculature, which may fit with studies suggesting perivascular smooth muscle cells and pericytes are a source for regenerating adult Leydig cells (Haider and Servos 1998, Davidoff et al 2004). However, this seems unlikely as COUP-TFII⁺ adult Leydig progenitor cells did not express the pericyte marker CD146, nor an endothelial cell marker CD31, one week post-EDS injection. Recent studies have suggested peritubular myoid cells may be a potential source for regenerating Leydig cells (Ge and Hardy 2007, Teerds et al 2007, Landreh et al 2013), however neither interstitial COUP-TFII⁺ adult Leydig progenitor cells or regenerating adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺/Insl3⁺) in the EDS model expressed the peritubular cell marker (SMA) in this study. However, the potential dedifferentiation of SMA⁺ PTM cells into adult Leydig cells, i.e. losing SMA expression but retaining COUP-TFII, is also a possibility.

This study cannot rule out the possibility that some adult Leydig cells might differentiate from cells other than COUP-TFII⁺ adult Leydig progenitor cells, as mentioned above. This is evident from the lineage tracing studies in which not all of the adult Leydig cells expressed GFP in adulthood. One explanation is that some adult Leydig progenitor cells might have suppressed the recombination of the loxP sites. Alternatively, as aP2Cre/GFP expression was localised to the

interstitium and peritubular layer, but not perivascular cells of the fetal testis, this may suggest that some adult Leydig cells could in fact, have differentiated from the perivascular layer, which fits with previous studies (Davidoff et al 2004, 2009). Nonetheless, the precise source(s) for regenerating/differentiating adult Leydig cells remains unknown. All that can currently be agreed upon, is that Leydig cells arise from undifferentiated progenitor mesenchymal cells located “within the interstitium” (Habert et al 2001, Mendis-Handagama and Ariyaratne, 2001), which is consistent with interstitial COUP-TFII⁺ cells in this study. Furthermore, as the majority of both normal and regenerating adult Leydig cells expressed COUP-TFII, and adult Leydig cells were shown to arise from aP2Cre/GFP⁺ cells which co-localised with COUP-TFII in the fetal testis, this strongly suggests COUP-TFII marks a population of adult Leydig progenitor cells. However, it is unclear whether all COUP-TFII⁺ adult Leydig progenitor cells have the potential to differentiate into adult Leydig cells, or only a subset.

Although COUP-TFII⁺ adult Leydig progenitor cells did not co-express any other Leydig cell markers e.g. 3 β -HSD, Insl3, LHR, SF1, a proportion of the progenitor cells post-EDS did however express GATA4, a transcription factor involved in Leydig cell development in humans and rodents (Ketola et al 2000, 2002, Ge et al 2006, Thurisch et al 2009). The COUP-TFII⁺ interstitial cells expressing GATA4 were located both within the interstitium and bordering the seminiferous tubules. These cells (COUP-TFII⁺/GATA4⁺) differentiated into adult Leydig cells two-three weeks post-EDS, as evident by their expression of the cytoplasmic adult Leydig cell marker, 3 β -HSD. An earlier study isolated interstitial “Leydig stem cells” from neonatal rats, which amongst other factors, expressed GATA4 and were capable of differentiating into steroidogenic cells (Ge et al 2006). In conjunction with the present findings, this points to a role for GATA4 in adult Leydig progenitor/stem cell differentiation but also strengthens the hypothesis, due to the co-expression of GATA4, that COUP-TFII marks a population of adult Leydig progenitor cells. These results also highlight the accuracy of the EDS model in recapitulating normal adult Leydig cell development as the differentiation pattern of GATA4 as an early differentiation

marker of adult Leydig cells, was also evident during puberty, as shown from immunostaining at pnd 15 and pnd 25 in the rat.

Deletion of COUP-TFII from pre-pubertal male mice prevented adult Leydig cell differentiation (Qin et al 2008), which potentially suggests the importance of COUP-TFII in this process. The current findings are in agreement with the previous authors, as evident from COUP-TFII expression in regenerating Leydig cells post-EDS and during normal development throughout puberty and adulthood. Quantification of adult Leydig progenitor cells throughout postnatal life in the mouse revealed a significant increase in cell number from fetal life, and more specifically between puberty (Pnd 12) to adulthood (Pnd 140). This indicates that there may not be a limiting number of these cells but rather a large progenitor pool. The number of adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) increased 20-fold, which corresponds with earlier data (De Gendt et al 2005) demonstrating a 26-fold increase in adult Leydig cell number during the same time frame. The increase in COUP-TFII⁺ adult Leydig progenitor cells during puberty is also consistent with an earlier study, which quantified ‘mesenchymal, non-Leydig interstitial cells’ based on morphology (Mendis-Handagama and Ariyaratne, 2001).

The study by Ge et al 2006, as previously discussed, showed that interstitial “Leydig stem cells” could be isolated from neonatal rats, which suggests that the adult Leydig progenitor cells are present during fetal life. The results of the present studies clearly demonstrate that COUP-TFII⁺ adult Leydig progenitor cells are abundant in fetal testes and conserved across species e.g. in human, marmoset, rat and mouse. These cells are a distinct population from fetal Leydig cells as they do not express the markers SF1 (Val et al 2003), MafB (deFalco et al 2011) nor do they express LHR (Chen et al 2009), which is consistent with studies that investigated LHR expression on Leydig “stem” cells (Ariyaratne et al 2000, Ge et al 2006, Teerds et al 2007). Quantification of adult Leydig progenitor cells during fetal development (e15.5 and e21.5) in the rat demonstrated that the number of these cells increased ~17-fold from e15.5 to e21.5 in control rats. This increase is coincident with increasing testosterone

levels during fetal programming/development (Welsh et al 2008, van den Driesche et al 2012), which may potentially point to a role for androgens in the proliferation of these cells. This raised the question as to whether the COUP-TFII⁺ adult Leydig progenitors express the androgen receptor (AR) i.e. are androgen responsive and might therefore be affected by altered levels of androgen, which the following chapter will address.

Since Leydig cell number in adulthood is a key determinant of testosterone levels in blood, and their function is vital for normal spermatogenesis and overall male health, the differentiation of adult Leydig cells is of great importance. Given the fact that adult Leydig cells rarely proliferate (Teerds et al 1989), it seems logical to assume that adult Leydig cells must develop from a pool of interstitial progenitor cells, which the present studies have identified. This chapter has demonstrated that adult Leydig progenitor cells express COUP-TFII and are present in the fetal testis across species, where they later differentiate into adult Leydig cells at puberty. This has been shown during normal development and in an EDS-induced ablation/regeneration model to recapitulate normal development. Furthermore, adult Leydig cell lineage tracing demonstrated that aP2Cre/GFP⁺ cells co-expressed COUP-TFII⁺ in fetal life, and a proportion of these cells differentiated into adult Leydig cells. The number of COUP-TFII⁺ adult Leydig progenitor cells increased significantly between puberty and adulthood, in parallel with the number of differentiating adult Leydig cells. The next chapter will address if adult Leydig progenitor cells are responsive and/or affected by manipulating fetal androgens using various animal models in order to determine if this may explain how fetal events predetermine adult testosterone levels.

Chapter 4 Androgen Regulation of Adult Leydig Progenitor Cells

4.1 Introduction

The previous chapter demonstrated that a population of COUP-TFII⁺ interstitial cells, present in the fetal testis, differentiate into COUP-TFII⁺ adult Leydig cells at puberty, and are thus taken to be adult Leydig progenitor cells. The experiments in this chapter aimed to investigate whether adult Leydig progenitor cells, present during fetal life, were androgen targets and might therefore be androgen-mediated. Various attempts to manipulate androgen levels/action in pre and post-natal life were undertaken to investigate whether altering fetal androgen action affects the number/function of adult Leydig cells via effects on their progenitors.

Androgens are vital for normal testis descent, however, they do not appear to be necessary for normal testis formation and organisation, as evident from ARKO mice (O'Shaughnessy et al 2002, De Gendt et al 2005, Verhoeven et al 2010). As Sertoli cells do not express AR in fetal life, it is interesting that fetal androgen deficiency results in a reduction of final Sertoli cell number as shown in ARKO but not SCARKO mice (De Gendt et al 2004, Tan et al 2005, O'Shaughnessy et al 2012). This suggests that androgen effects on Sertoli cells must be indirect, potentially via an AR⁺ cell within the fetal testis. Deletion of AR in PTM cells does not affect Sertoli cell number (PTMARKO mouse; Welsh et al 2009), which points to a role for another AR⁺ cell present in the fetal testis, potentially via COUP-TFII⁺ adult Leydig progenitor cells. Therefore, the first aim of this chapter was to investigate if adult Leydig progenitor cell number and thus adult Leydig cell function were affected by altered fetal androgen signalling via ubiquitous ARKO and cell-specific ARKO mouse models.

Previous studies that investigated the ablation of AR signalling in Sertoli cells (SCARKO), reported a 40% reduction in adult Leydig cell number (DeGendt et al 2005), although a more recent study reported a comparable number of the latter cells between control and SCARKO (O'Shaughnessy et al 2012). AR

ablation in PTM cells (PTMARKO), resulted in altered adult Leydig cell function (Welsh et al 2009). Ubiquitous knockout of AR (ARKO) results in a reduction in adult Leydig cell number (DeGendt et al 2005, O'Shaughnessy et al 2012). Thus, use of the ubiquitous ARKO or cell-specific mouse models were used to determine whether the adult Leydig progenitor cells were androgen-modulated and if alterations to the number of the progenitor cells might explain the altered number and/or function in adult Leydig cells.

Primary hypogonadism in men (also called hypergonadotrophic hypogonadism) can occur as a result of testicular failure due to genetic disorders (e.g. Klinefelter syndrome) or adult Leydig cell dysfunction and is associated with low-normal testosterone and high levels of LH and FSH (Tajar et al 2010). Secondary hypogonadism (also called hypogonadotropic hypogonadism) can occur due to hypothalamic-pituitary failure (e.g. Kallmann's syndrome) and is associated with low levels of testosterone and normal-low levels of LH and FSH (Tajar et al 2010). The main difference between primary and secondary hypogonadism is an increase or decrease in LH levels respectively. ARKO male mice are a useful model for human AIS individuals with primary hypogonadism, which manifests as compensated adult Leydig cell failure. This is evident from increased LH and low-normal testosterone levels in ARKO males (Yeh et al 2002, Matsumoto et al 2003, Notini et al 2005, De Gendt et al 2005).

DBP-induced lowering of fetal testosterone in rats has been used to model human testicular dysgenesis syndrome (TDS) (Mahood et al 2005, 2006, Sharpe & Skakkebaek 2008, van den Driesche et al 2012). These studies illustrate the importance of normal fetal androgen action and suggest that fetal androgen deficiency can cause life-long abnormalities of the male reproductive tract (Barlow and Foster 2003). Using DBP to lower fetal androgen levels provides a useful model for investigating whether fetal androgens affect adult Leydig progenitor cells and their development into normal functioning adult Leydig cells.

The next aim of this chapter was to investigate whether postnatal androgens could regulate adult Leydig progenitor cell development. Flutamide, a potent AR antagonist, has previously been used to block androgen action prenatally (Imperato-McGinley et al 1992, McIntyre et al 2001, Welsh et al 2008) and postnatally (Auharek et al 2010, van den Driesche et al 2011) which disrupted testis function. Postnatal flutamide treatment of fetally DBP-exposed animals was also investigated to determine whether androgen action during the pre- or postnatal period was more important for adult Leydig progenitor cell development.

Fetal TP or DHT exposure has previously been reported to masculinise female offspring, which was evident at birth and in adulthood (Wolf et al 2002, 2004, Welsh et al 2008, 2010, Dean et al 2012). This suggests that the elevation in fetal androgen exposure was sufficient to masculinise females. However, as TP was administered via injection into the pregnant dam, it passes via the placenta to the developing fetus and thus introduces the confounding factor of increased aromatisation of TP into oestradiol. Therefore, administration of the more potent and non-aromatisable androgen, dihydrotestosterone, (DHT) was also investigated. These treatments were initially chosen to investigate whether an increase in fetal androgen level/action might potentially affect adult Leydig progenitor cell development.

However, as both the TP and DHT models may not effectively increase intratesticular testosterone levels by much, a third approach to potentially raise endogenous intratesticular testosterone was utilised. Inducible nitric oxide synthase (iNOS) is expressed constitutively in adult Leydig cells (and other testicular cells) and suppresses testosterone production (O'Bryan et al 2000, Lee and Cheng 2004) under normal physiological conditions in the testis (Ishikawa & Morris 2006). iNOS levels are increased in aging rats, which coincides with the suppression of testosterone production (Sokanovik et al 2013). A recent study reported that in adult iNOS^{-/-} mice, AGD was significantly increased in comparison with respective wild-type controls. Furthermore, adult iNOS^{-/-} mice showed a 60% increase in testis weight, along with a 16% increase

in the number of adult Leydig cells per testis, whereas body weight was unchanged (Auharek et al 2011). During puberty in iNOS^{-/-} mice, the number of adult Leydig cells was significantly increased by 60% and 40% at pnd 10 and 15 respectively. Coinciding with these time points examined, AGD was significantly increased in iNOS^{-/-} mice. These results suggest that there was an effective increase in fetal androgen exposure, in comparison to wild-type controls (Auharek et al 2012). Thus, the iNOS^{-/-} mouse was chosen as a potentially effective model to increase endogenous fetal intratesticular testosterone levels and thus investigate potential effects on adult Leydig progenitor cell development.

This chapter aimed to answer three fundamental questions. Firstly are adult Leydig progenitor cells AR-modulated, especially in fetal life. Secondly, does altering fetal and/or postnatal androgen levels affect adult Leydig progenitor cell development and thus potentially adult Leydig cell function. Thirdly, is it possible that in a potential 'gain of adult Leydig cell function' model, the number of adult Leydig progenitor cells are increased as a consequence of increased fetal androgen exposure.

4.2 Materials and Methods

4.2.1 Generation of complete or cell-selective AR knockout (ARKO) mice

Ubiquitous and cell-specific androgen receptor knockout models were generated as described in Chapter 2. Groups of 5-11 wild-type, ARKO, SCARKO and PTMARKO mouse models were analysed at pnd 2, 12, 20, 50 or 140 for experiments described in this chapter.

4.2.2 Human fetal testis tissue

Second trimester (14-18 weeks) human fetal testes were obtained following termination of pregnancy. Women gave consent in accordance with UK national guidelines (Polkinghorne 1989), and ethical approval was obtained from the Local Research Ethics Committee, as described previously (Mitchell et al 2013).

4.2.3 Tissue collection

To recover fetal samples (e21.5) for investigations in this thesis, control and treated pregnant dams were killed by inhalation of CO₂ and subsequent cervical dislocation. Postnatal animals were sacrificed in the same manner, and dissected tissue was subsequently fixed in Bouins, as previously described in chapter 2.

4.2.4 Di-*n*-butyl phthalate treatment

To investigate whether reduced fetal testosterone levels affects the development of adult Leydig progenitor cells, time-mated pregnant rats were administered di-*n* butyl phthalate (DBP; Sigma-Aldrich Co. Ltd., Poole, Dorset, UK) at 500mg/kg/day by oral gavage in 1 ml/kg corn oil from e13.5-e21.5. Testosterone production commences at e15.5 in rats (Warren et al 1973, Habert and Picon 1984), therefore the treatments were initiated just prior to this time point. A dose of 500mg/kg/day was chosen as this treatment regime reduces fetal intratesticular testosterone by 40-80% during the treatment period (van den Driesche et al 2012). Groups of 6-8 vehicle- and DBP-exposed rats were

killed at e21.5 or at postnatal (Pnd) days 6, 10, 15, 25 or 75 days (Chapter 2). Sections of control and DBP-exposed animals were then used for immunohistochemistry and stereology.

4.2.5 DBP and/or postnatal flutamide treatment

To investigate the modifying effects of androgen deprivation during the postnatal period prior to adult Leydig cell differentiation (~Pnd15), the AR antagonist 'flutamide' (Sigma-Aldrich) (DMSO; Sigma-Aldrich) was administered to nursing dams by oral gavage in 1ml/kg corn oil from Pnd1-Pnd15. In some animals, prenatal DBP treatment (as above) was combined with postnatal flutamide treatment. DBP disrupts fetal testosterone production (van den Driesche et al 2012), while flutamide blocks the action of testosterone and DHT by competitively binding to the AR in target tissues (Welsh et al 2008, 2010). Groups of 5-8 vehicle- and DBP and/or flutamide-exposed rats were killed in early puberty (Pnd25) when adult Leydig cells are differentiating, or in adulthood (Pnd90) and used for experiments described in this chapter.

4.2.6 Hormone analyses

Plasma LH and testosterone were measured using radioimmunoassays as previously published (van den Driesche et al 2012, Mitchell et al 2013) and were performed by Chris Mc Kinnell in our research group, to whom I am extremely grateful. All samples from each experiment were run in a single assay for each hormone and the within-assay coefficients of variation were <10%.

4.2.7 Testosterone propionate treatment

To investigate the modifying effects of increased androgen levels during fetal development, pregnant rats were administered testosterone propionate (TP) at 20 mg/kg/day maternal bodyweight from e14.5-e21.5 and fetuses were killed at either birth (e21.5) or adulthood (Pnd90). Previous studies have shown that such treatment is sufficient to masculinise the female offspring (Wolf et al 2002,

Welsh et al 2008). To investigate the effects of TP during the postnatal period, prior to adult Leydig cell differentiation (~Pnd15), TP was administered by subcutaneous injection at 20 mg/kg/day every third day from pnd 1-24 as described previously (Welsh et al 2010). Male offspring from control and treated groups were examined at e21.5, pnd 25 and 90.

4.2.8 Dihydrotestosterone treatment

A second model to investigate the modifying effects of increased fetal androgen levels on male offspring via dihydrotestosterone (DHT) treatment to pregnant dams was used. Pregnant rats were administered either 10mg/kg DHT or corn oil (controls) via subcutaneous injection daily during fetal development (e15.5-e21.5). A dose of 10mg/kg/day of DHT has previously been shown to masculinise females (Dean et al 2012), however the effect on adult Leydig cells or their progenitors was not investigated. Testis sections from control and DHT-exposed animals were analysed at e21.5 and pnd 75.

4.2.9 Inducible nitric oxide synthase (iNOS^{-/-}) knockout model

As exogenous TP/DHT treatment may not effectively increase intratesticular testosterone levels by much, a better model would be one in which endogenous ITT levels were raised. One potential model for this was the generation of an iNOS^{-/-} mouse, as described in Chapter 2. The iNOS^{-/-} mouse model was previously reported to exhibit significantly increased testis weight and AGD from puberty onwards (Auharek et al 2011, 2012), suggesting an increase in fetal androgen exposure. Thus, this model was used to investigate whether potentially increased fetal ITT levels might affect adult Leydig progenitor cell development.

4.2.10 Immunofluorescence

Triple immunofluorescence was carried out as described in chapters 2 and 3 on testis sections from human and rodents at varying ages, as described below.

Antibodies, their dilution and visualisation method employed, are listed in table 4.1. For co-localisation studies of different proteins in this chapter, normal chicken serum (NChS) was used as a blocking serum to prevent cross-reaction with mouse/rabbit/goat antibodies and the protocol was continued as detailed earlier (Chapter 2).

Table 4.1 Primary and secondary antibodies used for immunofluorescence

Primary	Species Raised	Dilution	Secondary	Visualisation
Ki67	Mouse	1:200	C α M	Tyr-Cy3 (Red)
AR	Rabbit	1:200	C α R	Tyr-Cy3 (Red)
COUP-TFII	Mouse	1:1,000	C α M	Fluorescein Green
3B-HSD	Goat	1:1,000	C α G	Tyr-Cy5 (Blue)

4.2.11 Triple colorimetric immunohistochemistry

In order to quantify both the adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) and fetal or adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺), colorimetric triple immunohistochemistry was carried out as described in chapters 2 and 3. Primary and secondary antibodies used are described in Table 4.2.

Table 4.2 Primary and secondary antibodies used for triple immunohistochemistry

Primary	Species raised	Dilution	Secondary	Visualisation
SMA	Mouse	1:3,000	R α M	Fast Red
COUP-TFII	Mouse	1:100	R α M	DAB
3B-HSD	Goat	1:100	R α G	Fast Blue

4.2.12 Quantification of adult Leydig progenitor and adult Leydig cell number

Quantification of adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) and adult Leydig cells (COUP-TFII⁺/3 β -HSD⁺) was carried out using stereological techniques, as described in chapters 2 and 3.

4.3 Results

4.3.1 AR expression in COUP-TFII⁺ adult Leydig progenitor cells

As androgens are essential for fetal masculinisation and COUP-TFII⁺ adult Leydig progenitor cells are increasing in number during this time (between e17.5-e21.5) (Chapter 3), the first experiment aimed to establish whether or not the COUP-TFII⁺ adult Leydig progenitor cells were in fact proliferating, using the proliferative marker, Ki67. This was carried out using triple immunofluorescence for COUP-TFII (green), Ki67 (red) and 3 β -HSD (blue). A proportion of COUP-TFII⁺ adult Leydig progenitor cells co-expressed Ki67, which shows that these cells are proliferating (Figure 4.1). Therefore, the next aim was to determine whether the adult Leydig progenitor cells were androgen targets i.e. express the androgen receptor (AR), and thus whether androgens might modulate their proliferation. Triple immunofluorescence using COUP-TFII (green; adult Leydig progenitor cells), AR (red; interstitial cells) and 3 β -HSD (blue; fetal Leydig cells) was carried out on sections from fetal rat (e21.5) and human (14 weeks) testis tissue. The tiled and higher power images (Figure 4.2), in rat and human fetal testis, showed that the majority of COUP-TFII⁺ (green) adult Leydig cell progenitors co-expressed AR (red), as depicted by the yellow nuclear co-staining (COUP-TFII⁺/AR⁺). A small proportion of fetal Leydig cells (blue) in the rat at e21.5 expressed AR, but none were found to express COUP-TFII.

COUP-TFII / Ki67 / 3B-HSD

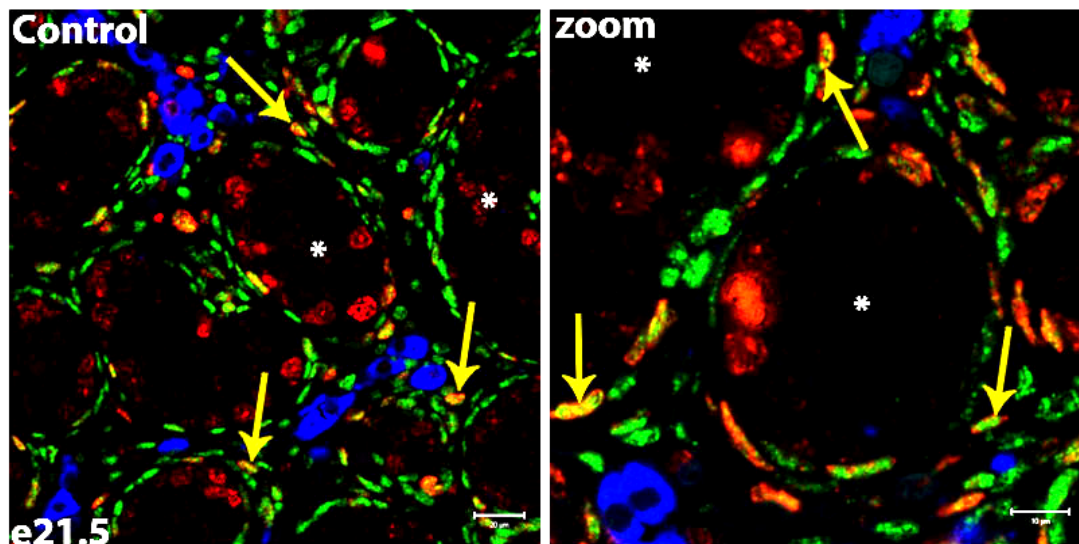


Figure 4.1 Proliferation of COUP-TFII adult Leydig progenitor cells during fetal life in the rat. A population of COUP-TFII⁺ (green) adult Leydig progenitor cells express Ki67 (red), as indicated by the yellow nuclear costaining (arrows) in both images at e21.5 in the rat. Ki67 is also evident in Sertoli cells within the seminiferous cords (cords denoted by an asterisk). Fetal Leydig cells (3 β -HSD⁺; blue) are negative for COUP-TFII and Ki67. Scale bars = 20 μ m.

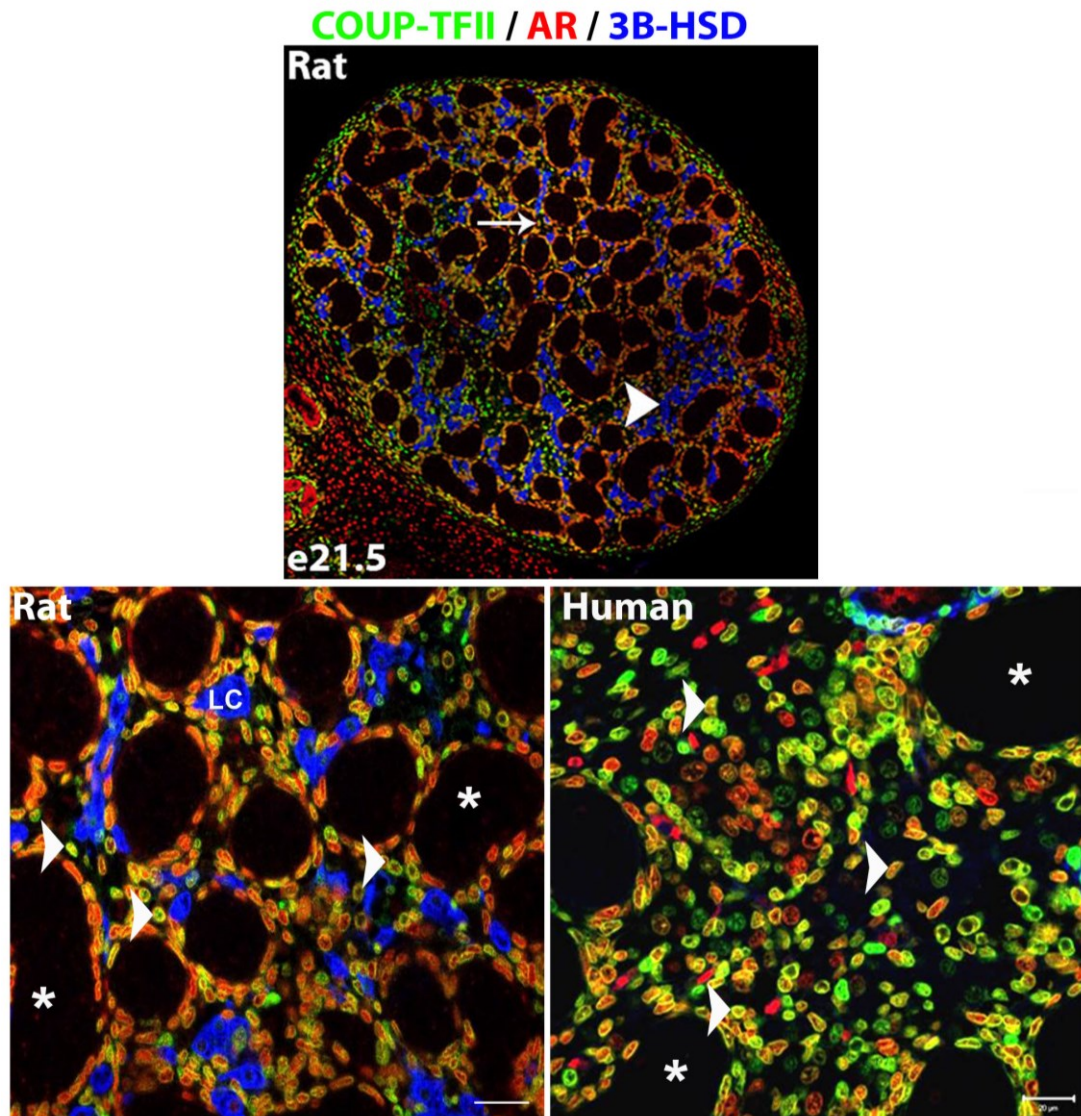


Figure 4.2 Co-localisation of COUP-TFII and AR in human and rat fetal testis. In rat and human fetal testis, the majority of COUP-TFII⁺ (green) Leydig progenitor cells co-express the androgen receptor (AR; red). COUP-TFII and AR co-localisation is evident by yellow nuclear staining (arrowheads). The majority of fetal Leydig cells, (3 β -HSD⁺; blue cytoplasmic staining: LC), are negative for COUP-TFII with a small proportion positive for AR. Note that fetal Leydig cells are not evident in the human sample shown (16 weeks gestation). Asterisks indicate seminiferous cords. Scale bars = 20 μ m.

4.3.2 Effect of AR knockout on adult Leydig progenitor cell development

As COUP-TFII⁺ adult Leydig progenitor cells appear to be androgen targets (COUP-TFII⁺/AR⁺), the next aim was to investigate whether knockout of the AR in COUP-TFII⁺ adult Leydig progenitor cells would affect their development.

Unfortunately, there is no way of specifically targeting the AR in the interstitial COUP-TFII⁺ adult Leydig progenitor cell population, given the fact that both these markers (COUP-TFII and AR) are also expressed in PTM cells. Therefore, the development of adult Leydig progenitor cells was investigated in complete AR knockout (ARKO) mice. Triple immunofluorescence for COUP-TFII (green), AR (red) and 3 β -HSD (blue) was undertaken on mouse testis sections from control (PGK-Cre only) and ARKO, around birth (e18.5) and during puberty (Pnd 12, 20). COUP-TFII⁺ (green) adult Leydig progenitor cells co-expressed AR (red) in control animals, as evident from yellow nuclear interstitial cell staining (white arrow) in the tiled and higher magnification images of fetal (Figure 4.3) and pubertal (Figures 4.4) mouse testes. Androgen receptor (red) expression was absent within the testes of ARKOs (Figures 4.3; 4.4). Fetal Leydig cells (3 β -HSD; blue) were negative for AR and COUP-TFII (Figure 4.3). During adult Leydig cell development, adult Leydig cells (3 β -HSD, blue) in wild-type mice expressed both COUP-TFII (green) and AR (red) staining (Figure 4.4).

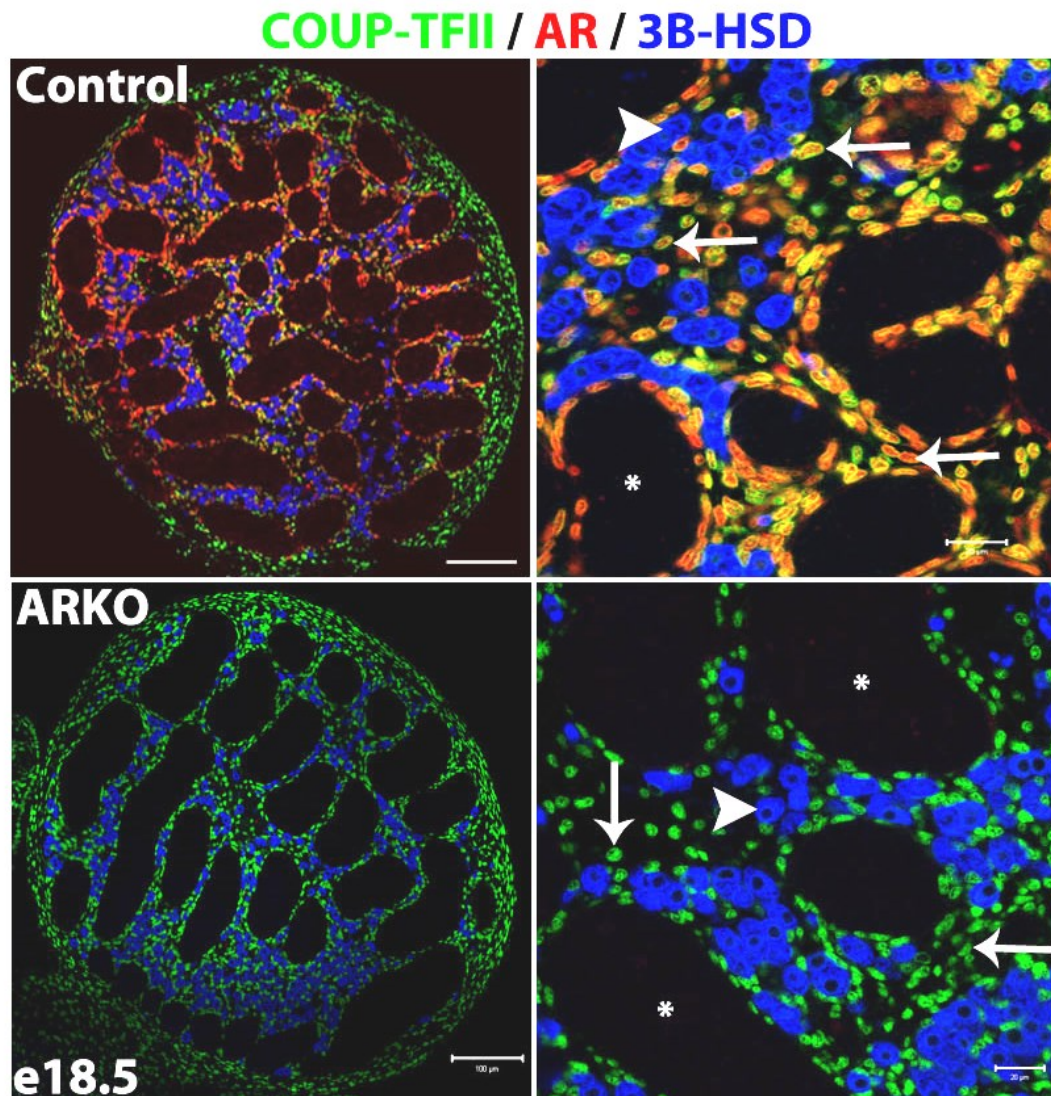


Figure 4.3 Ubiquitous knockout of AR in fetal mouse testis at e18.5. In the control (PGK-Cre only), fetal mouse testis (e18.5), COUP-TFII⁺ (green) adult Leydig progenitor cells co-express AR (red), as indicated by the yellow nuclear staining. AR staining in the ARKO mouse testis is absent while COUP-TFII⁺ adult Leydig progenitor cells (green; arrows) are present in the interstitium. Fetal Leydig cells (blue; arrowheads) are negative for AR and COUP-TFII. Scale bars in tiled image = 100 μ m, higher magnification = 20 μ m.

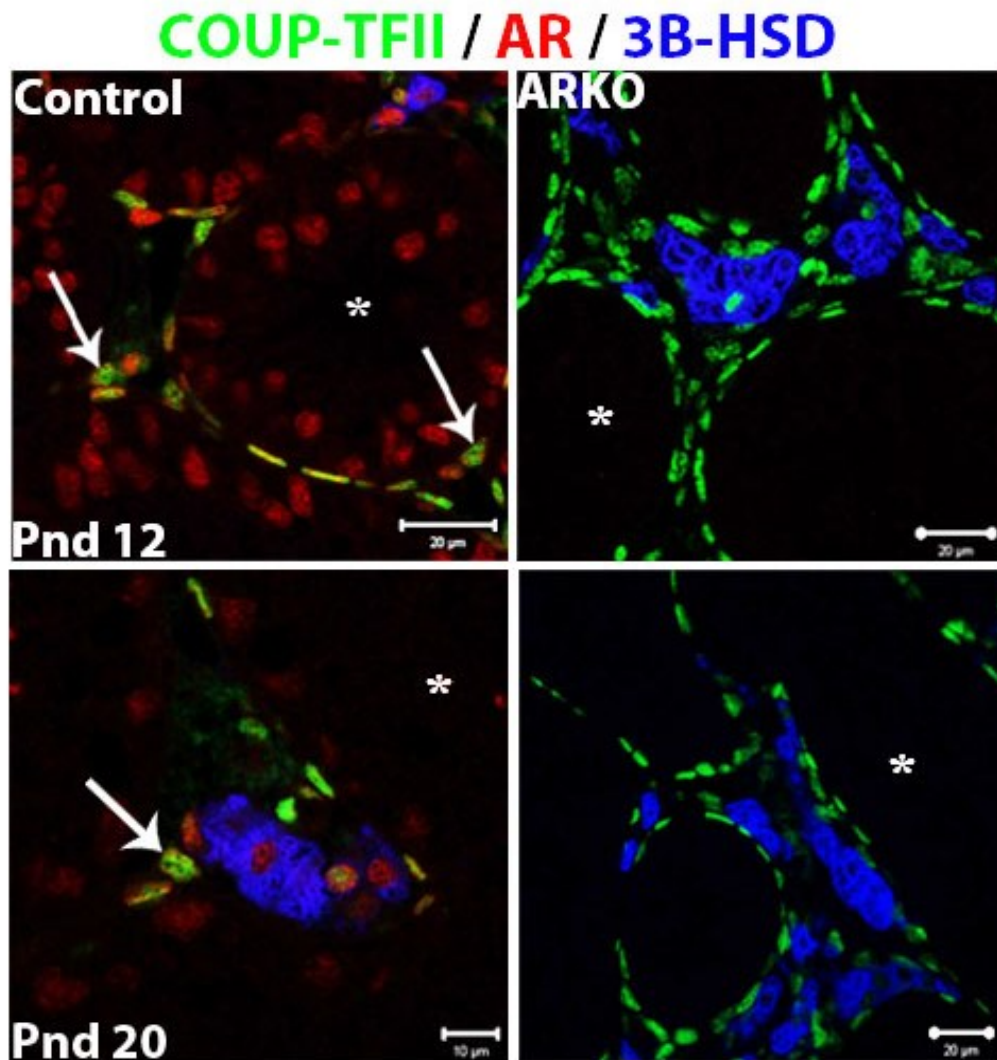


Figure 4.4 Expression of AR in COUP-TFII⁺ adult Leydig progenitor cells in control and ARKO mice during puberty. Adult Leydig progenitor cells (COUP-TFII⁺/AR⁺/3β-HSD^{neg}) are present in the interstitium (arrows; yellow colocalisation). Note absence of AR staining in ARKO samples. Control samples are PGK-Cre only. Adult Leydig cells are shown in blue. Scale bars = 20 µm, bottom left image = 10 µm.

4.3.3 Quantification of adult Leydig progenitor cells in ARKO mice

The next aim was to quantify the number of adult Leydig progenitor cells (COUP-TFII⁺/3β-HSD^{neg}) in ARKO mice, in comparison to their respective wild-type controls throughout development; samples were therefore investigated at pnd 2, 12, 20, 50 and 140. Knockout of AR specifically in Sertoli cells (SCARKO) has been reported to affect the development of adult Leydig cells (De Gendt et al 2005), while knockout of AR in PTM cells (PTMARKO) affects only their function

(Welsh et al 2009). Therefore, knockout of AR in these specific cell-types was also investigated for any effect on the development of the COUP-TFII⁺ adult Leydig progenitor population. As in chapter 3, the use of colorimetric immunohistochemistry was undertaken in order for stereological analysis of three separate proteins to be undertaken i.e. COUP-TFII (brown), 3 β -HSD (blue) and SMA (red) on testis sections from control and ubiquitous/cell specific ARKO mouse models throughout development. Representative images from wild-type control, SCARKO and ARKO mice after birth (Pnd 2), during puberty (Pnd 12) and in adulthood (Pnd 50) are shown below (Figure 4.5).

Staining for SMA (a marker for differentiated PTM cells) in ARKO males at pnd 2, was weakly expressed in comparison to controls (Figure 4.5; top). Also, from initial observations, it appeared that adult Leydig cell hyperplasia had occurred in the adult ARKO compared to controls; however the fact that testis weight was reduced by about 90% in ARKOs (Figure 4.6), and tubule diameter was also reduced, most likely explains this 'illusion'. Quantification of adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) after birth (pnd 2) demonstrated these cells were reduced in number following complete AR knockout (ARKO), while fetal Leydig cell number was unchanged (Figure 4.7). A reduction in adult Leydig progenitor cells (approximately 40%) was further evident through postnatal life into adulthood in ARKO males, in comparison to their respective wild-type controls (Figure 4.8). There was a parallel reduction in adult Leydig cell (COUP-TFII⁺/3 β -HSD⁺) numbers in ARKO males, in comparison to wild-type controls (Figure 4.8). In contrast, cell-selective knockout of AR, as in SCARKO and PTMARKO models, had no significant effect on numbers of adult Leydig or their progenitor cells during development (Figure 4.8 and 4.9).

COUP-TFII / 3 β -HSD / SMA

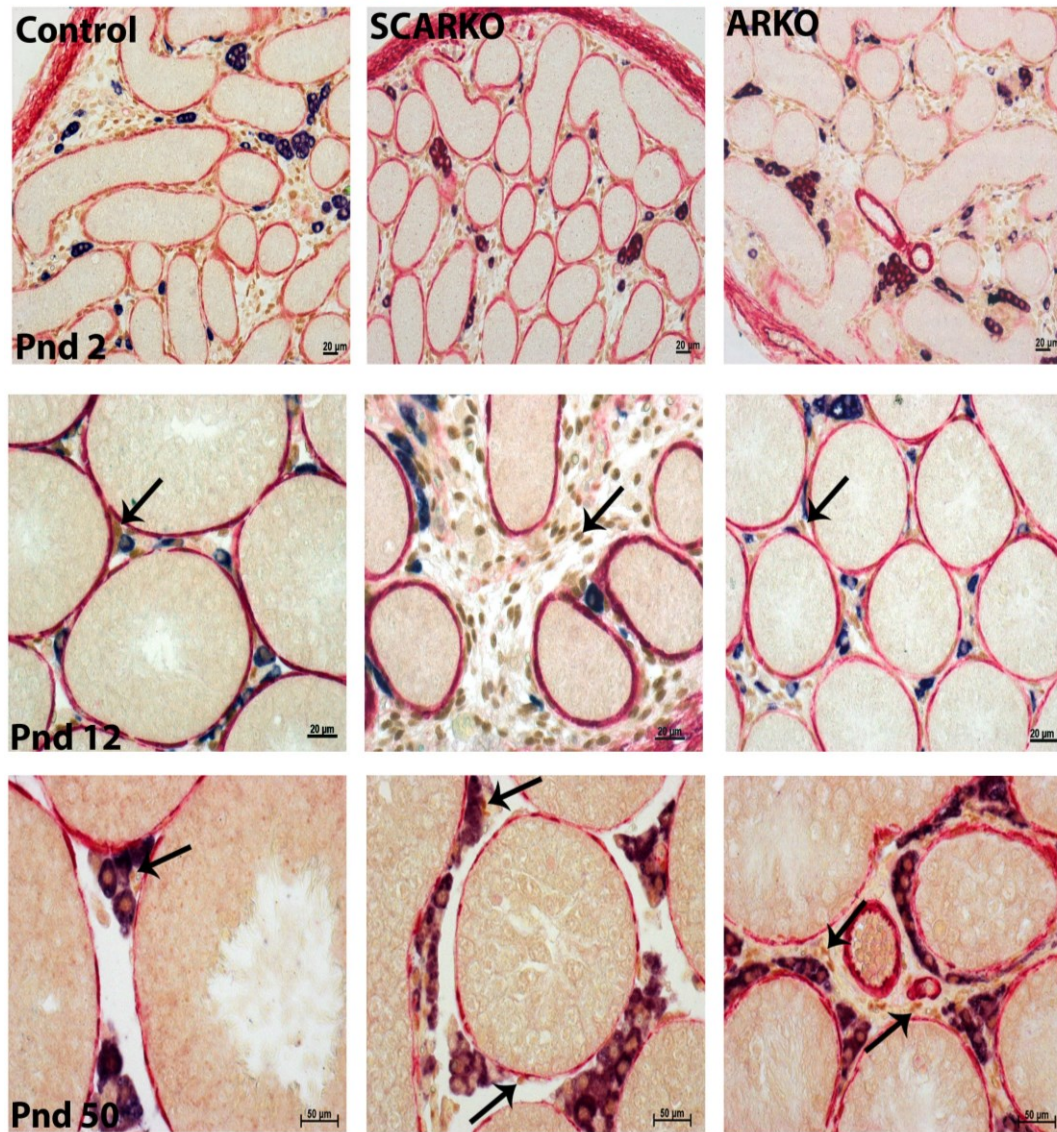


Figure 4.5 Gross testis morphology and occurrence of adult Leydig cells and their progenitors across development in control, SCARKO and ARKO mice. Adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}; arrows) are located in the interstitium throughout development. Leydig cells are shown in blue (3 β -HSD⁺). Control samples are PGK-Cre only. Note weak SMA staining in PTM cells in ARKO mice at pnd 2. Scale bars = 20 μ m (Pnd 2, 12) and 50 μ m (Pnd 50).

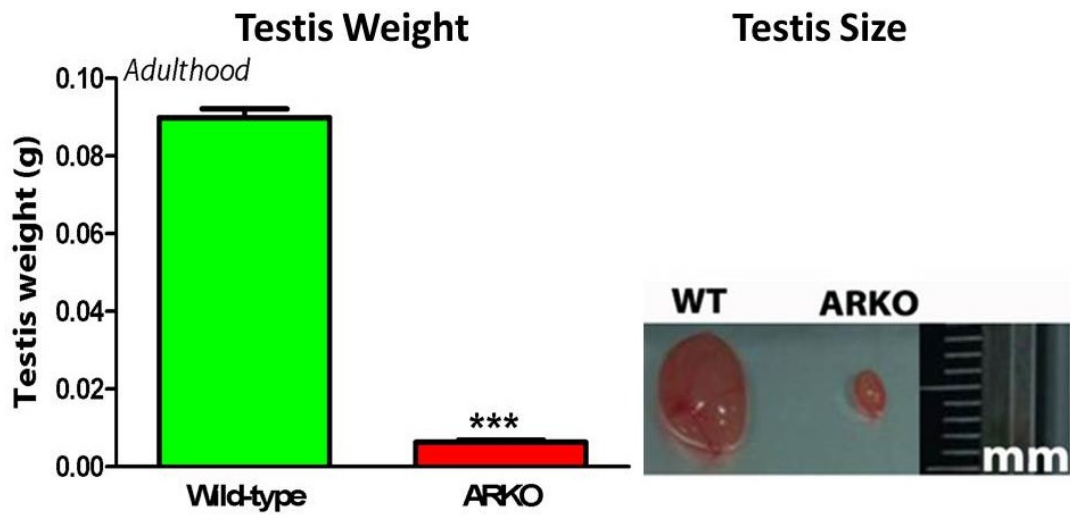


Figure 4.6 Effect of complete ARKO on adult mouse testis weight and size in comparison to wild-type controls. Means \pm SEM for $n=5-6$. Data were analysed using an unpaired t-test. *** $p<0.001$ in comparison to wild-type controls.

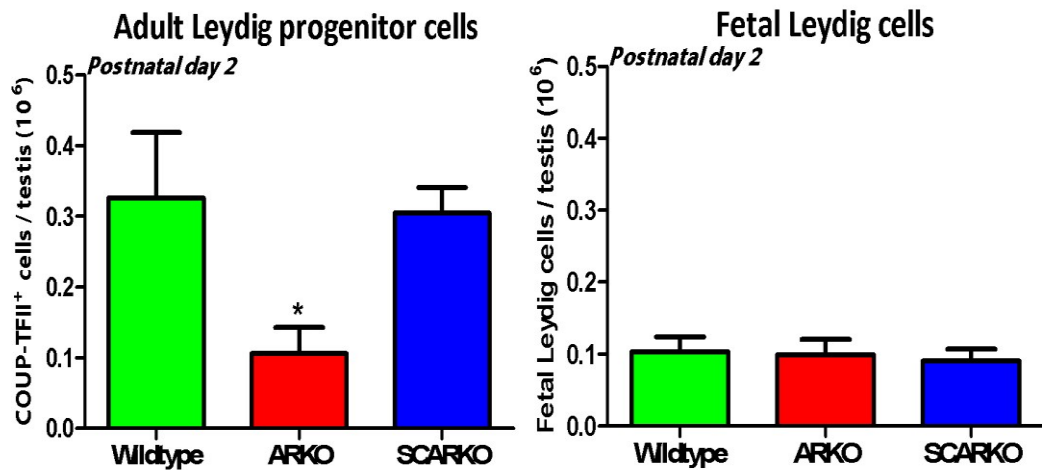


Figure 4.7 Adult Leydig progenitor cell number is decreased in ARKO mice at pnd 2. Adult Leydig progenitor cell (COUP-TFII⁺/3 β -HSD^{neg}) number was reduced in ARKO males, in comparison to wildtype controls, unlike in the SCARKO model in which cell number was unchanged. Fetal Leydig cell number was comparable across all knockout models. Means \pm SEM for $n=5$. Data were analysed using an unpaired t-test; * $p<0.05$, in comparison to wildtype controls.

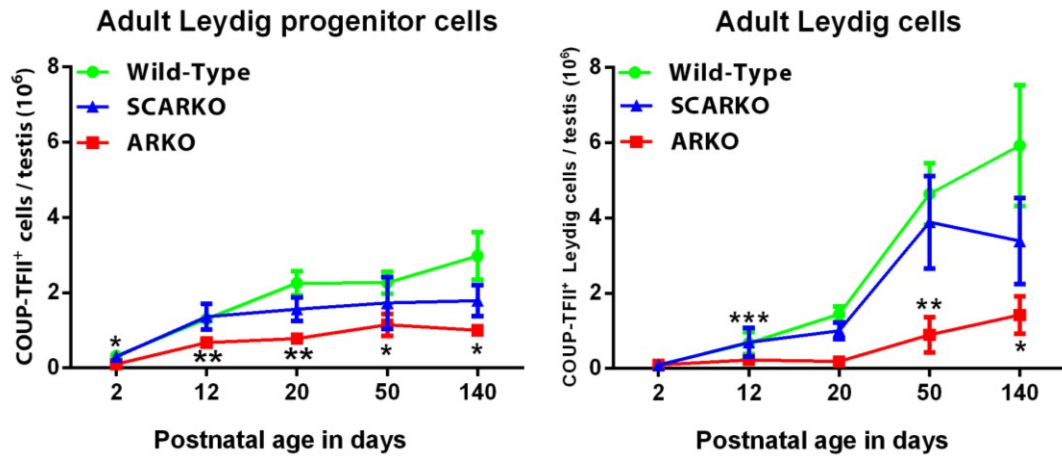


Figure 4.8 Change in COUP-TFII⁺ adult Leydig progenitor and adult Leydig cells across development in wild-type, SCARKO and ARKO mouse models. Adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) were reduced in number at all ages in ARKO males, in comparison to wild-type controls. A parallel reduction in adult Leydig cell numbers (COUP-TFII⁺/3 β -HSD⁺) was also evident in ARKOs, in comparison to controls. Means \pm SEM for n=7-10 mice, at each age. Data were analysed using an unpaired t-test; *p<0.05, **p<0.01, ***p<0.001, in comparison with respective wild-type controls.

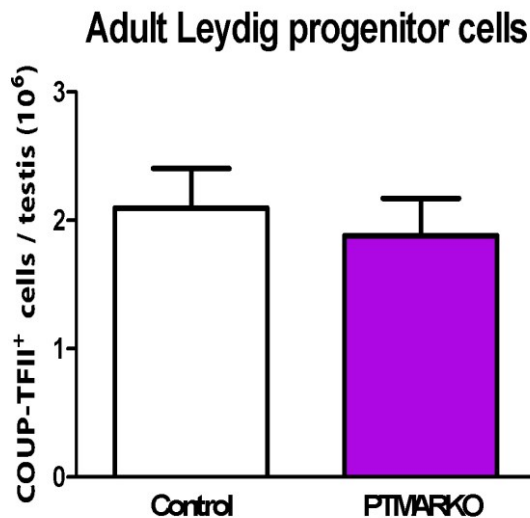


Figure 4.9 Adult Leydig progenitor cell number is unchanged in adult mouse testes in AR cell-selective knockout in PTM cells (PTMARKO). Adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) were quantified during adulthood (Pnd 50 and 140). Means \pm SEM for n=7-11 mice. Data were analysed using an unpaired t-test and found to be not significant.

4.3.4 Evidence for compensated adult Leydig cell failure in ARKO males

To determine adult Leydig cell function in ARKO males, plasma levels of LH and testosterone were measured in adulthood, and compared with wild-type controls. This provided evidence for ‘compensated Leydig cell failure’ in ARKO males, as evident from the significantly increased LH and grossly distorted blood LH:testosterone ratio, in comparison to wild-type controls (Figure 4.10), confirming previous findings (De Gendt et al 2005).

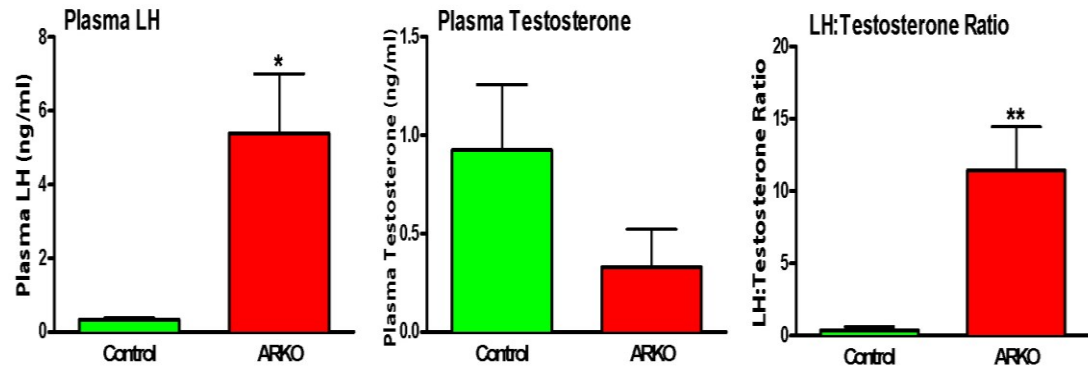


Figure 4.10 Plasma hormone levels in adulthood in control and ARKO mice. Plasma hormone levels, as a measure of adult Leydig cell function, are shown for LH (increased) and testosterone (decreased) in ARKO adult mice, in comparison to wild-type controls, resulting in an abnormal LH:testosterone ratio. Means \pm SEM for $n=5$. Data were analysed using an unpaired t-test; * $p<0.05$, ** $p<0.01$.

Given the significant reduction in number of adult Leydig cells and their progenitors throughout development in ARKO mice, plus the evidence for compensated adult Leydig cell failure in adulthood, it suggests that fetal androgen action plays a key role in adult Leydig progenitor cell development and normal adult Leydig cell function. However, the fact that AR is knocked out ubiquitously in this model plus the fact that all testes are cryptorchid, are potentially confounding factors. Therefore, the next aim was to investigate a model in which fetal intratesticular testosterone was selectively reduced, in order to investigate whether this would affect the development of adult Leydig and/or their progenitor cell population, as it appeared to do in the ARKO model.

4.3.5 DBP-Induced reduction of fetal intratesticular testosterone production

Exposure to DBP (500mg/kg/day) during fetal life (e13.5-e21.5) has been shown to lower fetal intratesticular testosterone by ~70% (van den Driesche et al 2012). Therefore, DBP treatment was used in this study to investigate the effect that lowered intratesticular testosterone might have on the development of adult Leydig cells and/or their progenitor cells across development. The timing of treatment and postnatal ages of analyses, are indicated in the schematic below (Figure 4.11).

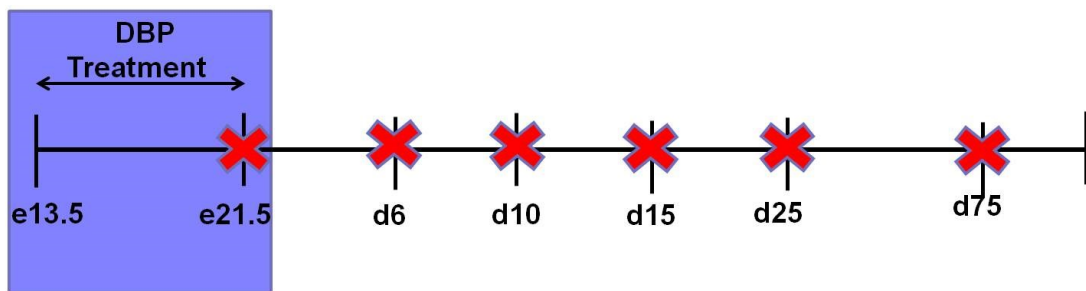


Figure 4.11 Schematic indicating the timing of fetal DBP exposure in rats. The time-points at which adult Leydig cells and their progenitors were investigated are marked by red crosses.

DBP-exposed males had lower intratesticular testosterone (ITT) levels at e21.5, (Figure 4.12; top left). As before, colorimetric immunohistochemistry was carried out prior to stereological analysis on testis sections throughout development at e21.5, pnd 6, 10, 25, 75 and 90. Quantification of adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) indicated a ~40% reduction at e21.5 in DBP-exposed animals in comparison to their respective (vehicle) controls (Figure 4.12; top right), a deficit which persisted throughout postnatal development into adulthood (Figure 4.12; middle left). DBP-exposed males showed a significant reduction in the number of developing adult Leydig cells at pnd 25 but this shortfall in adult Leydig cells was not evident in adulthood (Pnd 90) (Figure 4.12; middle right). However, normal adult Leydig cell number does not necessarily reflect normal Leydig cell function (Welsh et al 2009), therefore

to investigate adult Leydig cell function in DBP-exposed males, plasma LH and testosterone blood levels were measured in adulthood and compared with controls. Despite the presence of normal adult Leydig cell numbers, these findings suggest there is evidence for compensated adult Leydig cell failure, as evident from significantly increased LH and decreased testosterone levels, resulting in a distorted LH:testosterone ratio (Figure 4.12; bottom).

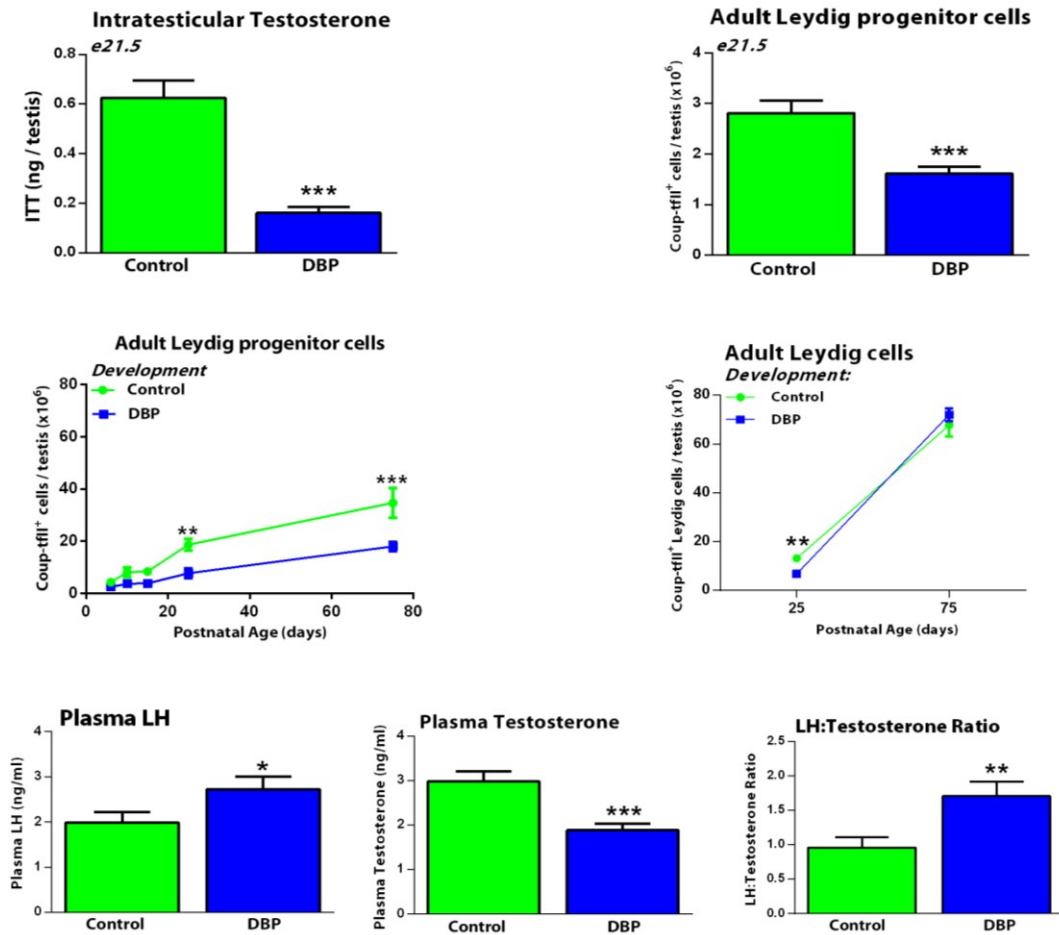


Figure 4.12 DBP-induced lowering of fetal ITT reduces the number of adult Leydig progenitor cells and alters adult Leydig cell function. At the end of fetal life in rats (e21.5), ITT was significantly reduced, as was the number of adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) throughout postnatal development. Adult Leydig cell (COUP-TFII⁺/3 β -HSD⁺) number was unchanged, except at Pnd 25. During adulthood (Pnd 90), plasma levels of LH were significantly increased whereas testosterone levels were reduced, resulting in a distorted LH: testosterone ratio. Means \pm SEM for n=6-8 (stereological analyses) and n=29-32 (hormone analyses). The results shown in the bar charts in the first and third rows were analysed by unpaired t-tests. Initially, unpaired t-tests were also used to compare cell numbers between DBP-treated and vehicle controls at different ages (graphs in second row; results not shown). However, to avoid multiple pairwise tests, the analyses were repeated using two-way ANOVAs. For adult Leydig progenitor cells (left graph), this showed significant differences between treatments (p<0.0001) and among ages (p<0.0001) and the interaction between ages and treatments was also significant (p=0.0115). Bonferroni post-hoc tests demonstrated that differences in cell numbers between DBP-treated and vehicle controls were significant at pnd 25 and 75, as shown in the figure. For adult Leydig cells (right graph), the overall difference between treatments was not significant (p=0.6729) but the two ages differed significantly (p<0.0001) and the interaction between ages and treatments was also significant (p=0.0447). Bonferroni post-hoc tests showed no significant difference between treatments at either age. *p<0.05, **p<0.01, ***p<0.001.

4.3.6 Effect of postnatal androgen action on adult Leydig progenitor cell development

The number of adult Leydig progenitor cells increased during postnatal development, albeit to a far lesser degree in the absence of normal androgen level/action (Figure 4.8 and 4.12). This posed two questions, firstly, whether blockade of postnatal androgen action prior to puberty (pnd 1-15) via an AR antagonist flutamide, would affect adult Leydig cells and/or their progenitor cell development, as outlined in the schematic (Flutamide; Figure 4.13). Secondly, whether postnatal administration of flutamide to DBP-fetal-exposed rats would affect adult Leydig progenitor cell development compared to fetal DBP exposure alone, as outlined in the schematic (DBP+Flutamide; Figure 4.14).

To perform stereological quantification in the various outlined treatment groups at Pnd25 and 90, triple colorimetric immunohistochemistry for COUP-TFII, 3β -HSD and SMA was initially carried out, as before. Stereological quantification revealed that postnatal flutamide treatment only, significantly reduced the number of adult Leydig progenitor cells at pnd 25 (Figure 4.15; top left). There was a similar reduction in adulthood, but this just failed to reach statistical significance (Figure 4.15; top right). Adult Leydig cell number (Figure 4.15; top right) and function (Figure 4.15; middle and bottom) were unaffected by postnatal flutamide treatment.

In contrast, postnatal administration of flutamide to DBP-exposed animals, resulted in comparable changes to the effects of DBP treatment alone, as shown earlier (Figure 4.12). Postnatal administration of flutamide to DBP-exposed animals, significantly reduced the number of adult Leydig progenitor cells at pnd 25 (Figure 4.15; top left), although in adulthood a similar reduction did not reach statistical significance (Figure 4.15; top right). The DBP+Flutamide experimental group showed evidence for compensated adult Leydig cell failure i.e. significantly increased LH and reduced testosterone with a distorted LH:testosterone ratio (Figure 4.15; bottom). These results suggest that fetal androgen action (e13.5-e21.5) is more important than postnatal androgen

action (Pnd1-15), in affecting adult Leydig progenitor cell development and in influencing adult Leydig cell function.

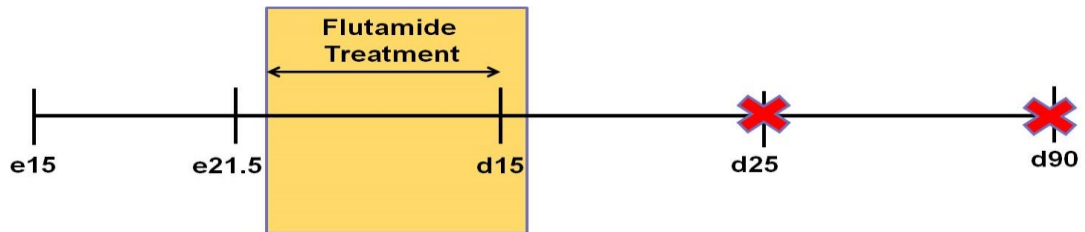


Figure 4.13 Schematic indicating the timing of postnatal flutamide treatment in rats. The time-points at which adult Leydig cells and their progenitors were investigated are marked by red crosses.

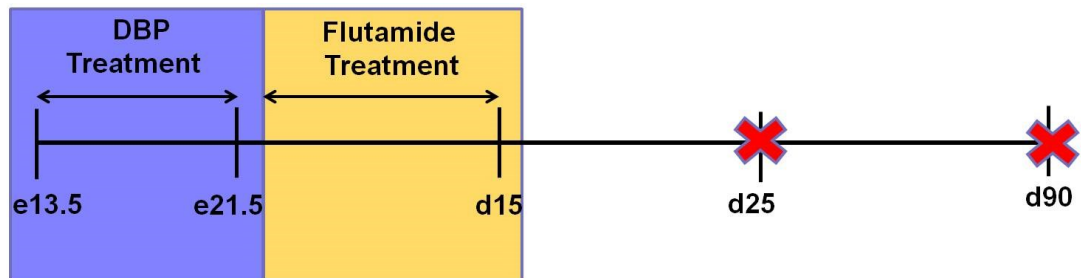


Figure 4.14 Schematic indicating the timing of postnatal flutamide treatment in DBP-exposed rats. The time-points at which adult Leydig cells and their progenitors were investigated are marked by red crosses.

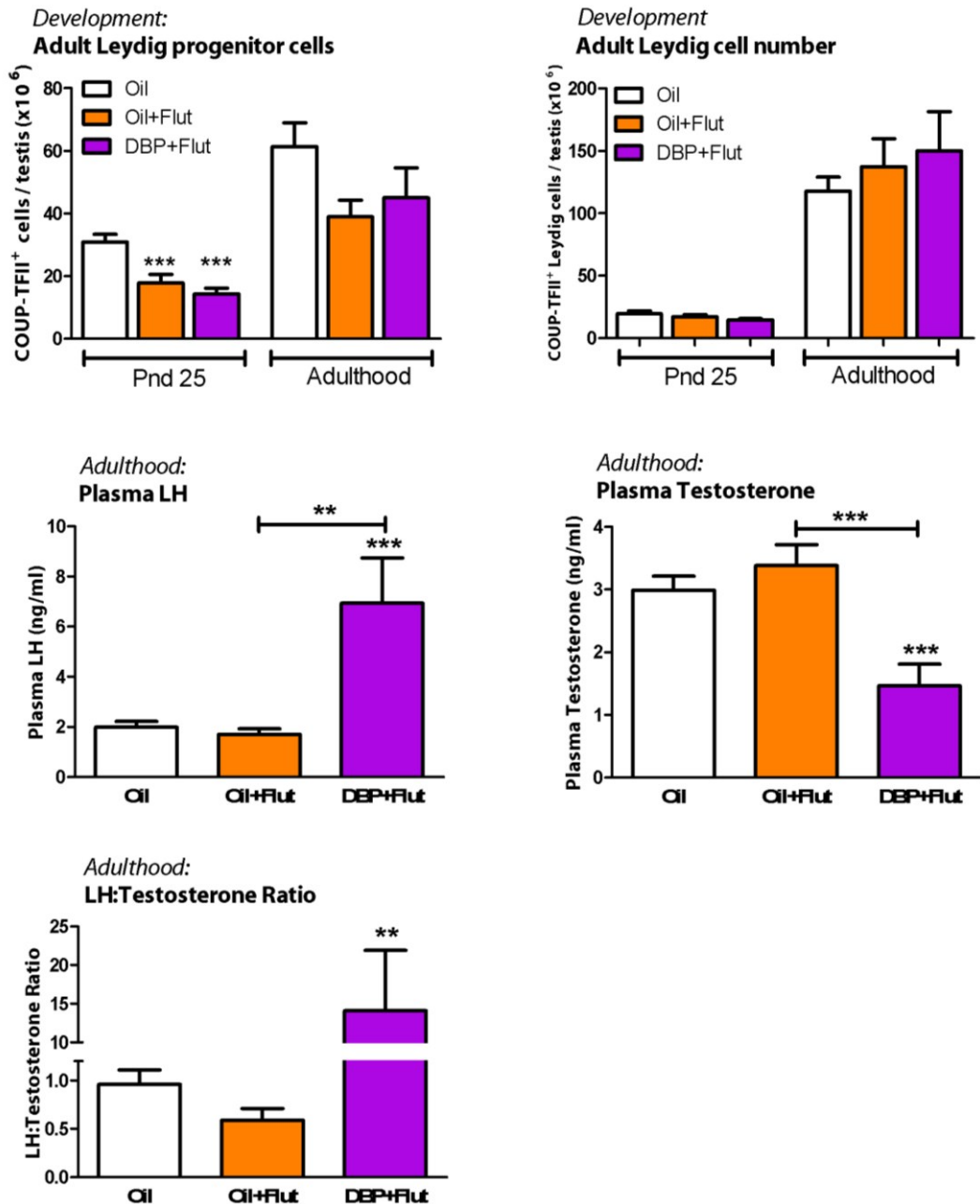


Figure 4.15 Effect of prenatal exposure to DBP and/or postnatal exposure to flutamide on the development of adult Leydig progenitor cells in relation to adult Leydig cell function in rats. Adult Leydig progenitor cells were reduced in both experimental groups (Oil+Flut and DBP+Flut) at pnd 25 but not in adulthood (Pnd 90). There was no change in the number of adult Leydig progenitor cells in adulthood (Pnd 90). For adult Leydig cells, there was no change in their number between experimental groups at pnd 25 or adulthood (Pnd 90), in comparison to respective control animals. Plasma LH was increased in DBP+Flut group, in comparison to both Oil and Oil+Flut group. Plasma testosterone was reduced in comparison to both Oil and Oil+Flut group. This resulted in a distorted LH:T ratio, but only in the DBP+Flut group, not in the Oil+Flut group. Means \pm SEM for $n=5-8$ (Pnd 25) or $n=5-11$ (adulthood) rats per group. Data were analysed using an unpaired t-test. ** $p<0.01$, *** $p<0.001$.

Effect of exogenous testosterone propionate exposure on adult Leydig progenitor cells

The previous results in this chapter have shown that lowering fetal androgen level/action results in a reduction in number of adult Leydig progenitor cells and somehow affects their functional competence when they differentiate into adult Leydig cells. Therefore, the next aim was to investigate whether increasing fetal androgen levels would have the opposite effect on adult Leydig progenitor cell development resulting in a potential "gain of adult Leydig cell function". Given that testosterone propionate (TP) exposure during fetal life has been shown to increase testosterone levels and masculinise female offspring (Wolf et al 2002, Welsh et al 2008) and the fact that normal testosterone production commences at e15.5 in the rat (Warren et al 1972), this study investigated the administration of 20mg/kg/day of TP during fetal life from e14.5-e21.5 (Figure 4.16). To investigate if postnatal administration of TP affected adult Leydig progenitor cell development, a second experimental group in which TP was administered postnatally (d1-25) was also investigated (Figure 4.18). The dose of TP used in this study can induce dystocia (Welsh et al 2008), so to prevent this, TP-exposed fetuses were caesarean-derived and cross-fostered to untreated/control dams, that had previously delivered within 6 hours. As before, testis sections underwent triple colorimetric immunohistochemistry for COUP-TFII, 3 β -HSD and SMA, prior to stereological analyses.

Fetal TP-exposure resulted in significantly increased numbers of adult Leydig progenitor cells, as evident in adulthood (Figure 4.17; bottom). However, this did not appear to stem from prenatal changes because, at birth, the number of adult Leydig progenitor cells was not increased, and if anything, was reduced (Figure 4.17; top). Postnatal TP administration did not affect the number of adult Leydig progenitor cells at pnd 25 or 90, although it did appear to significantly decrease the number of adult Leydig cells at pnd 25 only (Figure 4.19; top). Although these experiments aimed to increase intratesticular testosterone (ITT) levels via TP exposure, it was evident that other confounding factors might have occurred as a result of this treatment. For example,

testosterone can be aromatised into oestradiol, thus increasing levels of the latter, which could have resulting adverse effects on fetal growth. In fact, a significant reduction in testis weight was evident at birth (e21.5), which might indicate an oestrogenic effect (Bartholomeusz et al 1999) (Figure 4.17). Also, altered development of the hypothalamic-pituitary-gonadal (HPG) axis might have been affected due to TP administration, resulting in permanent changes to LH secretion and/or negative feedback control (Grober et al 1998, Micevych et al 2010). Bearing these factors in mind, it seemed apparent that the best way to investigate if increased ITT levels could increase adult Leydig progenitor cell numbers is via specifically elevating ITT and not altering peripheral levels greatly. Therefore, fetal administration of a non-aromatisable androgen, dihydrotestosterone (DHT) was investigated next.

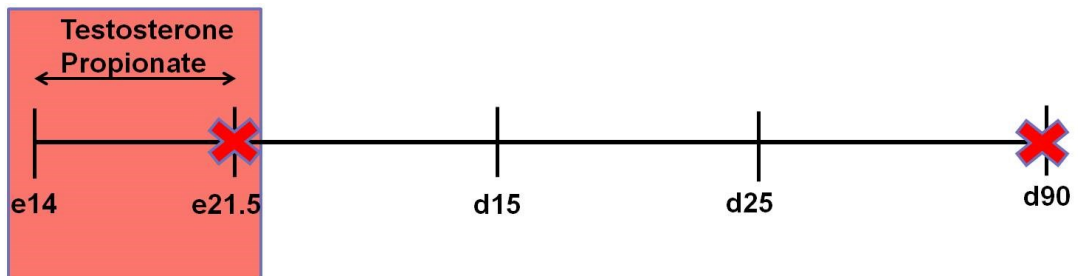


Figure 4.16 Schematic indicating the timing of fetal TP-exposure in rats. The time-points at which adult Leydig cells and their progenitors were investigated are marked by red crosses.

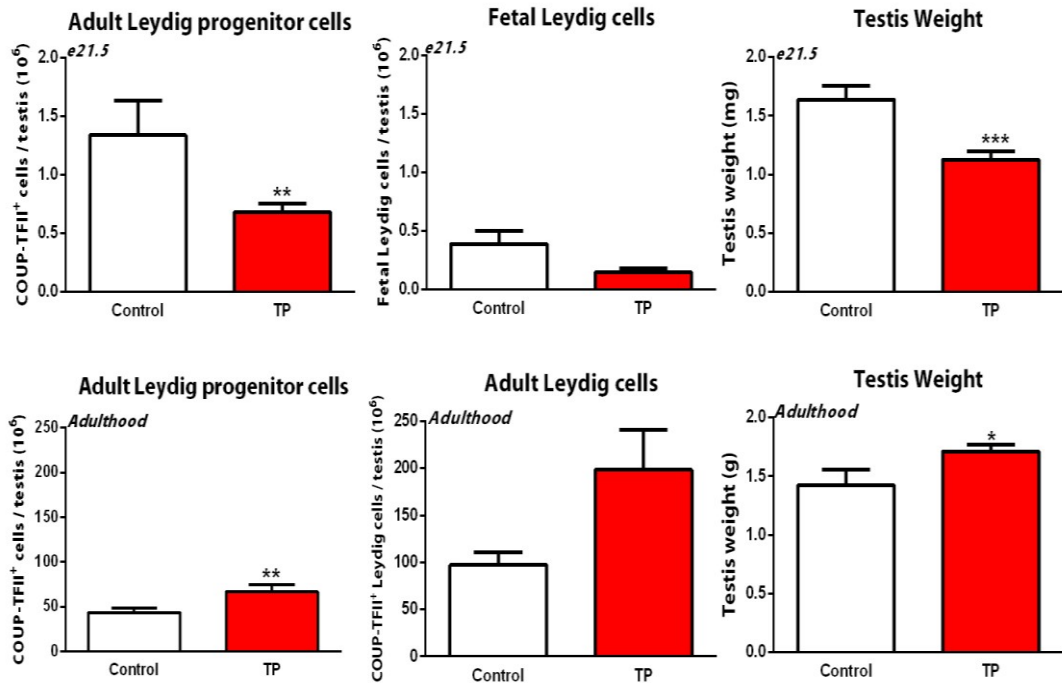


Figure 4.17 Effect of fetal TP exposure (e14.5-e21.5) on adult Leydig progenitor cell numbers at e21.5 and pnd 90 in the rat. Fetal TP exposure caused a reduction in testis weight at e21.5 and an apparent increase in adult Leydig progenitor cells and testis weight during adulthood. Means ± SEM for n=5. Data were analysed using an unpaired t-test; *p<0.05, **p<0.01, in comparison with respective control.

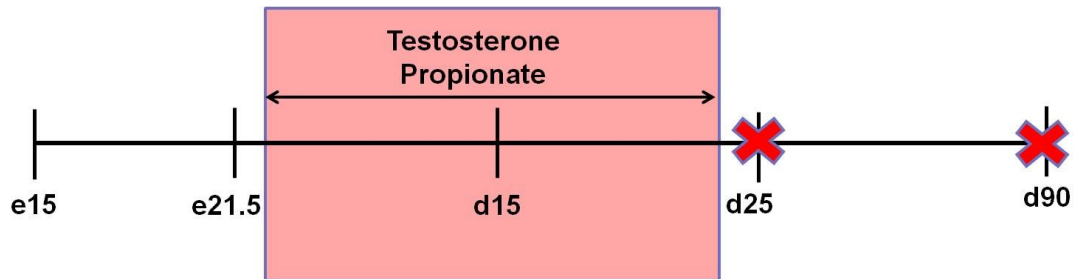


Figure 4.18 Schematic indicating the timing of postnatal TP administration in rats. The time-points at which adult Leydig cells and their progenitors were investigated are marked by red crosses.

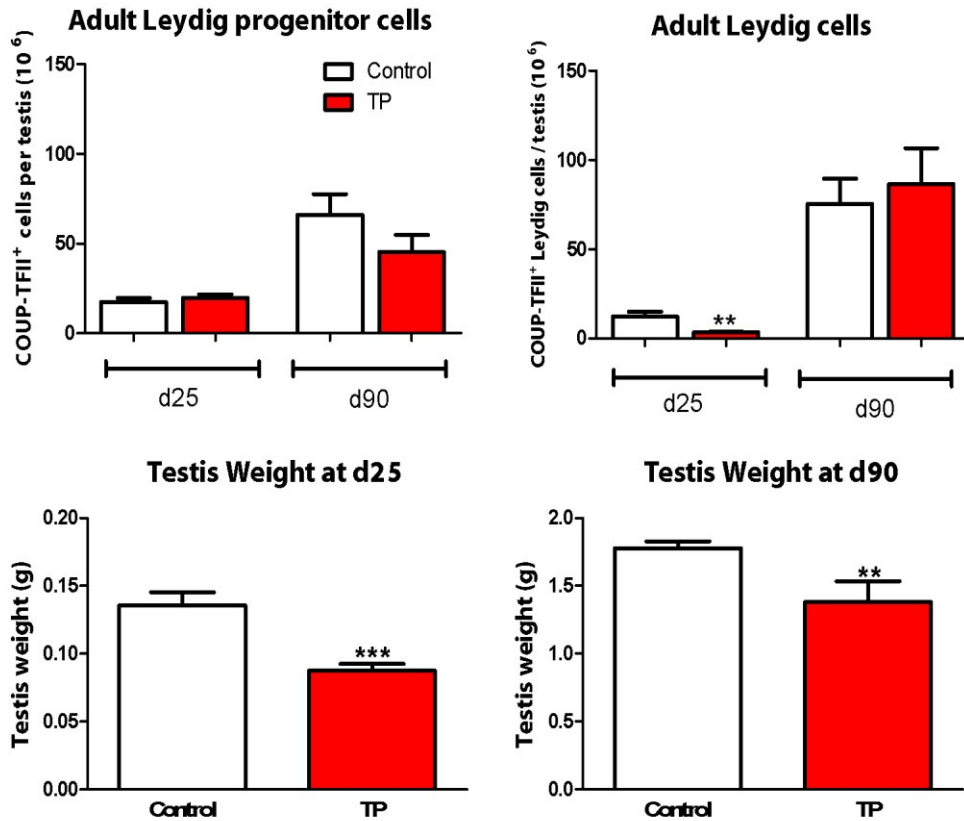


Figure 4.19 Effect of postnatal TP administration (Pnd1-25) on adult Leydig progenitor cell numbers and testis weight at pnd 25 and 90 in the rat. Postnatal TP administration did not affect the number of adult Leydig progenitor cells, in comparison with respective controls. Testis weight was reduced at pnd 25 and 90 and delayed differentiation of adult Leydig cells. Means are \pm SEM for $n=5$. Data were analysed using an unpaired t-test; ** $p<0.01$, *** $p<0.001$.

4.3.7 Effect of fetal DHT exposure on adult Leydig progenitor cells

Dihydrotestosterone (DHT) was used in these experiments as, unlike TP, it cannot be aromatised into oestradiol, thus eliminating potentially confounding results via this pathway. Pregnant dams received a dose of 10mg/kg DHT as used in an earlier study in the lab (Dean et al 2012) between e15.5-e21.5 (Figure 4.20), to investigate whether this might increase adult Leydig progenitor cell proliferation. This dose did not cause any toxic effects to the dams (this study and Dean et al 2012). The overall aim was to investigate whether increasing fetal androgen levels via exogenous DHT exposure, would

affect adult Leydig progenitor cell development in male offspring, which were analysed after birth (e21.5) and in adulthood (Pnd 75). Females were included in this study as a control parameter of DHT exposure i.e. to determine whether DHT treatment had been correctly administered and was capable of masculinising females, as previously shown (Dean et al 2012).

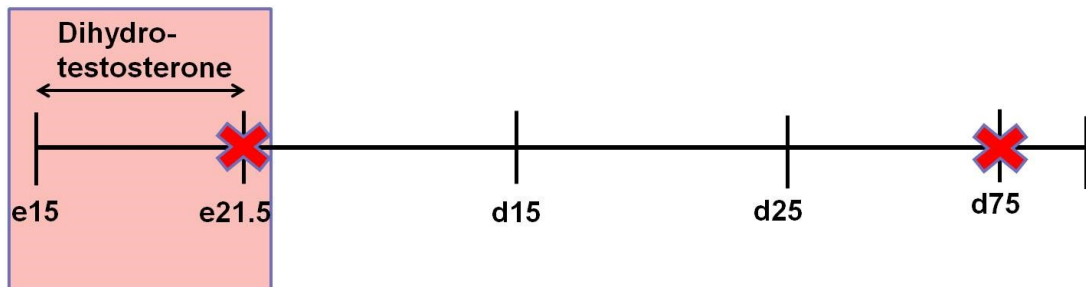


Figure 4.20 Schematic indicating the timing of fetal DHT-exposure in rats. The time-points at which adult Leydig cells and their progenitors were investigated are marked by red crosses.

Fetal DHT exposure had no effect on the number of adult Leydig progenitor cells or fetal Leydig cells at e21.5, whereas fetal TP-exposure (above) had reduced the number of both adult Leydig progenitor and fetal Leydig cells (Figure 4.21). Similarly, whereas DHT-exposed animals had comparable testes weight to wild-type controls at birth (e21.5), testis weight was reduced in TP-exposed fetal animals (Figure 4.21). Analysis of DHT-exposed female offspring in adulthood (Pnd 90), indicated that fetal DHT exposure was successful in inducing masculinisation, as evident from an increased AGD (Figure 4.23), lack of a vaginal opening and absence of nipples (Table 4.3). Furthermore, the presence of a rudimentary prostate gland was found in some females, or gross distension of the uterus (hydrometrocolpos) (Figure 4.22) (this study and Dean et al 2012), overall indicating that DHT treatment had been effective in masculinising female offspring. However, there was no evidence for an 'enhancing effect' on adult males, as neither AGD nor testis weight were increased, in comparison to their respective wild-type control animals (Figure 4.23). This experiment aimed

to increase fetal intratesticular testosterone levels in males, in order to assess whether this could potentially increase the number of adult Leydig progenitor cells. However, it appeared that fetal DHT administration, in this study, was not sufficient in effectively increasing fetal androgen exposure in males, although this was not formally checked. Thus, adult Leydig progenitor cell number was not evaluated as it seemed apparent that exogenous fetal TP/DHT administration would not effectively elevate ITT levels enough to provide a true model of "gain of adult Leydig cell function". A better model of the aforementioned, would be one in which endogenous ITT levels might potentially be increased, which is discussed in the next section.

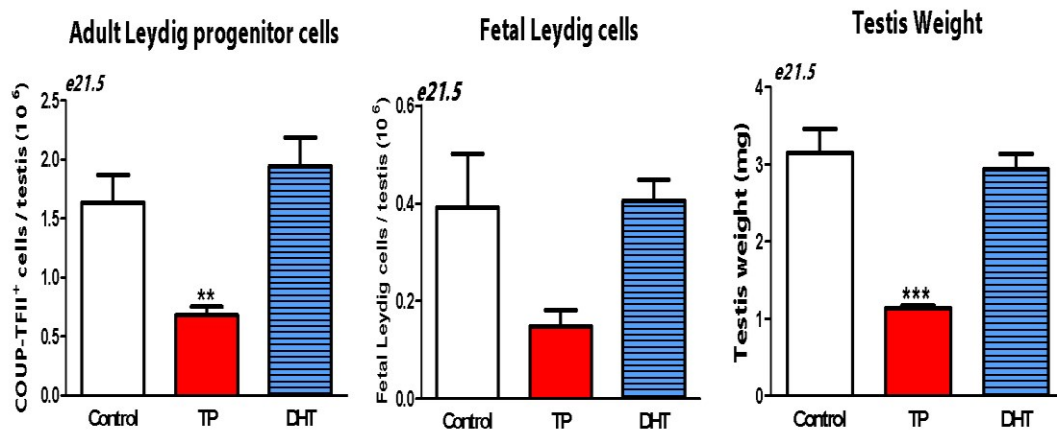


Figure 4.21 Comparative effects of fetal exposure to TP or DHT, on fetal rats before birth (e21.5). Following fetal TP exposure (e14.5-21.5), the number of adult Leydig progenitor cells, fetal Leydig cells and testis weight were reduced before birth, in comparison to wild-type controls. Fetal DHT-exposed animals (e15.5-e21.5), were comparable to wild-type controls at birth. Means \pm SEM for n=5-9. Data were analysed using an unpaired t-test; ***p< 0.001 **p<0.01. Data for TP-exposed animals is reproduced from figure 4.17 for purposes of comparison.

Table 4.3 External masculinisation in adult females following fetal DHT exposure.

<u>External masculinisation parameter</u>	<u>DHT 10 mg/kg</u>
Vaginal Opening	Absent 9/11
Nipples	Absent 11/11

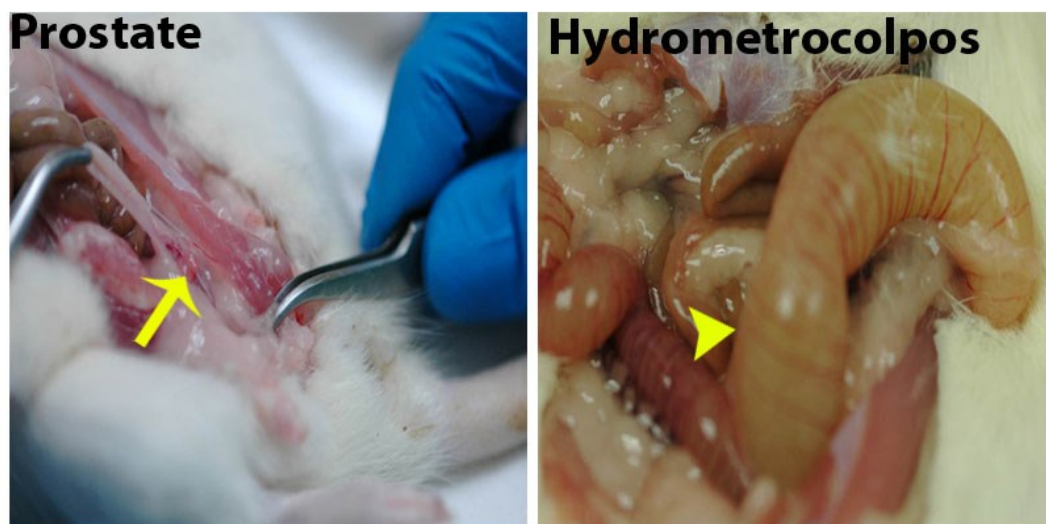


Figure 4.22 Fetal DHT exposure causes female masculinisation and hydrometrocolpos in the rat. Following DHT exposure during fetal life (e15.5-e21.5), females were examined during adulthood (Pnd75), to investigate whether DHT could induce masculinisation. A rudimentary prostate is evident (left image; yellow arrow). Increased fluid in the uterine horn (hydrometrocolpos) is also shown (right image; yellow arrowhead), as a result of fetal DHT exposure.

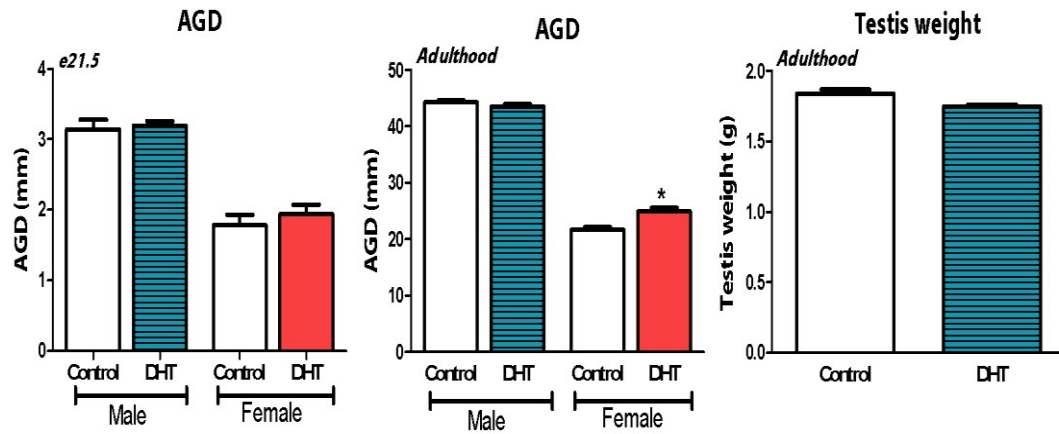


Figure 4.23 Effect of fetal DHT exposure in rats on AGD of fetal offspring before birth and AGD and testis weight in adulthood. Fetal DHT exposure (10mg/kg/day between e15.5-21.5) did not increase male AGD after birth (e21.5), or in adulthood (Pnd 90), in comparison with wild-type controls. Testis weight was unchanged in adulthood following fetal DHT exposure. Female AGD was increased in adulthood, in comparison to wild-type controls. Means \pm SEM for $n=5-11$. Data were analysed using an unpaired t-test; * $p<0.05$.

4.3.8 Effect of iNOS knockout on adult Leydig progenitor cells

The iNOS^{-/-} mouse model has previously been shown to exhibit a 60% increase in testis weight, along with a 16% increase in the number of adult Leydig cells per testis during adulthood, whereas body weight was unchanged (Auharek et al 2011). During the pubertal period in iNOS^{-/-} mice, the number of developing adult Leydig cells was significantly increased by 60% and 40% at pnd 10 and 15 respectively. Coinciding with this, a significant increase in AGD was reported in iNOS^{-/-} mice at the same time-points, suggesting that there was an effective increase in fetal androgen exposure (at least peripherally), in comparison to wild-type controls (Auharek et al 2012). Given the increase in adult Leydig cell number in puberty and adulthood in iNOS^{-/-} mice as previously described, and the fact that LH blood levels appeared to be lower in the face of normal testosterone levels in adult iNOS^{-/-} mice (Lue et al 2003), the evidence suggested that iNOS^{-/-} mice might potentially have increased fetal ITT levels and potentially depict a "gain of adult Leydig cell function" model. Thus, adult Leydig progenitor cell number (COUP-TFII⁺/3 β -HSD^{neg}) was investigated at birth

(e18.5), pre-puberty (Pnd 10, 15) and adulthood (Pnd 70). There was an increase in adult Leydig progenitor cells at pnd 10, 15 and 70, but not at e18.5, in comparison with wild-type controls (Figure 4.24). The number of adult Leydig cells did not differ between *iNOS*^{-/-} and their respective wild-type controls (Figure 4.24).

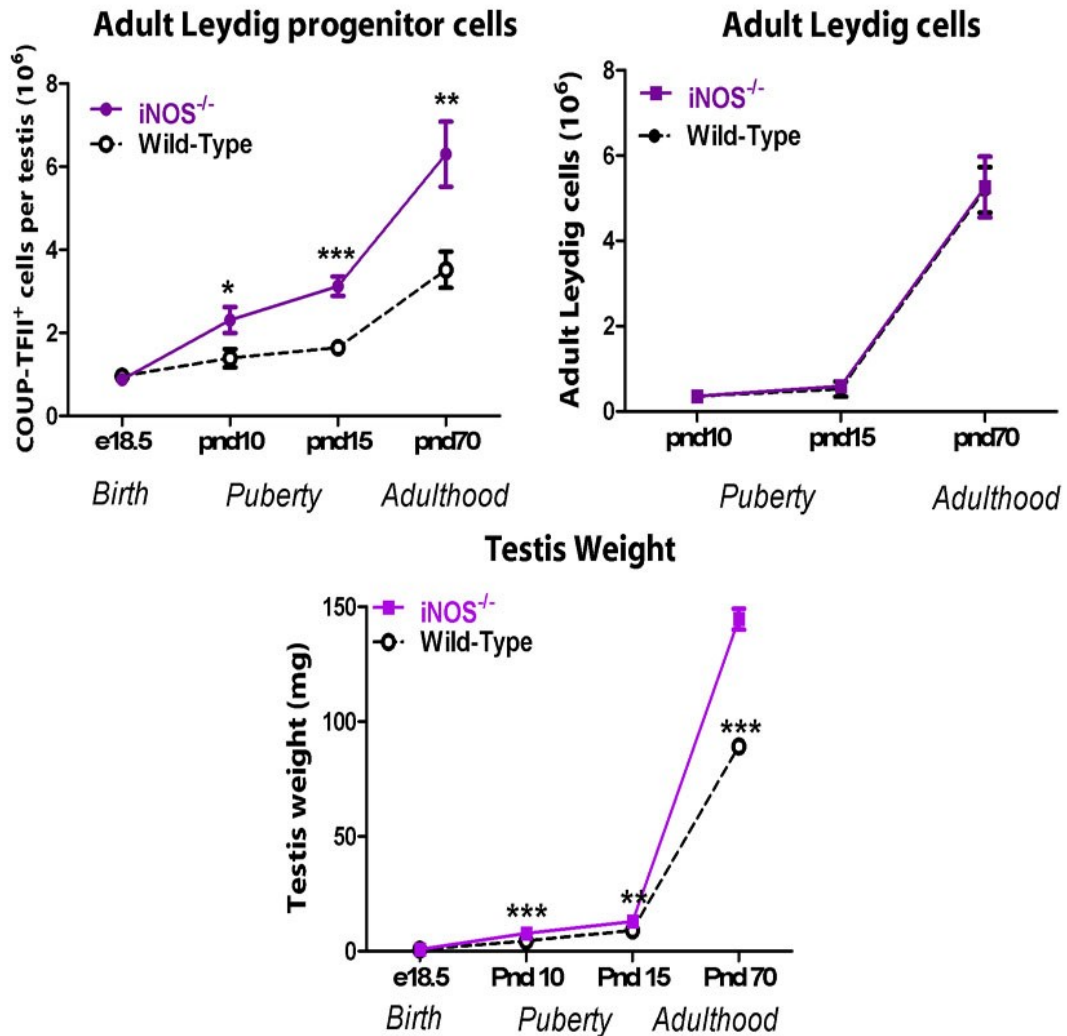


Figure 4.24 Effect of *iNOS*^{-/-} on adult Leydig cells and their progenitors throughout development in the mouse. Testis weight and adult Leydig progenitor cell number were increased at pnd 10, 15 and 70, in comparison with wild-type controls. There was no difference in the number of adult Leydig cells between *iNOS*^{-/-} and wild-type controls, at any age studied. Means \pm SEM for $n=4-10$. Data were analysed using an unpaired t-test; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ in comparison with respective wild-type controls.

4.4 Discussion

This chapter has established that COUP-TFII⁺ adult Leydig progenitor cells, present in the fetal testis, are androgen targets, as shown by their expression of AR in human and rodents. Deficient fetal androgen exposure via experimental reduction of androgen production/action primarily in fetal life in rodents (via transgenic and chemical manipulations), resulted in corresponding reductions in progenitor cell numbers which was accompanied by compensated adult Leydig cell failure.

Previous studies have shown that AR is expressed in the fetal rat testis in non-steroidogenic interstitial and peritubular myoid (PTM) cells from e17.5 to e21.5 (Majdic et al 1995) and at 10 weeks in the human (Sajjad et al 2004). In postnatal life, (Pnd 5), AR expression was localised again to non-steroidogenic interstitial cells and PTM cells in the rat (Majdic et al 1995). The authors suggest these cells may represent adult Leydig progenitor cells, and that extensive morphological and marker analysis was needed to investigate this further (Eacker and Braun 2007). The current findings agree with the previous observations and demonstrate that the majority of COUP-TFII⁺ adult Leydig progenitor cells express AR in fetal human and rodent testes. COUP-TFII⁺/AR⁺/3 β -HSD^{neg} adult Leydig progenitor cells are also present in the interstitium during postnatal life where they appear to differentiate into adult Leydig cells (COUP-TFII⁺/AR⁺/3 β -HSD⁺) around puberty in the rat.

To assess the role of androgens in adult Leydig progenitor cell development, the most direct approach would be to knock out AR specifically in the COUP-TFII⁺ adult Leydig progenitor cell population. However, this was not feasible as firstly the only known markers for these cells (from the studies in this thesis), are AR and COUP-TFII and, secondly, COUP-TFII is expressed not only in the progenitor cells, but also in PTM cells, and initially in fetal Leydig cells (van den Driesche et al 2012). It would have been useful to investigate the aP2-Cre;AR^{flox/flox} mouse model as a specific way of targeting AR on adult Leydig progenitor cells, however tissue for this model was not available at the time these studies were undertaken. Therefore, the ubiquitous ARKO mouse model (De Gendt et al

2005), was used to investigate any effect that non-functioning AR in adult Leydig progenitor cells might have on their development and on adult Leydig cell development/function. The use of other cell selective ARKO models, Sertoli cell (SC)-ARKO (De Gendt et al 2005) and a peritubular myoid (PTM)-ARKO (Welsh et al 2009) were also included in this study to evaluate any effect androgen signalling via these cells may have on adult Leydig progenitor development. Quantification of adult Leydig progenitor cells (COUP-TFII⁺/3 β -HSD^{neg}) in ARKO mice, revealed a significant reduction after birth (Pnd 2) and a 40% deficit in number throughout postnatal life, in comparison to respective wild-type controls. This reduction was paralleled by a reduction in adult Leydig cell number throughout development in ARKOs. This finding is in agreement with previous studies that investigated the role of androgen signalling in adult Leydig cell development. ARKO mice were reported to exhibit an 83% reduction in adult Leydig cell number throughout postnatal development (De Gendt et al 2005), and in adulthood, adult Leydig cells were reduced by 60% (O'Shaughnessy et al 2002, 2012). The fact that adult Leydig progenitor cells were also reduced in parallel adds a new dimension to the aforementioned studies. Fetal Leydig cell number was comparable in control, ARKO and SCARKO models at pnd 2, consistent with the fact that fetal Leydig cell development and function is androgen-independent (O'Shaughnessy et al 2002).

However, the fact that AR is knocked out ubiquitously in the ARKO model plus the fact that all testes are cryptorchid, are all potentially confounding factors. Therefore, a model in which fetal intratesticular testosterone was selectively reduced, via DBP, was investigated to determine whether this would affect the development of adult Leydig and/or their progenitor cells, as it appeared to do so in the ARKO model. Fetal DBP-exposure reduced intratesticular testosterone levels in the rat by 40-80% (Mylchreest et al 1999, 2000, 2002, Scott et al 2007, van den Driesche et al 2012) and in this study. DBP was chosen as the prenatal 'anti-androgen' instead of flutamide (Imperato McGinley et al 1992, Welsh et al 2008, 2010), because it is the most direct way of reducing androgen action on the progenitor cells by reducing fetal intratesticular testosterone levels, rather

than trying to block androgen action, as is the case with flutamide. Treatment with the latter may simply not be enough to block the actions of the already high intratesticular testosterone levels present during fetal development. DBP-exposure resulted in a significant reduction (40%) in adult Leydig progenitor cell number at the end of gestation and throughout postnatal development into adulthood. The fact that knockout/inactivation of the AR in mice resulted in a similar magnitude of reduction in numbers of adult Leydig progenitor cells across development, led to the conclusion that this may have resulted from deficient androgen action. The reduction in adult Leydig cell number in ARKO, could potentially reflect the lower number of adult Leydig progenitor cells present. However, a similar deficit in adult Leydig cell numbers was not found in DBP-exposed males (in which androgen level/action was only reduced in fetal life). One explanation is that knockout of AR in other cell types e.g. Sertoli or PTM cells might have contributed to the reduction in adult Leydig cell number, as reduced adult Leydig cell numbers were reported in SCARKO mice (De Gendt et al 2005) and abnormal adult Leydig cell differentiation, in PTM-ARKO mice (Welsh et al 2009). In this regard, there was no significant difference in the number of adult Leydig progenitor cells in SCARKO and PTMARKO models in the present study, in comparison to wild-type controls.

The main findings in this chapter demonstrate that fetal androgen deficiency (ARKO and DBP-exposed males) results in a deficit in adult Leydig progenitor cell number and compensated adult Leydig cell failure, i.e. normal/reduced blood testosterone in the face of elevated LH, and thus an altered LH:testosterone ratio, in comparison with wild-type controls. This finding is in agreement with earlier studies of ARKO males, in which LH serum levels were significantly increased while testosterone levels were decreased/normal (Yeh et al 2002, Matusumoto et al 2003, Notini et al 2005, De Gendt et al 2005). In humans, it has been suggested that fetal androgen deficiency can result in adult Leydig cell dysfunction and thus 'primary hypogonadism' (Tajar et al 2010, Andersson et al 2004). Patients with symptoms in adulthood symptomatic of TDS, commonly display abnormal/compensated adult Leydig cell failure, as

evident from increased LH and decreased testosterone (Joensen et al 2008, Lardone et al 2012). For example, Increased LH levels and low-normal testosterone levels appear to be a common finding in infertile men (Glass & Vigersky 1980, Stanwell-Smith et al 1985, Andersson et al 2004), indicative of compensated Leydig cell failure. In long-term follow up studies of males with disorders of sexual development (DSD), analysis of their serum hormone levels demonstrated an increase in LH and FSH with low-normal testosterone, again indicative of compensated adult Leydig cell failure (Kojima et al 2009, van der Zwan et al 2013). This phenotype is comparable to the ARKO and DBP models presented in this study, potentially as a result of lowered fetal androgen action.

Overall, this suggests the importance of normal androgen signalling during fetal life for the development (numerically and functionally) of adult Leydig progenitors when they differentiate into adult Leydig cells. In a large clinical study of idiopathic infertile men, on average, LH was increased by 19% and testosterone serum levels were reduced by 18%, in comparison to fertile men (Andersson et al 2004). The authors suggested that adult Leydig cell dysfunction might have resulted from testicular dysgenesis during fetal development, which the results in this study provide evidence for. In fact a subgroup of the infertile men who had a history of cryptorchidism, had lower sperm concentration and an even poorer Leydig cell function, as reflected by an increased LH:testosterone ratio, in comparison with the fertile men without a history of cryptorchidism (Andersson et al 2004). In line with this, a shortened AGD in newborn males is associated with cryptorchidism and hypospadias, which highlights the role of fetal androgen action in these disorders (Eisenberg et al 2012, Jain & Singal 2013, Dean & Sharpe 2013). Newborns who present with cryptorchidism or hypospadias, potentially as a result of testicular dysgenesis, have a higher risk for other TDS conditions to manifest during postnatal life, e.g. adult Leydig cell dysfunction (Toppari et al 2010).

The findings presented in this study demonstrate that a deficiency in fetal androgen level/action results in adult Leydig cell dysfunction in adulthood in rats. However, there could be other interpretations for these findings, such as,

that fetal androgen deficiency could have induced other changes which could secondarily lead to altered adult Leydig function. As sex steroids can regulate the expression of GnRH neurons involved in normal signalling in the HPG axis (Garcia-Galiano et al 2012), it might seem plausible that the reduction in fetal androgens could have altered sensitivity of the HPG axis, involved in the androgen negative feedback pathway. This could then affect the number of adult Leydig progenitor cells. However, this seems unlikely as LH is not a key mediator of testis function during fetal life (Scott et al 2009, O'Shaughnessy and Fowler 2011), thus cannot be responsible for the clear reduction in adult Leydig progenitor cell number in the rodent models used in these studies.

The present findings show that the number of adult Leydig progenitor cells increases substantially during postnatal life, prior to and during puberty, consistent with an increase in 'mesenchymal, non-Leydig interstitial cells' (Mendis-Handagama et al 2001). This prompted the investigation into whether or not these cells were affected by altering androgen levels/action during different periods of potential susceptibility i.e. fetal or postnatal life. Flutamide treatment prior to puberty in the rat (Pnd 1-15), resulted in a reduction of the number of adult Leydig progenitor cells at pnd 25, in comparison with wild-type controls. These findings are consistent with a recent study which highlighted the importance of postnatal androgens on Leydig progenitor cell proliferation (Guo et al 2013). In the latter study, a GnRH antagonist (NalGlu), was used to block LH stimulation of androgen production at pnd 14 in the rat, and resulted in a reduction (83%) of 'adult Leydig progenitor cells'. In the present study, postnatal flutamide treatment of fetally DBP-exposed animals, resulted in a significant reduction in adult Leydig progenitor cell numbers. While it appeared a similar reduction was evident in adult Leydig cells, this did not reach statistical significance, perhaps due to a smaller sample size in this group. In terms of adult Leydig cell function, postnatal flutamide treatment of fetally DBP-exposed animals resulted in compensated adult Leydig cell failure, which was comparable to that found in DBP-exposed animals in the absence of postnatal

flutamide treatment. This highlights that androgen deficiency in the fetal period, but not in the postnatal period, results in adult Leydig cell dysfunction.

Previous studies have reported that fetal TP exposure resulted in masculinised female offspring (Wolf et al 2002, Welsh et al 2008), which suggests that exogenous fetal TP administration increases fetal androgen exposure during fetal life, although whether this extends to increasing intratesticular androgen levels in fetal males is unlikely based on an earlier study (Scott et al 2007). Nevertheless, it prompted the use of this model in order to investigate whether or not adult Leydig progenitor cells were affected by increased fetal exposure to TP. Furthermore, effects on adult Leydig progenitor cells were also investigated following postnatal TP treatment, to determine whether or not a potential increase in postnatal testosterone levels could 'advance' the development of adult Leydig progenitor cells. Fetal exposure to TP appeared to increase the number of adult Leydig progenitor cells at birth, and there was a tendency for the same change in adulthood, whereas there was no comparable effect following postnatal TP treatment. However, upon examination across development, the reduced testis size and fetal growth restriction following fetal TP exposure, similar to that found in previous studies (Wolf et al 2002, 2004, Welsh et al 2008), suggested that TP treatment of pregnant dams might have introduced confounding factors. Any results obtained from these experimental groups could not be confidently attributed as due to an androgenic response, as such effects could also have been due to potential aromatisation of excess TP into oestradiol.

As dihydrotestosterone (DHT) is a more potent and non-aromatisable androgen, it was administered to pregnant dams, instead of TP. Fetal DHT exposure resulted in masculinisation of female offspring, as evident by an absence of nipples, absence of a vaginal opening and the presence of a vestigial prostate. However, in terms of enhancing male reproductive parameters, there was no affect evident on reproductive organ size in DHT-exposed males, in comparison with controls. Both AGD and the number of adult Leydig progenitor cells were comparable to controls at e21.5. This is in agreement with other studies that

failed to find an increase in male reproductive development following fetal exposure to androgens (Wolf et al 2008, Welsh et al 2008, Dean et al 2012). A higher dose of 20 mg/kg of DHT was also administered to pregnant dams during these studies, which neither enhanced nor advanced any reproductive organ size or AGD in male offspring examined in adulthood. Although DHT has a higher affinity than testosterone for AR, it seems likely that intratesticular testosterone levels are already high enough to induce maximal androgen effects locally, and thus cannot be added to, although this was not formally checked. However, it seemed apparent that exogenous TP/DHT exposure, in this study, could not effectively demonstrate a gain of function (in terms of adult Leydig progenitor cells numbers and or adult Leydig cell function) due to exogenous androgen exposure. Therefore, a more convincing model would be one in which endogenous ITT levels might potentially be increased, namely the iNOS^{-/-} mouse.

Previous studies of the iNOS^{-/-} mouse model reported a 40% and 16% increase in adult Leydig cell number at puberty and adulthood respectively, and increased AGD at both ages (Auharek et al 2011, 2012). Increased AGD suggests an increase in fetal androgen exposure which might in turn reflect elevated intratesticular testosterone levels (Welsh et al 2008, Auharek et al 2011, 2012). The current findings show that the number of adult Leydig progenitor cells in fetal life (at e18.5) of iNOS^{-/-} mice, is comparable to their respective wild-type controls. In fact, it is only in postnatal life, that an increase in adult Leydig progenitor cells is evident (Pnd 10, 15 and 70). This is in contrast to the earlier studies described herein, which demonstrated that androgen action in the pre-pubertal period is less important (than fetal life) for altering adult Leydig cell function. In adult iNOS^{-/-} males, plasma levels of testosterone are normal whilst LH levels are decreased (Lue et al 2003), which suggests that lower LH is required to stimulate testosterone production in this model. Unfortunately, plasma levels of LH and testosterone for the iNOS^{-/-} model, in which the present studies were undertaken, were unavailable from our collaborators. Also there was no difference in adult Leydig cell number in iNOS^{-/-} mice, at any age

examined, in comparison to control animals, which is contrast to earlier studies (Auharek et al 2011, 2012). This can potentially be explained by the difference in stereological techniques employed, as the previous study identified adult Leydig cells by morphology, unlike the present study. The previous study also reported that the size of individual adult Leydig cells was significantly reduced, thus suggesting that iNOS is important for steroidogenic cell function (Auharek et al 2011), so it may not be an effective gain of function model. In any case, vital data from the iNOS^{-/-} model in this study are missing, e.g. the measurement of fetal intratesticular testosterone and adult hormone measurements (e.g. testosterone and LH), which could determine whether this model exhibits a true "gain of adult Leydig cell function." Without this key data, any effect on adult Leydig progenitor cell development, as a result of potentially increased intratesticular levels, can only be deemed as speculation.

Smooth muscle actin (SMA) is a marker for differentiated peritubular myoid cells, which envelop Sertoli and germ cells and delineate the seminiferous cords/tubules, but also stain blood vessels. An interesting observation from these studies was that, compared with control testes at pnd 2, ARKO-males exhibited reduced SMA staining in the PTM cells, whereas this was normally present in the blood vessels of the same sample, indicating it was not due to any technical error. Testosterone has been shown to induce peritubular cell differentiation (Schlatt et al 1993) in prepubertal monkeys which suggests that androgens play a role in the differentiation of peritubular cells. Weak SMA staining in PTM cells in ARKOs at pnd 2, suggests there might be a delay in their differentiation, presumably due to the absence of fetal androgen signalling in ARKO males. A similar reduction in SMA expression in PTM cells was noted at e17.5 in *Tfm* mice with an inactivated AR (Merlet et al 2007). In relation to adult Leydig progenitor cells, the absence of AR signalling might affect/compromise their differentiation into adult Leydig cells. Compared to control mice in which the number of adult Leydig cells increased between pnd12 and 20, coincident with adult Leydig cell differentiation, this increase appeared to be delayed in ARKO males and occurred between pnd 20 and 50. One potential explanation is

that abnormal androgen signalling compromises the differentiation of adult Leydig progenitor cells into adult Leydig cells (Ivell et al 2013).

A recent study that investigated adult Leydig cell function in DBP-exposed rats, using *Insl3* as a marker, reported a disruption of normal adult Leydig cell development, as evident from a prolonged period of adult Leydig cell differentiation (Ivell et al 2013). This is similar to the current findings, which show that DBP-exposed rats exhibit a significant reduction in adult Leydig cell number at Pnd 25, but reach normal numbers in adulthood. Thus, differentiation of adult Leydig progenitor cells in DBP-exposed males between d15-25 appears somewhat delayed, in comparison to the controls. This potentially reflects a prolonged/delayed differentiation period of adult Leydig progenitor cells into adult Leydig cells.

An earlier study reported that in adult ARKO males, there was no difference in the number of adult Leydig cells, in comparison to control animals (Notini et al 2005). However, as there is no indication of how or if adult Leydig cell quantification was carried out, this might simply be a misleading observation made by the authors, due to the smaller diameter of the seminiferous tubules (which the authors had highlighted). Initial observations, within the present study, of adult Leydig cell hyperplasia in ARKOs were dismissed, once adult Leydig cells were quantified (as above), which highlights the importance of cell quantification for accurate results (O'Shaughnessy et al 2002).

The present findings in this chapter provide new insight into a potential role for androgens in regulating adult Leydig progenitor cell development numerically and their functional capability when they differentiate into adult Leydig cells, as evident from the ARKO and DBP rodent models investigated. In humans, infertile men with a history of cryptorchidism, exhibit compensated adult Leydig cell failure i.e. low-normal testosterone levels and high luteinising hormone to maintain a normal testosterone level (Andersson et al 2004). The authors suggest that compensated adult Leydig cell failure is a symptom of TDS, which is believed to have a common fetal origin, namely due to fetal androgen

deficits (Skakkebaek et al 2001, Sharpe & Skakkebaek 2008). The results in this study provide direct evidence for this and suggest that fetal androgen deficiency affects the adult Leydig progenitor cells. Precisely how fetal events can predetermine adult testosterone levels has thus far not been elucidated, however, a potential mechanism using the DBP rat model to mimic human TDS, is investigated in the next chapter.

Chapter 5 Fetal Programming of Adult Leydig Progenitor Cells

5.1 Introduction

The main findings from the previous chapter demonstrated that fetal androgen deficits, led to a reduction in the number of adult Leydig progenitor cells, and resulted in adult Leydig cell dysfunction. This chapter aimed to investigate the potential mechanisms how this might have occurred, by analysing expression of genes involved in the steroidogenic pathway in adulthood and investigating an epigenetic change to explain one potential mechanism involved.

The previous chapter demonstrated that fetal DBP-exposure reduced testosterone production during fetal life, and in adulthood this resulted in 'compensated Leydig cell failure'. The exact mechanism via which DBP exerts its effects on fetal Leydig cells is unknown but DBP exposure clearly inhibits the rate limiting step in steroidogenesis, *StAR* (Clark et al 1995, Manna et al 2009, Papadopoulos and Miller 2012, van den Driesche et al 2012), as well as enzymes involved further downstream in the steroidogenic pathway (Schultz et al 2001, Barlow et al 2003, Lehmann et al 2004, Thompson et al 2004, Liu et al 2005, Plummer et al 2007, van den Driesche et al 2012). A recent study which used DBP (500mg/kg) to lower fetal testosterone levels showed that in adulthood, the levels of testicular 3 β -HSD and 17 β -HSD enzymatic activity were reduced, which was associated with compensated adult Leydig cell failure (Giribabu et al 2012). This indicates an altered/abnormal steroidogenic pathway, which will be investigated in this chapter.

Epigenetic changes can be meiotically and/or mitotically heritable, altering gene expression potential without altering the gene sequence (Wu and Morris 2001), and can occur early during cell proliferation and development (Klein et al 2005). The proximal promoter region in *StAR* was found to have reduced H3K27me3 upon *StAR* transcription, as induced by hCG injection in granulosa cells (Lee et al 2013). This specific region has also been shown to be important for *StAR*

transcription in MA-10 cells (Hiroi et al 2004) and granulosa cells (Christenson et al 2001) and will be investigated in this chapter.

Transcription of *StAR* is crucial for regulating steroidogenesis. Epigenetic factors can alter gene transcription including methylation of DNA, microRNAs and histone modifications (Bibikova et al 2008). The latter refers to modifications to chromatin structure, which is composed mainly of DNA wrapped around histones. Histone modifications cause chromatin remodelling which controls gene transcription (Kadonaga 1998, Shen et al 2009). Transcriptional activation can occur via histone methylation e.g. of lysine 4 at histone 3 (H3K4) or transcriptional repression by methylation of lysine 27 at histone 3 (H3K27) (Lee et al 2005). Tri-methylation at lysine 27 of histone 3 (H3K27me3) is one such histone modification, the expression of which commonly acts as a repressive mark (Kirmizis et al 2004, Barski et al 2007, Sui et al 2012) by condensing the chromatin, thus impeding the binding of transcription factors to response elements in the promoter and thus preventing transcription (Li et al 2007). These studies prompted the investigation into the hypothesis that compensated adult Leydig cell failure is 'programmed' in DBP-exposed animals by increasing histone methylation in adult Leydig progenitor cells which manifests as repressive *StAR* transcription in adult Leydig cells.

5.2 Materials and Methods

5.2.1 RNA extraction and RT-PCR

Briefly, total RNA was isolated from frozen testes from control and DBP-exposed rats followed by RNA extraction and cDNA synthesis (Chapter 2).

5.2.2 Quantitative gene expression analysis using Taqman PCR

Quantitative PCR was performed for genes listed in Table 5.1 using primers and probes listed in Table 5.1 and an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems; California, USA). This was run using optimised standard conditions and taqman probes (Chapter 2).

Table 5.1 Primers and probes used for gene expression analysis.

<i>Gene</i>	<i>Forward Primer</i>	<i>Reverse Primer (Probe No.)</i>
<i>Sox9</i>	<i>CTGAAGGGCTACGACTGGAC</i>	<i>TCTTGATGTGCGTTCTCTGG (63)</i>
<i>LHR</i>	<i>CTGGAGAAGATGCACAGTGG</i>	<i>CTGCAATTTGGTGGAAAGAAATA (107)</i>
<i>StAR</i>	<i>TCACGTGGCTGCTCAGTATT</i>	<i>GGGTCTGTGATAAGACTTGGTTG (83)</i>
<i>CYP11a1</i>	<i>TCACATGCAGAATTTCCAGAAG</i>	<i>AGGATGTAAACTGACTCCATGTTG (7)</i>
<i>3β-HSD</i>	<i>TCATCTGATTTTGAACAATTTAGC</i>	<i>CTCCTGCTCCTGTCACCAG (105)</i>
<i>CYP17a1</i>	<i>CATCCCCACAAGGCTAAC</i>	<i>TGTGTCCTTGGGACAGTAAA (67)</i>
<i>17β-HSD3</i>	<i>AATATGTCACGATTGGAGCTGA</i>	<i>AAGGAATCAGGTTTCAGAATTATCG (5)</i>

5.2.3 ChIP assay

In order to isolate DNA which contained the histone of interest for this study, chromatin immunoprecipitation (ChIP) was carried out, which used whole rat testis DNA. Detailed methods are found in Chapter 2. All methods used to perform ChIP were initially validated as this was a novel technique in our research lab. The schematic illustrates the method employed (Figure 5.1). Briefly, DNA was cross linked to histones using formaldehyde. The DNA-histone

complex was then broken into smaller fragments via sonication. An antibody against the histone of interest, H3K27me3, was used to isolate the chromatin fragments to which H3K27me3 had attached. The cross-links were then reversed and the 'released' DNA was separated by protease digestion and purified. Purified DNA was then quantified via PCR, as described below.

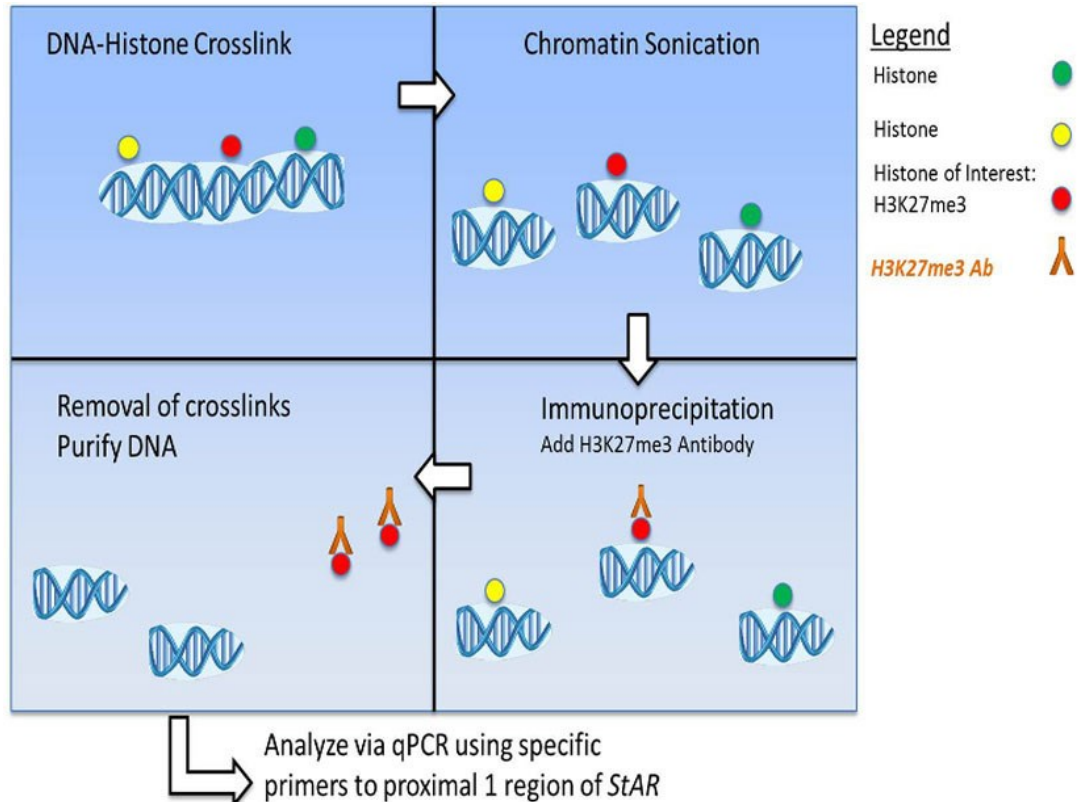


Figure 5.1 Schematic of the methodology of a CHIP assay

5.2.4 Primer design and validation

Primers were specifically designed for the proximal promoter region (85bp upstream) of *StAR*, (Table 5.2; 167bp) using the websites 'UCSC Genome Bioinformatics' (<http://genome.ucsc.edu/>) and 'primer3' <http://primer3.ut.ee/>. Primers were purchased from Eurofins, MWG Operon (Germany) and validated using a standard curve (Figure 5.2). Slopes in the range of -3.10 to -3.60 are considered acceptable for real-time PCR. The slope of the standard curve in this study was -3.19, which correlates to a high amplification efficiency of the PCR

reaction. A melt curve step was included to determine the specificity of the primers designed (Chapter 2) which demonstrated their high specificity for this study (Figure 5.2). PCR reaction was carried out as described in chapter 2.

Table 5.2 Primers used to target the proximal promoter region of *StAR*

<i>StAR</i>	AGGCAATCATTCCATCCTTG	GCTCCCAGGGATTGTCTT
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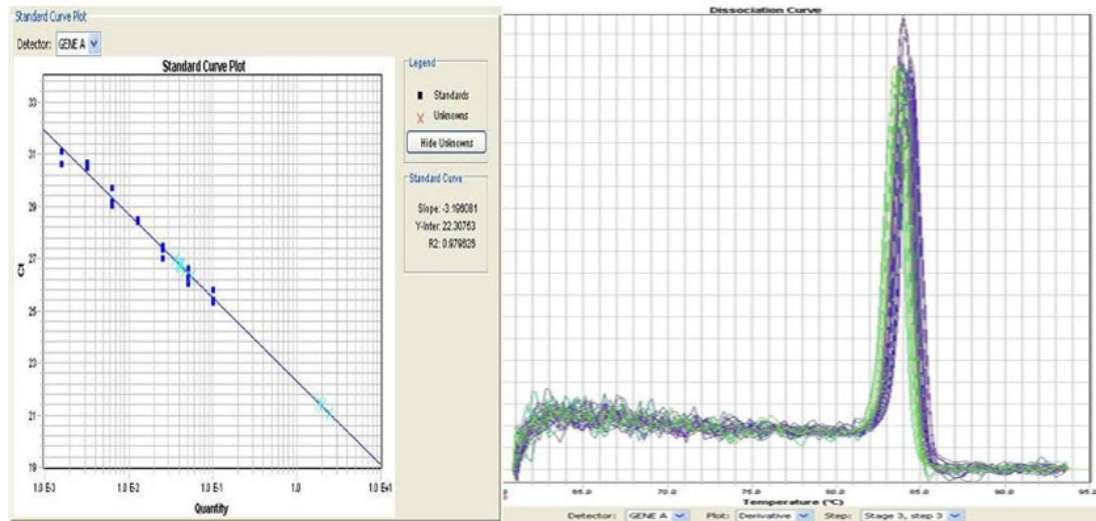


Figure 5.2 Standard curve (left) and melt curve analysis (right) of primers used

5.2.5 Gel electrophoresis and densitometry

As further validation of the findings from the ChIP assay, PCR products underwent gel electrophoresis, followed by densitometric analysis using 'ImageJ' (Chapter 2). DNA bands from control animals are shown at the top adjacent to green labels and on the same gel, DNA bands from DBP-exposed animals are shown next to blue labels, as shown in a representative sample (Figure 5.3). The software in 'ImageJ' computed the level of intensity of all the bands and this was compared to the input control.



Figure 5.3 Densitometric analysis of purified DNA after ChIP

5.2.6 Immunofluorescence

Double/triple immunofluorescence was carried out in order to determine whether the results obtained from the ChIP experiments, could also be demonstrated by antibody detection via immunofluorescence. A detailed protocol is provided in chapter 2. The primary antibodies used in these experiments are listed below (Table 5.3).

Table 5.3 Antibody dilutions and detections used for immunofluorescence

Primary	Species Raised	Dilution	Manufacturer	Visualisation
COUP-TFII	Mouse	1:1,000	Perseus Proteomics Inc	Fluorescein (Green)
3 β -HSD	Goat	1:1,000	Santa Cruz	Tyr-Cy3 (Red)
H3	Rabbit	1:5,000	Abcam	Tyr-Cy3 (Red)
H3K27me3	Rabbit	1:5,000	Active Motif	Tyr-Cy3 (Red)
H3K4me3	Rabbit	1:5,000	Active Motif	Tyr-Cy3 (Red)

5.3 Results

5.3.1 Steroidogenic gene analysis in adulthood following fetal DBP-exposure

DBP-exposed animals had reduced fetal and adult testosterone levels, which alongside elevated blood LH levels, indicated 'compensated adult Leydig cell failure', as shown in the previous chapter. To further investigate adult Leydig cell function, specific genes involved in the steroidogenic pathway to produce testosterone were analysed. As fetal exposure to DBP can result in cryptorchidism and thus affect testicular cellular composition, in particular loss of germ cells (and their mRNA), the expression of each gene in each testis sample was expressed relative to the expression of Sox9, a specific Sertoli cell marker. This was chosen as an appropriate control as the number of Sertoli cells is unchanged in adulthood between scrotal or cryptorchid testes of DBP-exposed animals (Hutchison et al 2008). The Leydig-cell specific genes examined included *Lhr*, *StAR*, *Cyp11a1*, *3 β -hsd*, *Cyp17a1*, and *17 β -hsd3* which all contribute to the end product, testosterone. There was a significant reduction in *StAR* and *3 β -HSD* mRNA expression in adulthood following fetal DBP exposure, without any significant effects evident for other steroidogenic genes examined (Figure 5.4). The next aim was to investigate the protein expression of both StAR and 3 β -HSD.

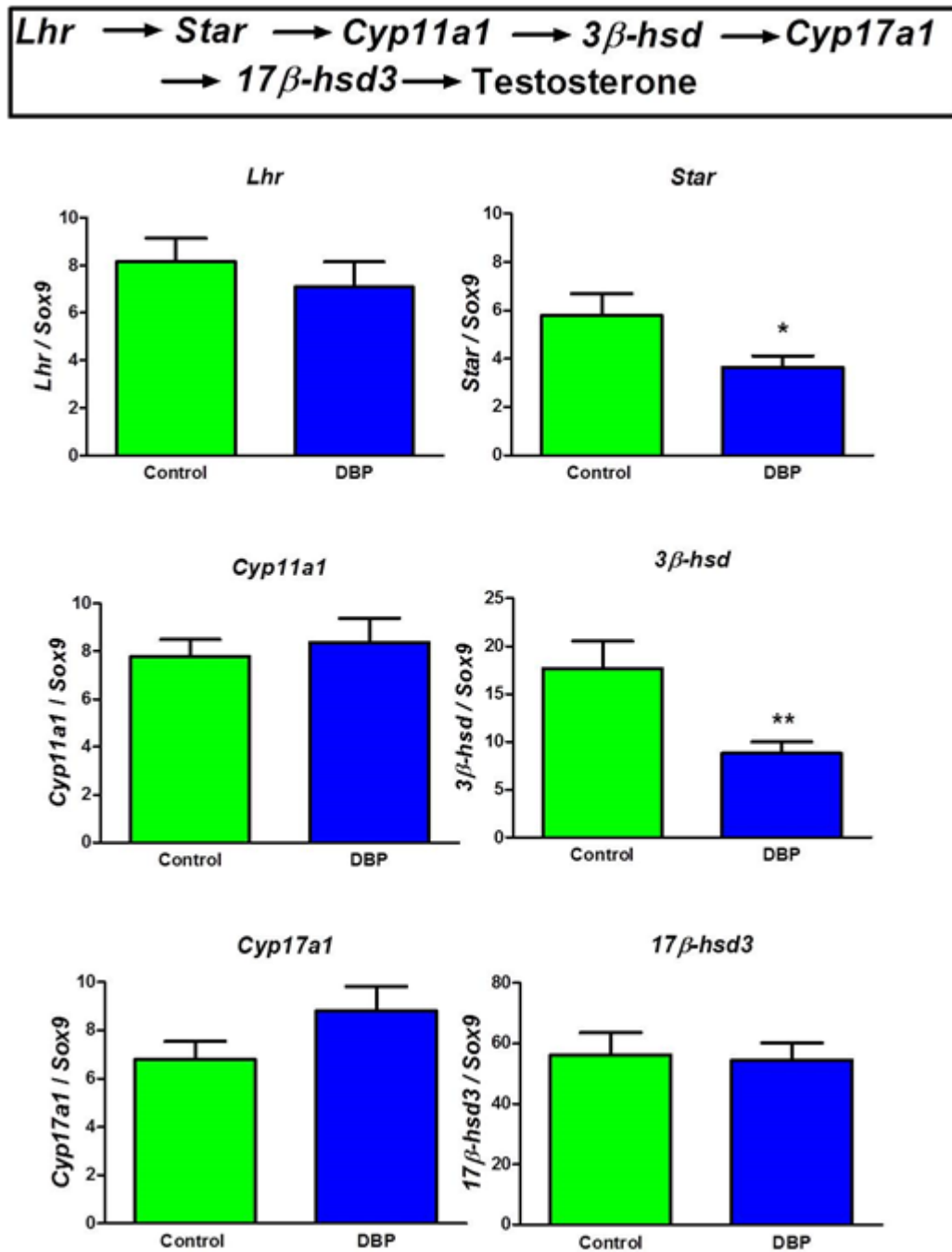


Figure 5.4 Effect of prenatal DBP exposure in rats on adult Leydig cell function as monitored by expression of Leydig cell-specific genes in the steroidogenic pathway. Values are relative mRNA levels as determined by quantitative PCR. The testosterone synthesis cascade is shown in the panel at the top, starting with the LH receptor (*Lhr*) and finishing with 17β -hydroxysteroid dehydrogenase type 3 (17β -*hsd3*). Values are Means \pm SEM for n=11-12 rats in each control and DBP-exposure group. Data were analysed using an unpaired t-test; *p<0.05, **p<0.01 in comparison with control (vehicle) animals.

5.3.2 Expression of StAR in adulthood following fetal DBP exposure

As *StAR* and *3 β -HSD* mRNA levels were decreased in DBP-exposed animals (Figure 5.4), the next experiment investigated the protein expression between control and DBP-exposed adult rats. Triple immunofluorescence was carried out using COUP-TFII (green), StAR (red) and 3 β -HSD (blue) in adult testes of control and DBP-exposed rats. Both StAR and 3 β -HSD were expressed in the cytoplasm of adult Leydig cells (arrowhead) with some Leydig cells expressing COUP-TFII in their nuclei in control and DBP-exposed animals (Figure 5.5). COUP-TFII⁺/3 β -HSD^{neg}/StAR^{neg} adult Leydig progenitor cells (arrow) were also present in the interstitium in both groups (Figure 5.5). Overall, from the immunohistochemistry experiments, there was not a convincing or consistent difference in 3 β -HSD or StAR protein expression in adult Leydig cells, which may be as a result of the subtle alteration/reduction in their gene expression (Figure 5.4). Although either change might theoretically lead to reduced steroidogenic output, StAR is one of the factors involved in cholesterol transport into the mitochondria (Fan & Papadopoulos 2013), which is rate-limiting for steroidogenesis (Miller & Bose 2011), so this change was considered the most significant. Given the reduced *StAR* expression found in DBP-exposed animals in this study, this prompted its further investigation to potentially explain how *StAR* transcription is repressed, as a result of fetal androgen deficiency.

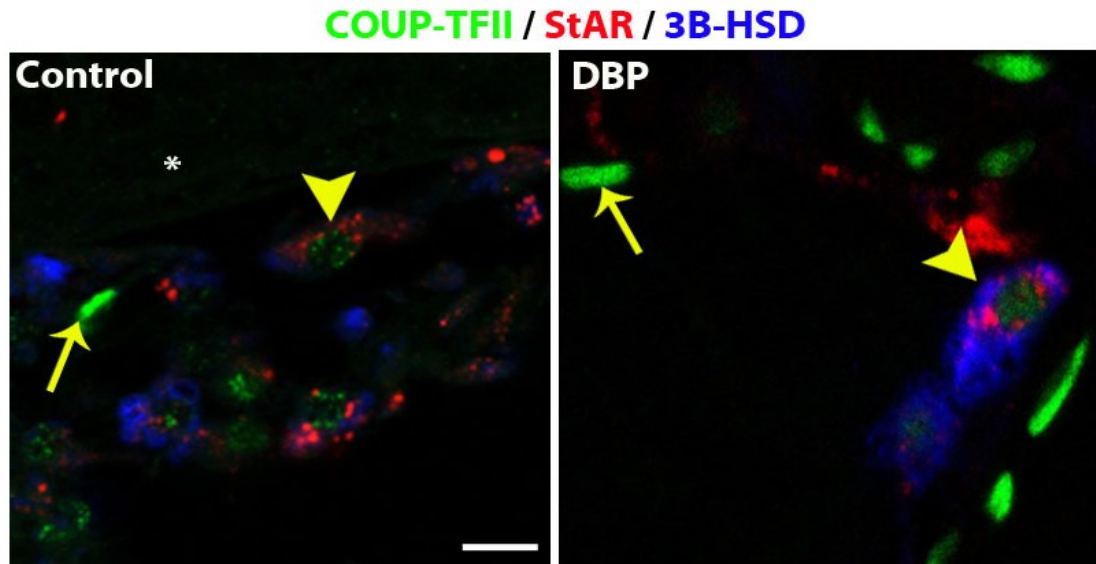


Figure 5.5 COUP-TFII⁺ adult Leydig cells express StAR and 3 β -HSD in adult control and DBP-exposed rats. COUP-TFII⁺ (green) adult Leydig cells express StAR (red) and 3 β -HSD (blue) in adulthood in both groups (arrowhead). COUP-TFII⁺/3 β -HSD^{neg}/StAR^{neg} adult Leydig progenitor cells are also evident (arrow). Scale bar =20 μ m.

5.3.3 Increased Histone 3 methylation in the proximal promoter region of *StAR*

These experiments aimed to investigate the hypothesis that a histone modification may result in repression of *StAR* transcription, as shown above (Figure 5.4). Specific primers for the proximal promoter region of *StAR* were designed and validated, as described earlier (Figure 5.2). This region is important for regulation of *StAR* (Sandhoff et al 1998, Silverman et al 1999, LaVoie et al 2005, Lee et al 2013) and is shown in the schematic (Figure 5.6). Chromatin immunoprecipitation (ChIP) was used to isolate the repressive histone, H3K27me3, prior to PCR being undertaken using primers specific to the proximal promoter region of *StAR*. This demonstrated a significant increase in the level of H3K27me3 within the promoter region of *StAR* in DBP-exposed animals, compared with controls (Figure 5.6). Gel electrophoresis of the PCR products was undertaken, which firstly indicated the presence of a single PCR product (as expected from the earlier primer validation) and, secondly, allowed for densitometric analysis, as in a recent study (Jia et al 2013). The findings from the ChIP assay were confirmed by densitometric analysis (Figure 5.6), thus

demonstrating increased levels of H3K27me3 in the *StAR* promoter region of DBP-exposed rats, compared with controls (Figure 5.6). Therefore, the next aim was to examine the immunoexpression of H3K27me3 in DBP-exposed animals compared with controls, to investigate if this histone modification was evident during fetal life and continued through to adulthood.

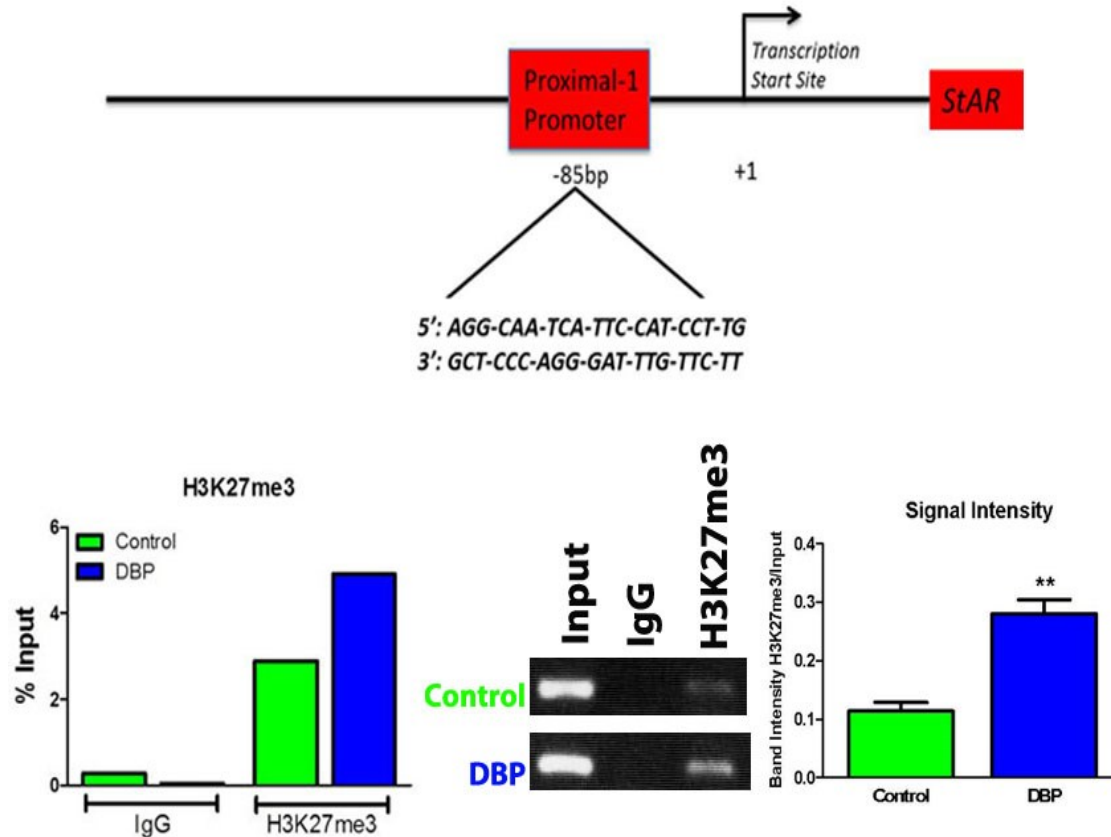


Figure 5.6 Increased methylation of histone 3 at lysine 27 (H3K27me3) in the proximal promoter of *StAR* in DBP-exposed rats. The schematic shows the region of the *StAR* promoter which was targeted during adulthood; 85bp upstream of the transcription start site, and amplified by PCR using primers as outlined (167bp in length). Levels of H3K27me3 were increased at the proximal promoter of *StAR*, as analyzed by ChIP and densitometric analysis of PCR products. Normal rabbit IgG was used as a negative control. Data were calculated using the percentage total genomic input method and shown as an average value (bottom left graph). The input DNA Ct value (i.e. DNA which did not undergo immunoprecipitation) was used to normalize the ChIP data in place of typical house-keeping gene values used to normalize real-time PCR (RT-PCR) reactions. The average threshold cycle (Ct) for triplicate assays was used in all subsequent calculations. Values are Means \pm SEM for n=3. Data were analysed using an unpaired t-test; **p<0.01, compared to (control) vehicle value.

5.3.4 Increased H3K27me3 in adult Leydig progenitor cells of DBP-exposed rats in fetal life

As fetally DBP-exposed animals in adulthood exhibit altered steroidogenesis, potentially as a result of increased histone repressor H3K27me3 at *StAR*, this posed the question as to whether this alteration was present in adult Leydig progenitor cells, during fetal life and manifested in adulthood in dysfunctional adult Leydig cells after their differentiation. To investigate this hypothesis, double immunofluorescence was carried out using an antibody against the repressive histone of interest (H3K27me3; red), and a marker of the adult Leydig progenitor cells (COUP-TFII⁺; green) in the rat at e21.5. In the control fetal testis, there was minimal H3K27me3 expression in the interstitium at this antibody dilution, whereas there was expression in the nuclei of adult Leydig progenitor cells in DBP-exposed animals, indicated by the yellow nuclear co-staining (yellow arrows) which is more apparent in the higher magnification image (Figure 5.7). Expression of H3K27me3 in Sertoli cells seemed to be more strongly expressed in DBP-exposed animals as compared with control animals, however this finding was not further investigated within this study (Figure 5.7). To ensure these findings were representative of increased methylation of histone 3 (H3) and not an increase in expression of the histone itself, single immunofluorescence was carried out on the same control and DBP-exposed animals using an antibody against H3. The immunohistochemistry experiments demonstrated clearly that there was no change in the expression of H3 between control and DBP-exposed animals at e21.5 in the rat (Figure 5.8).

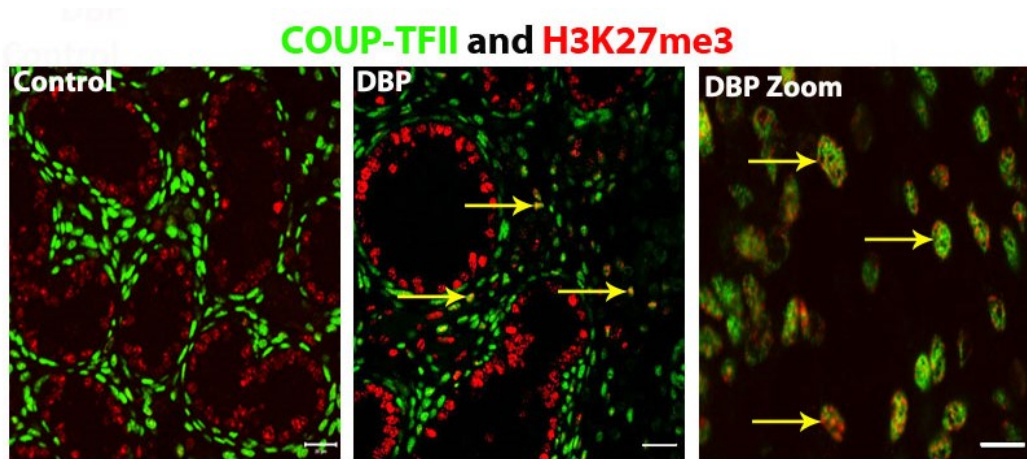


Figure 5.7 Increase in repressive H3K27me3 in adult Leydig progenitor cells of DBP-exposed rats at e21.5. A proportion of COUP-TFII⁺ adult Leydig progenitor cells co-express COUP-TFII (green) and H3K27me3 (red) in their nuclei (yellow arrows) in DBP-exposed rats at e21.5 (middle and zoom on right). In controls (left), H3K27me3 was absent from adult Leydig progenitor cells, at this antibody dilution and detected only in Sertoli cells. Scale bars = 20 μ m

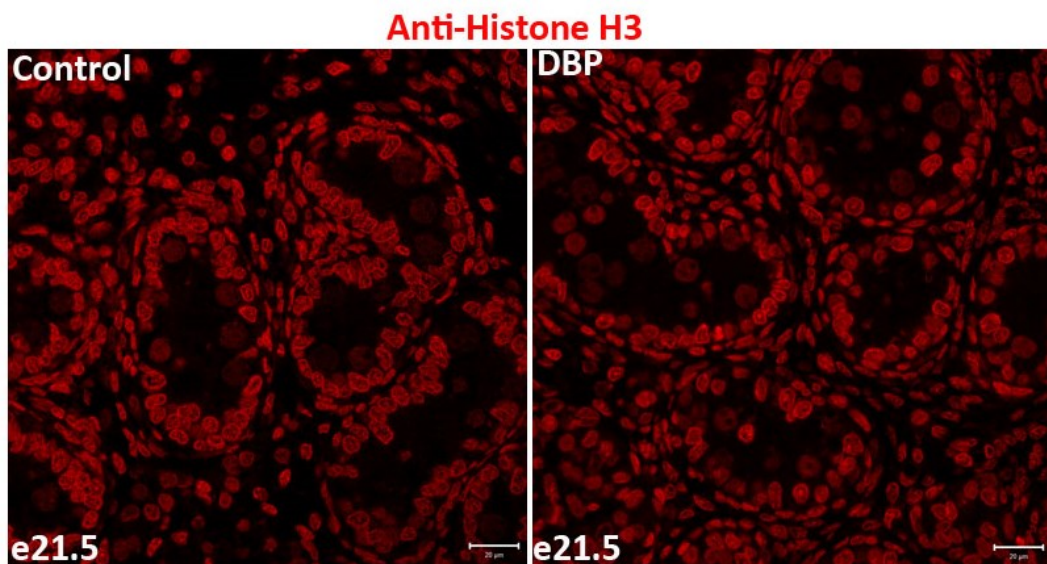


Figure 5.8 Expression of histone 3 at e21.5 in control and DBP-exposed rats. Histone 3 (red) is comparable in the testes of control (left) and DBP-exposed (right) rats at e21.5. Scale bars = 20 μ m.

5.3.5 Increased H3K27me3 in adult Leydig cells of DBP-exposed animals in postnatal Life

As the adult Leydig progenitor cells in DBP-exposed animals expressed increased levels of H3K27me3 compared with controls during fetal life, the next experiment aimed to investigate whether this was evident during puberty and adulthood when Leydig cells have differentiated. Double immunofluorescence was carried out using H3K27me3 (green) and 3 β -HSD (red) on pnd25 and pnd90 testes from control and DBP-exposed rats. DAPI (blue) was used as a nuclear counterstain. These findings demonstrated that at puberty (top right) and in adulthood (bottom right), adult Leydig cells (3 β -HSD⁺; red cytoplasm) clearly expressed higher levels of H3K27me3 (green nuclei; arrows) in DBP-exposed rats compared with their age-matched controls (left panels) (Figure 5.9).

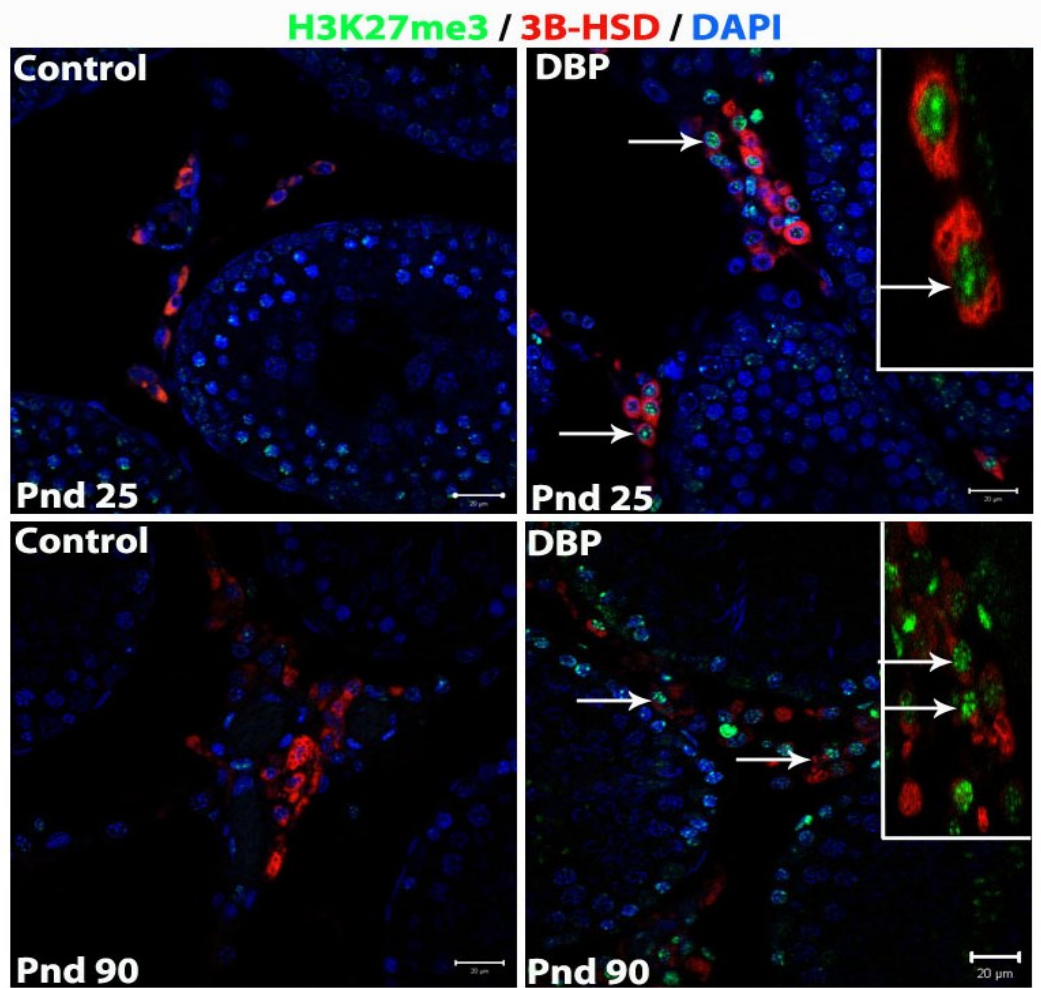


Figure 5.9 Increase in repressive H3K27me3 in adult Leydig cells of DBP-exposed rats. During puberty (Pnd25) and in adulthood (Pnd 90), adult Leydig cells (3 β -HSD⁺; red cytoplasmic staining) in DBP-exposed rats expressed higher levels of H3K27me3 in their nuclei (green; white arrow) compared to their age-matched controls in which it was minimal/absent (left panels), at this antibody dilution. Scale bars = 20 μ m.

As before, to ensure these findings were representative of increased methylation of histone 3 (H3) and not an increase in expression of the histone itself, single immunofluorescence was carried out on the same control and DBP-exposed animals using an antibody against H3. The level of H3 was comparable between control and DBP-treated animals at pnd25 and 90 in the rat (Figure 5.10).

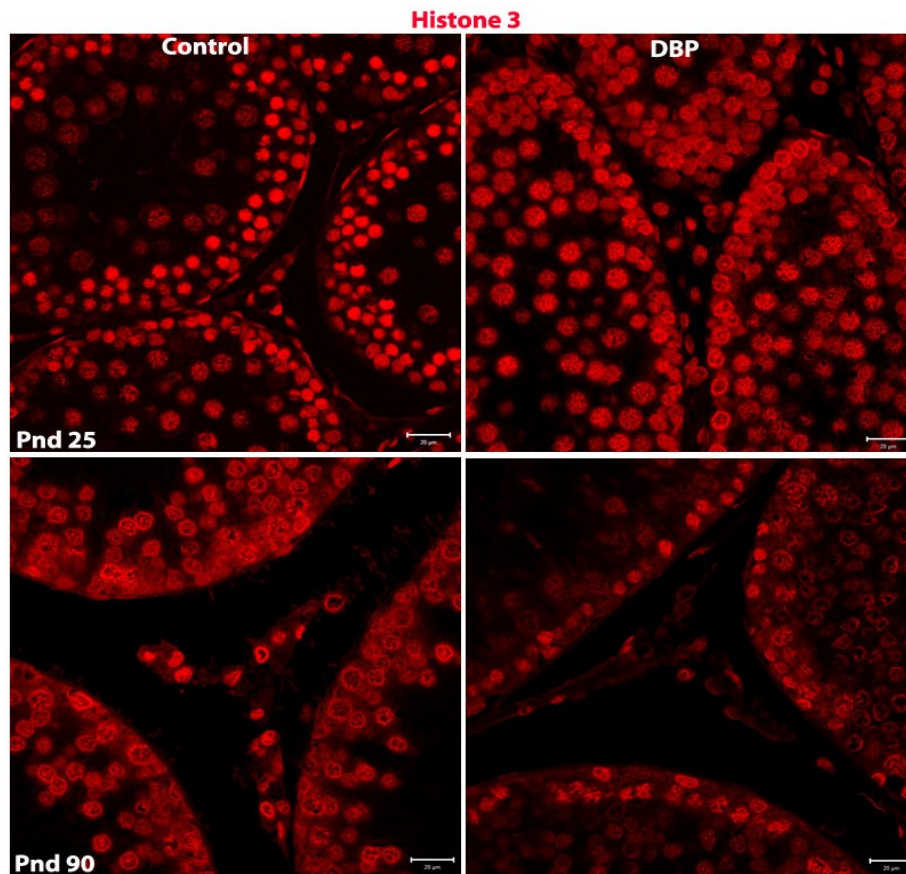


Figure 5.10 Expression of histone 3 in the testis of pnd 25 and pnd 90 rats. Histone 3 (red) is comparable in the testes of control (left) and DBP-exposed (right) rats at pnd 25 (top panel) and pnd 90 (bottom panel). Scale bars=20 μ m.

5.3.6 H3k4me3 is absent in DBP-exposed animals in adulthood

Promoters of genes marked only with H3K27me3 are "stably repressed" (Bernstein et al 2007, Mikkelsen et al 2007) while with H3K4me3 are termed 'active' (Lee et al 2006). Therefore to confirm that the histone modifications in the interstitial area were indicative of transcriptional repressive marks, and not active (H3K4me3) in DBP-exposed animals, double immunofluorescence was carried out using COUP-TFII (green) and H3K4me3 (red) on adult rat testes from control and DBP-exposed animals. Staining for H3K4me3 in COUP-TFII⁺ adult Leydig progenitor cells and in the interstitial area itself was minimal in control and absent in DBP-exposed animals in adulthood (Figure 5.11). This fits with the above findings (Figure 5.9) that an increase only in H3K27me3, in DBP-exposed animals, provides evidence for transcriptional repression.

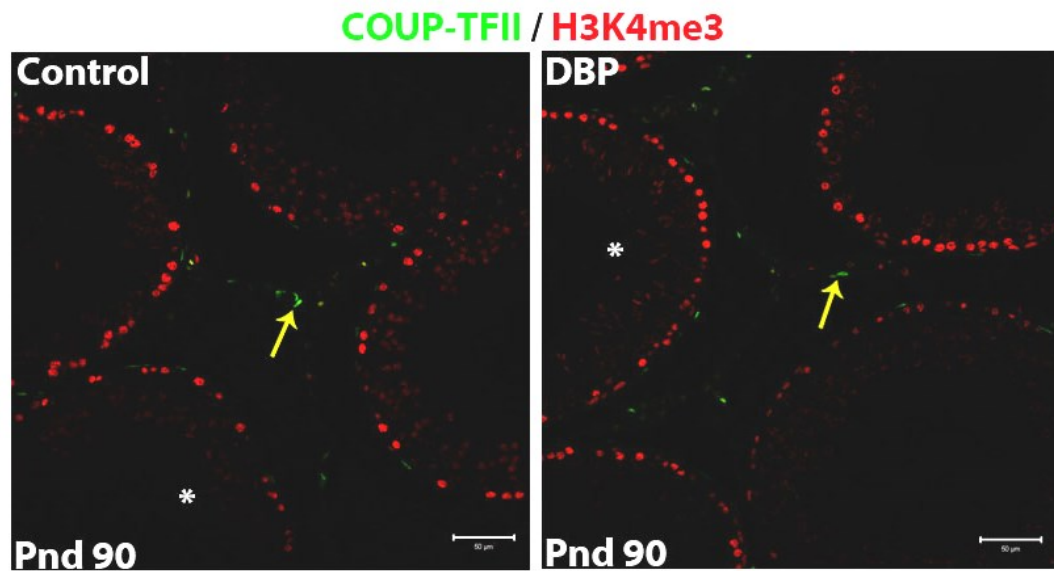


Figure 5.11 Absence of H3K4me3 staining in the interstitium of DBP-exposed rats. H3K4me3 (red), a marker of active gene transcription is absent in COUP-TFII⁺ adult Leydig progenitor cells (green; arrows) and the interstitial area of DBP-exposed adult rats while is minimal in the interstitium of controls. Expression of H3K4me3 is comparable in Sertoli cells between control and DBP-exposed rats. Scale bars = 50µm.

5.4 Discussion

This chapter aimed to investigate a mechanism to potentially explain how adult Leydig cell dysfunction occurs as a result of fetal androgen deficiency. Following DBP-exposure to lower fetal testosterone, adult rats showed evidence for 'compensated Leydig cell failure' i.e. low testosterone with increased LH, as in the previous chapter. This chapter demonstrates how this might occur via altered Leydig-cell specific gene expression in the steroidogenic pathway, as shown by reduced *3 β -HSD* and *StAR* expression. More specifically, histone modifications (increased tri-methylation of lysine 27 at histone 3 (H3K27me3) at the proximal promoter region of *StAR*, represses its transcription resulting in altered steroidogenesis and lowered testosterone in adulthood. The adult Leydig progenitor cells expressed increased H3K27me3 during fetal life, as did adult Leydig cells in DBP-exposed animals during postnatal life, compared with controls. Thus the epigenetic changes (programmed histone modifications) to adult Leydig progenitor cells may potentially affect adult Leydig cell function via their differentiation from 'programmed' progenitor cells.

Leydig cell-specific genes in the steroidogenic pathway were investigated in adulthood following fetal testosterone reduction (DBP-exposure). As some DBP-exposed animals were cryptorchid, this meant that Leydig cell mRNA may be over-represented due to the depletion of germ cells in cryptorchid testes. However, as Sertoli cell number is unchanged between control and DBP-exposed animals (scrotal or cryptorchid testes) (Hutchinson et al 2008), this provided a control parameter i.e. the use of a Sertoli cell marker (*Sox9*), to control for target gene expression. The mRNA expression of *StAR* and *3 β -HSD* were significantly reduced in DBP-exposed animals as compared with controls, while there was no significant change to the expression of *Lhr*, *Cyp11a1*, *Cyp-17a1* or *17 β -HSD3*. A more recent study that investigated the effects in adulthood after fetal DBP-exposure demonstrated 'compensated Leydig cell failure', (evident from decreased testosterone and increased LH levels) and a reduction in the enzymatic activity of *3 β -HSD* and *17 β -HSD* (Giribabu et al 2012), which suggests reduced steroidogenesis. However, the authors did not

examine mRNA expression levels of steroidogenic genes, as in the present study. Given the significant reduction in the rate-limiting step of steroidogenesis, *StAR*, which is crucial for transportation of the steroid hormone precursor cholesterol, to the inner mitochondrial membrane (Manna et al 2009, Papadopoulos and Miller 2012), it was chosen for further investigation in the present study.

To investigate whether histone modifications were one potential mechanism for *StAR* repression, a ChIP assay followed by quantitative PCR was carried out, and these results were further confirmed by densitometric analysis of the purified DNA after gel electrophoresis. The proximal promoter region contains crucial factors for regulating *StAR*, including transcription factors and histone modifications (Sugawara et al 1997, Silverman et al 1999, Christenson et al 2001, Hiroi et al 2004, Lee et al 2013) which is conserved across species (Manna et al 2009), demonstrating its importance in transcriptional regulation. These experiments demonstrated increased tri-methylation of lysine 27 at histone 3 (H3K27me3) in the proximal promoter region of *StAR* in DBP-exposed animals, in comparison with control animals. This suggests that histone modifications to *StAR* in DBP-exposed animals might be one potential mechanism via which *StAR* expression is repressed, which could result in reduced steroidogenesis.

In a recent study investigating the mechanisms involved in down-regulation of *inhibin a* (Meldi et al 2012) after the normal LH surge during ovulation (Woodruff et al 1989), the authors demonstrated an increase in repressive H3K27me3 and H3K9me3 in the proximal promoter of *inhibin a* which corresponded with its repression. This demonstrates a role for H3K27me3 in gene regulation and repression. More relevantly in relation to the *StAR* gene, there are limited studies to date on histone modifications regulating its transcription, with the majority of research based on female tissue. One such study investigated *StAR* regulation in granulosa cells (Christenson et al 2001) which is normally activated by the mid-cycle LH surge (Kiriakidou et al 1996), which the authors found, amongst other factors, occurred as a result of increased acetylation of histone 3 (H3) in the proximal promoter of *StAR* (Christenson et al 2001). In other recent studies investigating *StAR* regulation,

its transcription was induced by hCG injection in granulosa cells (Lee et al 2013) or by exposure of MA-10 cells to 8-Br-cAMP (Hiroi et al 2004), and in both cases, increased *StAR* transcription was associated with a reduction in H3K27me3 in the proximal promoter region of *StAR*. Combining the previous studies with the current findings in this thesis, suggests that histone modifications, mainly an increase in H3K27me3 at the proximal promoter region plays a key role in regulation of the *StAR* gene resulting in its repression. This would explain the reduction in *StAR* transcription in DBP-exposed animals, when compared with controls.

Methylation marks, particularly H3K27me3, have been suggested to stably function as a true epigenetic signal (Trojer and Reinberg 2006) as it is inherited by daughter cells after cell division (Hansen et al 2008, Margueron et al 2009, Walker et al 2011). In DBP-exposed animals, the increased immunoexpression of repressive H3K27me3 in adult Leydig cells (coinciding with an absence of activating H3K4me3) in adulthood and in their progenitors during fetal life, suggests that adult Leydig cells may have 'inherited' the programmed H3K27me3-induced modification of the *StAR* promoter from their progenitor cells, despite the huge proliferative changes that occur in the progenitor cells pre- and post-natally.

These findings also suggest an altered responsiveness of the *StAR* gene, as its mRNA expression is usually positively regulated by LH, and normally induces increased mRNA expression levels (Clark et al 1995, Lee et al 2013). In contrast, in DBP-exposed animals which had significantly increased LH blood levels (previous chapter) and normal *Lhr* expression (this chapter), a decrease rather than an increase, in *StAR* mRNA expression was found. This could be explained by the increased H3K27me3 altering its regulation/responsiveness.

Histone modifications to the distal region of the *StAR* promoter were not investigated in this study, mainly due to technical issues in validating the primers designed for this region. This seems irrelevant given the significance of the proximal promoter region in *StAR* regulation, as above, but also previous

studies have shown that the distal promoter may not be as important for regulation of *StAR*, at least in relation to histone modifications (Hiroi et al 2004, Lee et al 2013). Furthermore, the distribution of H3K27me3 in 512 genes in the mouse was localised close to the transcription start site (Boyer et al 2006), which highlights a role for H3K27me3 in transcriptional regulation at the proximal promoter region.

To further investigate the mechanism of *StAR* repression via increased H3K27me3, it would be interesting to examine precisely how trimethylation of Lysine 27 on histone 3 (H3K27me3) is mediated. Several studies have shown that the polycomb-repressive complex 2 (PRC2) plays a role, which is comprised of histone methyltransferases including enhancer of zeste (Ezh1/2) (Margueron et al 2008, 2009, Cao et al 2002, Muller et al 2002, Shen et al 2008) catalysing the methylation of H3K27 (Cao and Zhang 2004, Schuettengruber et al 2007). It could thus be insightful to investigate whether *StAR* transcription could be increased in DBP-exposed animals with the administration of a specific histone methyltransferase inhibitor to prevent trimethylation of lysine 27 at histone 3 (H3K27me3) e.g. adenosine dialdehyde (Adox) or 3-deazaneplanocin A (DZNep), as previously shown (Miranda et al 2009). Lysine-specific demethylase (LSD1) removes repressive lysine-methylation in favour of gene transcription, as shown by LSD knockdown resulting in a decrease in transcriptional activation of the androgen receptor gene (Metzger et al 2005). Specific demethylases of H3K27 include UTX and JMJD3 (Agger et al 2007, De Santa et al 2007, Lan et al 2007 and Lee et al 2007) which may prove useful for future investigations into removing histone repression of gene transcription.

This study is the first, to my knowledge, to investigate histone modifications to the proximal promoter of *StAR* in testis tissue and highlights their importance in altering the chromatin structure of promoters to impede/assist gene transcription. The findings in this thesis fit with the hypothesis that disorders/abnormalities such as adult Leydig cell dysfunction in the human (Andersson et al 2004, Eisenberg et al 2012) have a common fetal cause (Sharpe and Skakkebaek et al 2008). Importantly, these studies add a new layer to the

current understanding of fetal programming by potentially providing one mechanism of how compensated adult Leydig cell failure can be programmed as the result of histone methylation at the proximal promoter of *StAR*. Although there are other factors, epigenetic or otherwise, which might be involved in this process, these findings demonstrate one way in which fetal androgen deficits can adversely programme adult Leydig cell dysfunction via their progenitor cells.

Chapter 6 Final Discussion

The experimental work in this thesis mainly stemmed from the TDS hypothesis, which suggests that common male reproductive disorders of newborn boys and young men, have a common fetal origin, namely fetal androgen deficiency (Skakkebaek et al 2001). Elucidating how this occurs, might help to address the topical issue of hypogonadism in aging men, which is associated with 'Western disorders', morbidity and mortality (Tivesten et al 2009, Traish et al 2011). Data from human and experimental animal studies have established that androgen exposure can programme adult testosterone levels, particularly if exposure occurs within the masculinisation programming window (Welsh et al 2008, Dean & Sharpe 2013). But the mechanism for how this occurs, has been unknown, as adult Leydig cells do not differentiate until puberty. Thus, the main hypothesis underpinning this thesis, was that fetal androgen deficiency can programme adult testosterone levels, via effects on adult Leydig progenitor cells, which are present in the fetal testis. Consequently, the main aims were firstly to identify/characterise adult Leydig progenitor cells in an adult Leydig cell ablation/regeneration model and throughout normal pubertal and adult Leydig cell development. Secondly, to determine if adult Leydig progenitor cells are conserved across species in the fetal testis. Thirdly, to establish whether adult Leydig progenitor cells are androgen-modulated via several androgen receptor knockout models and altered testosterone production/action rodent models. Fourthly, to investigate potential 'gain of function' models (in terms of adult Leydig progenitor numbers and/or adult Leydig cell function). Finally, to investigate a mechanism of fetal 'programming' of adult Leydig progenitor cells, which may explain how fetal events programme adult testosterone levels/Leydig cell function.

The presence of adult Leydig stem/progenitor cells in the fetal testis has long been a source of intrigue for researchers, as a common marker for these cells has not been identified. More recent studies have identified and isolated cells at pnd7/8 in the mouse, which expressed PDGFR α , GATA4, LIFR, c-KIT and were capable of differentiating into steroidogenic cells in the presence of

differentiation-inducing medium *in vitro*, and in an adult Leydig regeneration model *in vivo* (Ge et al 2006, Landreh et al 2013). However, these studies did not examine whether the 'Leydig stem/progenitor cells' were present during fetal life, which this study addresses. The current findings demonstrated that COUP-TFII⁺ adult Leydig progenitor cells, present in the fetal testis, differentiate into adult Leydig cells during puberty. This was also evident in the EDS model which recapitulates normal adult Leydig cell development via adult Leydig cell ablation/regeneration. Similar to the aforementioned published studies, COUP-TFII⁺ adult Leydig progenitor cells expressed GATA4 during their differentiation into adult Leydig cells during puberty and in the EDS model. Furthermore, COUP-TFII⁺ adult Leydig progenitor cells express AR, and these non-steroidogenic COUP-TFII⁺/AR⁺ cells were shown to be conserved across species in the fetal testis (Chapter 3).

An interesting study, following on from the identification of these cells, would be to establish which other factors regulate adult Leydig cell differentiation. Some of the key players include LIFR, c-KIT and PDGFR α , which have been reported to mark putative 'Leydig stem cells' (Ge et al 2006, Landreh et al 2013). Various attempts by me, to localise LIFR in the rat testis failed, most likely due to the non-specificity of the antibody used. Future experiments are therefore needed to investigate whether COUP-TFII⁺ adult Leydig progenitor cells express LIFR. A more recent study demonstrated that PDGFR α ⁺ cells isolated from pnd 8 rat testis, which belong to the peritubular myoid cell (PTC) lineage, expressed pluripotency markers and also expressed genes encoding steroidogenic enzymes *in vitro* (Landreh et al 2013). The present study also investigated PDGFR α expression in COUP-TFII⁺ adult Leydig progenitor cells, in fetal life (e21.5) and during adult Leydig cell development one week post-EDS in the rat, which yielded equivocal results. Some, but not all, COUP-TFII⁺ adult Leydig progenitor cells appeared to express PDGFR α , but there was large variation in PDGFR α expression between samples. Pre-absorption of the PDGFR α antibody used in this study, demonstrated that the antibody was specific, even though apparent non-specific interstitial background staining, was

still evident in samples. Given the fact that the previous studies have isolated PDGFR α ⁺ 'adult Leydig stem cells' just prior to or during the onset of puberty in the mouse at pnd 7/8, this may suggest that PDGFR α is increased in expression specifically during this time, in comparison to fetal life. It is interesting therefore to speculate that the majority of COUP-TFII⁺ adult Leydig progenitor cells only begin to express PDGFR α just prior to the onset of puberty. In line with this, it might also be possible that more COUP-TFII⁺ adult Leydig progenitor cells expressed PDGFR α immediately post-EDS, as PDGFR α expression was only examined one week post-EDS, in this study. In line with this, an earlier study investigating gene expression post-EDS, demonstrated that mRNA levels of *pdgfra* increased dramatically and transiently one day post-EDS (O'Shaughnessy et al 2008). Future experiments to investigate PDGFR α expression using different PDGFR α antibodies and different time-points are needed to fully elucidate the role of PDGFR α in adult Leydig cell development.

The precise structure or ligand regulation of COUP-TFII has not yet been elucidated. A previous study investigated the conformational status of COUP-TFII and described it as having an 'autorepressed conformation', as the activation binding sites are inaccessible due to its folded conformation (Kruse et al 2008). Retinoic acid, a vitamin A metabolite, can alter this conformational state thus promoting COUP-TFII to recruit co-activators and increase its transcriptional activity (Kruse et al 2008). This is intriguing as retinoic acid has been reported to play a role in adult Leydig cell development, as described below. Male offspring of pregnant dams who were fed a vitamin A deficient diet, were reported to have reduced testosterone production by fetal Leydig cells (Livera et al 2004). This study showed that during fetal Leydig cell differentiation, retinoic acid negatively regulated steroidogenesis, potentially via down regulation of *Cyp17a1*, as demonstrated in cultured cells from fetal rat testes (Livera et al 2004). The role for retinoic acid in negatively regulating steroidogenesis during fetal Leydig cell differentiation (Livera et al 2004) appears somewhat similar to COUP-TFII, which has also been proposed to negatively regulate steroidogenesis in differentiating fetal Leydig cells (van den

Driesche et al 2012). Of the six retinoic acid receptors, NR2B1 in particular is expressed in adult Leydig cells (Boulogne et al 1999). Taking the latter fact into account and the role for retinoic acid in altering the conformational state of COUP-TFII (Kruse et al 2008), might suggest that retinoic acid could potentially play a role in the onset of differentiation of adult Leydig progenitor cells via effects on COUP-TFII. The precise role and interplay between these factors postnatally, regarding adult Leydig progenitor cells differentiation, would be an interesting path to investigate.

Androgen effects might be mediated in part by the control of retinoic acid concentrations (Eacker et al 2007, O'Shaughnessy et al 2007). In the latter study, androgen regulated genes were investigated in a large study comparing transcript levels between control and ARKO mice. Interestingly, 50% of downregulated 'androgen genes' in ARKOs, were associated with vitamin A metabolism, amongst other factors (O'Shaughnessy et al 2007). Upregulation of these genes by testosterone administration to *hpg* mice confirmed their regulation by androgen (O'Shaughnessy et al 2007). This study suggests a link between androgen action and retinoic acid action (Eacker et al 2007, O'Shaughnessy et al 2007). Even more interesting is that these genes were not identified in SCARKO mice, which suggests that Sertoli cells are not the main target cells for androgen stimulated upregulation of genes involved in vitamin A metabolism. It is tempting to speculate that this could occur via adult Leydig progenitor cells, as AR is still functioning in the latter cells in SCARKOs. This might further tie together retinoic acid and COUP-TFII⁺ adult Leydig progenitor cells, in regulating adult Leydig cell development.

Insulin-like growth factor 1 (IGF1) enters the testis via the systemic bloodstream and is also produced within the testis by both Sertoli and Leydig cells, under the control of gonadotrophins (Smith and Walker 2014). Its receptor (IGF1R) is found on Leydig, Sertoli, PTM and germ cells (Yagci and Zik 2006, Froment et al 2007), and also in progenitor and immature Leydig cells (Hu et al 2010). Administration of a specific inhibitor of IGF1R (picropodophyllin), resulted in a blockage of the anti-apoptotic effect of IGF1,

during Leydig cell development, which highlights the role of IGF1 in Leydig cell survival (Colon et al 2007). IGF-1 treatment of cultured rat Leydig cells in vitro from pubertal and adult animals demonstrated an increase in intracellular steroidogenesis and testosterone production, with a greater response from the pubertal cells than adult, suggesting a role for IGF-1 in Leydig cell maturation (Gelber et al 1992). IGF-null mice exhibit a reduction in adult Leydig cell number, a reduction in the proliferative labelling index of progenitor and immature adult Leydig cells, and potentially as a consequence, an 80% reduction in serum testosterone levels, in comparison to wild-type controls (Baker et al 1996, Wang et al 2003, Hu et al 2010). The deficit in proliferative labelling index in progenitor and immature Leydig cells could be rescued by recombinant IGF-1 (Hu et al 2010). Interestingly, this deficit in proliferative capacity was not evident in 'stem' Leydig cells, in which proliferation was comparable to controls. This might suggest that during postnatal life, IGF1 is required for the postnatal proliferation of adult Leydig progenitor cells. The expression of IGF1 was not examined in this study, but it would be worthwhile to investigate whether COUP-TFII⁺ adult Leydig progenitor cells begin to express IGF during their proliferation in puberty. Also, IGF-II has previously been shown to regulate steroidogenesis in rat Leydig cells (Colon et al 2005). Whether or not IGFII can increase adult Leydig progenitor cell proliferation, via IGF1R, would also be worth investigating.

Desert hedgehog (Dhh)-null mice exhibit a failure of adult Leydig cell development (Clark et al 2000), which highlights a role for Dhh in adult Leydig cell development. Sonic, a hedgehog family member, acts via a common pathway to Dhh (Jenkins, 2009), and has been reported to regulate the expression of COUP-TFII in neurons (Krishnan et al 1997). Thus, it could be plausible that Dhh could act on COUP-TFII in the testis, to regulate differentiation of the COUP-TFII⁺ adult Leydig progenitor cells into adult Leydig cells (Qin et al 2008). This merits further investigation in future experiments.

Previous studies have reported an important role of thyroid hormone (thyrotrophin) in adult Leydig cell development (Rijntjes et al 2009, 2010). An

isoform of thyroid hormone receptor (TR α 1), was found abundantly expressed in unidentified spindle shaped interstitial cells in the developing mouse testis (Pnd 0-5) and in adult testis (Pnd 63) (Buzzard et al 2000). It would therefore be interesting to investigate whether TR α 1 is expressed in COUP-TFII⁺ adult Leydig progenitor cells in which thyrotrophin could bind to and potentially play a role in their differentiation into adult Leydig cells.

Studies that investigated adult Leydig cell development in ARKO males have demonstrated that androgens are required for adult Leydig cell development, as evident from the 80% reduction in cell number (De Gendt et al 2005, O'Shaughnessy et al 2002, 2012). Quantification of adult Leydig cell number in the ARKO mouse, confirmed the previous findings, and demonstrated that in the complete absence of androgen action, adult Leydig cells were significantly reduced by ~70% (Chapter 3). Furthermore, the current findings have added a new dimension to previous understanding, in that adult Leydig progenitor cells were also reduced in number as a result of ablating androgen action. Fetal exposure to DBP, results in a reduction in testosterone production (Mylchreest et al 2000, Fisher et al 2003, van den Driesche et al 2012), which the present data confirmed. Similar to ARKOs, fetal androgen deficiency via DBP-exposure, resulted in a ~40% reduction in adult Leydig progenitor cell number throughout development. There was evidence for compensated adult Leydig cell failure in both ARKO and DBP-exposed animals, which highlights the importance of androgen action during fetal development, rather than postnatal, for adult Leydig cell function (Chapter 4).

Several attempts were made in this study, to investigate whether or not exogenous fetal androgen exposure could potentially increase intratesticular testosterone levels during fetal development and thus provide a "gain of function" model (in terms of adult Leydig progenitor cells numbers and or adult Leydig cell function) (Chapter 4). Fetal androgen exposure (TP/DHT), did not appear to have any enhancing effect on the number of adult Leydig progenitor cells, potentially due to the fact that intratesticular androgens are already high enough in the fetal testis to induce maximal androgen effects locally, and thus

cannot be added to. Lack of key data in the iNOS^{-/-} model from collaborators, hindered any interpretation of the findings thus far. An earlier iNOS^{-/-} study, reported that adult Leydig cell cytoplasmic volume was reduced (Auharek et al 2012), which suggests that steroidogenesis is affected by knockout of iNOS^{-/-}. In fact, preliminary data from our collaborators have reported that steroidogenic genes (e.g. *StAR*) are downregulated in iNOS^{-/-} mice, which taken together, might suggest that this is not a good model for the purpose of the studies described in this thesis. Irrespective of this, measurement of adult hormone levels, are crucial before any interpretation can be made regarding adult Leydig cell function in the iNOS^{-/-} mouse.

Fetal DBP-exposure resulted in compensated adult Leydig cell failure (Chapter 4), and a reduction in *StAR* expression, potentially due to increased expression of histone repressor H3K27me3 in the proximal promoter region (Chapter 5). Adult Leydig progenitor cells at e21.5 and adult Leydig cells in adulthood, both expressed increased H3K27me3 expression, in comparison with wild-type controls (Chapter 5). What further ties these two cell types together, is the fact that H3K27me3 is inherited by daughter cells after cell division (Hansen et al 2008, Margueron et al 2009, Walker et al 2011). It would be interesting to investigate whether other genes involved in steroidogenesis e.g. *3β-HSD*, also displayed increased repressive histone methylation, as *3β-HSD* mRNA levels were also decreased following fetal DBP exposure in this study (Chapter 5).

To confirm that the increased repressive H3K27me3 mark in DBP exposed animals was not simply a DBP effect, but rather a result of fetal androgen deficiency, immunolocalisation of H3K27me3 in adult ARKOs would have been useful. In fact, this experiment was carried out during the completion of this thesis and demonstrated increased H3K27me3 expression in adult Leydig cells in ARKOs. This demonstrates that increased repressive histone methylation in adult Leydig cells, is evident in both fetal androgen deficient models (ARKO and DBP-exposed), which potentially provides a mechanism for how fetal events can result in adult Leydig cell dysfunction.

The next logical step to advance the previous findings, would be to establish how the androgen-driven epigenetic change in adult Leydig progenitor cells is mediated e.g. trimethylation of H3K27. The histone methyltransferase enhancer of zeste (Ezh1/2) has been reported to catalyse the methylation of H3K27 (Cao and Zhang 2004, Schuettengruber et al 2007), which would be worth investigating. Another demethylase, namely Tet1, has been shown to be a regulator of H3K27me3 (Sui et al 2012). Preliminary studies within the lab indicate that COUP-TFII⁺ adult Leydig progenitor cells also express Tet1 at e21.5 in the rat. Furthermore, a microarray analysis would also prove beneficial to compare the expression profiles of adult Leydig cell progenitors from control and DBP-exposed fetuses, which might reveal androgen target genes and/or identify potential new markers of adult Leydig progenitor cells. This would require the development of methods for the isolation of a highly enriched population of the adult Leydig progenitor cells.

DBP treatment to lower fetal androgen production was administered to animals during fetal life, particularly between e15.5-e21.5, which encompassed the MPW (Welsh et al 2008), and resulted in adult Leydig cell dysfunction (Chapter 4). It would be interesting to investigate whether DBP-exposure prior to or after the MPW, results in compensated adult Leydig cell failure, in order to determine the importance of this fetal period in determining long-term function of adult Leydig cells. Preliminary studies within the lab following the completion of this thesis, have demonstrated that fetal DBP-induced androgen suppression only within the MPW, causes compensated adult Leydig cell failure in adulthood. It might also be worth investigating whether lower doses of DBP exposure, which would result in a more subtle reduction of fetal testosterone production, might also affect adult Leydig progenitor cell number and adult Leydig cell function.

Transplantation of COUP-TFII⁺ adult Leydig progenitor cells into DBP-exposed animals prior to puberty would be an intriguing experiment to investigate whether this could rescue adult Leydig dysfunction via differentiation from the transplanted cells. Of course, problematic issues would need to be addressed first, i.e. specific COUP-TFII⁺ adult Leydig progenitor cell isolation, along with

the potential rejection of the transplanted cells in the animal. Perhaps isolation of adult Leydig progenitor cells could be successfully achieved during fetal life, as this is when the cells are most plentiful in the testis.

An earlier clinical study that investigated male infertility, reported that a portion of infertile men who had a previous history of cryptorchidism, were associated with poorer adult Leydig cell dysfunction and a lower sperm quality, in comparison to those without a history of cryptorchidism (Andersson et al 2004). More recently, a study from close collaborators (in press), has reported that in a large-scale investigation of male fertility in 3,000 young men from the normal population, 15% of those who had low sperm counts, also exhibited compensated adult Leydig cell failure, which is consistent with the data presented in this thesis and the previous study (Andersson et al 2004). This strengthens the hypothesis that adult Leydig cell dysfunction is associated with other TDS disorders, as a result of fetal androgen deficiency (Figure 6.1).

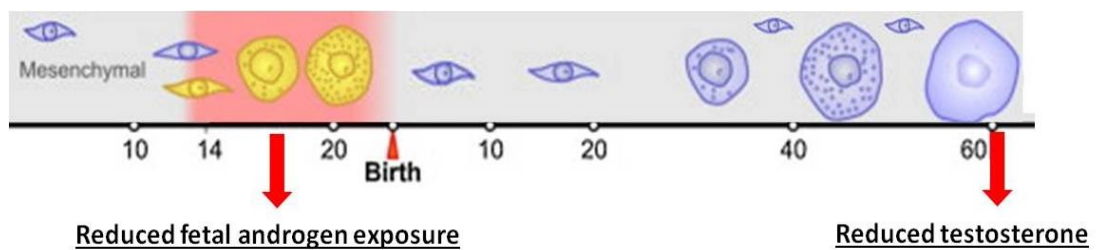


Figure 6.1 Fetal androgen deficiency results in adult Leydig cell dysfunction, as shown in the rat. Red shading depicts the period of reduced fetal androgen exposure (via DBP). Blue spindle-shaped cells represent adult Leydig stem/progenitor cells present during fetal and postnatal life. Larger round cells represent adult Leydig cells during their stages of differentiation into adult Leydig cells. Yellow cells represent the separate generation of fetal Leydig cells. (Adapted from Martinez-Arguelles et al 2013).

The current findings add a new dimension to the substantial body of evidence in that fetal androgen deficiency leads to a range of adverse changes in reproductive function and disorders in boys/men (Sharpe and Shakkebaek 2008, Dean & Sharpe 2013). It also has wider health implications in relation to the aging related decline in testosterone levels (Travison et al 2007, Tajar et al 2010, Traish et al 2011, Finkelstein et al 2013).

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