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Androgens and the Masculinisation Programming Window

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For my Mum and Dad

Thank you for allowing me to follow my heart and encouraging me to pursue my dreams

Declaration

The studies described in this thesis is the sole work of the author, except where acknowledgment 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.

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Bismillah ir-Rahman ir-Rahim

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Abstract

The commonest reproductive disorders of young men (namely low sperm counts, testicular germ cell cancer) may originate in fetal life similar to established disorders (cryptorchidism, hypospadias) that manifest at birth. These disorders are interlinked and may comprise a testicular dysgenesis syndrome (TDS), a concept supported by animal model studies. The latter have identified the likely time-frame within which TDS disorders may be induced, namely within the so-called masculinisation programming window (MPW). During this critical period, sufficient testosterone (androgen) must be produced by the fetal testis to program the male reproductive tract so that it will differentiate and grow normally after the MPW. Impaired androgen production or action within the MPW can result in smaller reproductive organs and their abnormal formation and function (e.g. cryptorchidism, hypospadias).

The MPW is thus of fundamental importance in determining normal, or abnormal, male reproductive development and function for later life. There are two big unanswered questions about the MPW. First, what determines its timing? Second, what mechanisms are controlled by androgens specifically within this time-window and not at later time points? Three approaches were undertaken to address the first question experimentally in rats. First, investigation of whether the availability of androgens and or androgen receptors (AR) plays a role in determining the onset or 'opening' of the MPW. Second, investigation of whether the expression of AR co-regulators was a factor in determining androgen sensitivity during the MPW. Third, investigation of whether prostaglandins played a role in mediating androgen action in the MPW, as studies in the 1980s had suggested this possibility. To address what mechanisms are controlled by androgens specifically within the MPW, the expression of selected genes in the genital tubercle was investigated before, during and after the MPW in fetuses that had been exposed to treatments that modulated androgen action. Selection of genes was based on microarray studies and data reported in the literature (ie candidate genes).

The studies reported in this thesis show that neither availability of androgens nor the AR are important in determining onset of the MPW, and providing exogenous androgens either prior to or during the MPW does not advance or enhance masculinisation. These studies also showed that females may have a slightly different window of susceptibility to androgen action than do males. Key AR co-regulators have been characterized in the male reproductive tract for the first time, two of which (BRG1, CBP) show changes in expression through development of the testis consistent with a role in Sertoli cells. Another AR co-regulator, RWDD1, was found to switch off in the absence of androgen action in the genital tubercle, pointing to a potential role during and/or after the MPW. Studies involving gestational exposure to indomethacin (a compound which inhibits prostaglandin synthesis) during the MPW showed no detectable effect on masculinisation. Finally, evaluation of candidate genes for mediating androgen action in the genital tubercle during the MPW, failed to identify their key involvement, thus they are unlikely to be involved in penis development and disorders such as hypospadias.

Presentations relating to thesis

Poster Presentation at 5th Workshop on Endocrine Disruptors 2011, Copenhagen, Denmark: ***'Molecular mechanisms of androgen induced programming of masculinisation in the fetal rat genital tubercle'.***

Oral Presentation at Testis workshop, 2010, Elba, Italy: ***'Regulation and importance of the masculinisation programming window.'***

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Abbreviations

Abbreviation	Definition
µg	Microgram
3β-HSD	3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase
17β-HSD	17β-hydroxysteroid dehydrogenases
AF1	Activation function 1
AF2	Activation function 2
AGD	Anogenital distance
AMH	Anti-Müllerian hormone
AR	Androgen receptor
ARE	Androgen responsive elements
ARKO	Androgen receptor knockout
BRG1	Brahma related gene 1
CAIS	Complete androgen insensitivity syndrome
CAH	Congenital adrenal hyperplasia
cAMP	Cyclic adenine mono-phosphate
CBP	CREB binding protein
CREB	cAMP response element binding
COX	Cyclooxygenase
DAB	3,3 DiAminobenzidine
DAX	Dosage-sensitive sex reversal
DBP	DI (n-butyl)phthalate
DES	Diethylstilboestrol
Dhh	Desert hedgehog
DHT	Dihydrotestosterone
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid
DNMT	DNA methyl transferase
DNMT1	DNA methyl transferase 1

DNMT3A	DNA methyl transferase 3A
DNMT3B	DNA methyl transferase 3B
DNMT3L	DNA methyl transferase 3L
e	Embryonic day
ERα	Oestrogen receptor α
ERβ	Oestrogen receptor β
ERKO	Oestrogen receptor knockout
EW	Early window of treatment (e11.5-e14.5 in rat)
FGF	Fibroblast growth factor
FSH	Follicle stimulating hormone
GATA4	GATA-binding protein 4
GnRH	Gonadotrophin releasing hormone
GC	Germ cell
GR	Glucocorticoid receptor
GT	Genital tubercle
GTF	General transcription factors
hCG	Human chorionic gonadotrophin
HDL	High density lipoproteins
HSD	Hydroxysteroid dehydrogenase
HPG	Hypothalamic-Pituatry-Gonadal (axis)
hsp	Heat shock protein
IGF 1R	Insulin-like growth factor 1 receptor
Insl3	Insulin-like growth factor 3
IUGR	Intra-uterine growth factor
kg	Kilogram
L	Litre
LDL	Low density lipoproteins
LH	Luteinizing hormone
MD	Müllerian duct

mg	Milligram
mm	Millimeters
MPW	Masculinisation programming window
mRNA	messenger Ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NLS	Nuclear localization signal
OPT	Optical projection tomography
p450c17	17α-hydroxylase/C17-20lyase
p450scc	Cholesterol side-chain cleavage
PAIS	Partial androgen insensitivity syndrome
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
PG	Prostaglandin
PGD2	Prostaglandin D2
PND	Postnatal day
PTM	Peritubular myoid (cells)
Q RT-PCR	Quantitative real-time polymerase chain reaction
RNA POLII	RNA polymerase-II
ROBO1	Roundabout homologue 1
SC	Sertoli cell
SEM	Standard error of mean
Sf1	Steroidogenic factor 1
SHBG	Sex hormone binding globulin
SHH	Sonic hedgehog
SLIT2	Slit homologue 2
SOX9	Sry related HMG box 9
SRY	Sex determining region on the Y chromosome
StAR	Steroidogenic acute regulatory protein
SW1/SNF	Switch / sucrose non fermentable

TBP	TATA box-Binding Protein)
TBS	Tris buffered saline
TDS	Testicular dysgenesis syndrome
TGCT	Testicular germ cell tumor
TP	Testosterone propionate
UGS	Urogenital sinus
UGT	Urogenital tract
VASA	Vasa/Mouse Vasa homologue/DDX11
WD	Wolffian duct
Wnt4	Wingless-related MMTV integration site 7
WT1	Wilms tumor

NB: Throughout this thesis, all embryonic ages stated are for the rat unless otherwise stated. For gene nomenclature, the following convention is used, when referring to the protein, capitals are used, i.e. PROTEIN and italics for gene, i.e. *Gene.*

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2. Materials and methods

3. Effects of DHT before and during the MPW

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1. Literature Review

Mammalian embryos, like most species, are initially sexually indifferent and can follow one of two developmental courses; becoming a male or a female. This fate is determined by the paternal sperm which can carry either a Y or an X chromosome. This adds to the maternal X chromosome to produce an XY embryo (male) or an XX embryo (female) (Welshons and Russell 1959). Fetal sex development can be divided into three sequential stages. The first of these is the indifferent stage when the primitive structures develop. The second stage involves gonadal differentiation into either the testes in the male or the ovaries in the female. The final stage of sex development is the differentiation of the internal and external reproductive organs which in the presence of testicular hormones in the male will lead to the development of the penis, scrotum, prostate, seminal vesicles, vas deferens and epididymis (Fig.1.1). In the absence of testicular hormones, irrespective of the existence of the ovaries, the females develop a uterus, fallopian tubes, cervix, clitoris and labia. Thus the female sex development pathway is considered to be more of a default pathway of development, whereas to become a male requires the intervention of testicular hormones.

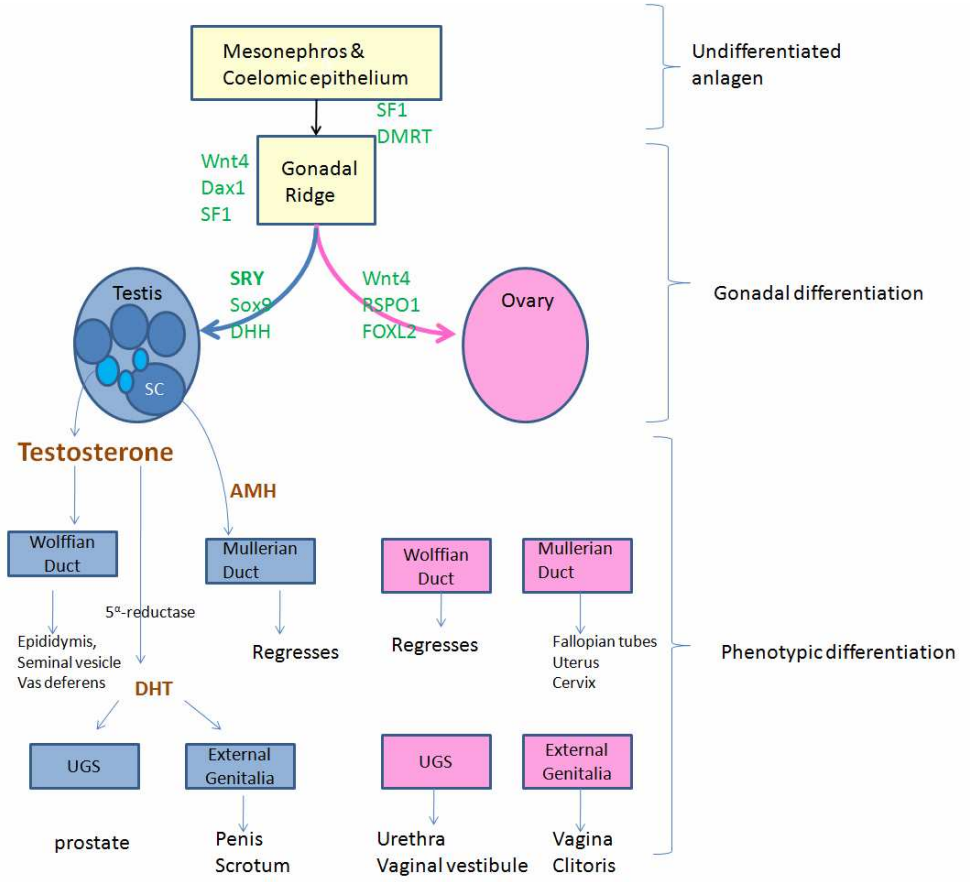


Figure 1.1 Brief representation of the three stages of normal sexual differentiation. Stage 1 shows the undifferentiated anlagen of gonads and external genitalia, identical in both sexes. Stage two illustrates gonadal differentiation and the genes (in green) involved in the formation of the testis and the ovary. The third stage is an overview of genital differentiation, which in males is under the influence of hormones (brown), namely testosterone, DHT or AMH. In the absence of testicular hormones, the female phenotype develops.

There are several disorders of the male reproductive tract and some studies suggest they are increasing in prevalence. The widely accepted Barker hypothesis (Barker, 1998) proposes that some common human diseases in adulthood are related to growth patterns during early life. Indeed there is much evidence emerging that fetal reproductive tract development is a highly controlled process which when disturbed can lead to disorders later in life. Understanding the molecular mechanisms which are employed by testicular

hormones in correct male reproductive tract development should aid our understanding of how male reproductive tract disorders occur and if they can be avoided or prevented.

This review will discuss key male reproductive tract disorders, and then the timing in fetal life when they are thought to originate. This will be followed by a detailed discussion of the key testicular hormone, testosterone (i.e. androgens), the receptor to which androgens bind for effect, and their role in masculinisation of the fetus. Androgens are C-19 steroid hormones produced by the testes in males and the ovaries in females and to a lesser extent also by the adrenal glands in both sexes. Androgens are vital in male sexual differentiation. The two main androgens required for virilisation of the fetus are testosterone and its derivative DHT (dihydrotestosterone). Testosterone elicits differentiation of the Wolffian duct into the seminal vesicles, vas deferens and epididymides (Berman et al, 1995) (section 1.9.1). DHT is required in the urogenital tract to differentiate the prostate and external genitalia (section 1.9.2). Later in life, correct androgen action is required at puberty and also in the adult male. Despite the existence of different androgens and their role in different tissues (Luttge and Whalen 1970), each exerts their effect through one common androgen receptor (section 1.4).

The review will highlight gaps in our current knowledge of androgens and masculinisation and the aims at the end of this chapter will outline how some of these gaps have been investigated in the studies presented in this thesis.

1.1 Testicular dysgenesis syndrome (TDS)

Almost exactly a decade ago, a theory was published proposing that certain male reproductive tract disorders have a common origin in the fetal period. This syndrome of disorders was termed the testicular dysgenesis syndrome (TDS), initiated by abnormal fetal testis development, leading to somatic cell/hormonal malfunction, which then causes deficiencies in male reproductive tract differentiation (Skakkebaek et al, 2001). Currently, there are four disorders which are considered to share this common origin

(Sharpe and Skakkebaek, 2008), although it may be possible that other disorders are also part of the TDS. Of the identified four disorders, two manifest at birth; hypospadias and cryptorchidism, and the other two present in adulthood; namely low sperm counts and testicular germ cell tumours (TGCT). It has been suggested, based on epidemiological studies (Joergensen et al, 2001; Boisen et al, 2004; Boisen et al, 2005), that each of these disorders are risk factors for each other, consistent with them having a common origin, and may possibly be increasing in incidence (Sharpe and Skakkebaek, 2008).

1.1.1 Hypospadias

Hypospadias is a congenital defect of the penis which consists of an abnormal location of the urethral opening (meatus) (Duckett and Baskin 1996). Hypospadias can be relatively 'mild' with the urethral opening located on the glans (near the tip) of the penis, in 'moderate' cases anywhere along the shaft, or in 'severe' cases in the perineal region (Baskin et al, 2001). Hypospadias is also commonly associated with abnormal foreskin or penile curvature (Catti et al; 2008). This disorder occurs when the urethral folds of the penis fail to fuse correctly during penile development. There are probably multiple mechanisms via which this failure can occur (Baskin et al, 2001), but the main cause is thought to be a defect in the action of the main hormone involved in masculinisation; androgens. The mechanisms by which this might arise are discussed in section 1.9.3.

Many countries in Europe and cities in the US, Australia and China have reported an increase in occurrence of hypospadias (Paulozzi, 1999) but the data from registries is often considered unreliable. This is due to under-reporting, particularly of mild cases; furthermore some forms of hypospadias can be masked under the foreskin and may not be obvious until puberty (Toppari et al, 2010). The current epidemiology data suggests that hypospadias occurs in 1 in every 125 live births (Baskin et al, 2001) and is usually identified at time of delivery; although more recently it can be determined prenatally by ultrasound.

Mutational analysis has identified genes which may be involved in hypospadias (Lin et al, 2009; Miyagawa et al, 2011; Wang et al, 2004). In addition, the importance of correct androgen action in penile development is well established and this will be discussed further in section 1.9.3. However it should be noted that for most hypospadias patients, there is no identifiable cause which raises the possibility that hypospadias can be induced by environmental factors (Baskin et al, 2001; Gray et al, 2001; reviewed by Kalfa et al, 2011). It is of general consensus that hypospadias is a highly heterogeneous condition subject to multiple genetic and environmental factors. Indeed, many environmental chemicals, such as pesticides, fungicides and phthalates display anti-androgenic properties and have been shown to induce hypospadias in rodent studies (Foster and Harris, 2005; Imperato-McGinley et al, 1986; McIntyre et al, 2001). The role of environmental chemicals as endocrine disruptors and inducers of hypospadias are discussed later.

1.1.2 Cryptorchidism

Cryptorchidism is the failure of one or both testes to descend into the scrotum and is the most common reported congenital disorder in baby boys. The position of the cryptorchid testes can vary from non-palpable (abdominal) to high scrotal (Boisen et al, 2004). Spontaneous testicular descent occurs in up to 75% of crytorchid testes during the first three months of life, during the period when testosterone production is elevated (Boisen et al, 2004; reviewed in Toppari et al, 2010). Cryptorchidism is reported to occur (on average) in 2-4% of boys, but it may be as high as 9% in Denmark and 6% in the UK (Toppari et al, 2001; Virtanen et al, 2007; Boisen et al, 2004; Hughes and Acerini, 2008). Although some registries are reporting an increase in occurrence of cryptorchidism, it is not clear if incidence is changing across the globe. This uncertainty arises from differences in standardising the classification of cryptorchidism and also the spontaneous resolution of cryptorchid babies requires standardization of the time of diagnosis (Paulozzi, 1999).

The descent of the testes from the abdomen into the scrotum is thought to be mediated either directly or indirectly by androgen action. The exact mechanism of descent, including the genes involved will be reviewed in section 1.2.2.3. However it should be noted that similar to hypospadias, it is believed that the aetiology of cryptorchidism is multifactorial and endocrine disruptors in the form of environmental chemicals may also play a role in this disorder.

1.1.3 Sperm count and quality

Concern about 'falling sperm counts' was raised as early as 1974 by Nelson and Bunge and further established by Carlsen et al in 1992, which reviewed 61 studies to reveal that average sperm counts appeared to have fallen from 113 million/ml to 66 million/ml between 1938 and 1991. Further studies published by Swan et al in 2000 updated this and confirmed that sperm counts had fallen in Europe, between the 1940's and 1966 from 170 to < 60million/ml and in North America between the 1930's and 1996 from 110 to 60 million/ ml. Both studies were met with scepticism based on criticisms such as non-uniformity of laboratory methods or statistical issues and many argue these studies do not prove a real decline (Jegou et al 1999). Despite the scepticism and unknowns, it is evident from all counts being done, there are no reports of an increase but only ever a decrease in sperm count. This, added to the many recent reports of an increased demand for assisted fertility suggests there is an adverse change occurring in sperm counts.

1.1.4 Testicular germ cell tumours (TGCT)

Of less controversy is the rise reported in TGCT, the most common cancer of young men. TGCT arises from carcinoma-in-situ cells (CIS) which are thought to be derived from primordial germ cells which did not undergo normal differentiation during the embryonic period (Rajpert-De Meyts et al, 1998). Many reports have shown an increase in TGCT occurrence in European countries and in particular in Caucasian men. 500,000 new cases were reported worldwide in 2002 (Bray et al, 2006). TGCT is six times more common in developed rather than developing countries.

1.1.5 Anogenital Distance

During development, the genital tubercle and genital swellings differentiate into either the penis and scrotum or the clitoris and labia majora in males and females respectively, whereas the cloacal membrane develops into the anus, giving rise to the perineum and the distance between the genitalia and the anus (Hsieh et al, 2008). In most mammalian species, this distance is longer in males than in females, suggesting it is under hormonal control (Salazar-Martinez et al 2004). Indeed, when females are exposed to androgens, the AGD increases and in males when androgen action is blocked, AGD is found to be decreased (Swan at al, 2005). There have been numerous experimental studies in animals showing that anti-androgens decrease AGD in males as well as affecting development of androgen dependent reproductive tissues (Gray et al, 1999; Imperato-McGinley et al 1986, Ostby et al 1999; You et al 1998). Furthermore, it has been shown that AGD can be correlated with androgen action during a specific time in gestation when TDS disorders are thought to originate, this time being termed the masculinisation programming window (MacLeod et al, 2010; Welsh et al, 2008).

1.1.6 The masculinisation programming window (MPW)

In order to gain understating into the origins of reproductive disorders, studies have been conducted in which reproductive disorders are induced in fetal rodents (Clark et al, 1990; Clark et al, 1993, Foster and Harris, 2005; McIntyre et al, 2001; Welsh et al, 2008). Such studies, combined with investigation of fetal chemical exposure in humans have provided insight into the time frames of critical periods of sexual differentiation in fetal life. It is now known that there is a specific time-frame in rats and mice, in which correct androgen action is necessary for normal male reproductive tract development. When androgen action is disturbed during this time frame, TDS disorders can result. The reporting of such experiments has continued to grow and now the literature has narrowed down the time period in sexual differentiation to be the ‘critical period of development’ for the male reproductive tract, during which androgen action in the male is most susceptible to perturbations (Welsh et al, 2008; MacLeod et al, 2010).

Androgens are first produced at approximately e14.5-e15.5 in rats, consequently, the initial rodent studies which investigated androgen action used chemicals to block androgen action from e12.5 until the end of gestation (e21.5). These studies provided insight into the differential effects of blocking androgen production by the testis and blocking androgen action at the receptor. These experiments also provided insight into the differential roles of the two main androgens; testosterone and its more potent metabolite dihydrotestosterone (DHT), in masculinisation. Testosterone is converted to DHT by the enzyme 5α-reductase.

An example of one such study exposed rats to 17β-N,Ndusopropylcarbamoyl-4-aza-5αandrostan-3-one, also called the aza steroid, which is a competitive inhibitor of the 5α-reductase enzyme, and thus inhibiting the formation of DHT which is important in masculinisation of the fetus. When rats were exposed to the aza steroid from e12.5-e21.5, normal inhibition of nipple development in male rats was blocked and hypospadias was found (Imperato-McGinley et al, 1986).

Flutamide is an anti-androgen which competitively binds to the androgen receptor, and so blocks androgen action (section 1.6.1.4.2). It was reported that exposing rats to flutamide from e12.5-e21.5 caused nipple retention and induced hypospadias in males (McIntyre et al, 2001). McIntyre et al also reported a significant decrease in AGD in males exposed to flutamide. This study also suggested that flutamide-mediated changes in AGD and nipple retention are not sensitive predictors of altered testosterone development in males, but more of the development which is dependent on the more potent androgen, dihydrotestosterone (DHT).

Clark et al (1993) narrowed the window of treatment and exposed rats to finasteride, which also inhibits 5α-reductase, with the aim of investigating the origins of hypospadias. Initially this study exposed rats from e15.5 until postnatal day (PND) 21. Surprisingly this study found that although AGD was decreased on PND1, this was reversed by PND22. Animals were reported to have hypospadias in addition to cleft

prepuce and reduced prostate weight. However, the same study then exposed animals to an approximately seven times higher dose of finasteride administered on successive 2 day periods during late gestation and identified that the most sensitive period or 'critical period' was at e16.5 and e17.5, in which the most TDS like defects, for example hypospadias, cleft prepuce and decreased anogenital distance, were induced by the finasteride (Clark et al, 1993).

Further studies have been conducted to investigate this critical period and insight has been gained by blocking androgen action using a single dose of flutamide, on particular days of gestation. This helps to elucidate which tissues of the male reproductive tract are being programmed on particular days of gestation and when this programming is most susceptible to disturbance (Foster and Harris, 2005). These studies found that blocking androgen action on e16.5 in rats induced a high incidence of retained nipples, epispadias and missing epididymal components in the male offspring. When exposed at e17.5, hypospadias, vaginal pouch, cleft prepuce and missing prostate lobes were reported more predominantly than after any other day of treatment. Reduced prostate size and abnormal kidneys were found after flutamide exposure on e18.5, and after exposure on e19.5 abnormal seminal vesicles were noted (Foster and Harris, 2005). There were few male offspring from this study which were found to have cryptorchidism, which suggests this disorder occurs when androgen action is blocked over multiple days of gestation. Nonetheless, even six years ago, it was clear that androgens act at very specific times during fetal life to regulate normal development of male reproductive tract tissues.

The most defining studies with regards to androgen action in the development of the male reproductive tract perhaps are those published by Welsh et al in 2008. The initial aim of these studies was to block androgen action in four different gestational time windows in the rat. Using flutamide, androgen action was blocked, during an early window, e15.5-e17.5, a middle window, e17.5-e19.5 a late window, e19.5-e2.5 and a

full window from e15.5-e21.5. The results found that androgen driven masculinisation of all male reproductive tract tissues is mediated during the early programming window (e15.5-e17.5) and that deficient androgen action during this window induced hypospadias, cryptorchidism, and decreased AGD. This study also underlined AGD as being a lifelong read-out of androgen action during just e15.5-e18.5. In these studies, phallus length and weight were found to be differentially regulated. In parallel, another set of animals were exposed to testosterone during the same four windows and this study concluded that excess exposure to testosterone could not advance or enhance masculinisation and that exposure to females during the early window induced a male phenotype, with increased AGD. The exposure to testosterone during the various windows demonstrated that not only is androgen action able to virilise the female after exposure in a particular window, but furthermore, presumptive excess androgen in this time window had no discernible effect on males. This suggests that in vivo androgen action is at its maximum capacity during the critical time for male reproductive tract differentiation (Welsh et al, 2008).

Another study by Welsh et al, again using flutamide, showed that adult penile size depends critically on androgen action during the MPW but subsequent growth depends on later androgen exposure after the MPW. Fetal or postnatal testosterone exposure did not increase adult penile size above its pre-determined (in the MPW) length, although the growth towards this could be advanced by pre-pubertal testosterone treatment (Welsh et al, 2010).

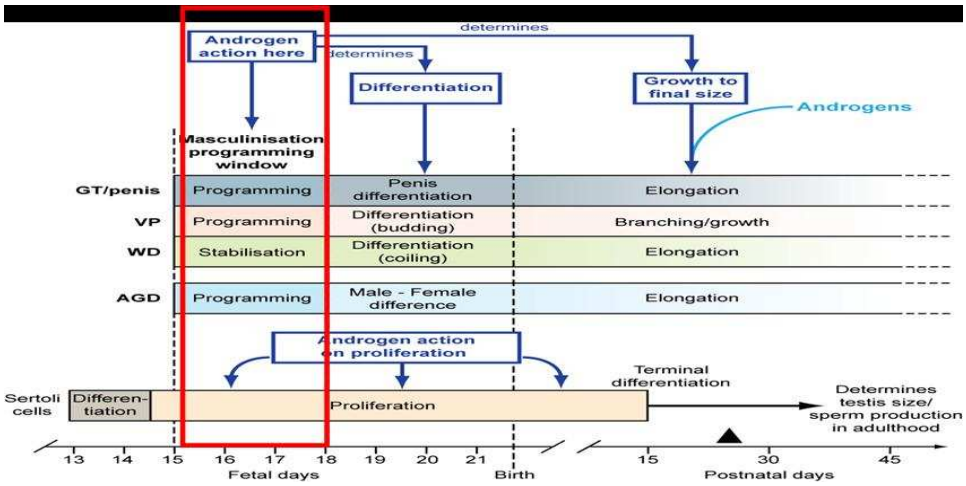


Figure 1.2 Illustration of the timing of events in male reproductive tract programming, differentiation and growth in the rat with the actions of androgens during the MPW highlighted in the red box. Note that the (GT) genital tubercle; (VP) ventral prostate; (WD) Wolffian duct; (AGD) and anogenital distance are all programmed by androgens during the MPW. Adapted from Macleod et al, 2010.

These foundation studies have been built upon and it is now widely accepted that a masculinisation programming window (MPW) exists in rats within the period e15.5-e18.5 (Fig1.2). During this critical period, sufficient testosterone (androgen) must be produced by the fetal testis to bring about 'programming' of the male reproductive tract so that it will subsequently differentiate and grow normally after the MPW. It is during the MPW that correct androgen action is required to program the genital tubercle (later penis), the Wolffian duct (later vas deferens, seminal vesicles and epidiymis), the AGD and the prostate (MacLeod et al, 2009), although postnatal androgen action is required to reach this full potential. Any impairment of androgen production or action within the MPW is likely to result in smaller reproductive organs and their abnormal formation and function (e.g. cryptorchidism, hypospadias). The MPW is an early event, occurring immediately after testis differentiation, so androgen production and action at this time can be difficult to measure directly, especially in humans (Scott et al, 2009). However, anogenital distance (AGD), which is longer in males than in females, provides a life-long read-out of androgen exposure just within the MPW (Welsh et al, 2008; MacLeod

et al, 2010). Based on AGD, emerging evidence for the human points towards similar importance of the MPW (~8-14 weeks' gestation; (Welsh et al, 2008)) and a critical role for deficient androgen action in the MPW as a cause of TDS disorders (Swan et al, 2005; Hsieh et al, 2008; Eisenberg et al, 2011; Mendiola et al, 2011).

The MPW is now commonly used as the time frame to investigate TDS disorders in rodent studies and this has provided an invaluable platform upon which investigations can be based. However there are many gaps in our knowledge about this relatively newly defined time-frame. The studies conducted to date address that impaired androgen action during the MPW causes a failure in programming of the reproductive tract. However the molecular mechanisms driven by androgens during this time in normal and abnormal development remain to be elucidated. Evidently, there must be something unique about androgen action during this time frame which does not occur at any other point. Furthermore, the regulation of this time frame remains to be understood. The presumption to date is that the onset of the MPW is due to the time of onset of testosterone production by the testis, which happens at around e14.5 in the rat, which is a very reasonable presumption. However if this is true, and androgens are the substance controlling the 'opening of the window', what might be involved in 'closing' the MPW? It is well established that androgens continue to be produced after the MPW and throughout much of postnatal life, and development of male reproductive organs cannot be induced during this time frame only during the MPW. It is interesting to note that there is little reported in the literature about the availability of the androgen receptor prior to the MPW. Furthermore, if AR is present, would excess exposure to androgens affect the timing of the MPW?

1.2 Determination of gonadal sex of an embryo

Although the genetic sex of an embryo is determined at fertilization, the sexual differentiation of the fetus actually only begins during the period of organogenesis when the gonads differentiate (Brennan and Capel, 2004; Swain and Lovell-Badge, 1999). The

gonads initially develop in a non-sex specific manner, and are identical in XY and XX embryos until e12 in mice, e13.5 in rats and week 6 in humans (Brennan and Capel, 2004; Lambrot et al, 2006). The development of the gonads can be divided into two phases, formation of the bipotential gonad and then its development into a testis or an ovary.

1.2.1 Formation of the bi-potential gonad

The urogenital system arises from the intermediate mesoderm layer of the fetus which lies on either side of the embryo, filling much of the coelomic cavity between the limb buds. The mesoderm layer consists of three regions from anterior to posterior, the pronephros, mesonephros (from which the gonads arises) and the metanephros, from which the kidney eventually forms. (Wilhelm et al, 2007).

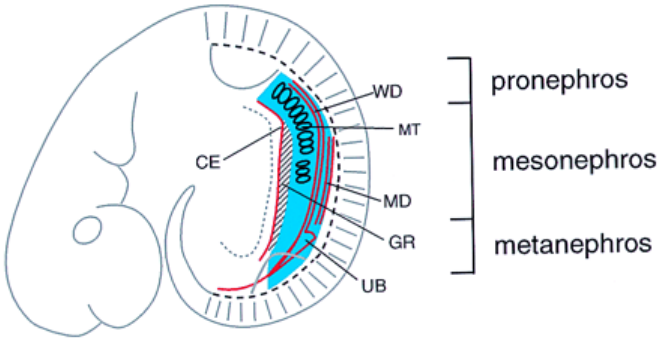


Figure 1.3 Diagrammatic representation of the urogenital system (mouse at e10). Epithelial structures are in red and mesenchymal structures are in blue. The genital ridge is represented by the striped region. WD represents Wolffian duct; MT represents mesonephric tubules, MD represent Müllerian duct, UB represents ureteric bud and CE represent coelomic epithelia. Diagram from Swain and Lovell-Badge, 1999.

The gonads develop from a single layer of coelomic epithelium which is located on the ventrolateral surface of the mesonephros (Swain and Lovell-Badge, 1999 Wilhelm et al, 2007). The coelomic epithelium thickens and proliferates to give rise to the genital ridge, also called the bipotential gonad. Genetic analysis in rodents and human has identified some key gene pathways which are involved in the early formation of the indifferent

genital ridges. These include *Wilms Tumour suppressor 1* (*Wt1*) (Nachtigal et al, 1998), *Steroidogenic factor-1 (Sf1)* (Achermann et al, 1999; Achermann et al, 2002), *GATA-binding protein 4 (Gata4)* (Tevosian et al, 2002), *Limhomebox protein 9* (Birk et al, 2000), *empty spiracles homologue 2 (Emx2*) (Miyamoto et al, 1997) and *insulin-like growth factor 1 receptor (Igf1r)* (Brennan and Capel, 2004). *Wt1* is expressed widely throughout the urogenital ridge, the kidney and the gonad. *Wt1* encodes a nuclear zinc finger protein that can function as both a transcriptional activator or repressor depending on cell type and promoter context (Wilhelm et al, 2007). *Steroidogenic factor-1 (Sf1)* is a nuclear receptor expressed in the developing urogenital ridge but also in the hypothalamus and pituitary, and is thought to be important in the formation of the hypothalamic-pituitary-gonadal axis early in development (Sadovsky et al, 2000, Shinoda et al, 1995). Gonads of *Sf1* mutant embryos do not develop beyond the early indifferent stage and fail to undergo masculinisation in XY animals (Wilhelm et al, 2007). *Limhomeobox protein 9 (Lhx9)* and empty *spiracles homologue 2 (Emx2)* are both homebox genes also crucial for the initial development of the bipotential gonads. It has been shown that mice lacking *Lhx9* show a similar phenotype to those lacking *Sf1* and furthermore *Lhx9* can bind to the promoter of *Sf1* (Luo et al, 1994). Mutants of *Emx2* lack a thickening of the coelomic epithelium and later lack gonads completely (Miyamoto et al, 1997).

Originally the bipotential gonad consists of two types of cells; the somatic progenitor cells, which have migrated from the mesonephros and the coelomic epithelium and the primordial germ cells, which arise from a population of epiblast cells in the extraembryonic mesoderm, near the base of the allantois. The primordial germ cells migrate via the hindgut to the genital ridge and can give rise to the spermatogonia in the testes and the oogonia in the ovary (De Felici et al, 2004).

1.2.2 Testes Development

The XY bipotential gonad begins differentiation into a testis when a particular gene, expressed only on the Y chromosome, termed the *Sry* gene, is expressed (Koopman et al, 1991). In mice this expression begins at e10, in the rat around e12.5 and in the human around week 6 of pregnancy. This leads to a cascade of events which results in four main cell types of the testis, the Sertoli cells, the Leydig cells, the peritubular myoid cells and the germ cells as well as formation of two compartments, the seminferous cords and the interstitial space and also the formation of the testis vasculature (Wilhelm and Koopman, 2007).

1.2.2.1 SRY

The testis determining gene, *Sry,* discovered in 1990 (Gubbay et al, 1990), has been shown to be the only gene required from the Y chromosome to establish male development. The loss of *Sry* function in XY embryos results in ovary development and the gain of *Sry* function in an XX embryo leads to the development of testes (Lovell-Badge et al 1990, Koopman et al 1991, Berta et al 1990, Jager et al 1990).

The *Sry* transcription factor encodes a nuclear high mobility group (HMG) domain protein which can alter chromatin structure (Brennan and Capel 2004). Outside of this domain, *Sry* exhibits little sequence conservation between species. Mutations of *Sry* which produce a clinical phenotype have always been found within this domain, implying its importance.

The main function of *Sry* is to induce the differentiation of Sertoli cells but the exact molecular mechanisms involved in achieving this are poorly understood (Swain and Lovell-Badge 1999). It is believed that *Sry* induces Sertoli cell differentiation by regulating other transcription factors, the most investigated of which is the *Sry-like HMG-box protein 9 (Sox9)* (Wilhelm and Koopman, 2007; Wilhelm et al, 2007). *Sox9* is critical in male development, and studies have shown that *Sox9* alone is sufficient to

induce testes formation in mice (Bergstrom et al, 2009; Vidal et al, 2001). Conversely, null mutations in *Sox9* prevent testis differentiation (Barrionuevo et al, 2005). *Sox9* expression persists in Sertoli cells throughout life after transient expression of *Sry* has ceased.

In addition to *Sox9*, the following genes have also been shown to be crucial in testes development, namely *Fgf9, Wnt4, Ptgds and Gata4* (Wilhelm et al, 2007; Brennan and Capel 2004). These genes contain potential *Sry* binding sites in their promoters but their direct interaction with *Sry* requires further investigation (Wilhelm and Koopman, 2007). It is also suggested that *Sry* represses a negative regulator that functions at the top of the genetic cascade to suppress testes development, and some studies propose this negative regulator to be *Dax1* (Bardoni et al, 1994; Jordan et al, 2001; Swain et al, 1996). The literature concerning the role of *Dax1* is contradictory. There is consensus that this X-chromosomal gene probably interacts with *Sf1* and with both oestrogen receptors α and β, and the androgen receptor (AR) and progesterone receptor (Conde et al, 2004; Park et al, 2005; Zhang et al, 2011). It has been shown to be expressed in the developing adrenal gland, gonads, hypothalamus and pituitary, with expression patterns correlating with *Sf1* (Ikeda et al, 1996). *Dax1* switches off in the male mouse testis at around e12 but then re-appears at around e15-17 (Wilhelm and Koopman, 2007). In contrast, it is constantly expressed in the ovary (Swain et al, 1996). Based on duplications in humans that lead to XY sex reversal, some literature states this to be an ovarian determining or anti-testis gene which, in the male, is suppressed by *SRY*. However, loss of function of *Dax1* in female mice has no reproductive consequences whereas testicular development in males was impaired which is why some scientists do not classify this as an ovary determining gene (Yu et al, 1998; reviewed in Wilhelm and Koopman, 2007).

Transient *SRY* expression occurs in a wave that begins in the centre of the genital ridge (e10) and then encompasses the entire gonad reaching peak expression at e11 in the mouse. The expression declines first in the centre, then at the poles and is undetectable

by e12 (Wilhelm and Koopman, 2007). The exact molecular mechanisms which regulate this expression are not yet fully understood. However, mice mutants have shown that splice variants of *Wt1, Gata4* and the *insulin receptor family* may be involved. The timing of peak *Sry* expression is crucial and delayed expression can lead to a range of phenotypes from complete XY sex reversal to unilateral or bilateral ovotestes (gonads which have both ovarian and testicular tissue/cells) (Wilhelm et al, 2009).

1.2.2.2 Sertoli Cells

The Sertoli cells are important not only because they are essential for early testes development (Swain and Lovell-Badge 1999) but also later and throughout life for germ cell support and spermatogenesis. Furthermore the initiation of the male pathway depends on the development and the threshold number of Sertoli cells (Brennan and Capel 2004). Once sufficient Sertoli cells have differentiated, morphological changes can be seen in the gonad, as the Sertoli cells and gonocytes arrange themselves into seminiferous cords and the proliferation of both cell types in parallel increases testes size. The formation of the seminiferous cords involves the Sertoli cells undergoing mesenchymal to epithelial cell transformation and polarization which allows aggregation around the germ cells. It was originally believed that the Sertoli cells then produced a trigger which signaled the mesonephric cells from the adjacent mesonepehros to migrate into the developing testis giving rise to the pertitubular myoid (PTM) cells (Brennan and Capel 2004), which begin to express AR during this migration (Sharpe, 2006). However, more recently it is believed that the PTM cells may arise from pre-existing cells in the gonad (Cool et al, 2008). The PTM cells collaborate with the Sertoli cells to induce the basement membrane, also called the basal lamina (Swain and Lovell-Badge, 1999). This finalises compartmentalization of the testes into the seminiferous cords that house the germ and Sertoli cells and the interstitium which houses the Leydig cells responsible for producing masculinising hormones.

The Sertoli cells also have two further roles. The first of these is to produce paracrine factors to initiate differentiation of the Leydig cells. In addition, and very importantly, Sertoli cells also secrete anti- müllerian hormone (AMH) at around e12 in the mouse and the action of this hormone is vital in masculinising the fetus and inducing apoptosis of a female structure, the müllerian ducts (Sajjid, 2010). AMH is responsible for regression of the müllerian ducts in the male fetus, which in females, gives rise to the fallopian tubes, the uterus and upper third of the vagina. It is proposed that *Sf1, Wt1* and possibly *Dax1* are involved in the regulation of AMH (Shen et al, 1994; Nachtigal et al, 1998).

1.2.2.3 Fetal Leydig Cells

Approximately 24 hours after Sertoli cell differentiation, the Leydig cells begin to differentiate in the interstitium in the mouse and this is thought to be regulated by paracrine factors from the Sertoli cells. These paracrine factors include *desert hedgehog (Dhh)* (Canto et al, 2005; Yao et al, 2002), *platelet derived growth factor (Pdgf)* (Brennan et al, 2003) and *aristaless-related homeobox gene (Arx)* (Kitamura et al, 2002), and defects in these factors can lead to abnormal Leydig cell differentiation (Barsoum and Yao et al, 2006). The Leydig cells have two distinct lineages, the fetal and adult Leydig cells. Fetal Leydig cells are present in the testes from e12 in the mouse (Brennan and Capel 2004) until shortly after birth and function primarily to produce testosterone to virilise both the internal and external genitalia of the fetus. The production of androgens is discussed in section 1.3. The Leydig cells also produce insulin like growth factor 3 (INSL3), a hormone which is essential for migration of the testes within the abdomen and for normal gubernaculum development (Barsoum and Yao, 2006).

An important difference between the male and female gonads is their final location within the body. The testes descend into the scrotum whereas the ovaries remain abdominal, close to the kidney. The descent of the testes is a pre-requisite for normal spermatogenesis and fertility in adulthood (Klonisch et al, 2004). Testes descent is

dependent upon the androgen and INSL3 mediated formation of the gubernaculum (an embryonic structure which connects the testes to the scrotum) and occurs in two phases (Barteczko and Jacob, 2000). In the first phase, testes move across the abdomen to the entrance of the inguinal canal and this involves both testosterone and INSL3. The next stage is believed to involved androgens in the production of a peptide called calitonin gene related peptide (CGRP) which produces rhythmic contractions of the gubernaculum that guide the testes along the inguinal canal and into the scrotum (Momose et al, 1993; Terada et al, 1994;). CGRP is a 37 amino acid neuropeptide, well classified to be involved in neurotransmission and in this area is known to elicit effects through a G-protein coupled receptor (Poyner et al, 2002; Rosenfeld et al, 1983). CGRP is produced mainly in the dorsal root ganglia that contain the cell bodies of sensory nerves (Supowit et al, 2001). Mutations in genes such as *Hoxa10, Hoxa11* and *Dhh* have been shown to disrupt the second phase of testicular descent whereas Insl3 mutations cause disruption of the initial phase (Hutson et al, 1997; Ivell et al, 2003). Thus the descent of the testes is clearly dependent on several different factors and when this descent fails to occur completely, cryptorchidism ensues. It is interesting that mice that are null for *Insl3*, have complete failure of testicular descent due to lack of development of the gubernaculum and over-expression of *Insl3* in females results in ovaries 'descending' and inguinal hernias (Sharpe, 2006).

1.2.2.4 Formation of the vasculature

Following *SRY* expression, vascular endothelial cells migrate into the gonad to form the coelomic blood vessel on the surface of the testis and the blood vessels that give rise to side branches in between the seminiferous cords (Jeays-Ward et al, 2003). This arterial network, which is identified by the arterial marker ephrin B2 and elements of the Notch signalling pathway, routes the blood flow through the testes (Brennan et al, 2004) and is important in providing oxygen to the testes as well as transporting testosterone produced by the Leydig cells to target organs in the body (Brennan et al, 2002). It has also been

suggested that formation of the vasculature plays a role in both seminiferous cord formation and Leydig cell differentiation (Tang et al, 2008).

1.2.3 Ovary development

The development of the ovary is well documented in the literature (Brennan and Capel, 2004; Hirschfield, 2008; Loffler and Koopman, 2002) and will not be reviewed in detail here. In brief and of relevance are two key independent signaling pathways which are believed to work in a complementary manner to promote ovary development and repress male signaling and testicular development. The first of these is the R-spondin / Wnt4/ β-catenin pathway. *R-spondin* is required for *Wnt4* expression in XX gonads and this exerts its functions via stabilization of *β- catenin* to promote ovarian fate and block testes development (Chassot et al, 2008). Loss of function of *R-spondin* leads to sex reversal in an XX female. Deletion of *R-spondin* in mice impairs ovarian differentiation, triggers development of ovotestes, results in testes-like vascularisation, and the appearance of Leydig-like cells. *Wnt4* is believed to act at the top of the ovarian cascade, and is expressed in the bipotential gonad and becomes ovary specific at e11.5 (Nef and Vassalli, 2009). *Wnt4* signalling has been implicated in repression of the testes pathway, and null mutations of *Wnt4* in XX mice results in partial masculinsation of the gonad (Vanio et al, 1999).

In addition, the *Foxl2* gene has been shown to be crucial for granulosa cell differentiation and ovarian maintenance. The expression of *Foxl2* is first seen at e12.5 in the pre-granulosa cells. Loss of *Foxl2* function leads to differing effects in different species. In goats, it causes sex reversal, in mice only partial sex reversal and in humans causes premature ovarian failure (Garcia-Ortiz et al 2009). Recently it has been reported that the absence of *Foxl2* (in knock out models) allows for an increase in expression in *Cyp26b1* which up-regulates *Sox9* and *Sf1* known to be involved in testis development (Kashimada et al, 2011).

Combined loss of *R-spondin* and *Foxl2* results in extensive gonadal XX reversal, where testes-like seminiferous cords form that express high levels of SOX9 and AMH and contain well differentiated spermatogonia (Ottolenghi et al, 2007). However, even the loss of both of these key pathways, does not induce complete sex reversal which suggests that additional factors are involved in ovarian promotion and testes suppression (Cederroth et al 2007). These factors may include *Dax-1*, which has been shown to be up-regulated by *Wnt4*, but of more relevance for this study, it has been shown to be an SRY repressor. Increased *Wnt4* and *Dax1* expression have been suggested to be involved in XY sex reversal (Domenice et al, 2004; Jordan et al, 2001).

1.3 The production of androgens

Steroid hormones are produced by a process called steroidogenesis in the specialised streoidogenic cells of the adrenals, gonads, placenta and brain (Stocco, 2001). The first step of steroidogenesis is shared between glucocorticoids, mineralcorticoids and sex steroids (Hiort and Holterhus, 2000). As this review is concerned with male sexual differentiation, it will focus on the synthesis of androgens, in particular testosterone.

1.3.1 Steroidogenesis

Cholesterol, the precursor of all steroid hormones can be synthesised de novo (from acetate) in the testis or acquired from serum lipoproteins, in the form of high density lipoproteins (HDL) or low density lipoproteins (LDL) (Cao et al, 2000). In the rat, HDL is the main form of circulating cholesterol whereas in the humans, LDL is considered the principal form of circulating cholesterol (Cao et al, 2000; Carr et al, 1983; Landschulz et al, 1996).

Cholesterol requires to be transferred from the outer mitochondrial membrane to the inner membrane. This requirement is due to the fluid filled space between the inner and outer membrane, filled with aqueous fluid which only allows the passage of water soluble molecules (Thomson et al, 2004; reviewed by Scott et al 2009). Scavenger

receptor class B type 1 (SRB1) is a cell surface HDL receptor, which is expressed in the mouse testis and which can import cholesterol from the outer mitochondrial membrane. Principally however, the steroidogenic acute regulatory protein (StAR) translocates the cholesterol from the outer mitochondrial membrane (Arakane et al, 1998; Miller, 2002; Stocco and Clark, 1996). *Sf1* has been shown to be involved in the regulation of both SRB1 and StAR, as well as two enzymes which are involved in the de novo synthesis of cholesterol, namely HMG-CoA synthase and HMG-CoA reductase (Mascaro et al, 2000; reviewed in Scott et al, 2009).

The conversion of cholesterol to testosterone requires two main categories of enzymes, the cytochrome p450 enzymes and the hydroxysteroid dehydrogenase (HSD) enzymes (Payne and Hales, 2004). The cytochrome p450 enzymes include CYP11a1 and CYP17a1 and these enzymes catalyse the hydroxylation and cleavage of the steroid substrate by using nicotinamide adenine dinucleotide phosphate (NADPH) as an electron donor. The HSD enzymes include 3β-HSD and 17β-HSD and catalyse the oxidation and reduction of steroid hormones and require NAD/NADP as electron acceptors (Payne and Hales, 2004).

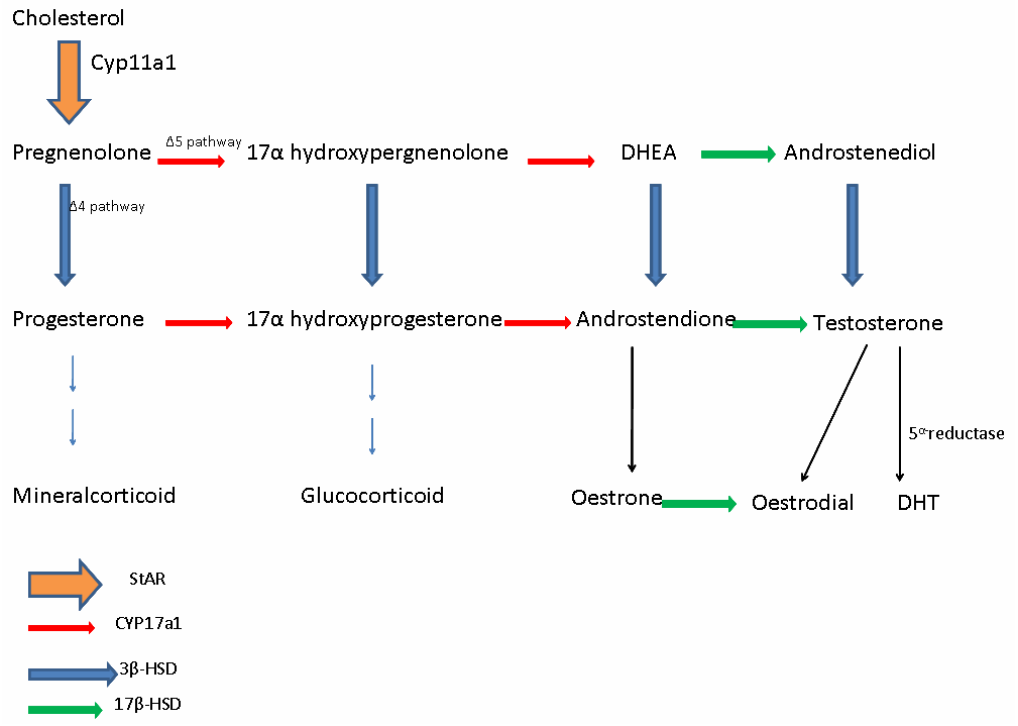


Figure 1.4 Simplified testosterone biosynthesis pathway in the mammalian Leydig cell, outlining the Δ4 and Δ5 pathways and enzymes involved.

On the inner mitochondrial membrane, the first of the enzymatic reactions in steroidogenesis can begin. This is catalysed by CYP11a1 which cleaves a six carbon chain from cholesterol to generate the steroid pregnenolone (Thomson et al, 2004). Pregnenolone then moves to the endoplasmic reticulum where the remaining steroidogenic reactions occur. Pregnenolone can be converted to androstenedione through one of two pathways, termed Δ5 and Δ4 (Coffey et al, 1972, reviewed in Scott et al, 2009). In the Δ5 pathway, the pregnenolone is catalysed to 17α-hydroxypregnenolone and then to dehydroepiandrosterone by CYP17a1 and then to androstenedione by 3β-HSD. It is thought the Δ5 pathway is the predominant steroidogenic pathway in human fetal (Fluck et al, 2003) and adult testes (Hammar et al, 1986; Rey et al; 1995). In the Δ4 pathway, pregnenolone is converted to progesterone and then to 17-α-hydroxyprogesterone and finally to androstendione, and each conversion is catalysed by CYP17a1. It is believed that the Δ4 pathway is the most prominently used pathway in rats (Bell et al, 1968; Kwan et al, 1988). However it is believed that it is possible to converge from the Δ5 pathway to the Δ4 pathway. In each of the pathways, androstenedione is finally converted to testosterone by 17β-HSD. There are multiple isoforms of 17β-HSD (O'Shaughnessy et al, 2000), of which type 3 is involved in testis function (Geissler et al, 1994), although type 5 has also been found in the fetal testis (Pelletier et al, 1999). It is interesting to note that the fetal LCs of the mouse do not express 17β-HSD type 3; however it is expressed in the seminiferous tubules. Consequently in the mouse, it is thought that androstendione is secreted by the fetal LCs and is then converted into testosterone in the seminiferous tubules (O'Shaughnessy et al, 2000).

It should be noted that there is controversy regarding the difference in concentration of serum testosterone between males and females. In the humans, it is believed the LCs begin to produce testosterone at 8 weeks of gestation and the levels peak at weeks 12-14 of pregnancy (reviewed in Scott et al 2009). Some studies report during week 12-20, testosterone levels are 3-8 fold higher in the male than female (Rodeck et al, 1985;

Takagi et al, 1977). However, some studies have found that at the individual level, this difference is not always apparent (Zondeck et al, 1977). Furthermore, some studies suggest there are no differences in blood testosterone between the two sexes at birth (Forest and Catchaird, 1975; Takagi et al, 1977) and again, some scientists disagree (Simmons et al, 1994). In the male rat, plasma testosterone levels are reported to be 4 fold higher than in females.

1.3.2 The differential roles of testosterone and DHT

Once produced in the fetal Leydig cells, testosterone is secreted into the bloodstream where in humans it binds to sex hormone binding globulin (SHBG) and in rodents to albumin (Alfsen, 1963; Grishkovskay et al, 1999). In addition, testosterone can reach the Wolffian duct in relatively high concentrations whereas in other tissues, when bound to SHBG or albumin, it is believed to reach targets in relatively low concentrations.

Once released from the testis, testosterone can be converted in target tissues into its more potent form, dihydrotestosterone (DHT) by the enzyme 5α-reductase. There are two isoforms of 5α-reductase, type I which is expressed in the skin and liver and type II which is expressed in the male reproductive tract. Defects in type II can cause pseudohermaphroditism and this is discussed later in section 1.6.1. Experiments have shown that 5α-reductase is regulated by testosterone; for example, castrated rats have a decreased expression of this enzyme in the prostate which can be recovered by exposure to testosterone (Dadras et al, 2001;Wright et al, 1999).

Testosterone and DHT are differentially effective in different response systems (Luttge and Whalen, 1970; Bentvelsen et al, 1995). Testosterone mediates Wolffian duct stabilisation while differentiation of the urogenital sinus and external genitalia depends more upon DHT action. The differing roles of the two hormones are clearly reflected in individuals with 5α-reductase deficiency and this is reviewed in section 1.6.1. It should be noted that in the absence of DHT, testosterone, at high concentrations, can

differentiate the external genitalia which implies both androgens work through a similar mechanism but less is required of the more potent DHT.

1.3.3 Hypothalamic-pituitary-gonadal axis (HPG axis)

As stated above, the Leydig cells, whether fetal or adult are responsible for testosterone production. Testosterone production is part of a feedback loop involving the hypothalamus, the pituitary and the gonads. In brief, the hypothalamus secretes gonadotrophin releasing hormone (GnRH), which travels to the pituitary and stimulates the production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) that are released into the bloodstream (Kaiser et al, 1995). The steroidogenic Leydig cells of the male have receptors for LH and respond by producing testosterone (Griffin et al, 2010). FSH binds to its receptors on the SCs and initiates and helps maintain spermatogenesis (Walker and Cheng, 2005). Once the testes have produced sufficient testosterone, testosterone itself acts as the negative feedback signal to decrease LH production, in males. Furthermore, in response to FSH, Sertoli cells can produce Inhibin B, a hormone which also acts as a specific negative feedback regulator of FSH (Goulis et al, 2008).

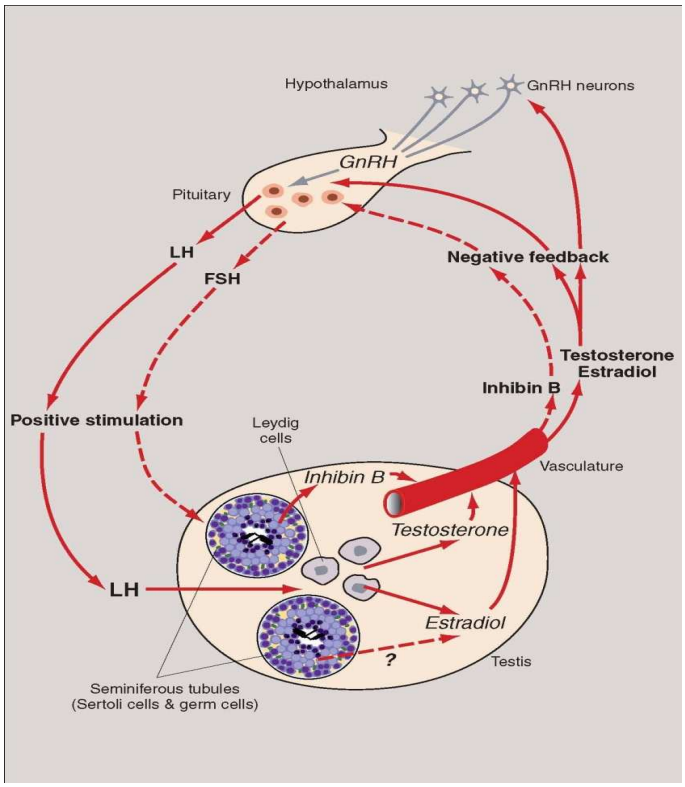


Figure 1.5 Diagrammatic representation of the feedback loops of the HPG axis. GnRH produced by the hypothalamus signals the production of gonadotrophins from the pituitary which stimulate the Leydig and Sertoli cells of the testis which in their turn produce negative feedback hormones (testosterone and inhibin B respectively)

In humans, testosterone is produced in the fetus at approximately week 8 of pregnancy, with a peak in production between weeks 11-14 (Scott et al, 2009). It is interesting to note that the LH receptor is first reported to be detectable in the testis at week 10 in humans and LH is first secreted at week 12. Human chronic gonadaotrophin (hCG) is a glycoprotein hormone similar in structure to luteinizing hormone that is produced by the placenta (in primates) and peaks in production between weeks 8-12 of pregnancy. Thus it is accepted that testosterone production by the fetal testis is initially not under the control of LH but is regulated by hCG and perhaps also by paracrine factors (Scott et al,

2009). Rodents do not produce hCG and the standing hypothesis is that testosterone production by the fetal testis in rodents is regulated initially by paracrine factors. In rats, testosterone is first produced at e14.5, and despite the presence of LH receptor, LH is not secreted until e17.5 and production peaks at e20.5-e21.5, suggesting that LH-driven testosterone production by the fetal rat testis is not operative until the end of gestation (Aubert et al, 1985, Scott et al, 2009).

Although the exact mechanism of early regulation of steroidogenesis has not yet been identified, several factors have been identified that can stimulate fetal LC steroidogenesis in vitro, and these include, IGF-1, VIP, ANP, CNP and BNP and the receptors for these have been shown to be present in the rat fetal testis from e15.5 (Scott et al, 2009). It should be noted that some studies have suggested that retinoic acid may be involved in early human steroidogenesis as it has been shown to stimulate testosterone production by fetal human testis explants in vitro (Lambrot et al, 2006). However, in fetal rodents, retinoic acid inhibits steroidogenesis (Livera et al, 2000). Of relevance are studies showing that absence of LH function (through decapitation) does not affect steroidogenic production in fetal life at least in the mouse, rat and rabbit (George et al, 1979; Habert and Picon, 1982; Pakarinen et al, 2002;). Chemicals which interfere with steroidogenesis are discussed in section 1.6.1.1.

1.4 Androgen receptor

Steroid hormones exert their effect by binding to receptors and altering gene expression. Steroid receptors are a large super-family of ligand activated transcription factors and this family includes oestrogen, progesterone, glucocorticoid, mineralocorticoid and androgen receptors (Beato and Klug, 2000). These receptors share a common molecular structure (Giguere, 1999; Whitfield et al, 1999) and this review will focus on the androgen receptor.

The gene for AR is located on the long arm of chromosome X at position Xq11-q12 in humans. It spans 90 kilobase pairs and consists of 8 exons (McEwan, 2004). Together, these encode a protein of 919 amino acids with a molecular mass of 110kDa (Patrao et al, 2009). The AR protein, like other members of its family, has four distinct functional domains which together mediate the genomic actions of androgens in target tissues. These domains are the N-terminal domain, the DNA binding domain, the ligand binding domain and a hinge region (Tilley et al, 1989; Heemers and Tindall, 2007).

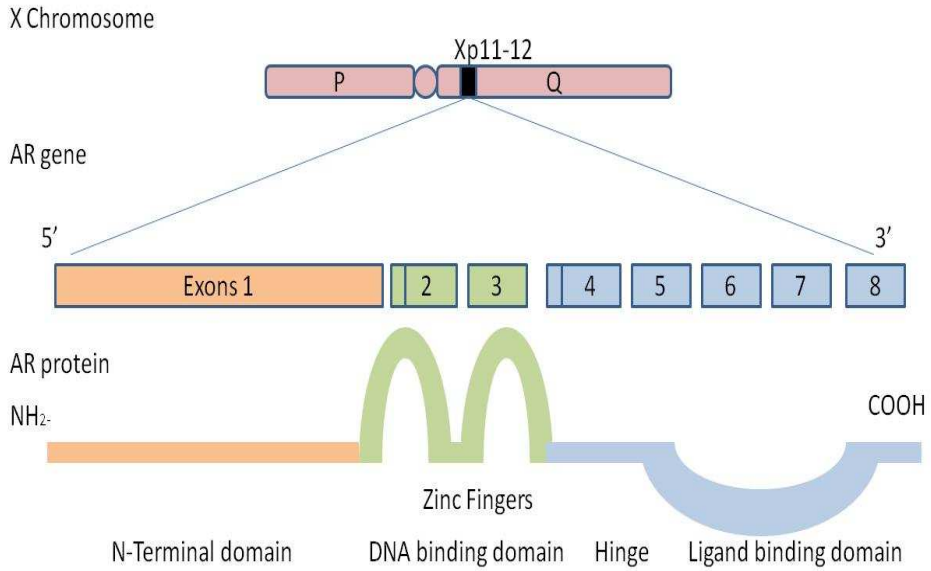


Figure 1.6 Diagrammatic representation of the AR gene and protein, showing the N-terminal domain in orange, the DNA binding domain in green, including the zinc fingers, and the hinge and ligand binding domain in blue.

The N-terminal domain, encoded by exon 1 is relatively long, representing almost half of the functional receptor. The N-terminus is responsible for transcriptional activity and contains the activation function 1 (AF1) domain (Betney and McEwan, 2003). AF1 is responsible for almost all AR transcriptional activity and also mediates interactions with

some co-regulators as well as with the basic transcriptional machinery. Analysis of the amino acid sequence of the AR N-terminal domain from a diverse range of organisms has revealed the presence of three areas of sequence conservation; amino acids 1-30, 224-258, and 500-541 (McEwan, 2004). The first 30 amino acids of the N-terminus are believed to be important for interaction with the AR ligand binding domain, perhaps forming a loop that is important for functional activation (Steketee et al, 2002). The second conserved area, 224-258 lies within the AF1 region and this sequence is highly conserved from fish to primates (Betney and McEwan, 2003).

The DNA binding domain is encoded by exons 2 and 3 is cystein-rich and is the most conserved region within the nuclear receptor family, with identical amino acid sequences in humans, rats and mice (Heemers and Tindall, 2007). This domain contains two zinc finger motifs and a c-terminal extension (Heinlein and Chang, 2002). The first zinc finger mediates specific DNA recognition and the second finger is involved in AR homodimerization as well as DNA/receptor complex stabilization (Centenera et al, 2008). Both the DNA-binding region and the hinge region contain nuclear localisation signals (NLS) suggesting these regions are involved in nuclear translocation of AR.

The C-terminal ligand binding domain is encoded by the remaining 5 exons and mediates high affinity interactions between AR and its steroidal and non-steroidal ligands. The LBD consists of 12 α-helices that form a ligand binding pocket (Heemers and Tindall, 2007; MeEwan, 2004). Upon ligand binding, a conformational change is induced in the LBD which results in the stabilization of α-helix 12, resulting in a more compact structure and the formation of a functional activation function 2 (AF2) on the surface of this domain. The AF2 region may play a role in dimerization and also in stabilization but this is weak in comparison to the AF1 region (McEwan, 2004).

1.4.1 Androgen mode of action

Once testosterone or DHT reaches its target cells, it diffuses through the cell membrane and interacts with the AR which, in the un-bound form, is located in the cytoplasm attached to heat shock proteins and immunophillin chaperones, including HSP90, HSP70, HSP56 and p23 (Eder et al, 2001; Heemers and Tindall, 2007; Kimmins and MacRae, 2000; McEwan, 2004). Studies suggest that the HSP chaperone machinery in eukaryotes functions to prevent AR misfolding and aggregation and promote refolding of denatured polypeptides (Chen et al, 1998; Fliss et al, 1999). Once the ligand has bound to the AR, the receptor undergoes conformational changes, allowing its release from the multiprotein complex, unmasking the AR NLS, causing increased phosphorylation, nuclear translocation and homodimerization. In the nucleus, the activated AR-ligand homodimer complex binds to specific DNA sequences in genes, termed androgen response elements (ARE's). AREs are most commonly localised in the promoter regions of androgen regulated genes, such as prostate specific antigen (PSA) (Cleutjens et al, 1997; Luke and Coffey, 1994). ARE's can also be present in intronic regions of AR regulated genes. The consensus ARE sequence has been identified as a partial pallindromic 15 basepair element consisting of two imperfect 6 base pair indirect repeats with a 3 base pair space (5'-GG(A/T)ACANNNTGTTCT-3; (Patrao et al, 2009, McEwan 2004).

AR mediated transcription of target genes requires the recruitment of RNA polymerase-II (RNA Pol II) to the promoter sequence of the target gene (Roeder, 1996). RNA Pol II recruitment is mediated through the assembly of GTFs (general transcription factors) and this forms the pre-initiation complex. The first of these GTFs is TBP (TATA box-Binding Protein) which binds near the transcriptional start site (McEwan and Gustafsson, 1997). TBP is part of a multi-protein complex, which also contains general and promoter-specific TBP-associated factors (TAFII). TBP binding induces DNA bending, which causes sequences of the TATA to interact with the GTFs and steroid receptor-coregulator complexes. Other transcription factors, namely TFIIB and TFIIB

bind to TBP and function to recruit the RNA Pol II complex (Heinlein and Chang, 2002).

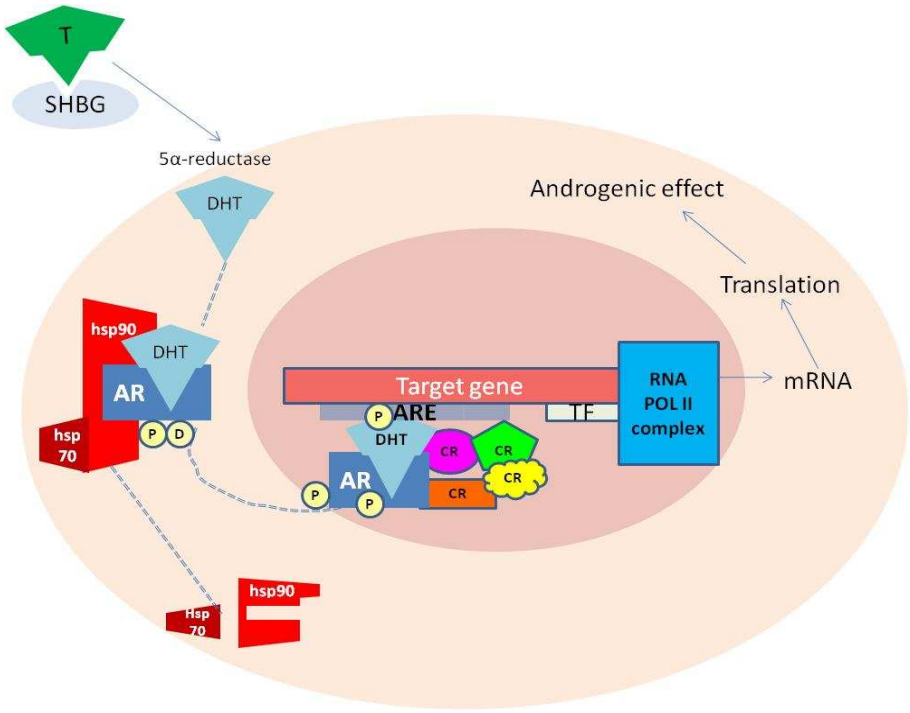


Figure 1.7 Summary of androgen receptor mode of action. Androgens disassociate from SHBG and bind to AR in the cytoplasm. This causes the disassociation of AR from chaperone proteins and the ligand-receptor complex translocates to the nucleus where it binds to target genes to modulate transcription via the involvement of GTFs, RNA pol II and various co-regulators

This classic genomic action of androgens is a mechanism which requires approximately 40 minutes to alter gene expression and hours if a new gene is to be expressed (Walker, 2009). There is increasing evidence that androgens may also initiate cellular processes through rapid non-genomic mechanisms, in a matter of minutes (Foradori *et al*, 2008). This is reviewed in section 1.5.

1.4.2 AR expression

The expression of androgen receptor in the developing reproductive tract has been investigated through immunohistichemical studies. In the male and female rat fetus, AR

is present at equal levels until the onset of differentiation (Bentvelsen et al, 1995) and the same appears to be true in humans (Sajjid et al, 2003; Wilson and McPhaul, 1996). It is interesting that both males and females have similar AR expression prior to masculinisation of the reproductive tract as this suggests that sex specific AR expression does not appear to be a factor in masculinisation. It should be noted that there are some differences in the literature which report the timings of AR expression in rat studies, this may be due to differences in rat strains and or antibodies which have been used (Bentvelsen et al, 1995; Majdic et al, 1995).

In the male reproductive tract of rats, AR is generally first expressed in the mesenchymal cells and later in the epithelial cells (Shannon and Cunha, 1983). An exception to this is displayed in the efferent ducts which express AR from e14.5 throughout gestation in both the mesenchymal and epithelial cells (Bentvelsen et al, 1995). In the WD, the mesenchyme is AR positive from e14.5 throughout gestation, however the epithelial cells only become AR positive at around e18.5 (Bentvelsen et al, 1995; Hannema and Hughes, 2007). Furthermore the epididymal tail is AR positive in the mesenchyme from e18, but epithelial cells only become AR positive from postnatal day 2. The distal WD (later vas deferens) does not express AR until e20.5 in the mesenchymal cells and, similar to the epididmyal tail, epithelial cells become positive at PND2. The mesenchymal cells of the prostate anlagen express AR at e16 with a majority of epithelial cells only becoming AR positive at PND5 (Bentvelsen et al, 1995).

The interstitial cells of the testis become AR positive at around e17.5 in the rat but some studies have reported staining as early as e15.5 in the interstitium (You and Sar, 1998); a similar pattern of staining has been reported in the human testis at corresponding ages (Boukari et al, 2009). SCs remain negative for AR until PND 5 in the rat and expression increases in SCs as the animal develops towards puberty (You and Sar, 1998; Bremner et al, 1994). The lack of AR in SC correlates with high AMH expression in the fetal period and, in contrast, when AR is switched on postnatally, AMH expression decreases

(Rey et al, 2009). The absence of AR in SCs in fetal life may be to protect the cells from precocious maturation and onset of pubertal spermatogenic development.
The folds and swellings in and around the urogenital tubercle express AR at e14.5 in the mesenchymal cells and during development, in the male GT, the number of mesenchymal cells expressing AR increases progressively. The epithelial cells in the skin of the genital tubercle express AR from PND5 in the rat (Bentvelsen et al, 1995). Both the gubernaculum and perineal region express AR abundantly from e18.5 in the rat.

In females, the Müllerian ducts are AR negative throughout development, after differentiation, females have low levels of AR expression in comparison to males in the urogenital sinus and tubercle (Bentvelsen et al, 1995).

The expression patterns of AR in the male reproductive tract suggest that androgens induce epithelial differentiation of the genitalia via action in the mesenchymal cells. Although a full review of mesenchymal-epithelial interactions is outwith the scope of this review, it is accepted as fact that mesenchymal-epithelial interactions play a central role in the development of the male reproductive tract (Cunha et al, 1991; Donjacour and Cunha, 1993; Kurzrock et al, 1999) and these will be referred to where appropriate in the review of phenotypic differentiation.

Of particular interest is the relative lack of data available for AR expression prior to the onset on androgen production in the fetus in both rodents and humans. One study of AR in the urogenital system of a marsupial reported that AR mRNA is present just before the genital ridge develops, but AR protein investigations were only carried out just prior to the end of gestation (Butler et al, 1998). To the best of my knowledge, there is little data showing the availability of AR protein in the developing reproductive tract prior to the production of androgens.

1.4.3. Regulation of AR expression

Given the importance of AR in the various actions of androgens it is not surprising that the AR protein is regulated at multiple levels. Furthermore, AR expression is differentially regulated in different tissues. Perhaps the simplest way to review this is to separate transcriptional regulation and post-transcriptional regulation of the AR protein.

At the transcriptional level, AR is auto-regulated by androgens, which have been shown to reduce AR mRNA in both rat tissues and LNCaP cells (Tan et al, 1998; Krongard et al, 1991). The stability of AR mRNA is important in the regulation of AR protein expression and when RNA-binding proteins bind to a conserved UC rich element of the 3' untranslated region of AR mRNA, AR protein expression is altered (reviewed in Yeap et al, 2004). In 1994 Bentvelsen et al demonstrated fetal exposure to androgens in the female up-regulated AR expression and that exposure to anti-androgens during the fetal period, down regulated the expression of AR in males in comparison to respective controls (Bentvelsen et al, 1994). Another example of androgens regulating AR expression, is the age-dependent down-regulation of AR levels in the rat penis, which has been shown to be androgen dependent (Takene et al, 1990). Furthermore, androgens can regulate the expression of co-regulators which in turn potentiate AR signalling, providing another level of regulation (Urbanucci et al, 2008). Co-regulators are discussed further in section 1.7.1. However it should be noted that some of these co-regulators are involved in post-translational regulation of the AR protein as well as enhancing or repressing AR activity. It is also important to remember that changes in AR mRNA do not necessarily mean a corresponding change in AR protein and generally the latter is increased by testosterone action.

The binding of steroids to their receptors to elicit a response, is a cyclical event and degradation of the receptor after this event is important in order to regulate subsequent rounds of transcription. Ubiquitin dependent proteolysis is an important mechanism for controlling protein turnover and is well established as a method of regulation of various

transcription factors (Freiman et al, 2003, Nawaz et al, 2004). For example, both the oestrogen and glucocorticoid receptors have been shown to be ubiquitinlyated and destroyed after binding to their respective ligands, as a form of regulation. This is also true for AR, which can be destroyed via the 26S proteosome (Sheflin et al, 2000). Another example of AR being a target of ubiquitination regulation / destruction is *Mdm2*, the proto-oncogene which has been reported to work with *histone deacetylase 1 (HDAC1*) to de-stabalise the AR protein and reduce AR mediated transcription (Gaughan et al, 2005).

1.5 Oestrogens in the male

Oestrogens are one of the sex steroid hormones, produced by steroidogenesis and historically are thought of as the 'female' hormone whereas androgens are considered the 'male' sex steroid hormone. Over the past couple of decades this perception has changed and it is imperative to note that not only are both sex steroids present in both males and females, but oestrogens can also be important for some aspects of 'normal' development of the male phenotype in some species. It is believed that it is not the qualitative differences in these hormones which are involved in sexual differentiation but the difference in hormone concentration and differential expression of hormone receptors. Indeed, several studies have reported that disturbance of the androgen-oestrogen balance can lead to disrupted development of the male phenotype (reviewed in Sharpe, 1998).

The importance of oestrogens in males was perhaps only really noted when mice in which the oestrogen receptor alpha had been knocked out (ERKO) were reported to be infertile (Lubhan et al, 1993; Korach et al, 1996). Further studies of the knockout model have reported that the efferent ducts in these males are incapable of reabsorbing luminal fluids from the testis and it is hypothesised that oestrogens are responsible for the regulation of fluid resorption and thus increasing sperm concentration prior to their entry into the epididymis (Hess et al, 1997). The role of oestrogens in the testis is probably

one of the more investigated roles of oestrogens in the male. Unlike androgens, which work through one receptor, oestrogens can work through one of two receptors, ER-α and ER-β and the latter is more widely expressed throughout the rodent and primate body (Saunders et al, 1998). ER-β is expressed in all of the cell types of the testis as well as in the epididymis, vas deferens, seminal vesicles and the prostate. ER-α is also expressed in the LCs, at least in rodents. Moreover, aromatase, which converts testosterone to oestradiol is expressed in many of the cell types in which ER-α and ER-β are expressed in the male reproductive tract as well as in adipose tissue, bone, the heart and the brain. Ironically, oestrogens produced in the brain are thought to be involved in masculinsing the brain and male sexual behaviour in rodents (Miesal and Sachs, 1994) but not in humans. As AR, ER and aromatase are expressed in many of the same cell types, a balance in action between androgens and oestrogens may be important for some aspects of male reproductive function. One of the older and more dramatic examples is a study in sheep (Bennetts et al, 1946). This showed that when clover (which contains weak phytoestrogens) was ingested by castrated rams, it led to death due to urinary retention caused by overgrowth of the bulbourethral glands. However, when animals which had not been castrated and consequently had high levels of circulating testosterone ingested clover, it had no effect. Another study which exemplifies nicely the balance between androgens and oestrogens, involved exposure of rats to diethylstilbestrol (DES) (a synthetic oestrogen discussed in section 1.6.1.1.1) in two different doses. In addition some rats were exposed to the low dose of DES in conjunction with a GnRH antagonist (GnRHa) to block androgen production or flutamide to block androgen action (Rivas et al, 2002). Treatment with the high dose of DES drastically reduced the expression of AR in investigated tissues, which was also seen when animals were exposed to the low dose of DES in combination with GnRHa or flutamide. However, administration of GnRH alone or flutamide alone did not have this effect. DES as an anti-androgenic compound is discussed in section 1.6.1.1.1. Another example of a requirement for the androgen-oestrogen balance is evidenced by gynaecomastia in males, which can be induced either

by exposure to oestrogens or by lowering androgens or androgen activity (which can occur in aging or in conjunction with abdominal obesity) (Gustafson et al, 2003).

Therefore it appears that the development of the correct male phenotype requires a fine balance of oestrogens and androgens in particular tissues. In addition there is some evidence that these sex steroids could work together. For example, oestrogens can trans-activate the AR/ARA70 complex at high concentration (Yeh et al, 2000) and using a two hybrid system, co-transfection of the two receptors into CV-1 cells has demonstrated a mutual ability of each receptor (AR and ER) to antagonise trans-activation mediated by ligand binding to the other receptor (Panet-Raymond et al, 2000)

1.6 Disorders of sex development in the male

As discussed in the introduction, the sex development of an embryo can be classified into three sequential stages, the indifferent stage, where primitive structures develop, gonadal differentiation and phenotypic differentiation. Disorders of sex development can occur as the result of deficiencies at any of these stages and it is important to review these to not only understand where androgens might fit aetiologically into these sex differentiation disorders but also to understand them in their possible relationship to TDS.

The exact molecular mechanisms involved in the indifferent stage of development of the urogenital tract remain to be fully understood. There are however, certain genes which have been identified to be crucial for this part of development, and mutations in these genes are reported to cause a variety of different disorders. For example, mutations in *Homeobox A13 (HoxA13)* have been associated with hand-foot-genital syndrome. This disorder is characterised by mild bilateral shortening of the thumbs and great toes and urogenital abnormalities including abnormalities of the ureters and urethra and Müllerian fusion in females and hypospadias of variable severity in males (Mortlock and Innis, 1997). Examples of genes which have been identified at the indifferent stage to be

involved in disorders of sexual development include: *GLI3*, associated with hypospadias, micropenis and Pallister-Hall syndrome (Bose et al, 2002; Narumi et al, 2010); *CFTR*, associated with congenital aplasia of the vas deferens (Zielenski et al, 1995); *ROR2*, associated with aphallia (absent phallus); Robinow syndrome, a disorder characterized by short-limbed dwarfism, abnormalities in the head, face, and external genitalia, as well as vertebral segmentation (Bacino, 2005; van Bokhoven et al, 2000).

During development of the gonad the most prominent disorder that has been reported to occur is hypogonadism, when functional activity of the gonads is low or absent. Hypogonadism can be due to whole testicular dysfunction or dysfunction of individual cell populations (Grinspon and Rey, 2010). Abnormal gonadal differentiation can cause hypogonadism and an example of this is 'streak' gonads, where the testes fail to produce testicular hormones. These individuals can have low or absent serum AMH and testosterone in conjunction with high gonadotrophin levels (Rey et al, 1999). Partial forms of this disorder can present with asymmetric gonadal differentiation or ovotestis (reviewed in Rey et al, 2011).

Mutations in certain genes at the indifferent stage can cause isolated testicular dysgensis, when non-reproductive organs form normally, for example mutation or deletions in *SRY*. However, most mutations do cause disorders elsewhere at this early stage. This is illustrated by mutations of *WT1*, which can result in at least three well known disorders, Denys-Drash syndrome (affecting the renal system as well as the gonads) (Heathcott et al, 2002; Little et al, 1993), Frasier syndrome (associated with nephropathy as well as gonadal dysgenesis) (Barbaux et al, 1997; Klamt et al, 1998) and WAGR syndrome (can cause mental retardation as well as gonadal dysgenesis) (Almind et al, 2009; Gessler et al, 1993). Other than affecting the gonads, mutations in *Sox9* have been shown to lead to skeletal malformations, also termed campomelic dysplasia (Kwok et al, 1995), micrognathia (Jakobsen et al, 2007) and cardiac (Akiyama et al, 2004; Montero et al, 2002) and renal defects (Reginensi et al, 2011) .

1.6.1 Androgen deficiency

Other than hypogonadism, caused by mutations in genes involved in formation of the testis, hypogonadism can also arise once the testis has formed and individual cell types do not function properly. An example of this is impaired LC function which leads to a decrease in virilisation due to lack of testosterone. This can mean that the WD is not stabilised or differentiated, the external genitalia is not masculinised, leading to varying severity of hypospadias and lack of testis descent. However, as the SCs continue to produce AMH, the Müllerian ducts and its derivatives are removed. LC dysfunction can be due to various factors, such as LH receptor mutations in humans, known as Leydig cell hypoplasia (Latronice et al, 1996; Mendonca et al, 2010), defects in steroidogenic enzymes such as StAR or CYP11a1 which causes lipid congenital adrenal hyperplasia (Miller et al, 2009). Defects of either of the HSD enzymes also result in LC dysfunction (Lee et al, 2007; Mendonca et al, 2010, reviewed in Rey et al, 2011).

Dysfunction of the Sertoli cells can lead to a lack of AMH and this can cause persistent müllerian duct syndrome (PMDS), characterised by the presence of a uterus and fallopian tubes in XY males (Josso et al, 2005). Such 'pseudohermaphroditic' individuals are usually virilised normally as the LC produce testosterone in the normal range (Rey et al, 1999; Thomson et al, 1994).

Incomplete masculinisation may also be caused by androgen deficiency downstream of the testis for example because of a lack of 5α-reductase in the target organ (Imperato-McGinley et al, 1974). Patients with a deficiency in 5α-reductase type 2 have low synthesis of DHT and at birth may have an under-virilised phenotype, with ambiguous genitalia, bifid scrotum, and a urogenital sinus opening on the perineum (Sultan et al, 2001). Due to normal levels of local testosterone, the WD derivatives are normal in these individuals. Over 40 mutations have been identified in 5α-reductase type 2 deficient patients but a very recent cohort investigation reported that the various

phenotypes displayed by patients with mutations in 5α-reductase type 2 could not be linked to particular genotypes (Maimoun et al, 2011).

So far the deficiency in androgen production has been discussed, however pseudohermaphroditism can also occur when the AR protein is defective and signalling is abnormal despite the presence of sufficient androgens. Three pathological situations are well associated with AR defects, androgen insensitivity syndrome (AIS), spinal and bulbular muscular atrophy (also known as SBMA or Kennedys disease) and prostate cancer (Brinkman, 2001).

Androgen insensitivity syndrome can present itself in two forms. The more severe phenotype is called complete androgen insensitivity syndrome (CAIS) and the less severe, partial androgen insensitivity syndrome (PAIS) (Ahmed et al, 2000; Quigley et al, 1995). CAIS patients have a completely non-functional AR and present with normal female external genitalia, a short blind ending vagina with no uterus or fallopian tubes, absence of WD derived structures, absence of prostate and development of normal female breast tissue at puberty (gynaecomastia) (Thiele et al, 1999; Quigley et al, 1995). In contrast, PAIS occurs when there is incomplete loss of AR function and can present with a range of phenotypes ranging from a predominantly female appearance as for CAIS, to ambiguous genitalia to a predominantly male phenotype with disorders such as micropenis, hypospadias and cryptorchidism (Jaaskenainen, 2011; Rey et al, 1994; Werner et al, 2010).

Over 400 different mutations leading to AIS have been reported (database can be found at http://www.mcgill.ca/androgendb) and in general these can be classified into two groups according to DNA and mRNA alterations. The first is loss or gain of genomic information such as macro and micro deletions and base pair insertions and the second is point mutations responsible for non-sense, mis-sense and splice mutations (Sultan et al, 2001). Each of these defects disturbs the ability of AR to regulate gene transcription. It

is interesting that the same mutation can lead to different phenotypes or alternatively different molecular defects can produce the same phenotype. This suggests that other determinants may influence androgen action within the target cell.

1.6.1.1 Anti-androgenic chemicals

There is growing concern that certain environmental chemicals can inhibit androgen production or action and affect virilisation (Fisher, 2004; Gray et al, 2006; Sharpe, 2001). This has been reported in wildlife in pollutant exposed fish that have ovotestes or intersex genitalia, in birds that display deformities of the reproductive tract and similar traits have also been found in amphibians and rodents (reviewed by Sharpe, http://www.chemtrust.org.uk/documents/ProfRSHARPE-MaleReproductiveHealth-CHEMTrust09.pdf). As discussed in section 1.1.4, TGCT are reported to be increasing in occurrence in humans and other TDS disorders are common and some believe that they may also be increasing in prevalence, in particular low sperm counts and associated infertility. The identification of sexual development disorders in wildlife and the increasing concern over human reproductive development has led to anti-androgenic environmental chemicals being studied intensively with the hypothesis that they may be involved in the origins of TDS and affect virilisation of the fetus. Although some genetic mutations have been found in patients with TDS, genetic abnormalities are not always found and other explanations for these disorders are sought. Moreover, TDS occurrence is highly linked to geography suggesting that environmental factors are involved in these disorders and/or that there are ethnic/genetic differences in susceptibility (Lopez-Teijon et al, 2008; Virtanen et al, 2005)

It is extremely difficult to accurately determine the exact level of exposure of the human fetus to environmental chemicals and the effects this may have. In order to prove that environmental chemical effects are linked to TDS, the level of exposure would have to be determined, this would have to be linked to a mechanistic effect and then to the outcome disorder. This obviously has incredible obstacles because of inaccessibility of

the fetus. Consequently, the majority of our understanding of environmental chemicals and their possible role in TDS has come from experimental rodent studies. One criticism of these rodent studies is that the dose of chemicals used to induce TDS disorders are far in excess of what humans are generally exposed to and any adverse outcomes of these chemicals in rodents are unlikely at exposure levels experienced by humans (Sharpe and Skakkebaek, 2008). However it can also be argued that humans are exposed to mixtures of many different chemicals, thus higher doses of an individual chemical may mimic the effects of more complex mixtures (Sharpe, 2008). Nevertheless the most relevant studies are those which expose rodents to mixtures of chemicals in much lower doses.

1.6.1.1.1 Diethylstilboestrol (DES)

DES is a synthetic non-steroidal oestrogen which was first synthesised in 1938 and was used therapeutically until 1971 to prevent pregnancy complications and miscarriage. DES was discontinued for use when it emerged that some females exposed in utero to DES developed clear cell adenocarcinoma of the vagina and fertility problems (Barclay, 1979; Fowler and Edelman, 1978; Herbst 1981). In sons exposed to DES in utero, testicular and reproductive tract abnormalities have been reported (Schrager and Potter, 2004; Wilcox et al, 1995).

Initially it was hypothesised that the adverse effects of DES in males was simply due to the oestrogen agonistic effect, but some studies suggest it is more complex than this. Thus, DES-induced disorders of the male reproductive tract may result more from the disturbance of the androgen-oestrogen balance rather than from oestrogen action alone (Rivas et al, 2002). Furthermore, it has been shown that altered gene expression induced by DES in fetal life can persist well after exposure is terminated and the permanent defect in gene expression does not necessarily coincide with spatial localisation of altered expression at acute exposure (Bromer et al, 2009). Thus, the typical endocrine regulation of gene expression may not account for these apparently epigenetic effects.

1.6.1.2 Phthalates

Phthalates are a group of chemicals mainly used as plasticisers to soften polyvinyl chloride in the manufacture of various different products including pharmaceuticals, cosmetics, perfumes, medical equipment and toys (Heudorf et al 2007). Examples of phthalate chemicals include Di-n-butyl phthalate (DBP) and di-2-ethyl hexyl phthalate (DEHP), but many more exist. The effects of phthalates on testosterone production in humans is conflicting. Some studies have reported a negative correlation between fetal AGD in humans and the levels of phthalates reported in maternal urine (Lottrup et al, 2006; Swan et al, 2005; Swan et al, 2008). However in contrast, other studies have found no association between phthalate exposure and AGD (Huang et al, 2009) although some effects have been noted on germ cells in vitro (Lambrot et al, 2009). Moreover, phthalates have no effects in vitro on steroidogenesis by human fetal testis explants (Hallmarkk et al, 2007; Lambrot et al, 2008) and, more convincingly, no effect on steroidogenesis in a fetal testis xenograft model after chronic phthalate exposure (Mitchell et al, 2011, submitted). This controversy however does not exist in rat studies, which provide unequivocal evidence that phthalates can inhibit fetal testosterone production (Gray et al, 2000; McKee et al, 2006; Parks et al, 2000; Wilson et al, 2004).

1.6.1.3 Fungicides and herbicides

Certain fungicides and herbicides have also been reported to affect androgen action. Ketoconazole is an anti-fungal agent used in the treatment of fungal skin conditions and also in Cushing's syndrome. Ketoconazole has been reported to inhibit CYP17 (Pont et al, 1982), vital in steroidogenesis and studies indicate that human LC steroidogenesis, which has a preference for the Δ5 pathway, is more susceptible to ketoconazole disturbance than are rat or dog Leydig cells (reviewed in Scott et al, 2009). Another fungicide of interest is prochloraz used in crop protection and shown in rodent studies to affect fetal steroidogenesis by inhibiting CYP17 conversion of progesterone to testosterone (Laier et al, 2006; Vinggaard et al, 2005).

Linuron is a urea-based herbicide and in rodents, can inhibit androgen action by reducing fetal testosterone production (Wilson et al 2004; Hotchkiss et al, 2004) but also by antagonising AR (McIntyre et al, 2000). Unlike the fungicides described above, the mechanism by which linuron might work to disturb androgen action has yet to be elucidated.

1.6.1.4 Pharmaceuticals

There has been substantial interest in the effect of therapeutic drugs on virilisation of the fetus. Glucocorticoids are used in the treatment of many different disorders, including those in pregnant woman, despite the fact that it is established that elevated glucocorticoids in response to stress can suppress testosterone production in adult males (Hardy et al, 2005).

16.1.4.1 Indomethacin

Indomethacin is a non-steroidal anti-inflammatory drug (NSAID) which inhibits the production of prostaglandins. PGs are reviewed in section 1.7.3. Indomethacin works by competitively blocking the COX1 and COX2 enzymes (non-selectively) which inhibits the synthesis of prostaglandins and their effect in inflammation (Mitchell et al, 1993). Blocking COX2 is what makes NSAIDs effective, as this reduces inflammation; however, blocking COX1 can cause unwanted side effects (Bateman et al, 1994; Vane, 1996). Prostaglandins, particularly of the E and F series are the main promoters of cervical ripening and myometrial contractility in pregnancy (Vladic-Stjernholm et al, 2009). Consequently indomethacin has been used in the treatment of pre-term labour (Norton, 1997). Many studies have been conducted on animals and humans investigating the effect of prenatal exposure to indomethacin with conflicting results. Some of these studies have reported indomethacin exposure in utero does not produce any malformations in offspring in mice and rats (Klein et al 1981; Randall et al 1987). However studies have been published which contradict these findings. Kusang et al (1977) reported that exposing mice to indomethacin causes skeletal defects as well as

fused ribs. In addition the studies reviewed in 1.7.3 showed that Gupta et al (1986) found that exposing male mice to indomethacin caused incomplete virilisation of the external genitalia as well as decreased AGD. When rabbits were exposed to indomethacin there were no birth defects in the fetuses which survived but an increase in resorption of developing embryos was reported (O'Grady et al, 1972). Studies using 8 rhesus monkeys reported that exposure to indomethacin was associated with oligohydramnios (deficiency of amniotic fluid) and also reported a 50% fetal mortality rate (Novy, 1978). Exposure at various doses, can also lead to constriction of the fetal ductus arteriosus (Arishima et al 1991; Sharpe et al, 1975).

In humans, exposure to indomethacin in the first trimester does not generally produce fetal malformations (Aselton et al, 1995; Katz et al, 1984) and it has been suggested that there is minimal transplacental passage of indomethacin in this trimester (Klein et al, 1981). In the second trimester, in humans, indomethacin crosses the placenta and reaches the fetus in concentrations close to those found in the mother (Moise et al, 1990). Some studies suggest there is a similar pattern in rats, such that indomethacin does not cross the placenta in pharmacologically significant quantities until close to parturition (Klein et al 1981). However, the vast number of publications which report adverse side effects in fetal rats when exposed to even low doses of indomethacin suggests otherwise. As prostaglandins play important roles in many different systems, it is inevitable that blocking PG action using indomethacin can cause various complications within the fetus. These include gastrointestinal complications, including perforation and bowel necrosis (Cassady et al, 1989; Krasna et al, 1992) and disorders of cerebral blood flow, such as cystic brain lesions and intracranial haemorrhage (Baerts et al, 1990; Norton et al 1993). In addition to the effects in utero, indomethacin can cause gastrointestinal ulceration in adults as well as acute renal failure (Schlondorff et al 1993). Thus it seems the effect of indomethacin depends on the species being treated, the dose being administered and the timing in gestation/development.

Although the ability of indomethacin to block PG synthesis by inhibiting COX enzymes is well documented, it is important to note that indomethacin may also have other mechanisms of action, which are independent of COX inhibition (Hawcroft et al, 2001). For example, indomethacin can directly activate the transcription factor peroxisome proliferator activated receptor γ (PPARγ) (Lehman et al, 1997). Furthermore, Zhang et al (1999) have shown that some of the antiproliferative and antineoplastic effects of NSAIDs are independent of the inhibition of either COX1 or COX2.

It should be mentioned that some of the chemicals discussed above are used regularly within the laboratory to elucidate the mechanisms of androgen perturbation. In addition, AR antagonists are often used to elucidate AR molecular mechanisms and flutamide is one such chemical.

1.6.1.4.2 Flutamide

Flutamide is a non-steroidal AR antagonist which competes with testosterone and DHT (Sufrin and Coffey, 1976). Flutamide blocks the interactions between the N-terminus and C-terminus of AR thus preventing transcriptional activation. Once absorbed orally, flutamide is converted in the liver to the biologically active metabolite, 2-hydroxyflutamide (Xu and Li, 1998) and has a half life of only 6 hours in humans; there is no evidence that flutamide or its metabolite accumulate in any tissues. Flutamide does not affect the testicular production of testosterone or its conversion to DHT (Peets et al, 1974). However, in order to be a competitive antagonist of these androgens, flutamide must be administered 500-1000 fold higher than circulating testosterone levels in order to induce antagonism (Simard et al 1986). Even at such high doses, complete antagonism is not always achieved against the competing testosterone and DHT, although DHT-dependent tissues are more susceptible to complete antagonism, whereas within the testis (where testosterone levels are extremely high) the opposite is the case.

1.6.2 Androgen receptor knockout models

Other than the use of chemicals, knockouts of the AR in mice have also been an invaluable tool for the study of androgen action. This has been done using Cre-lox technology which will not be reviewed here but can be found elsewhere (Sauer et al, 1987; Sauer et al, 1988; Orban et al, 1992). The first of the AR knockout (ARKO) models employed this technology to knockout AR in all cell types. The male phenotype was reported to be female, i.e. the genetic males were phenotypically female in outward appearance. ARKO males were reported to have decreased anogenital distance, micro-phallus with hypospadias and the vas deferens, epididymis, seminal vesicles, and prostate were absent. In addition, the testes were far smaller than in wild type males and were cryptorchid and serum testosterone was low. ARKO males also displayed a decreased bodyweight (Yeh et al, 2002). When published, these studies only reported early findings of the effect of ARKO in females but stated that female fertility was not affected. However more recent studies have shown that ARKO females have intra-ovarian defects and are subfertile (Walters et al, 2009).

AR has also been knocked out of particular cell types and the effects reported. In 2004, AR was knocked out of SCs in the testis (SCARKO). In contrast to the cryptorchid and small testis reported in ARKO mice, the SCARKO model was found to have normal development of the male reproductive tract and normal descent of the testes. The testis weight was reduced however in comparison to respective controls, and it was found that the number of spermatocytes and spermatids were greatly reduced or absent and all animals were infertile. This study concluded that AR is required within the SC for androgen maintenance of complete spermatogenesis (De Gendt et al, 2004).

Attempts to knockout AR from LCs have been reported and these animals were infertile, and spermatogenesis was arrested at the spermatid stage (Tsai et al, 2006; Xu et al, 2007). These animals also had reduced levels of testosterone and increased levels of LH and FSH demonstrating the effects on the HPG axis. However, it has been shown that

AR knockout in this model may not be specific to LCs and some AR may have been knocked out of SCs also and so to date, no LC specific AR knockout model has been reported (De Gendt and Verhoeven, 2011).

There have been two notable attempts to knockout AR in PTM cells (PTM-ARKO). The first of these reported decreased testis size and oligozoospermia, but fertility was stated to be normal (Zhang et al. 2006). A more recent PTM–ARKO model reported grossly normal reproductive tract development, but the males were azoospermic and infertile. These animals had normal levels of testosterone, LH and FSH but had impaired SC function (Welsh et al, 2009). The latter study also showed that the former knockout (Zhang et al, 2006) had not in fact knocked out AR in PTM cells, but rather only in the smooth muscle of arterioles.

1.6.3 Excess exposure to androgens

In addition to deficiencies in androgen action during the fetal period having a variety of affects, excess androgen exposure can also be detrimental, particularly in females. In humans this is the most common endocrine disorder in woman of reproductive age. Androgens are produced by the ovaries and adrenal glands and excess production can lead to a range of virilisation phenotypes including a masculinised urogenital tract and external genitalia, hirsutism, acne, menstrual irregularity and fertility problems (Abott et al, 2005). Excess androgens in females can be due to congenital adrenal hyperplasia (CAH) (Barnes et al, 1994; Kalaitzogou and New, 1993) or polycystic ovarian syndrome (PCOS) (Abott et al 2005; Dumesic et al, 2005).

CAH is an autosomal recessive disorder which occurs in most cases when there is a mutation in 21-hydroxylase (also called CYP21), one of the 5 enzymes required by the adrenal glands to synthesize cortisol (White and Speiser, 2000). This enzyme is one of the cytochrome p450 enzymes. Deficiency in CYP21 results in the adrenal cortex compensating by over-producing other cortisol precursors which leads to the excess

synthesis of androgens (White and Speiser, 2000). CAH females usually have normal internal reproductive tracts, but may display ambiguous genitalia. In addition as these females have high circulating androgens but not locally produced testosterone, WD derivatives are not present (reviewed in Merke and Bornstien, 2005). Other symptoms include abnormal menstruation, deepening of voice and hirsutism. Males can also be affected by CAH and these individuals may undergo puberty as early as 2-3 years of age (reviewed by Merke and Bornstien, 2005).

PCOS is an autosomal dominant condition in which the ovaries (and sometimes adrenal glands) over-produce androgens mediated by hyperinsulaemia and LH pulses (Rosenfield, 1999). As well as the expected virilisation, females with PCOS also display abnormal menstruation and reproductive abnormalities. In addition, many of these patients are found to have other disorders such as insulin resistance, central obesity, dyslipidemia, hypertension, glucose intolerance and type 2 diabetes (reviewed by Dunaif, 1997). The aetiology of PCOS remains unknown but one study has suggested that excess androgen exposure in utero can cause the female offspring to develop PCOS in adulthood (Xita and Tsatsoulis, 2006). Furthermore a microarray study reported PCOS theca cells to have abnormal expression of CYP17 (involved in steroidogenesis) and also in GATA-6, known to be involved in embryonic development and Wnt signalling (Wood et al, 2003).

Several studies have investigated excess androgen exposure using laboratory rodents not only to understand disorders such as CAH and PCOS but also to address increasing concern about androgenic chemicals in the environment. Exposing rats to excess androgens, in the form of testosterone has been the most common experimental approach and this has been shown to lead to various degrees of virilisation of the female fetus. The effects reported in this approach depend very much on the dose of testosterone administered, the timing of administration and the strain of rats used. For example a study which used three different doses of testosterone in Sprague Dawley rats

found that although dams had a lower bodyweight and an increase in embryonic lethality, the reproductive tract of the female offspring was normal (Fritz et al, 1984). It is interesting to note that this study exposed rats from e13.5- e16.5. Most studies report females to be susceptible to masculinisation by excess testosterone exposure from e15.5 onwards. For example, studies published by Rhees et al in 1997 exposed female rats to testosterone from e16.5 onwards and reported females to have an increased AGD, abnormal vaginal development and opening. Interestingly, LH surges were not affected (Rhees et al, 1997). In general, when exposed in utero to testosterone, the female offspring display male-like genitalia (Welsh et al, 2008; Wolf et al, 2002), increased AGD (Kawashima et al, 1978; McCoy and Shirley, 1992; Rhees et al, 1997) delayed puberty, early constant estrus (McCoy and Shirley, 1992; Slob et al, 1983) and increased infanticide in rats (Rosenberg and Sherman, 1974). The WD can be stabilised in females exposed to testosterone and its derivatives have also been found in postnatal female rats. Some studies have found prostates in females after excess testosterone exposure but the same studies have not found the WD to be stabilised. In contrast, some studies have not only reported the stabilising of WD but have also provided evidence for its coiling (differentiation) (Welsh et al, 2009). The latter study reported that WD derivatives persisted into adulthood despite the lack of exogenous androgens postnatally. Furthermore, WD of control females which regress were reported to have apoptotic activity in contrast to the WD stabilised in testosterone exposed females which have cell proliferative activity. Of particular significance is the timing of WD stabilisation reported. Welsh et al (2009) concluded that degeneration of the WD in males in which androgen action was ablated experimentally happened later than in control females and that a male female time difference in programming of the WD may exist.

An extensive study was published in 2001 by Wolf et al who exposed rat fetuses to various doses of testosterone propionate from e14.5-e19.5. The exposure to TP in utero had no effect on the male pups at any dose, at the ages examined. At a dose of 0.5mg/kg TP, exposed females displayed increased AGD at weaning, reduced number of nipples, cleft phallus, small vaginal orifice, and the presence of prostatic tissue. At a higher dose

of 10mg/kg, virilisation of the females was more prominent, with no vaginal opening at all and animals also had bulbourethral glands as well as seminal vesicles. Some females also displayed a greatly distended, fluid filled uterus or hydrometrocolpos (Wolf et al, 2002). These studies also reported that TP treated dams were late with parturition, had abnormally small litter sizes as well as lower birth weights of pups. This is a well documented effect of TP in rats and some studies have resorted to caesarean section and use of foster mothers to overcome problems with dystocia (Welsh et al, 2008, 2010). In contrast injecting testosterone directly into the amniotic sac had a less severe effect (Swanson and Bosch, 1965). This is one of the reasons why the studies conducted in this thesis to investigate androgens, used DHT rather than testosterone. Further reasons for using DHT are discussed in chapter 3.

There have been reports that female rats display a homologue of the male prostate and this is termed the Skenes paraurethral gland. Some scientists have reported that Wistar rats (the strain used for the studies in this thesis) do not have this gland due to absence of sufficient testosterone during embryonic and neonatal periods, whereas others have reported that some Wistar females can have an underdeveloped but functional prostate (Biancardi et al, 2010). In our control Wistar female rats, no prostates have ever been reported previously and were not found in the control studies reported in this thesis.

1.7. Androgen biochemical associates

So far, this review has discussed androgens and AR. In transcription, this ligand-receptor complex does not work alone. AR has been shown to work with various transcription factors. Over the past 15 years, it has become established that correct AR transcriptional activity also depends on various factors which do not posses DNA binding affinity. These include, but are not limited to, co-regulators, methylation enzymes and prostaglandins.

1.7.1 Co-regulators

Co-regulators can be generally defined as proteins which interact with nuclear receptors to either enhance (co-activators) or repress (co-repressors) transcription. Co-regulators are not generally considered to possess specific DNA binding affinity and do not significantly alter basal transcription rate (McKenna et al, 1999). Almost 300 nuclear receptor co-regulators have been identified since the isolation of the first co-activator in 1995. In 1969, Britten and Davidson published a theory of 'master genes', a type of 'master RNAs' suggested to directly regulate transcription, but data could not be found to support this. Then, the vast studies focusing on RNA pol II and regulation of mRNA transcription discovered a new group of proteins. These proteins, co-regulators, were proven to be vital in transcription in addition to RNA pol II and general transcription factors (Lavery et al, 2006; Lee and Young, 2000; McEwan et al, 1997). The emergence of new co-regulators and our understanding of co-regulator function is growing progressively. It is now accepted that co-regulators are involved in virtually all of the reactions needed for control of gene expression. Many publications now term the genes which encode the co-regulator proteins, the true master genes of eukaryotes, proving Britten and Davidson were not completely incorrect.

Co-regulators that interact directly with nuclear receptors exist in large steady state complexes with multiple secondary co-regulator partners. Each component may have multiple enzymatic capabilities, for example acetyltransferase, ATPase activities, methyltransferase. In addition, co-regulator proteins are affected by phosphorylation, methylation and other modification statuses. Most co-regulators are expressed in a wide variety of cell types and can interact with more than one nuclear receptor and there may also be redundancy between or within different classes of co-regulators (Heinlein and Chang, 2002; Heemer and Tindall, 2007). Furthermore, multiple cofactors may regulate nuclear receptor function at any one time. This fundamentally complicates interpretation as the demonstration of co-expression of a co-regulator with a particular nuclear receptor

does not necessarily mean it is modulating the activity of that particular receptor at that time.

There are many ways in which this large group of proteins can and have been categorized. In this review, co-regulators will be classified and briefly discussed based on functional properties, followed by discussion of key (for this thesis) co-regulators.

1.7.1.1 Components of the chromatin remodelling complex

Gene transcription occurs on a chromatin template, in which DNA is wound around a core of four basic histone proteins (H2A, H2B, H3 and H4), which form nucleosomes. Regions of DNA between adjacent nucleosomes are linked by histone H1 (Wolffe, 2001; Hayes and Hansen, 2001). DNA-histone interactions limit the accessibility of the nucleosomal DNA to transcription factors, thus forming an obstacle to transcription. Chromatin remodelling complexes alter and unwrap the histone-DNA contacts in an ATP dependent manner (catalysed by ATP), leading to re-organisation of the nucleosomal structure, thus making the chromatin status more permissive to transcription (Trotter and Archer 2008).

There are several AR co-regulators identified as components of the chromatin remodelling complex. Androgen receptor interacting protein 4 (ARIP4) was one of the first proteins which illustrated chromatin remodelling with AR. ARIP4 interacts with the zinc finger region and stimulates AR dependent transactivation, shown in co-transfection studies (Rouleau et al, 2002). After this initial discovery, an array of chromatin remodelling complexes which work with AR have emerged, for example SRG3 (Hong et al, 2005) and SRCAP (Monroy et al, 2003). Perhaps the most established (in the literature) chromatin remodelling complex is the SW1/SNF family which contains the ATP-ase Brahma related gene (BRG1), shown to be a potent AR regulator.

1.7 .1.1.1 BRG1

SW1/SNF compromise a family of large, multi-subunit complexes containing 8 or more proteins present in a cell which contain one of two core ATP-ases; BRG1or hBRM as well as numerous BAFs (BRG1 associated factors) (Trotter and Archer 2008). Human SW1/SNF complexes usually contain core subunits of BRG1 (or hBRM), BAF170, BAF155 and BAF47, BAF60, BAF57 and BAF53 as well as actin (Wang et al, 1996). These complexes regulate transcription factors by re-organising the chromatin structure through one of two processes, either by facilitating nucleosome condensation, which induces repression of transcription or by nucleosome dispersion which assists in activation of transcription. BRG1 has been identified to interact with many different proteins and some of these are summarised in Fig 1.8.

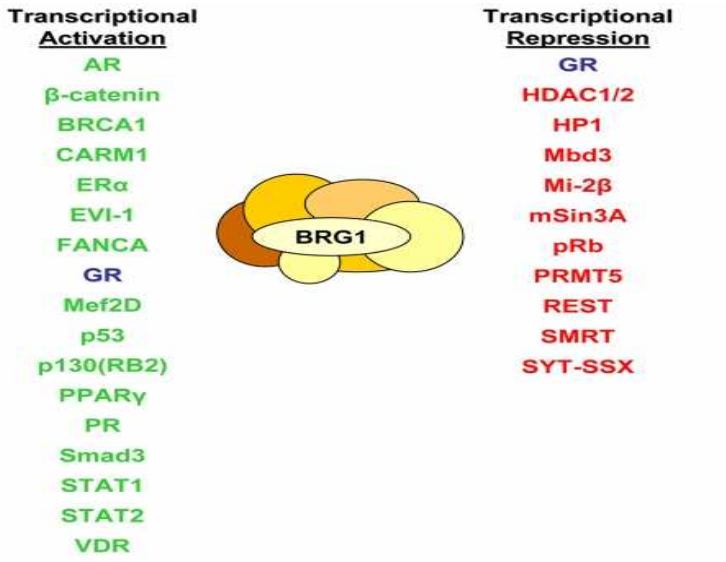


Figure1.8 Summary of proteins which BRG1 co-regulates in either transcriptional activation or transcriptional repression. Diagram adapted from Trotter and Archer, 2008.

BRG1 and *hBRM* share a high degree of sequence identity (74%) and in vitro, display similar biochemical activities (Khavari et al 1993; Randazzo et al 1994), however, in vivo are believed to have different roles in proliferation and differentiation (Kadam and

Emerson, 2003; Reyes et al, 1998). Although the exact mechanisms employed in the different roles are yet to be elucidated, knock out models have provided insight into the importance of these ATP-ase units. BRG1 deficient mice die at the pre-implantation stage suggesting it is vital for early development. *BRG1* heterozygous mice are thought to be more susceptible to tumour development (Glaros et al, 2008). Conversely, hBRM null mice are found to be viable and fertile, although they do express *BRG1* at higher levels than controls in particular tissues and display aberrant cell cycle regulation (Reyes et al, 1998). The *BRG1* gene is composed of multiple domains which include an ATP-ase domain, a C-terminal bromodomain, AT-hook motif and an N-terminal region which houses three further domains; QLQ, HSA and BRK domains (Khavari et al 1993).

The large SW1/SNF multimeric assemblies are recruited to specific gene targets through association with DNA-binding transcription factors, co-regulators or by general transcriptional machinery. Furthermore, various different interactions are thought to be involved in recruiting and stabilising the BRG1 unit to the hormone responsive elements in promoters (Trotter and Archer, 2008). Several SW1/SNF components including BRG1, BAF250, BAF60 and BAF57 have been shown to mediate interactions of nuclear receptors including glucocorticoid, oestrogen and androgen receptors (Link et al, 2005). Although the role of SW1/SNF is better established in oestrogen and glucocorticoid receptors, progress is being made to understand if this complex is important for androgen receptors. BRG1 has been shown to be involved in both co-activation and co-repression of AR (Trotter and Archer, 2008).

Probasin and prostate specific antigen (PSA) are two proteins which are predominantly expressed in the prostate. The gene for each of these has a strong promoter sequence for the target of AR. Cell culture experiments have recently shown the AR target promoter sequences of *probasin* and *PSA* require SW1/SNF for complete activation. In the absence of SW1/SNF, *PSA* failed to activate entirely and only a low level of activation was observed for *probasin*, illustrating that *probasin* can be at least partially activated

independent of chromatin remodelling. Both gene AR promoters were fully activated when *hBRM* was re-introduced into the cell culture. The re-introduction of *BRG1* however only weakly activated probasin and did not manage to activate *PSA* promoter by the androgen receptor. This data suggested *hBRM* was the preferred core ATP-ase of SW1/SNF for AR activity (Marshall et al, 2003).

Studies conducted by Dai et al (2008) found *BRG1* to be involved in AR antagonism in prostate cancer cells. Ablating androgen action using antagonists is an important method of treatment for prostate cancer; however the mechanisms by which the antagonists work are poorly understood. This study illustrated that *prohibitin*, which is a tumour suppressor, is essential in androgen antagonism. *BRG1* but not *hBRM* was demonstrated to be required for the repressive action of *prohibitin* on AR responsive promoters. The antagonist bound to AR is thought to induce recruitment of prohibitin, and BRG1 to endogenous AR responsive promoters to mediate transcriptional repression. At the PSA promoter, this happened by PSA disassociating from p300 (a histone modifier co-regulator) allowing BRG1 to remodel the chromatin. The p300 co-regulator is closely associated with CBP (CREB binding protein) and this co-regulator is discussed in detail below. However, it should be noted that CBP interacts with BRG1 and this interaction has been demonstrated to be vital at least in the transcriptional activation of the oestrogen receptor (Direnzo et al, 2000; Naidu et al, 2009). Studies have shown, in response to oestrogen, ER recruits BRG1, and targets BRG1 to the promoters of oestrogen responsive genes, in a manner that occurs simultaneously to histone acetylation, controlled by CBP (Direnzo et al, 2000). BRG1 mediated co-activation of ER signalling is regulated by the state of histone acetylation of the cell, controlled by CBP. Given the importance of both these co-regulators, and the various roles they play and their well documented roles in ER and GR activation, it is surprising that this mechanism has not yet been furthered explored with the AR.

Some studies have found higher levels of BRG1 in malignant prostate cancer tissue and this was associated with larger volume of tumour mass (Sun et al 2007), and yet the loss of *BRG1* (targeted knockout) actually potentiates lung cancer. (Glaros et al, 2008). It is hypothesised that different genes have different requirements for two ATP-ases with some relying on *hBRM* and others on *BRG1* for androgen regulation.

1.7.1.2 Histone Modifiers

More localised control over transcriptional events in the chromatin can be achieved by modifications of histone residues. Histone residues can be modified in a number of ways including acetylation, methylation, phosphorylation, ubiquitination, ADP-ribosylation and glycosylation. Overall, most of these modifications change the net charge of the nucleosome, inducing either a loosening or a tightening of the DNA histone interactions (Heemers and Tindall, 2007). Several co-regulator proteins function in this pathway in either co-activation of AR, by recruiting histone acetylase, or co-repressing AR activity by de-acteylation, rendering the chromatin environment transcriptionally repressed.

1.7.1.2.1 CREB binding proteins (CBP)

The CREB binding protein (CBP) was initially identified as a co-activator for CREB, the cAMP response element binding protein (Chirivia et al, 1993). These studies used protein interaction assays and determined that CBP can bind to CREB when CREB is phosphorylated by protein kinase A. Similar protein assays, using an adenoviral transforming protein E1A, discovered *p300,* a homologue of *CBP* as both co-regulators share a highly conserved sequence (Stein et al, 1990; Eckner et al, 1994). Since then, CBP and p300 have been reported to bind to a number of different proteins involved in transcriptional regulation. Their intrinsic histone acetyltransferase activity works to relax the chromatin and assist in transcription (Fronsdal et al, 1998) and some of the interactions that CPB/p300 are involved in are outlined in Fig 1.9. *CBP* and *p300* have similar structures. Both genes contain a nuclear receptor interaction domain, the CREB interaction domain, the cysteine/histidine regions, the interferon response binding

domain, the histone acetyletransferase domain and a bromodomain, which binds acetylated lysines. A PHD finger motif has also been identified but its function is unknown, thus the name is fitting (Shiama et al, 1997; Vo and Goodman, 2001).

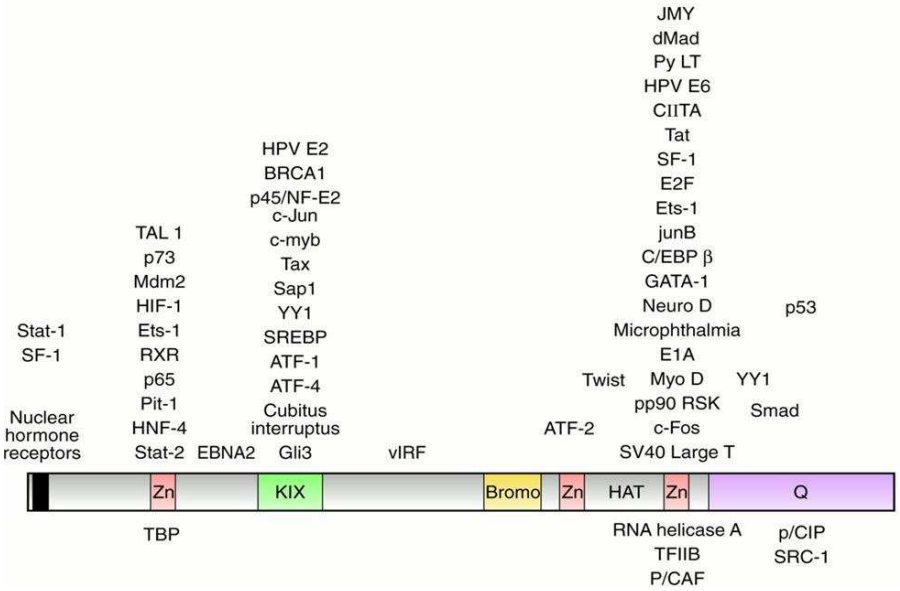


Figure 1.9 Domain structure of CBP/p300 gene indicating three zinc fingers (Zn), a bromodomain (Bromo), a CREB binding domain (KIX), a HAT domain and glutamine-rich domain (Q). Association with transcriptional activators is shown above the domains with which they interact and transcriptional repressors are indicated below. Diagram from Vo and Goodman, 2001.

CBP and p300 have been reported to assist in transcription using three main methods, condensing or relaxing the chromatin, recruiting basal transcription machinery or acting as adaptor molecules. Despite their similarities, however, it should be noted that CBP and p300 are not functionally interchangeable (Vo and Goodman, 2001). This can be observed from the different phenotypes which occur as a result of specific knockouts. For example, heterozygous inactivation of *CBP* in mice leads to haematological defects and a predisposition to cancer which is not displayed by mice with *p300* deficiencies (Kung et al, 2000). A similar scenario is observed in humans in whom insufficiency of CBP leads to Rubinstein-Taybi Syndrome (RTS), a disease characterized by mental retardation, craniofacial abnormalities, broad toes and thumbs as well as an increased

incidence of malignancy (Blobel, 2000). An identical mutation in one p300 allele however does not produce the same haemotological or cancer pre-disposition effects (Goodman and Smolik, 2000). Homozygous mutations in either *CBP* or *p300* however result in embryonic lethality and furthermore, *CBP/p300* double hetrozygotes are invariably lethal which suggests that their function may overlap to some extent (Vo and Goodman, 2001).

The complexity of CBP and p300 protein interactions has led to them being described as molecular integrators rather than co-regulators. This is exemplified by the vast number of publications outlining the various different factors that CBP/p300 have been shown to interact with and their various modes of action. Historically, the adenovirus, E1a has played a large role in understanding CBP/p300. E1a is a multifunctional pleiotrophic protein that mediates biological effects through transcriptional activity of target genes. It affects proliferation and cellular differentiation to produce an oncogenic phenotype (Shiama et al, 1997). The N-terminal of E1a can bind to and block CBP/p300 which causes the cell to enter the S phase of the cell cycle. Two methods have been proposed for how CBP/p300 action might be blocked. The first of these proposes that E1a can bind to the CBP/p300 region which interacts with RNA polymerase II and TFIIB, thus preventing these essential (for transcription) factors from interacting with the co-activators (Felzien et al, 1999). In addition, E1A binding to the third zinc finger domain of CBP/p300 has been reported, blocking the histone acetyl activity essential for CBP/p300 mode of action (Chakravarti et al, 1999; Perissi et al, 1999). Other than E1a, CBP/p300 has been shown to interact with many other viral oncoproteins, including human T-cell leukemia virus (HTLV-1), V-myb and human papilomavirus E6 (Goodman and Smolik, 2000). In addition to oncoproteins and CREB, CBP/p300 also works with other transcription factors such as c-myb, Sap1a, Elk1 and NFKb (Aarnisalo et al,1998) and many of the members of the nuclear receptor family.

CBP/p300 has been reported to interact with the oestrogen receptor, glucocorticoid receptor, retinoic acid receptor, thyroid hormone receptor and the androgen receptor

(Aarnisalo et al, 1998). Using cell culture techniques, it has been shown that the N-terminus of *CBP/p300* works with both the N-terminal domain and DNA binding domain of AR. Furthermore, it has been suggested that the limiting amount of CBP in the cell might mediate transcriptional interference with AP-1. AP-1 is Activator Protein - 1, a transcription factor which is involved in regulation of genes involved in differentiation, proliferation and apoptosis.

As briefly mentioned in section 1.7.1.1.1, it has been shown that CBP and BRG1 work together with oestrogen and glucocorticoid receptors (Direnzo et al, 2000; Naidu et al, 2009). Given the various different pathways each of these co-regulators is involved with, and the importance of their expression during fetal life, it is surprising that their role with androgens during the fetal period has not gained more attention. To my knowledge, no such studies have been published in the literature.

17.1.3 Components of the ubiquitination/ proteasome pathway

Ubiquitination is a post translational modification of cellular proteins in which ubiquitin (a 76 amino acid polypeptide) is attached to lysines in target proteins. Ubiquitination allows proper progression through rounds of transcription and the appropriate assembly of the necessary proteins. Ubiquitination involves the activation of an E1 enzyme, which transfers ubiquitin to a conjugated E2 enzyme and this recruits E3 ligase, which transfers the ubiquitin to the intended target protein (Heemers and Tindall, 2007).

AR co-regulators which employ ubiquitination in order to regulate AR activity include E6-AP (Khan et al, 2006), Mdm2 (Gaughan et al, 2005), PIRH2 (Logan et al, 2006), SNURF/RN4 (Poukka et al, 2000) and Chip (Rees et al, 2006). Each of these co-regulators employs the ubiquitination pathway, but all work via a different mechanism. For example, E6-AP is recruited to the promoter region of target genes upon androgen binding to its receptor and interacts with the N-terminal to enhance transactivation (Khan et al, 2006). E6-AP may also be involved in regulating the level of AR protein. This is demonstrated in E6-AP null mice, which display increased AR levels, and

similarly, cell culture experiments in which E6-AP is over expressed causes a decrease in AR protein expression.

Proteins such as Mdm2 interact with the AR N-terminal domain and the DNA binding domain and this interaction is dependent on E3 ligase activity. Furthermore the Mdm2-AR complex actually recruits another AR co-regulator, HDAC1 and in response to androgen, both AR and HDAC1 become ubiqutinated. Mdm2 and HDAC1 work at target gene promoter sites to attenuate activity. Furthermore, this activity is attenuated by the histone acetyl transferase activity of another AR co-regulator, Tip60 (Gaughan et al, 2005). This is a fine demonstration of the interplay between various AR-co-regulators and the vast number of interactions which may be taking place, to direct the correct action of androgens through AR.

17.1.4 Components of sumoylation

SUMO, the small ubiqutin modifier protein is similar to ubiquitination in its employment of E1 activating enzyme, E2 conjugating enzymes and an E3 ligase enzyme, but differs due to the SUMO homologs, SUMO 1, 2, and 3. SUMO 1 has been shown to decrease AR activity (Poukka et al, 2000) whereas SUMO 2 and 3 enhance transcription (Zheng et al, 2006).

1.7 .1.5 Chaperones and co chaperones

In the absence of the ligand, AR is stabilised within the cytoplasm by being bound to molecular chaperones. Although ligand binding disassociates the AR from the majority of the heat shock proteins (reviewed in section 1.4.1), some chaperones remain attached to the AR. These act as co-regulators for downstream events such as translocation, transcription and even AR degradation. For example Hsp40 has been shown to be necessary for androgen binding to AR and defects of Hsp40 leads to problems in the formation of the AR–Hsp70 complex which causes AR folding defects (Fan et al, 2005).

In contrast, loss of Hsp70 in a mouse knock out model showed enhanced AR gene transcription which led to defects in spermatogenesis (Terada et al, 2005).

1.7.1.6 Cytoskeletal proteins

Conventionally, actin is thought of as a cytoplasmic organiser and a major component of the cell cytoskeleton. More recently, actin has been shown to be a player in transcriptional events also, including playing roles in chromatin remodelling (Ting et al, 2002). SUPERVILLIN is an actin binding protein which interacts with both the N-terminal domain and DNA-binding domain of AR and this association is enhanced by androgens but furthermore cooperates with other AR co-regulators such as ARA55 and ARA70 (Ting et al, 2004). Three different isoforms of actin cooperate with SUPERVILLIN to stimulate AR transactivation. Another example of an actin binding protein is GELOSOLIN which has been reported to interact with AR during nuclear translocation to enhance ligand dependent AR activity (Nishimura et al, 2003).

1.7.1.7 Endocytosis

Proteins which are involved in cell endocytosis can play multiple roles and are reported to interact with AR to influence AR mediated transcription. One example of this is huntingtin-interacting protein, HIP1 which associates with AR to reduce the rate of AR protein degradation. HIP1 is recruited to AREs upon androgen stimulation and it has been shown that over-expression of HIP1 enhances AR transcription whereas transcription is repressed when HIP1 is knocked down (Mills et al, 2005). G-associated kinase (GAK) is another example of a protein involved in endocytosis which has been shown to interact with the N-terminal domain of AR to enhance AF1 activity, in a ligand dependent manner (Ray et al, 2006).

1.7.1.8 Cell cycle regulators

There seem to be few components of the cell machinery which are not involved with androgen and AR action. Proteins which are involved in cell cycle regulation are also

involved in AR modulation. One such protein is CYCLIN E, which has been shown to bind directly to the N-terminal domain of AR to enhance AF1 transactivation function (Heemers and Tindall, 2007). Another example of a cell cycle regulator involved with AR transcription is CDC25B, which interacts directly with AR to enhance transcription, and the action of this complex is enhanced by interaction with CBP (Ma et al, 2001). Other cell cycle regulators inhibit AR activity. An example of this is CYCLIN D1 which can bind directly to the AF-1 region of AR, independent of the ligand, recruit HDACs and inhibit the N and C-terminal domains of AR. This activity has been shown to be independent of the role that Cyclin D1 plays in cell cycle regulation (Reuten et al, 2001; Petre-Draviam et al, 2003). The retinoblastoma protein (RB) is a protein which functions as a tumour suppressor and controls progression through cell cycles. RB can bind to AR to enhance transcriptional activity and it does this by co-operating with another AR co-regulator, ARA70 (Yeh et al, 1998).

1.7.1.9 Regulators of apoptosis

Proteins involved in the execution of apoptosis can also act as AR co-regulators. An example of this is seen with the pro-apoptotic caspase-8, which represses AR dependent gene expression by disrupting N and C terminal interaction of AR. This inhibits androgen induced nuclear localisation (Qi et al, 2007) which leads to transcriptional repression. An example of co-activation by apoptotic factors is displayed by Par-4, a pro-apoptotic protein which interacts with the AR DNA binding domain and is recruited to the promoter of AR driven genes. Here it enhances the association of AR with DNA and increases AR dependent transcription (Gao et al, 2006).

1.7.1.10 RWdd1

A newly discovered co-regulator is Rwdd1 which in the rat has also been called small androgen receptor interacting protein. Rwdd1 is an abbreviation of RWD containing 1; RWD is a domain named after three major RWD containing proteins, RING-Finger containing proteins, WD domain containing proteins and DEAD like helicases. This co-

regulator was originally identified in the thymus only 3 years ago (Kang et al 2008a) and its functions are yet to be fully understood. There is evidence suggesting that AR is related to thymus involution as testosterone inhibits thymocyte development and castrated animals display thymus enlargement. Moreover androgen deprivation can lead to T- cell augmentation. In an epithelial cell line it has been shown that Rwdd1 can enhance transactivation of AR. In localisation studies, in the thymus at least, Rwdd1 and AR may both be cytoplasmic and upon ligand binding, RWDD1 remains cytoplasmic whereas the AR complex becomes nuclear. FRET studies suggest there is no direct interaction between RWDD1 and AR in this organ (Kang et al 2008b).

1.7.2 Methylation

In mammals, DNA methylation is a vital cellular process (Fraga, 2009). DNA methylation is the covalent addition of methyl group to the 5'position of cytosines. This usually occurs on a cyotisne which is followed by a guanine and termed CpG dinucleotides (reviewed by Garcia-Carpizo et al, 2011). CpG dinucleotides occur in a low frequency in vertebrates but stretches of DNA with high CCG content with high frequency of CpG dinucleotides relative to the bulk genome have been identified and these are termed CpG islands (Gardiner-Garden et al, 1987). Most CpG dinucleotides are methylated but those located in CpG islands, found mainly in promoter regions of genes, are not usually methylated (Garcia-Carpizo et al, 2011). DNA methylation is responsible for genomic imprinting, X chromosome inactivation in females and for securing monoallelic gene expression by repressing expression of maternal or paternal genes. DNA methylation is catalysed by DNA methyltransferase (DNMT) enzymes and knockout mice lacking these enzymes die at the morula stage (Shock et al, 2011, Woroniecki et al, 2011). Generally, DNA methylation leads to the repression of gene expression whereby methylated genes are either not transcribed or transcribed at a reduced rate (reviewed by Garcia-Carpizo et al, 2011).

A full review of the role of gene methylation in the embryo and in postnatal life is outwith the scope of this review and can be found elsewhere (Walsh, 2006, chapter 5). Methylation and the enzymes involved have become an important focus of epigenetic research and the literature concerning methylation and AR is increasing. Very recently, histone methyltransferase (HMT) and histone demethyltransferase (HDM) have been shown to be involved in AR gene regulation. In particular, an HMT enzyme termed SET9 interacts with AR and associates with the PSA promoter in a ligand dependent manner. SET9 adds a methyl group onto a lysine residue within the hinge region of PSA and this modification is necessary for AR activity in prostate cancer cells. Thus, SET9 plays an important role in cancer progression and implicates another mode of AR regulation (Gaughan et al 2010). Another study done in prostate cancer cells has shown lysine specific demethylase 1 (LSD1 also known as BHC110) co-localises with AR in vitro and in vivo to stimulate AR expression. LSD1 can demethylate to control specific gene expression (Metzger et al 2005).

1.7.2.1 DNA methyltransferase like -3 (DNMT3L)

Four DNMT enzymes involved in catalysing DNA methylation have been characterised in the literature. DMNT3A and DNMT3B are de novo methyltransferases which function to establish DNA methylation patterns early in development and DNMT31 maintains methylation patterns during replication (Aapola et al, 2002). The fourth DNMT enzymes, is DNMT3L (DNMT3-like) and this member shares similarity in protein structure to DNMT3A, DNMT3B and DNMT31 and in addition, it has been shown that DNMT3L is required specifically for the establishment of maternal genomic imprints and is vital for de novo methylation (Aapola et al, 2002). However, DNMT3L lacks the catalytic motif required for methyltransferase and is believed to function as a regulator of methylation at imprinted loci rather than a cytosine methylatransferase enzyme (Aapola et al, 2002; Garcia-Carpizo et al, 2011). Although the mechanisms by which DNMT3L regulates methylation are not completely understood, it has been

shown that DNMT3L can mediate transcriptional repression through interaction by associating with histone deactylase activity (Aapola et al, 2002; Deplus et al, 2002).

Targeted disruption of *DNMT3L* expression in mice led to severe hypogonadism and Sertoli cell only phenotype with azoospermia. Heterozygous progeny of the *DNMT3L* deficient homozygous females failed to develop past 9 days of gestation due to embryonic defects resulting in biallelic expression of genes which are normally only expressed from one the of paternal origin (Bourc'his et al, 2001). Further studies have characterised the testis of *DNMT3L* knock out mice to understating the cause of the impaired spermatogenesis (Webster et al, 2005) and found GC in these mice were arrested and died around the early meiotic stage (Hata et al, 2006). Microarray expression profiling of the testis of *DNMT3L* knockout mice found a down regulation of gonad specific and or sex-chromosome linked genes (Hata et al, 2006).

1.7.2.2 Brother of the regulator of imprinted sites (BORIS)

CTCF is a conserved and ubiquitous 11 zinc finger protein which is involved in various aspects of gene regulation and forms methylation sensitive insulators that regulate X chromosome inactivation and expression of imprinted genes (Loukinov et al, 2002). More recently a paralogue for this gene was identified, BORIS, which has the same exons encoding the 11 zinc finger domain as the CTCF gene (Klenova et al, 2002). However BORIS expression has only been found in the testis and is predominantly expressed during embryonic male germ cell development; however, it is also expressed in tumours and consequently has been classified as a cancer-germline or cancer-testis gene (Nguyen et al, 2008; D'Arcy et al, 2008). Using cancer cell lines, it has been shown that disruption of DNA methylation induces BORIS expression. In addition the promoter site of BORIS has DNA hypomethylation and histone 3 modification patterns (Woloszynska-Read et al, 2007). Furthermore, the BORIS promoter is repressed by CpG methylation in a dose dependent manner which suggests a role for DNA methylation in BORIS transcriptional regulation.

1.7.3 Prostaglandins

Prostaglandins, the products of the arachidonic acid pathway, were originally isolated in 1935 from the seminal fluid of the prostate (Goldblatt 1933, Von Euler 1936). Prostaglandins are lipid compounds which are derived from essential fatty acids. They have been shown to be autocrine and paracrine mediators involved in a wide variety of physiological effects in various tissues and organs around the body. These physiological effects include activation of the inflammatory response (Williams and Peck, 1977), platelet movement, calcium regulation (Horribin et al, 1978) and regulation of hormonal secretions of the hypothalamic-pituitary-testicular axis (Vermes et al, 1979). The arachidonic acid pathway is summarised in Fig 1.10. In brief, an intermediate is created from phospholipase–A2, which is either catalysed down the cycloxygenase pathway to produce prostaglandins and thromboxanes or catalysed down the lipooxygenase pathway to form leukotrienes. The cyclooxygenase (COX) enzymes are crucial in the formation of prostaglandins. In order to inhibit the inflammatory process and consequent pain, COX-inhibitors, such as aspirin and indomethacin are used commonly as medicines.

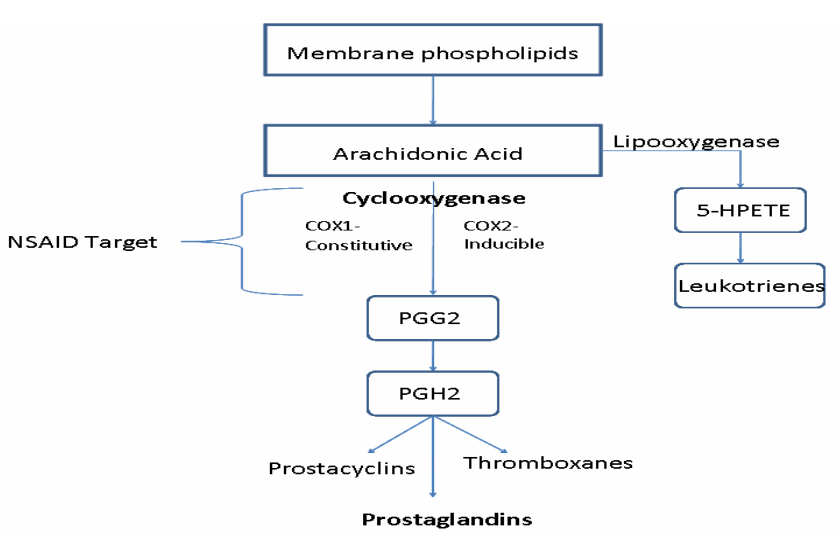


Figure 1.10 Summary of the production of prostaglandins from phospholipids and the involvments of COX1 and COX2.

COX1 is present in most tissues to maintain regular function whereas COX2 is mainly expressed in tissues upon their inflammation (Kuboto et al 2011). Perhaps surprisingly, therefore, COX2 is the predominant isoform expressed in the fetal male reproductive tract (Kirschenbaum et al, 2000). Using immunohistochemistry, it has been shown that COX1 levels are minimal in the male reproductive tract, with no expression detected in fetal life. COX2 has been found in the fetal and adult seminal vesicles, and also in the ejaculatory ducts (vas deferens), with expression varying and correlating with levels of testosterone (Kirschenbaum et al, 2000). A role for prostaglandins in regulating testosterone production is well documented (Sawada et al 1998, Romanelli et al 1995, Wang et al 2003), and will not be reviewed in full detail here. In brief, it should be noted that arachdonic acid is involved in the regulation of testosterone production in adult Leydig cells (Romanelli et al 1995). Furthermore, some reports suggest COX2 is involved in the regulation of steroidogenic acute regulatory gene transcription and hormone biosynthesis (Wang et al, 2003). Moreover, mice deficient in COX1 remain fertile whereas deficiencies in COX2 lead to infertility (Kirschenbaum et al, 2000).

A series of studies published in the late 1980's suggested that the arachidonic acid pathway was involved in the masculinising action of testosterone, particularly the embryonic external genitalia in mice. The initial studies blocked masculinisation using oestradiol 17-β or cyproterone acetate and animals displayed typical evidence of impaired androgen action, such as reduced anogenital distance and hypospadias. This effect was reversed by the administration of arachidonic acid and, furthermore, this reversal was blocked by administering COX inhibitors such as aspirin and indomethacin (Gupta and Goldman 1986). The mice in this study were exposed to treatments between e11-e14 and Gupta termed this time as 'a critical period for affecting anogenital distance'. Later studies by the same group investigated two key prostaglandins, PGE2 and 6-keto-PGF1. These studies reported male and female fetuses to have similar levels of these PGs before 'the period of sexual differentiation', which in this study is defined as e13-e17. During the period of sexual differentiation, these prostaglandins were found

to increase in the male genital tract but not in the female. Furthermore, when female fetuses were exposed to testosterone, their levels of these PGs were reported to increase, and in males exposed to an anti-androgen, the investigated PG levels decreased (Gupta, 1989). This study suggests three concepts which are important to this thesis; the first of these is that the period of masculinisation in mice is from e13-e17. This correlates with what we now call the masculinisation programming window (MPW), a time frame found in rats when correct androgen action is critical in the development of the male reproductive tract. In rats this time frame is suggested to be e15.5-e18.5 (Welsh et al, 2008). The MPW was discussed in detail in section 1.1.6. The second important concept is that prostaglandins are important for male sexual differentiation. Finally, blocking PG synthesis using indomethacin can affect virilisation. In the last study published by this group in this field, an in vitro organ culture was used to determine the role that PGE2 played in differentiation of the testosterone dependent Wolffian duct (WD). This study found that blocking PG synthesis using an anti-PGE2 antibody, even in the presence of testosterone, blocked differentiation of the WD in a dose dependent manner. Furthermore, the differentiation of a WD in females as a result of exposure to testosterone in utero, was inhibited by exposure to indomethacin (Gupta and Bentlejewski, 1992). Thus a very convincing set of studies, demonstrating a critical time period for masculinisation, but also the importance of arachidonic acid and prostaglandins in producing the correct male (and female) phenotype. Investigations by other groups have also suggested PGs are important in the male reproductive tract.

1.8 Non genomic effects of androgens

The discussions so far in this review have centred around the genomic actions of androgens. However, there is growing evidence indicating that androgens are capable of triggering cellular processes through non-genomic, rapidly occurring mechanisms (Kousteni et al, 2001; Nazareth and Weigel, 1996; Peterziel et al, 1999). These actions usually occur within seconds or minutes and are often dependent on plasma membrane associated signalling pathways that lead to the activation of cytoplasmic second

messengers. The two most studied non genomic androgen pathways involve kinase associated intracellular signalling and modulation of intracellular calcium levels. The molecular mechanisms and, more importantly, physiological relevance of the non genomic effects remains largely unknown but recently more studies are being undertaken to gain insight into this.

The emerging literature suggests that non-genomic androgen action may be important in the fetal male reproductive tract. A rapid increase in intracellular calcium was observed when primary rat Sertoli cells, human androgen dependent (LNCaP) and androgen independent (PC3) human prostate cancer cells were exposed to testosterone, DHT or R881 (an AR agonist). In another study, testosterone was able to induce a rapid increase in concentration of the transcriptional factor CREB in rat primary Sertoli cell culture via MAPK pathways (Cheng et al, 2007). However, it is important to note that both rat Sertoli cells and LNCaP cells contain a population of plasma membrane associated AR which transiently increase in expression in response to testosterone stimulation. Therefore it is yet to be elucidated whether the non genomic actions of androgens are in fact taking place via the classical AR genomic mechanism, through membrane bound AR or perhaps by another membrane associated signalling pathway.

MAP kinases are proteins which transduce signals coming from cell membrane receptors after binding of growth factors such as epidermal growth factor (EGF) or insulin like growth factor 1(IGF-1). Yeh et al (1999) illustrated MAP kinases can phosphorylate and activate the AR, with the respective phosphorylation site being in the N terminal domain. Further studies have shown that not only can MAP kinases activate AR in the absence of androgens, but in the presence of androgens, AR can activate MAP kinases.
A different route of ligand independent AR activation is through cross talk with PKA pathways. Studies have shown that PKA can interact with the N-terminal domain of AR, and in addition activators of AR such as forskolin can induce AR activation and PSA secretion in the absence of androgens (Nazareth and Weigal, 1996).

1.9. The indifferent reproductive tract

The reproductive tract consists of the gonads, the internal genital system and the external genitalia. The development and differentiation of the gonads is primarily driven by genetics whereas differentiation of the internal and external genitalia is under the influence of hormones produced by the gonads and the appropriate receptors. During gastrulation, the embryo becomes tri-laminar with three germ layers; the ectoderm, the mesoderm and the endoderm (Carlson, 2004). Through a series of complex morphogenic movements, the tri-laminer embryo is converted into a tubular embryo containing a foregut, midgut and hindgut (Moore and Persuad, 2003). The hindgut terminates caudally in an expansion called the cloaca.

The mesoderm is the source of much of the urogenital system, and within this layer, three structures form; the pronephros, the mesonephros and the metanephros. The pronephros is the first to form and is a rudimentary, nonfunctional structure which gives rise to the pronephric duct. The pronephros regresses and is succeeded by the mesonephros; however, the pronephric ducts persist alongside the mesonephros and are called the mesonephric ducts, also termed the Wolffian ducts. The cranial end of the WD is connected to the gonads via the mesonephric tubules, which later in males form the efferent ducts. Later, and laterally to the WD, invaginations of the coelomic epithelium gives rise to paramesonephric ducts, also called the Müllerian duct. The MDs are positioned on the lateral side of the cranial WD but cross to the medial side at the caudal end. The WD gives rise to the internal reproductive tract of the male whereas the MD gives rise to the internal reproductive tract in the female and this is discussed in section 1.9.1.

The hindgut terminates caudally in an expansion called the cloaca which reaches a cloacal membrane on the ventral surface of the embryo (Yamada et al, 2003). The cloacal membrane and the oropharyngeal membrane are the only two sites of the embryo which are bi-layered with the absence of the mesoderm layer. The bi-layered cloacal

membrane retracts into the perineum due to the cranial and medial migration of mesodermal cells. These migrating mesodermal cells spread around the cloacal membrane and pile up to give rise to three swellings (Sajjid, 2010; Yamada et al, 2003). One swelling is the genital tubercle (GT) which appears in the midline between the cloacal membrane and the umbilicus. The GT, which will later form the phallus, at this stage is the same size in both males and females (Yamada et al, 2003). Distally, on either side of the cloacal membrane, two pairs of swellings appear, termed the urogenital folds and the urogenital swellings. A wedge of mesenchymal cells then forms the urorectal septum which migrates caudally to divide the cloaca into a ventral portion called the urogenital sinus (UGS) and a dorsal part called the anorectal canal (Baskin. 2004) The differentiation of the UGS is discussed further in section 1.9.2. The urorectal septum fuses with the cloacal membrane eventually causing a division between the urinary and genital systems (Yamada et al, 2003; Sajjid, 2010).

1.9.1 Development of the internal genitalia

The initially identical urogenital tract in both sexes consists of two duct systems, the WD and the MD. In males, the WD gives rise to the epididymis, vas deferens and seminal vesicles, and the MD regresses. In the females the MD is programmed to form the fallopian tubes, uterus, cervix and upper third of the vagina and the WD regresses (Jirasek, 1971; Hashimoto, 2003; Rey and Picard, 1998; Sajjid, 2010).

In males the MD regresses under the direction of AMH, which is secreted by the Sertoli cells, and binds to its receptor AMHR2 (Donahoe et al, 1977). This induces a signalling cascade that results in the production and secretion of matrix metalloptoteinase 2 (MMP2). MMP2 induces apoptosis in the epithelial cells of the MD (Roberts et al, 2002). Failure of this to occur in males leads to persistent MD syndrome (PMDS) (Thomson et al, 1994; Rey et al, 2011). Once the MD has regressed, testosterone from the LCs stabilise and differentiate the WD into the epididymis, vas deferens, and seminal vesicles and much like other male specific organs, this differentiation, is dependent upon

epithelial mesenchymal interactions (Xavier and Allard, 2003; Welsh et al, 2009). Testosterone can reach the WD in one of two ways, via the blood circulation, which is an endocrine interaction or via testicular fluid.

In rats, studies have illustrated that AR is expressed from e14.5 onwards specifically in mesenchymal cells of the WD whereas, AR does not switch on in the epithelial cells until e18.5 (Cooke et al, 1991) when the WD begins to elongate and convolute to produce a highly coiled epididymal structure. It is believed the effects of androgens on the epithelium are indirect and likely occur via the production of paracrine factors from the mesenchyme. Conversely, the paracrine factors produced by the mesenchyme and which differentiate the epithelium, stimulate production of growth factors and other signals which are essential for the growth of the mesenchyme; these include epidermal growth factor (EGF) (Gupta, 1999; reviewed by Archambeault et al, 2009), growth hormone (GH) (Nguyen et al, 1996), insulin-like growth factor 1 (Igf1) (Baker et al, 1996), transforming growth factor beta 2 (Tgfβ2) and fibroblast growth factor (Fgf) (Thomson and Cunha, 1999). Moreover, *Hox* genes, which are well established for their role as critical regulators of cell differentiation, positional arrangement, and patterning during the development of various organs, display regional specificity within the WD (reviewed in Hannema and Hughes, 2007). The precise role *Hox* genes play in WD development however is yet to be established.

In females the lack of testosterone causes the WD to regress and the lack of AMH allows the MD to persist (Jirsova and Vererova, 1993; Sajjid, 2010). Excess androgens in females in utero causes the WD to stabilise as discussed in section 1.3.3. It should be noted that the MD derivatives can develop even in the absence of the ovary contrasting the male WD which requires androgens produced by the testis for differentiation.

1.9.2 Differentiation of the UGS

The UGS forms in both males and females when the cloaca is subdivided by the urorectal septum. The UGS is divided into three sections, the upper, pelvic and phallic regions (Meeks and Schaeffer, 2011; Wilson, 1978). Like most structures of the reproductive tract, sexual dimorphism of the UGS is dependent on the presence or absence of androgen action. In males, the upper part of the UGS forms the future urinary bladder, the phallic part gives rise to the spongy part of the urethra and the urethral vestibules and the pelvic portion is where the prostate originates from (Carlson, chapter 16).

AR is expressed in the UGS mesenchyme and upon DHT binding, these cells induce differentiation of the epithelial cells though local paracrine effects of growth factors (Meeks and Schaeffer, 2011). This causes the outgrowth of solid buds from the UGS epithelium into the UGS mesenchyme. This epithelial-mesenchymal interaction results in cell differentiation and prostate branching morphogenesis (Chung and Cunha, 1983). Studies have shown that activation of AR by DHT in the mesenchymal cells results in the activation of at least the following pathways; Notch, FGF, TGF, Wnt and Shh (reviewed by Meeks and Schaeffer, 2010). Shh signalling causes the prostatic ducts to bud off the epithelium of the UGS and the extent of budding is controlled by the inhibitory action of BMP-4 (Pu et al, 2004). Furthermore, HOXA13 and HOXD13 are also involved in the positioning of the tissue and differentiation of prostatic buds (Podlasek et al, 1997; Warot et al, 1997).

It is interesting to note that the genes which control formation of the prostate are also involved in formation of the genital tubercle in the male. How the same set of control genes leads to the formation of two completely distinct structures is not yet understood. It is possible that interacting partners may be expressed differentially, or different thresholds or combination of expression may be the determining factors. In addition, other molecules such as CD44 and follistatin, not known to play a role in GT

development, have been identified in prostate development (Cancilla et al, 2001; Gakunga et al, 1997; Wang et al, 1999). Later, condensation of the mesenchyme, urethra and WD give rise to the adult male prostate, the function of which is to produce an alkaline fluid that contributes to seminal plasma to support sperm and ejaculation.

In females, the cranial and caudal portion of the pelvic area of the UGS gives rise to the urethra and vaginal vestibule respectively.

1.9.3 Differentiation of the male external genitalia

After the initial bipotential phase, between weeks 9-12 of human pregnancy, the genitalia becomes sex specific. In males, the GT elongates and a groove appears in the ventral aspects called the urethral groove. Initially, the groove and urethral folds only extend part of the way along the shaft of the elongating phallus and terminate in a solid plate of epithelial cells, termed the urethral plate, which extends into the glans penis (Carlson, 2004). The urethral plate then canalizes causing the urethral groove to extend distally into the glans (Yamada et al, 2003). The urethral folds grow towards each other and fuse in the midline, eventually causing the urethral groove to form a tubular penile urethra. Hypospadias occurs when the urethral folds fail to fuse, leading to an abnormal location of the urethral opening. The site at which the fusion fails, dictates where the hypospadias occurs on the penis and this can occur anywhere from the perineal region along the shaft of the penis (Yamada et al, 2003).

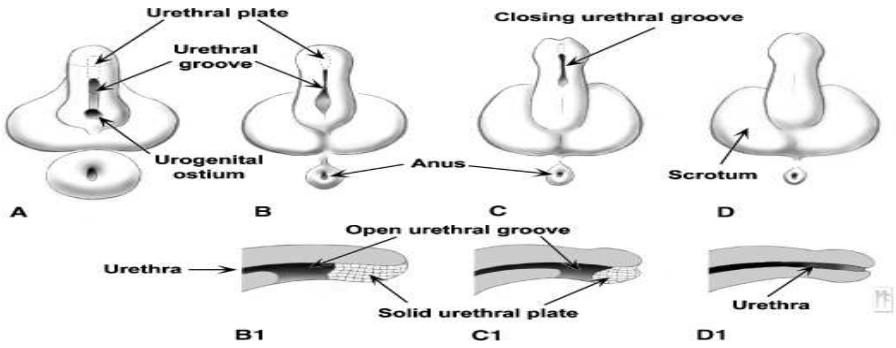


Figure 1.11 Diagrammatic representation of development of the urethral plate, urtheral groove, ostium, anus and scrotum. Figure from Yamada et al, 2003

The urethral groove and the urethral plate are thought to be lined by and derived from the endoderm (based on histological examinations) and some believe that the urothelium lining all of the urethra is endodermal in origin (Seifert et al, 2008; Yamada et al, 2003). The urothelium is a unique epithelium found only in the ureter, bladder and urethra with only the ureter urothelium being mesodermal in origin as it is derived from the embryonic WD. However, because the epithelium of the urethral folds is precisely at the interface of the ectoderm and endoderm, controversy exists amongst the experts and some believe the penile urethra could be ectodermal in origin (Larson, 2001). The ectodermal in-growth theory postulates that the glandular urethra is formed by an in-growth of epidermis.

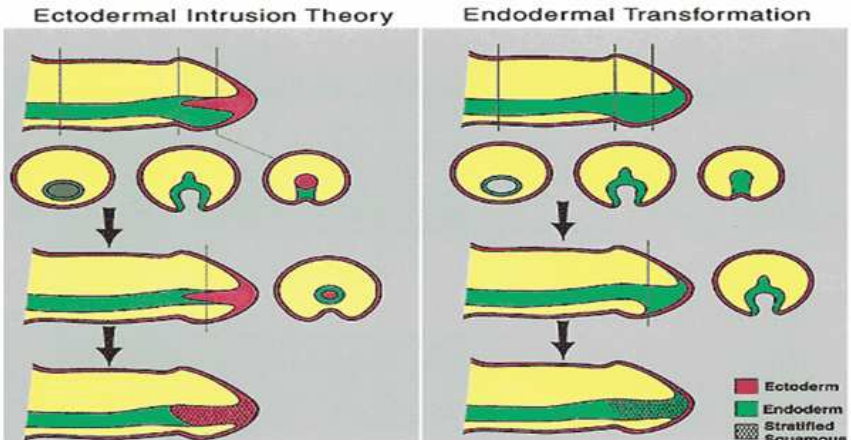


Figure 1.12 Diagrammatic representation of the two theories of how the penile urethra forms, with the ectodermal intrusion theory on the left and the endodermal transformation theory on the right. Figure from Wang and Baskin, 2008.

Despite the controversy about whether the urothelium is of ectodermal or endodermal orgin, it is widely agreed that the ectoderm will give rise to the penile skin (Yamada et al, 2003) and most substances of the penis are actually derived from the mesodermal cells which segregate into corporal bodies, connective tissue and dermis. Dense mesenchymal condensations give rise to corporal tissue within the shaft of the developing penis, and these differentiate further to give rise to the connective tissue

capsules termed the tunica albuginea (Baskin, 2004). Around the tubular urethra, the mesenchyme differentiates into smooth muscle of the mucosa and submucosa of the urethra. In humans, this is surrounded by erectile tissue of the corpus spongiosum, also termed the corpus urethrae. In some species, including rats and mice, the GT mesenchyme will also form an os penis composed of bone and cartilage (Rasmussen et al 1986) and the growth of the os is stimulated by androgens and signals from the epithelium (Murakami 1986). In both humans, rodents and indeed most species in which penile growth has been studied, there is dorsal-ventral patterning, illustrated by the corpus cavernosa being located dorsally and the urtheral plate, groove and urethra being located ventrally. This is also true for the os penis which develops between the corpus cavernosum (dorsal) and the urethra (ventral). The molecular mechanisms determining this pattering, also seen in the outgrowth of other limb appendages, are poorly understood, but it is widely hypothesized that mesenchymal-epithelial interactions play a critical role. It is thought that signals from the epithelium can determine both proximal-distal and dorsal-ventral polarity.

Although the initial ambisexual stage of GT outgrowth, described in section 1.9 is hormone independent, development of the male penis is heavily reliant on androgen action. Within the developing GT, testosterone is metabolised by 5α-reductase to the more potent androgen, DHT. AR receptors are found in all cells of the GT but are particularly dense in expression in the mesenchymal cells, and it is also in these cells where 5α-reductase type 2 is also localised, specifically around the urethra and urethral seam. Although the exact molecular mechanisms and downstream genes of AR activation in the GT at specific stages, still remain to be elucidated, an extensive number of studies have shown that the action of DHT and AR is fundamental for correct development.

Although the exact downstream triggers of AR are yet to be defined, certain genes have been shown to be vital in GT development and much of this information has been

obtained from mice knock-out models. The first of these to merit consideration is the fibroblast growth factor family which has 23 family members which exert their effects through four different Fgf surface receptors. Initially, in the cloaca, both Fgf8 and Fgf10 are expressed, and this later separates to Fgf8 being expressed in the distal urethral epithelium and Fgf10 in the surrounding mesenchyme (Haraguchi et al, 2000). Fgf10 acts as a paracrine factor that signals through Fgf receptor 2IIIb, which is expressed in the urethral plate and urethral epithelium. Fgf10 null mice produce a malformed GT, with failed fold fusion and in fact severe hypospadias, suggesting that Fgf10 has a critical role in GT development (Cohn et al, 1995; Min et al 1998). Another transcription factor which appears to be critical in GT development, and indeed in limb, tooth, foregut and prostate development, is sonic hedgehog (SHH) (Seifert et al, 2010). This glycoprotein is expressed in the cloaca, the urogenital sinus and later also in the urethral plate epithelium. SHH is vital for proximo-distal growth and in culture has been shown to control the growth of the GT through genes such as Patched 1 (Ptch1) (Lin et al, 2009), bone morphogenetic protein4 (Bmp4), Hoxd-13 and Fgf10. Although Shh is not required for initial outgrowth, it is necessary for sustained outgrowth and formation of a tubercle (Cohn, 2011). Shh mutant embryos form the initial genital swellings but these buds arrest immediately after initiation and fail to give rise to a tubercle. This can be partially rescued by over-expression of β-catenin. Given the importance of this gene, additional studies have removed Shh signalling at different stages and found the extent of genital tubercle outgrowth is determined by the duration of Shh signalling, with earlier ablation resulting in more severe truncation. Shh is also required for development of the closed urethral tube and for correct positioning of the urethral opening with deletions resulting in hypospadias as described above. Furthermore these studies have established that Shh primarily targets the centre distal urethral epithelium through Wnt and fgf8 signalling but also regulates Hoxa13 and Hoxd13 expression independent of distal urethral signalling.

Hox genes are also well established in their role in GT development and although four are known to be expressed in the GT, Hoxa13, Hoxd13, Hoxd11 and Hoxd12, it is thought that Hoxa13 and Hoxd13 play a more important role (Warot et al, 1997). Both of these are expressed in the mesenchyme of the GT and mice null for both of these genes fail to develop a GT or a cloaca. Mutations in Hoxa13 can induce a partially or completely divided uterus, septum vaginalis, and hypospadias (Morgan et al, 2003).

The expression and up-regulation of TGF-β receptor III and TGFβ1 selectively in the urethral epithelial plate suggests that TGFβ signalling is another important factor and regulator of urethral tube development. In the absence of TGFβ receptor III, palatal fusion is inhibited. The exclusive expression in the urethral epithelium is also true for Frizzled 1, the receptor for Wnt ligands, suggesting there are several signalling pathways required in urethral tube formation (Li et al, 2006).

A p53 homolog, p63 is also an important transcription factor in the epidermis and urothelium and null mice and mutations in humans lead to a micropenis phenotype (Yamada et al, 2003). Although the key genes involved in penile development have been discussed, it should be considered that there are many other genes, the exact molecular roles of which remain to be elucidated, but for which defects result in hypospadias. Such genes include Mamld1, Dicentric, Mid1, Rxfp2 and Atf3 (Matzuk and Lamb, 2008).

1.9.4 Differentiation of the female external genitalia

Development of the female external genitalia is essentially the default, promoted by the absence of androgens and possibly the presence of oestrogens. The distal end of the combined paramesonephric ducts contact the posterior wall of the urogenital sinus to form a pair of endodermal swellings called the sinovaginal bulbs (Sajjid, 2010). These structures are originally solid and later, when the lumen forms, develop into the lower two thirds of the vagina. The lumen of the vagina remains separated from the remaining urogenital sinus by a thin membrane, the hymen, whereas the urogenital sinus caudal to

the vaginal opening becomes the vestibule. Thus the vagina is made in part from the paramesonephric ducts and the sinovaginal bulb of the urogenital sinus. In the absence of testosterone, the urogenital folds and swellings do not fuse and form the labia minora and labia majora respectively. The genital tubercle develops into the clitoris and canalization of the vaginal plate results in the lower part of the vagina. The phallic portion of the urogenital sinus forms a vestibule in which the urethral meatus, the vaginal orifice and the ostium of the opening of the vestibular glands can be found. The anogenital distance does not increase, the rims of the urethral groove do not fuse and the labioscrotal swellings give rise to the labia majora. The dorsal commisure forms at their junction and the genital folds remain separate to become the labia minora.

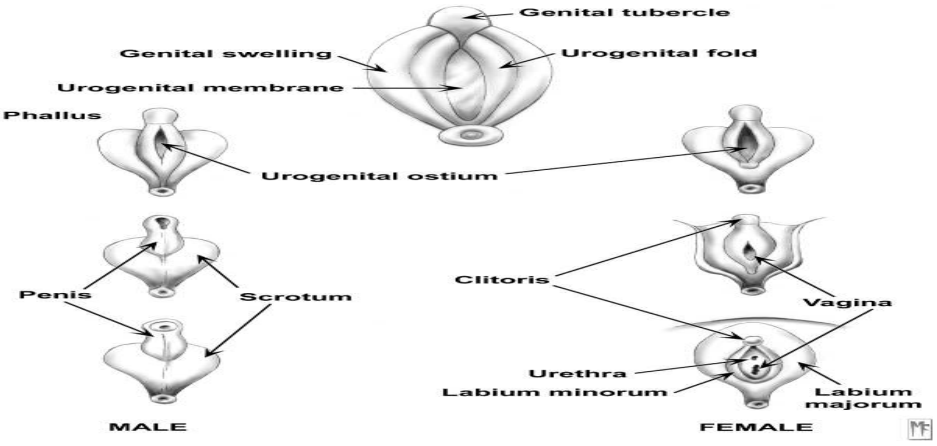


Figure 1.13 Diagrammatical representation summarising the development of the male and female external genitalia. Figure from Yamada et al, 2003

1.10 Conclusions

Masculinisation of the male fetus requires the testis to produce androgens and for these hormones to elicit a response through its receptor and an array of important associates. Furthermore, extensive studies summarised in this review have proven that the timing of androgen action is fundamental. Correct androgen action is required during the MPW in order for correct programming of the male reproductive tract. Deficits in androgen

action, caused by genetics or the environment can cause TDS disorders and malformations induced at this time cannot be corrected by postanatal androgens. Thus androgen action during the MPW is key in correct male development and identification of the mechanisms of androgen action during this time frame is required to further our understanding of the origins of TDS.

It is during the MPW that the Wolffian duct is stabilised, and the genital tubercle, prostate and anogenital distance are all programmed and yet what makes androgen action during this time period different to any other time period is a complete mystery. Androgen action begins once the Leydig cells produce testosterone, and remains active throughout the fetal period and for some time postnatally, with a surge at puberty and activity thereafter. However, the essential programming which takes place during the MPW cannot be induced during any other time period, as far as is known. More importantly, any deficit in correct androgen action during this time cannot be corrected later.

The possibility exists that it is not the events occurring within the window which may be unique, but rather what is ‘opening and closing the window’, or regulating the window. The events occurring before the MPW have had very little investigation and yet it would make sense that some sort of ‘pre-set up’ would be required for the androgen programming to take place. This simply could just be the availability of androgen as the production of testosterone almost coincides with the start of the MPW. If this is true, could providing androgens to the fetus, prior to the MPW initiate the MPW any earlier? There is the possibility that this may be restricted by AR expression. This is the focus of chapter 3.

As discussed in this literature review, the role of co-regulators in the activation or repression of androgen action is emerging to be critical. There are many co-regulators known to be essential in AR action, such as BRG1 and CBP, and yet their role in the

male reproductive tract, in the disorder of hypospadias or indeed in the MPW are completely un-characterised. The aim of chapter 4 was to provide some initial information to address this deficit.

The earliest studies, to my knowledge, which identify a 'critical time period' are those which found that arachidonic acid and the role of prostaglandins is involved in the masculinisation process, as reviewed in section 1.7.3. These studies had the MPW pin-pointed in the mouse and also found the phenotypes one would expect when androgen action is deficient just within the MPW, such as hypospadias, cryptorchidism and reduced AGD. Furthermore these studies suggested that prostaglandins are critical during the MPW. However, convincing as these studies are, they have never been repeated, not done in another animal model, or taken any further. The final aim of this thesis was to determine if prostaglandins are vital in the MPW and or even involved prior to this time frame. These results are presented in chapter 5, where the effects of blocking PGs before and during the window were examined in fetal life and at 3 postnatal time points. These time points of examination were also conducted in chapter 3 as any effects in fetal life either before or during the MPW, may not present until later in life.

Although many genes and downstream targets of androgen action have been identified, little has been noted to be unique in the presence or absence during the MPW, to elucidate why this time frame differs for androgen action. Repeated studies in rodents have shown that hypospadias occurs when androgen action is blocked during the MPW and not thereafter. The final results chapter of this thesis sought to identify candidate genes which may be androgen regulated in the GT during the MPW both to gain insight into genes involved in hypospadias but also to identify downstream targets which may be unique to the MPW.

The main aims of this thesis are:

To investigate the regulation of the MPW, specifically if the availability of androgens and AR are 'opening factors' and if the exposure to androgens before the MPW can initiate masculinisation earlier

To characterize expression patterns for co-regulators during and after the MPW, in the GT as well as in the developing testis. The co-regulators are known to be important in androgen action but their role and potential importance in the developing male reproductive tract is yet to be determined.

To investigate if prostaglandins are critical for masculinisation before and during the MPW.

To identify androgen regulated candidate genes in the MPW involved in urethral development and related hypospadias.

2. General Materials and Methods

2.1 Animal Work

All animal work was completed in accordance with the UK Home Office Animal Experimentation (Scientific Procedures) Act 1986 under project licence 60-3259. The animals used were Wister rats originally purchased from Harlan UK and then bred to generate stock within our own animal facility. A majority of licensed procedures were performed by Mark Fisken and William Mungal who also provided day to day animal husbandry. I performed some licensed procedures under my personal license 60-11992.

2.1.1 Welfare Conditions

Animals were housed in a room with a fixed 12 hours per day light cycle, from 7am-7pm. The room temperature was kept between 20-25°C and an average humidity of 55%. Fresh tap water and food (soya free) were available ad libitum. The rats were kept in clear sided, solid bottom cages and typically females would be housed in groups of six and stud males housed individually. For mating, a male and female were placed temporarily in a cage with a mesh grid bottom that allowed the copulatory plug to fall to the tray below.

2.1.2 Timed-mating

Time mating was used to determine the date of conception and gestational stage of pregnant rats. One male and one female were placed together in a grid bottomed cage, by Mark Fisken or William Mungal at the end of the working day (approximately 4pm) in order to allow mating to take place overnight. The following morning the tray beneath the cage was examined for a copulatory plug. If found, mating was presumed to have occurred and the date was recorded and designated as embryonic day e(0.5). The male would then be removed and the female housed separately. Females rats have an oestrus cycle of four to five days, so copulation could take up to five days to occur. To minimise the risk of non-pregnancy, whenever possible, sexually mature, proven fertile males and females were used, usually around 3 months of age but at least 10 weeks old.

2.2 In vivo treatments

The chemicals administered to pregnant female rats for studies in vivo were flutamide, testosterone proprionate (TP), dihydotestostoetrone (DHT), diethylstilboestrol (DES) or indomethacin and for each, the respective vehicle was administered to other females as a control. Flutamide was administered by oral gavage using a 10-12cm long 15-16G blunt ended steel gavage cannula, (Medicut, Sherwood Medical Industries Ltd, UK) attached to a disposable plastic 1ml syringe. DHT, TP and indomethacin were administered daily and DES every second day, by subcutaneous injection using a 25G, 0.5 x16mm needle (BD Microlance) attached to a plastic 1 ml syringe. Each pregnant female was weighed prior to dosing in order to administer correct volume of chemical for bodyweight. Treatments were administered between 0900 and 1100 hrs and animals were checked thereafter regularly for signs of toxicity or discomfort.

2.2.1 Flutamide

Flutamide is a synthetic anti-androgenic agent that competitively blocks the action of testosterone and DHT by binding to the androgen receptor (AR). To investigate androgen specific mechanisms which are critical during the masculinisation programming window (MPW, e15.5-e18.5) flutamide was administered to block these mechanisms, during this time window. For all studies in this thesis, flutamide was administered, at 100mg/kg per day during the MPW. Previous studies had shown this dose induces severe hypospadias in all exposed males (MacLeod et al, 2010). Due to insolubility in corn oil directly, flutamide was dissolved in 2.5% dimethyl sulphoxide (DMSO; Sigma) diluted in corn oil, then administered at 1ml/kg bodyweight using oral gavage. Flutamide was freshly prepared for each study, kept at room temperature between doses and disposed of appropriately at the end of each study. Control dams received only DMSO/ corn oil daily from e15.5-e18.5.

2.2.2 Testosterone Propionate (TP)

TP is an androgenic steroid and is used to manipulate circulating testosterone levels. To investigate the effect these androgens may have on both the developing male and female during the MPW, and to investigate mechanisms activated by androgens, TP was administered to the pregnant dams from e15.5 to e18.5 at 20 mg/kg per day. This dose was chosen based on studies in the literature (Welsh et al, 2008). TP is insoluble in water and therefore was dissolved in corn oil (20mg per ml corn oil) at the beginning of each study and administered at 1ml/kg bodyweight by subcutaneous injection, using a disposable plastic 1ml syringe, fitted with a 25G needle. Any remaining TP was disposed of appropriately at the end of each study. For control animals, female dams were administered only corn oil daily from e15.5-e18.5. TP has the ability to be aromatized into oestradiol, and so the effects seen in exposed pups could have been mediated by oestrogen effects. In order to ensure any effects seen were due to androgen action, TP treatment was only used for initial preliminary studies and for a majority of the project, DHT was used, which cannot be aromatized.

2.2.3 Dihydrotestosterone (DHT)

DHT, a more potent (~10 fold) androgen than testosterone, cannot be aromatised to oestradiol, therefore any effects seen following treatment would be through the androgen receptor. DHT was administered to pregnant dams in one of two gestational windows. Either daily between e11.5-e14.5 (the early window ((EW)) or daily during the MPW (e15.5 to e18.5). DHT was initially administered at 1mg/kg but when no effect was seen in the females (see chapter 3) this dose was increased to 10mg/kg. DHT was dissolved in corn oil at the beginning of each study and administered by subcutaneous injection using a disposable plastic 1ml syringe, fitted with a 25G needle, at 1ml/kg bodyweight. For control animals, female dams were administered only corn oil daily from either e11.5-e14.5 or e15.5-e18.5. Any remaining DHT was disposed of appropriately at the end of each study.

2.2.4 Diethylstilboestrol (DES)

DES is a synthetic nonsteroidal oestrogen and was used for studies in this thesis, to compare treatment effects with TP to assist in determining if effects seen were due to TP conversion to oestradiol and its binding to oestrogen receptors. The side effects of in utero exposure to DES are well documented (Herbst et al 1971) and previous studies have shown that an optimum dose of 100µg/kg should be administered on alternate days in order to avoid adverse effects on the dams (McKinnell et al, 2001; Williams et al, 2001). DES was administered to the pregnant dams on e15.5 and e17.5 (which would ensure DES was available throughout the MPW) at 100µg/kg. DES was dissolved in corn oil (100µg per ml corn oil) at the beginning of each study and administered at 1ml/kg bodyweight by subcutaneous injection, using a disposable plastic 1ml syringe, fitted with a 25G needle. For control animals, female dams were administered corn oil on the appropriate days. Any remaining DES was disposed of appropriately at the end of each study.

2.2.5 Indomethacin

Previous studies suggested blocking prostaglandins during the MPW, using indomethacin, may affect male reproductive tract development (Gupta and Goldman, 1986; Gupta, 1989; Gupta and Bentlekewski, 1992). Previous studies have used various doses (from 0.5mg/kg – 10mg/kg) in mice and rats (section 1.6.1.4.1) and an initial dose for studies in this thesis was chosen at 2mg/kg, administered by gavage, in order to see an effect in pups, with minimal side effects on the dam. However, even at this dose, dams were having difficulty with parturition, some giving birth late but most not giving birth at all. In addition, the oral route of administration was causing heavy gastric bleeding in the dams and so the decision was taken to administer indomethacin by subcutaneous injection, which was also the route used by Gupta (1989). Therefore a lower dose of 1mg/kg was chosen and administered to the pregnant dams from e15.5 to e18.5, by subcutaneous injection. The indomethacin was dissolved in corn oil (1mg per ml corn oil) at the beginning of each study and administered at 1ml/kg bodyweight by

subcutaneous injection, using a disposable plastic 1ml syringe, fitted with a 25G needle. For control animals, female dams were administered only corn oil daily from e15.5-e18.5. Any remaining indomethacin was disposed of appropriately at the end of each study.

2.3 Necropsy procedure and gross dissection

Pregnant dams were killed by inhalation of carbon dioxide followed by cervical dislocation under schedule 1 of the Animal (Scientific Procedures) Act 1986. Once deceased, the pregnant dam was placed on her back and the abdomen opened. The uterus was removed, each amniotic sac cut open and the umbilical cord severed. Once removed from the dam, foetuses were placed in ice-cold 0.01M phosphate buffered saline (PBS, Sigma). If late gestation (i.e. e19.5 and older) the foetuses were decapitated and then placed in the ice cold PBS.

Once placed in the chilled PBS, the foetuses were transported to the dissection area, on ice, to avoid degeneration. Pups taken at e21.5 were wiped cleaned and bodyweight and anogenital distance (AGD) were recorded prior to each pup being decapitated.

2.3.1 Fetal bodyweight and AGD

Bodyweight of e21.5 pups was evaluated using an electronic analytical balance (Handy H110, Sartorius). Anogenital distance (AGD) was measured as the distance between the midpoint of the anus and the base of the genital tubercle. This was measured on e21.5 pups using electronic digital callipers with a resolution of 0.02mm (Faithfull Tools, Kent). Under normal circumstances, the AGD of male rats should be 1.5-2 times greater than females from e21.5 onwards. If reduced AGD is seen in males, it suggests a deficit in androgen action and masculinisation during the MPW (MacLeod et al, 2010; Welsh et al, 2008).

2.3.2 Fine dissection

Fine dissection was carried out in a designated dissection area. Individual foetuses were placed on their backs and onto a bed of paper towels, dampened with PBS in an appropriately sized petri dish. The foetuses were then placed under a binocular dissecting microscope (Leica, MZ6) with a transilluminated stage. External lighting was provided by external cold lights (Leica CLS 150x) which are designed to cause minimal heat damage to the tissue they illuminate. The genital tubercle (GT) was dissected from the base from each foetus using fine tweezers.

If the tissue was being fixed in Bouin's, the tip was lightly marked using green tissue biomarker (Biostain Ready Reagents Ltd, Merseyside, UK), to facilitate embedding of the tissue in a consistent 'tip down' orientation. After GT removal, the abdomen was opened with a horizontal incision slightly below the umbilical cord. The intestines were pushed up and out of the abdominal cavity to access the gonads and urogenital tract. Gonads and attached ducts were removed and placed in a petri dish containing chilled PBS for microdissection. Gonads were separated from ducts using the bevelled edge of 27G needles (Monoject, sterile needles, 0.4mm x 12mm) attached to disposable plastic 1ml syringes.

2.3.3 Postnatal tissue collection

Postnatal animals were culled on either PND8, 25, 75 or 90. These animals were killed by inhalation of carbon dioxide followed by cervical dislocation under schedule 1 of the Animal (Scientific Procedures) Act 1986. The penis or clitoris were dissected, the length measured using electronic digital callipers with a resolution of 0.02mm (Faithfull Tools, Kent). The tissue was weighed prior to being either snap frozen or fixed in Bouin's fixative. Following phallus removal, the testis, prostate and seminal vesicles were removed from the males and the ovaries and occasionally, the uterus, was removed from females. Each tissue was individually weighed and either fixed in Bouin's fixative or

snap frozen. Where abnormalities were seen in the reproductive tract, these were photographed using a Nikon D70S digital camera.

2.3.4 Tissue fixation

Once GT, gonads and ducts had been microdissected, they were either fixed as outlined below or snap frozen on dry ice in 1.5ml Nunc tubes, appropriately labelled and archived at -80°C. Alternatively, depending on tissue requirements, tissue was fixed by immersion in Bouin's solution (BIOS), a formaldehyde based fixative containing glacial acetic acid, formalin, and picric acid, which forms cross links between proteins and aldehydes producing a stable structure. The tissue was submerged in appropriate volumes of Bouin's fixative in an airtight container before being transferred to 70% ethanol. Fetal tissue was left for fixing in Bouin's fixative for 1 hour before transfer to ethanol, and postnatal tissue was left for 4 hours. Postnatal testes were cut in half after 2 hours and placed back in the Bouin's fixative for the remaining 2 hours to allow the fixative to penetrate the entire tissue.

2.4 Fixed tissue processing

Following transfer to 70% ethanol, the tissue was processed by the histology support services. Briefly, the tissue was processed through a series of graded alcohols using an 18 hour automated cycle on a Leica TP-1050 (Leica UK Limited, UK) tissue processor, then embedded in molten paraffin wax by hand. These blocks were left to cool and stored at room temperature.

2.5 Investigation of protein expression

2.5.1 Immunohistochemistry

Protein expression was detected within specific cell(s) in tissue sections, fixed in Bouin's, using specific antibodies. In brief, this involved the following steps (Fig. 2.1) which are described in greater detail below:

- Tissue sections were cut at 5µm, and mounted on slides
- Tissue was dewaxed and re-hydrated
- Depending on the primary antibody being used, target antigen was retrieved
- Non specific antigens were blocked
- Tissue was incubated with the primary antibody
- Tissue was incubated with the secondary antibody
- The secondary antibody (and hence primary antibody) was detected using an amplification system
- Antibody visualized using a colour reaction (DAB)
- Non stained tissue was counterstained

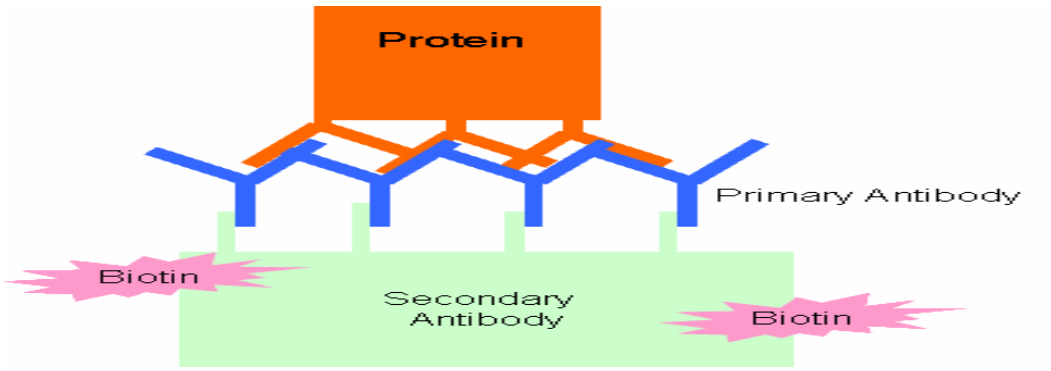


Figure 2.1 Schematic diagram representing the principles of determining protein expression within a cell using immunohistochemistry. The primary antibody binds to the antigens of the protein of interest within the cell. A secondary antibody conjugates to species specific regions of the primary antibody. The secondary antibody is conjugated to biotin and the entire enzymatic reaction binds to chromagen causing a colour change and allowing visibility of the protein of interest in specific cell types.

In order to allow reproducibility of results and provide data for accurate comparison of immunostaining between treatment groups, sections from control and treated animals were processed in parallel on at least two occasions: sections from at least three animals in each treatment group were run on each occasion. Appropriate negative controls were included, whereby the primary antibody was replaced by blocking serum alone, or when available, blocking peptide, to ensure that any staining observed was specific.

2.5.1.1 Sectioning

Fixed tissue embedded in paraffin blocks was chilled on ice in order to harden the wax and allow for easier cutting. 5µm thick sections were cut using a hand-operated microtome (RM 2135, Leica) and floated onto 30% industrial methylated spirit (IMS, Fischer Scientific), followed by water heated to 45-50°C, to allow for any creases to smooth out. Each individual section was then mounted onto pre-labelled (slide labeller, Leica IPS) charged glass slides (Superfrost Menzel GmbH & Co.) and placed in metal racks. Slides were dried overnight at 50°C.

2.5.1.2 Dewaxing and rehydration

Wax was removed from slides by submerging them in xylene for 5 minutes x2. Slides were then rehydrated by placing them for 20 seconds in each of the following alcohols; absolute alcohol x2, 95% alcohol, 80% alcohol and 70% alcohol.

2.5.1.3 Antigen retrieval

Bouins is a formaldehyde based fixative and can cause the formation of protein cross-links that may mask antigenic sites within a tissue. Antigen retrieval will break these cross-links which then allows the detection of the antigen. This is not always required for all antigens, but was required for all immunohistochemistry done in this thesis. Antigen retrieval required placing slides in a Tefal Clipso pressure cooker (Tefal) containing 2L of boiling buffer consisting of 0.01M citrate buffer (pH6). Once the lid was sealed, pressure was set at the highest setting and full pressure achieved, indicated

by onset of continuous steam release and the slides then left for 10 minutes. The pressure was then released and the cooker removed from heat. The slides were left for a further twenty minutes in the buffer, then removed and cooled in water and transferred to Tris-buffered saline (TBS) for 5 minutes x2.

2.5.1.4 Blocking

Slides were incubated in 3% hydrogen peroxide (BDH) in methanol for 30 minutes to block any endogenous peroxidase activity which could have caused non specific background staining. After this, slides were washed in tap water and then in TBS for 5 minutes x2. Excess TBS was wiped from around each tissue section. Slides were blocked in normal serum (Diagnostic Scotland) diluted 1:5 in TBS and 5% bovine serum albumin (BSA, Sigma) to block non specific binding sites. The serum used depended on the species the secondary antibody was raised in.

2.5.1.5 Primary antibodies

Titre runs were carried out for each primary antibody to establish the optimal working concentration. The antibody was diluted to the required concentration in the appropriate normal serum/TBS/BSA, placed on each tissue section on each slide and incubated at 4°C in a humidity chamber overnight.

Table 2.1 Primary antibodies used and their optimum working concentrations

Primary Antibody	Source	Retrieval	Species	Dilution
Androgen Receptor	Abcam, Cambridge	Citrate	Rabbit	1:500
BORIS	Abcam, Cambridge	Citrate	Rabbit	1:200
BRG1	Santa Cruz	Citrate	Rabbit	1:200
CBP	Sigma Life Sciences	Citrate	Mouse	1:150
COX2	Gift from Henry Jabbor	Citrate	Goat	1:50
DNMT3L	Abcam, Cambridge	Citrate	Rabbit	1:200
RWDD1	Abcam, Cambridge	Citrate	Mouse	1:100
VASA	Abcam, Cambridge	Citrate	Rabbit	1:200

2.5.1.6 Secondary antibodies

After overnight incubation with the primary antibody, slides were washed for 5 minutes in TBS x2 and incubated with biotinylated secondary antibody. The secondary antibody was chosen to be specific against the species in which the primary antibody was raised in, and to be raised in the same species as the normal blocking serum used. The biotinalyted secondary antibody was diluted 1:200 in normal blocking serum and placed on slides for 30 minutes.

Table 2.2 Secondary antibodies used and their optimum concentration

Secondary Antibody	Source	Dilution
Goat Anti Rabbit Biotinylated	VECTOR	1:500
Goat Anti Mouse Biotinylated	VECTOR	1:500
Rabbit Anti Mouse Biotinylated	Zymed	1:500
Chicken Anti Goat Bioinylated	Santa Cruz	1:500
Rabbit Anti Goat Biotinylated	DAKO	1:500
Chicken Anti Rabbit Biotinylated	Santa Cruz	1:500

2.5.1.7 Signal amplification and chromagen detection

After incubation with the secondary antibody, slides were washed in TBS for 5 minutes x2. The secondary antibody signal was amplified using steptavidin-horseradish peroxidise (Strep-HRP) (Vector Laboratories, Peterborough, UK) diluted 1:500 in TBS (ABC-HRP; DAKO, Ely, UK). Slides were then incubated with Strep-HRP for 30 minutes at room temperature.

Following Strep-HRP amplification, antibody localisation was visualised using 3,3'-diaminobenzidine (liquid DAB^{+}; DAKO). DAB chromagen was diluted in the specific buffer, as per the manufacturers' instructions of 1 drop per 1ml. Sections were incubated with DAB until staining developed to the required intensity (microscopically monitored) and this varied depending on the antibody and target tissue. The reaction was stopped by immersing the sections in TBS.

2.5.1.8 Counterstaining

Slides were counterstained with haematoxylin for 4 minutes, ensuring the saturation of all binding sites. Slides were then rinsed in tap water and briefly immersed in 1% acid alcohol to remove any non-specific background staining, then rinsed again in tap water. Slides were briefly immersed in Scott's tap water (approximately 20 seconds) to allow an alkaline environment which caused the haemotaxylin staining of the nuclei to turn blue. Scott's tap water was washed off the slides by placing them in tap water.

2.5.1.9 Dehydration and mounting

Slides were dehydrated through increasing concentrations of alcohol, briefly in 70% alcohol, 80%, 90% and finally in absolute alcohol x2. Slides were then cleared in xylene for 5 minutes x2. The slides were covered using glass coverslips (VWR International) and pertex, a solvent based glue (Cellpath, Hemel, Hempstead UK). Slides were left to dry at room temperature before being archived or imaged.

2.5.1.10 Imaging

Slides were examined using a Provis microscope (Olympus Optical., London, UK) and photographed using an attached digital DCS330 camera (Eastman Kodak, Rochester, NY). Captured images were compiled using Adobe Photoshop and Adobe Illustrator.

2.5.2 Fluorescent Immunohistochemistry

Fluorescent immunohistochemistry is a method for detection of more than one antigen simultaneously using different coloured flourophores. It allows better visualisation of some antigens and also allows co-localisation of antigens to be visualised. Similar to the DAB immunohistochemistry, protein expression was detected on sections of Bouins fixed tissue using specific antibodies. In brief, this involved the following steps:

- Tissue was dewaxed and re-hydrated
- The target antigen was retrieved

- Non specific antigens were blocked
- Tissue was incubated with the primary antibody
- Tissue was incubated with the secondary antibody
- Secondary antibody detected using fluorescent tyramide amplification system
- The above three steps were repeated where more than one protein was being detected (a different colour of fluorescence was used for each protein)
- Non stained tissue was counterstained with Dapi
- Slides mounted using Permaflour

2.5.2.1 Primary antibodies

Tissue was dewaxed, rehydrated, citrate retrieved (where necessary) and non specific antigens blocked exactly as described in section 2.5. Titre runs were carried out for each primary antibody to establish the optimal working concentration, and diluted to these concentrations in the appropriate serum. Slides were incubated with the primary antibody overnight at 4°C, and as with all immunohistochemistry, at least three slides for each experimental group were added to confirm staining and appropriate positive and negative control slides were added to each run.

Table 2.3 Primary antibodies used for immunofluorescence and their optimum concentrations

Primary Antibody	Source	Retrieval	Species	Dilution
Androgen Receptor	Abcam, Cambridge	Citrate	Rabbit	1:800
BORIS	Abcam, Cambridge	Citrate	Rabbit	1:800
BRG1	Santa Cruz	Citrate	Rabbit	1:800
CBP	Sigma Life Sciences	Citrate	Mouse	1:800
DNMT3L	Abcam, Cambridge	Citrate	Rabbit	1:800
RWDD1	Abcam, Cambridge	Citrate	Mouse	1:400

2.5.2.2 Secondary antibodies

The secondary antibody (table 2.4) used was peroxidase labelled and diluted 1:200 in the appropriate serum and incubated on slides for one hour before being detected by the Tyramide system outlined below.

Table 2.4: Secondary antibodies used for fluorescence immunohistochemistry

Secondary Antibody	Source	Dilution
Chicken Anti Goat Peroxidase	Santa Cruz	1:200
Chicken anti Rabbit Peroxidase	Santa Cruz	1:200
Chicken anti mouse Peroxidase	Santa Cruz	1:200

2.5.2.3 Detection system

The fluorescent detection system used was the Tyramide Signal Amplification (TSA) system (PerkinElmer, MA, USA). Tyramide (red, green or blue) was diluted 1:50 and incubated on slides for ten minutes before they were washed in PBS. Slides were covered where possible in tin foil thereafter to avoid light exposure and bleaching.

2.5.2.4 Counterstaining and mounting

Dapi (Molecular Probes, Invitrogen, Paisley, UK) was used as a nuclear counterstain for immunoflourscent runs. Dapi was diluted 1:1000 in PBS and placed on slides for ten minutes. Slides were then mounted using PermaFlour aqueous mounting medium (Thermoscientific) and left in the dark at 4°C overnight to set prior to being imaged.

2.5.2.5 Confocal microscopy

Immunofluorescence slides were imaged using a Zeiss LSM710 Confocal Microscope (Carl Zeiss Ltd, Hertfordshire, UK) and Zen software (Carl Zeiss Ltd)

2.5.2.6 Double immunofluorescence

If more than one antigen was being detected in the sections, after the initial tyramide detection, slides would be washed in PBS for 5 minutes x2 and blocked in normal serum

for 30 minutes. The primary antibody for the second antigen being detected was added to the tissue and left overnight at 4°C in a humidity chamber. As outlined above, slides would then be washed in TBS for 5 minutes x2, the appropriate secondary, peroxidase labelled antibody would be added to the slides for 30 minutes. The second antigen of interest would be detected using a different coloured tyramide. Slides were counterstained, mounted and imaged as outlined above (section 2.5.2.4-5)

2.5.3 Western blots

Western blotting is a technique which allows the separation of proteins according to their molecular size. This is done using protein electromobility on a gel with specifically sized pores. Western blot was attempted in this thesis to identify the androgen receptor protein, in rat fetal tissue, prior to the MPW.

In brief, the technique involved the following:

- Tissue was lysed to release protein
- Protein samples were prepared with appropriate running buffers
- Protein samples were loaded on to a gel and separated using electrophoresis
- The separated proteins were transferred from the gel onto a membrane
- Primary and secondary antibodies were incubated with the membrane
- Primary antibody for protein of interest was visualised

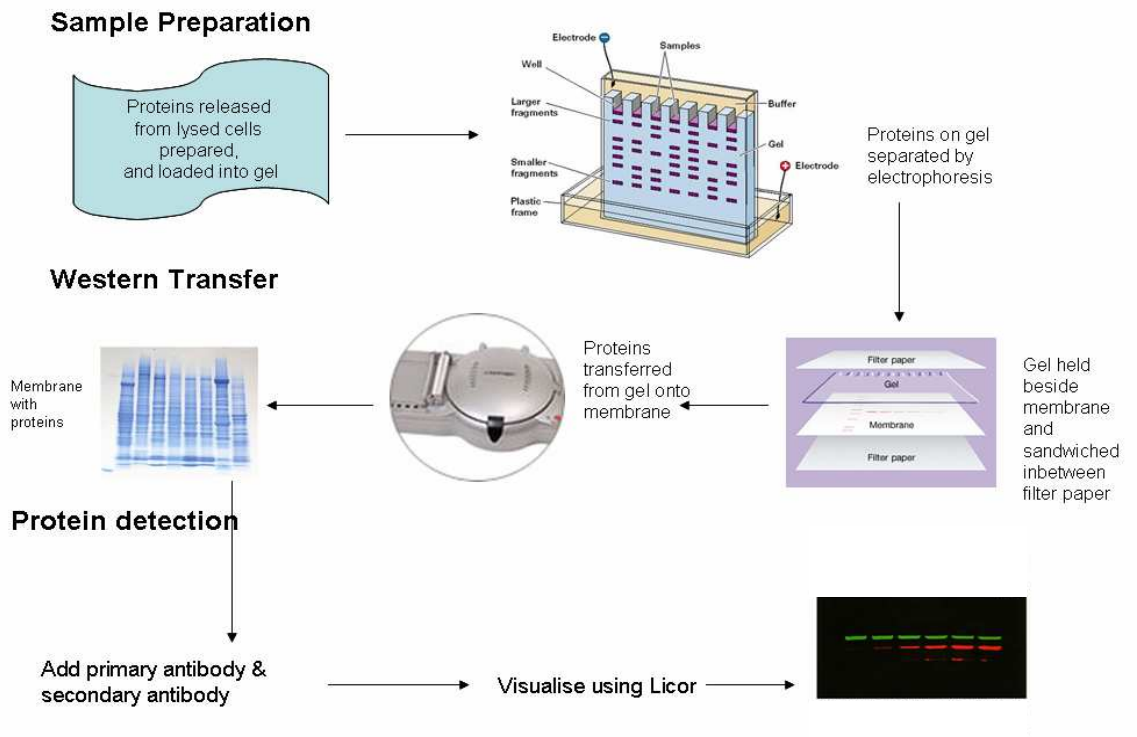


Figure 2.2 Schematic diagram summarising the processes involved in protein detection by Western blotting

2.5.3.1 Protein extraction

The tissue was broken open using lysis buffer, allowing the cells to release their proteins. The whole cell protein was harvested from frozen tissue using RIPA lysis buffer and in order to minimise degradation, tissue was kept frozen on dry ice until the protease inhibitor was added to the lysis buffer and then lysis buffer added to the tissue. In order to break the non-covalent bonds within the proteins and give the protein a negative charge to facilitate their separation in the acrylamide gels, SDS was added to the sample. Samples were then homogenised in 75µl RIPA buffer using a cordless handheld homogeniser (Sigma Z36,997-1) to disrupt the tissue and release the protein. The homogenate was incubated on ice for 1 hour prior to centrifugation at 2500rpm for 10 min. The protein enriched supernatant was collected in a fresh 1.5ml eppendorf and stored at -80°C until required.

2.5.3.1 Preparing samples

Ideally, 15µg of protein in a total volume of 20µlwould be used in each sample but this was restricted by the sample availability, concentration and finite volume of the wells in the gels. The appropriate volume of sample was added to 5µl of NuPage, 4x LDS Sample buffer (Invitrogen) and 2µl NuPage 10x reducing agent (Invitrogen) and made up to 20µl in de-ionised water, to allow for complete reduction of proteins. The protein was denatured by heating these samples to 70°C for 10 minutes followed by brief centrifugation prior to being loaded onto the gel.

2.5.3.2 Gel electrophoresis

Proteins were carefully loaded into the gel (12% acrylamide 1mmNuPage Novex bis-tris gels (Invitrogen)). These gels have a lower pH than standard SDS Page gels and allow for denaturing gel electrophoresis. NuPage SDS MOPS buffer (Invitrogen) was used as the running buffer with Nupage Antioxidant (Invitrogen) added to a cathode buffer chamber. This inhibits the reduced proteins from re-oxidising. A size marker, SeeBlue Plus2 (Invitrogen) was run alongside the gel, which contained ten proteins. The proteins were separated by running the gels at 200 volts for approximately 40 minutes.

2.5.3.3 Western transfer of proteins

Once the proteins were separated by gel electrophoresis, specific proteins were investigated. Proteins were transferred onto an immobilin-FL PVDF membrane (Millipore, Herts, UK) for fluorescent detection using a Hoefer TE22 transfer chamber. The protein gel plates were prised apart and the gel was carefully removed and placed in a petri dish with running buffer. A ‘sandwich; was made with the gel membrane set in between layers of blotting paper, sponges and then bound in a blotting cassette. The gel membrane, layers of blotting paper and sponges were equilibrated in NuPage transfer buffer (Invitrogen). The cassette was placed in the transfer chamber and transferred overnight at 20volts.

2.5.3.4 Primary antibody

Following transfer of the protein onto the membrane, the membrane was blocked in dried skimmed milk powder (20%w/v) in TBS +0.05% Tween-20 (Sigma) and then incubated overnight at 4ºC with primary antibody. The same primary antibodies were used as for immunohistochemistry but were diluted 10x more than for immunohistochemistry. An anti-ß-tubulin antibody (Sigma) was used as a standardization control. Membranes were washed in TBS+0.05% Tween and incubated at room temperature with an appropriate secondary antibody diluted TBS+0.05% Tween for one hour. The membrane was then washed in TBS alone before being visualised.

2.5.3.5 Imaging

The membranes were visualised and analysed using the Odyssey Infrared Imaging System (Li-cor Biosciences). Blots were scanned and viewed using the odyssey Software (Li-cor Biosciences).

2.6 Investigations of Gene Expression

To investigate gene expression in specific tissues, standard PCR and QRT-PCR were used. For both these techniques, RNA must be extracted from the tissue (which had been frozen at -80°C after dissection) and cDNA synthesised from the RNA, to be used for both PCR techniques.

2.6.1 RNA extraction

RNA was extracted from frozen tissue using the RNeasy mini extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. Briefly, the frozen tissue was homogenised in 2ml tubes with lysis buffer consisting of RLT buffer containing β-mercaptoethanol, using a hand homogeniser (Kontes Pellet Pestle) on ice. After homogenization, 350µl 70% ethanol was added to each sample and transferred into RNeasy mini columns and centrifuged for 15 seconds at 10,000rpm. The flow through was re-loaded into the column to maximise yield before being centrifuged again for 15

seconds at 10,000rpm. The flow through was then discarded and 350µl of RW1 buffer was added and samples centrifuged for 15 seconds at 10,000rpm. On column DNase digestion was performed by incubating with DNase (10µl DNase 1 stock and 70µl RDD buffer) for 15 minutes at room temperature. Following this incubation, 350µl of RW1 buffer was added and the column was centrifuged for 15 seconds at 10,000 rpm. The column was then transferred to a new collection tube and 500µl RPE buffer was added and the column centrifuged for 15 seconds at 10,000rpm. The flow through was discarded and 500µl 80% ethanol was added and centrifuged for 2 minutes at 10,000 rpm. The column was then transferred to a fresh collection tube and centrifuged at full speed (14,000 rpm) for 2 minutes in order to dry the membrane. The RNA was then eluted by adding 14µl RNase-free water directly onto the membrane and then centrifuging for 1 minute at 10,000 rpm to collect the RNA. RNA concentrations were measured using the Nanodrop spectrophotometer and stored at -80°C before use.

2.6.2 RNA quantification

The purity and concentration of RNA samples was analysed using the Nanodrop-1000 spectrophotometer (Nanodrop Technologies, Delaware, USA). The nanodrop only requires 1.5µl sample which is pipetted directly onto the measurement pedestal. The amount of 260nM UV light absorbed by the sample is measured and the Beer Lambert law is used to relate this to the concentration of the light absorbing molecule, ie the RNA.

The nanodrop also measures the absorption of light at 280nM, the wavelength absorbed by impurities such as protein, salt and solvents. Given the ratio of 260nM:280nM, the purity of the RNA sample can be ascertained, ideally the 260nM:280nM ratio should be 2, although values in the range of 1.9-2.1 are acceptable.

2.6.3 Preparation of cDNA for PCR- reverse transcription

The Invitrogen ROX Vilo kit was used to make cDNA from isolated tissue RNA. The following master mix was made:

Stock	1x (20 µl)
5x Vilo Reaction Mix	4
10x Superscript enzyme Mix	0.25
RNA 100ng/ µl	1
RNase free water	14.75

The reagents were added together in 0.2ml sterile thin walled PCR tube and cycled in a thermo-cycler on the following programme:

25 °C for 10 minutes

42 °C for 60 minutes

85 °C for 5 minutes.

cDNA was then stored at -20°C until required for use.

2.6.4 PCR

PCR is a technique which employs thermal cycling to separate a DNA strand and allow short, specifically designed, complementing DNA sequence primers, to bind to a particular sequence of interest. A heat stable DNA polymerase enzymatically constructs a new DNA strand. Repeated heating to a denaturing temperature and cooling to an annealing temperature, allows for this process to make millions of copies of the DNA sequence for particular genes and subsequent proteins of interest. In order to confirm that detectable, multiple copies of the correct sequence have been formed, the PCR product can be run on an agarose gel where applying voltage allows the DNA bands to be separated and visualised using UV light.

A master mixture was made using the following:

	1x (µl
5X Go Taq Flexi buffer	4
25mM MgCl2	1.2
10mM dNTPs	0.4
5µM Forward Primer	2
5µM Reverse Primer	2
Go Taq Polymerase	0.25
dH2O	9.15
cDNA	1

Each tube was briefly vortexted before being placed in the thermal cycler under the following programme:

95°C	5 minutes	
95°C	15 seconds	Repeat for 35 cycles
65°C	30 seconds	
72°C	1 minute	
72°C	10 minutes	
4°C	10 minutes	

Each sample was then loaded onto a 0.7% agarose gel. In brief, this was made by dissolving 1.4g of agarose in 200ml 1x TAE buffer and placing in a microwave for approximately 2 minutes before adding 20µl SYBR safe DNA gel stain. The solution was poured into a gel tank, in which a comb was inserted with appropriate well numbers. Once solidified, at room temperature, the gel was submerged in a solution of 1x TAE, in a tank connected to a power supply. 10µl of each PCR sample was loaded into individual wells and a voltage was applied of 70V for approximately 30 minutes, to allow for the negatively charged nucleic acids to move through the agrose gel. The separated DNA bands were viewed under UV light using Geneflash Syngene bioimaging UV light box (Syngene, Cambridge, UK) and photographed.

***Table 2.5**:* Sequences of genes investigated by standard PCR

Gene	Forward Primer 5'→3'	Reverse Primer 3'→5'
AR	AAG CAG GTA GCT CTG GGA CA	CGT TTC TGC TGG CAC ATA GA

2.6.5 Quantitative RT-PCR (QRT-PCR)

Quantitative RT-PCR is based on the principles of standard PCR described in section 2.6.4, however as well as amplifying the target DNA, QRT-PCR also simultaneously quantifies the target DNA. As with standard PCR, forward and reverse primers were designed for the target DNA sequence. In addition, in RT-PCR, a probe from the Universal ProbeLibrary (Roche) was assigned to each reaction which bound to a specific

location in between the forward and reverse primers. At the 5' end of the probe was a reporter dye, 6-carboxyfluorescein (FAM) and at the 3' end was a quencher dye, 6-carboxy-tetramethyl-rhodamine (TAMRA). The quencher would suppress the reporter dye while the probe is intact, but when the target sequence is present, the probe will anneal to the cDNA between the forward and reverse primers. Taq polymerases then cleave the probe so that the reporter dye (FAM) is no longer quenched and can emit a fluorescence signal. This occurs at every cycle and consequently the fluorescent activity increases as PCR product is formed and this signal is measured quantitatively by the sequence detection system.

2.6.5.1 Primer Design

Primers were designed for each gene of interest using the free Universal ProbeLibrary assay design centre at http://www.roche-applied-science.com/sis/rtpcr/upl/exhome/html. For each gene of interest this library allowed design of the primer sequences and provided the number of the probe compatible with the Universal ProbeLibrary (Roche). The primers were then purchased from MWG Biotech, London, UK, which were then diluted to 20µM with nuclease free water. Table 2.6 summarises the primer sequences and Universal Probe Library probes used for the studies in this thesis.

Table 2.6 Sequences and probe numbers of primers designed for QRT-PCR

Gene	Forward Primer 5'→3'	Reverse Primer 3'→5'	UPL Probe Number:
AR	CCT GGG CTT TTC CTT CTT TC	CAG CGG ATT CAA AAT GTG C	13
Asporin	AAG AAA ACC TCT CTT CTG ACA AGG	GCA CAC AGC CAA AAG CAG TA	56
BORIS	GAA GAA AAA GAA AGA TGC GGT CT	GAG ATC CGG CTC AGC ATT T	82
BRG1	ACG GTG TCC CAG CTA GAT TC	GCA TCC GCA TGA ACA TAC TTC	20
CBP/P300	GGC AAA GAG GAC CAG ACG	AAT CGG TGG GAA TTG ATG TC	22
COX1	GCT CTT CAA GGA TGG GAA ACT	TTC TAC GGA AGG TGG GTA CAA	42
COX2	CTA CAC CAG GGC CCT TCC	TCC AGA ACT TCT TTT GAA TCA AGG	5
DNMT3L	GAG GGT GTG GAG CAA CAT TC	GCT CTT CCT TAG GGG TCA GG	41
FKBP5	CTC AAA CCC CAA TGA AGG AG	GCA GTC AAA CAC CCT TCC A	66
ROBO1	GCA GAG AGG CCT ACA CAG ATG	CAC TGG GCG ATT TTA TAG CAG	10
RWDD1	ACA ACG CAA CGA GTT GGA G	GCT GGG TGG GTT TTC TGA TA	109
SLIT2	GCC ATT CAG GAC TTC ACC TG	CGA AGG ACA ACG GGA GAG	108
SNURF	AAC AGC ACG TCC CAG AGA GT	AGA ATG CCG TGG GTA ACA GT	85
SRY	TCA GCC TCA TCG AAG GGT TA	GCA ACT TCA CGC TGC AAA G	82
Transgelin	AGT GTG GCC CTG ATG TGG	TCA CCA ACT TGC TCA GAA TCA	5
VAV3	CCA TGG AGA AGT CGA ACC TG	TTC ACA TAT TGT GCC AAG TCC T	21

2.6.5.2 Q-RT-PCR reaction

Sample to be analysed using the Taqman system were run in triplicate in a 96 well MicroAmp optical reaction plate (Applied Biosystems). For each sample, a tube containing the following was prepared, totalling 45µl.

Reaction	1X (15 µl)
Taqman mastermix	7.5
18s (Applied Biosystem)	0.225
Forward primer (20µM)	0.15
Reverse Primer (20µM)	0.15
Universal Probe (Roche)	0.15
cDNA	1
dH2O	5.325

Each Taqman reaction mixture was vortexed and then divided into three wells, with 13.5µl in each. When each sample was loaded, the entire plate was sealed with a MicroAmp optical adhesive cover (Applied Biosystems) and then loaded onto the ABI7900 sequence detection system.

2.6.5.3 Analysis of results - comparative Ct method

The Taqman RT-PCR system provided the results as an amplification plot, which showed the amount of reporter dye/fluorescence generated during amplification, which is proportional to the amount of PCR product formed. The FAM Ct value is the cycle number at which the fluorescence rises above the threshold level, representing when the amount of amplified DNA becomes significantly above the background level. The threshold was determined at a point during the exponential increase of the PCR product. The FAM Ct value was dependent on the original amount of target mRNA in the reaction mixture, such that a difference in Ct value by one compared to a reference sample equated to a two-fold difference in RNA in the initial reaction mixture. 18s ribosomal RNA was used as an internal control, to adjust for mRNA variation between samples.

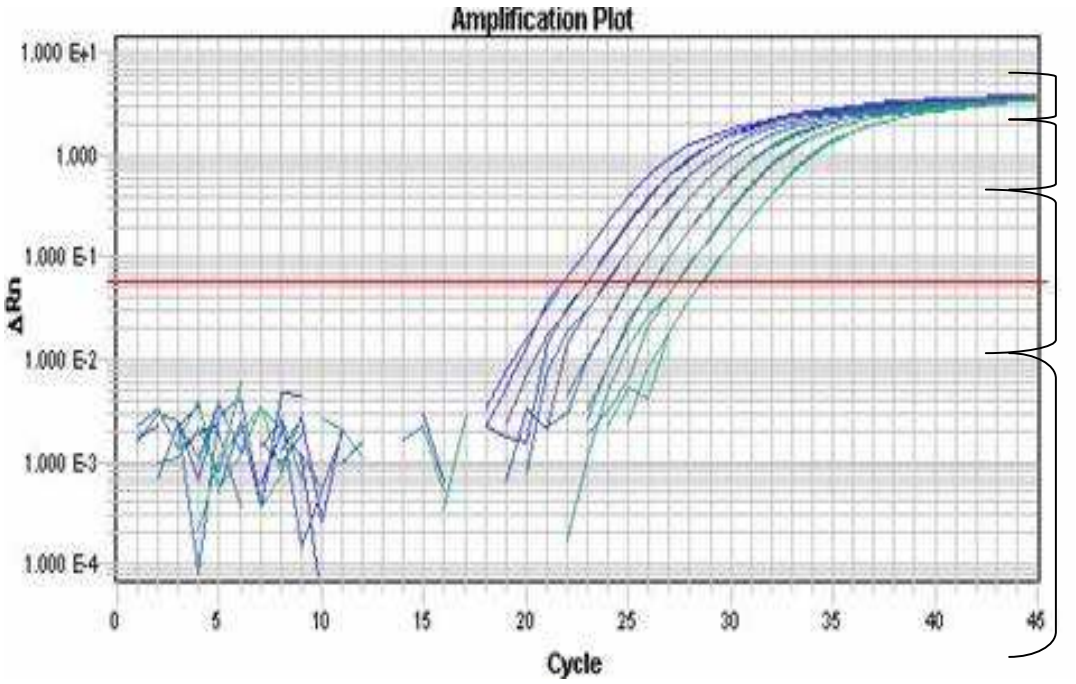


Figure 2.3 A typical QRT-PCR amplification plot. 1- plateau phase, 2 linear phase, 3 exponential phase, 4 background.

By analysing the differences in FAM Ct value compared to a reference control (usually adult rat testis), relative changes in RNA expression of the target gene were calculated using the comparative Ct method. This required the calculation of the ΔCt value, which was the difference between the FAM Ct and the 18s Ct value for each of the three 15µl reaction wells for each sample. From these triplicates, the mean ΔCt for that sample was calculated. Using the mean ΔCt, the ΔΔCt was then calculated, which was the difference between the mean ΔCt of each sample compared to the mean ΔCt of the reference sample. In most cases, the reference sample was adult rat testis total RNA, collected and processed in house.

The amount of amplified target was calculated as 2-ΔΔCt, which was based on the mathematical equation for the exponential amplification of the PCR reaction Xn=X0 x (1+Ex)n, where Xn is the number of target molecules at the threshold at cycle n, X0 is the initial number of target molecules, (1+Ex) is the efficiency of the target

amplification and n is the number cycles. If the efficiencies of the target and internal control reactions are equal, the 2-ΔΔCt value provided a measure of relative quantification, showing the fold increase or decrease in mRNA expression in samples in relation to the reference sample, which always has a 2-ΔΔCt value of one.

2.7 Optical Projection Tomography (OPT)

OPT is a 3D optical microscopy technique developed by the MRC Human Genetics Unit. In brief, it is a machine in which up to 1cm^3 of tissue is mounted and rotated. Whilst being rotated, light from a lamp is converted into an even illumination by a diffuser. The light then passes through the tissue specimen and is focused by the optics of the microscope onto a CCD imaging chip. The light rays do not follow straight paths through the specimen but the intensity recorded at each position on the camera chip approximates a projection through the focused part of the specimen. 400 images are taken per tissue specimen and reconstructed using a 'back project algorithm' which builds a 3D image of the internal structure of the tissue.

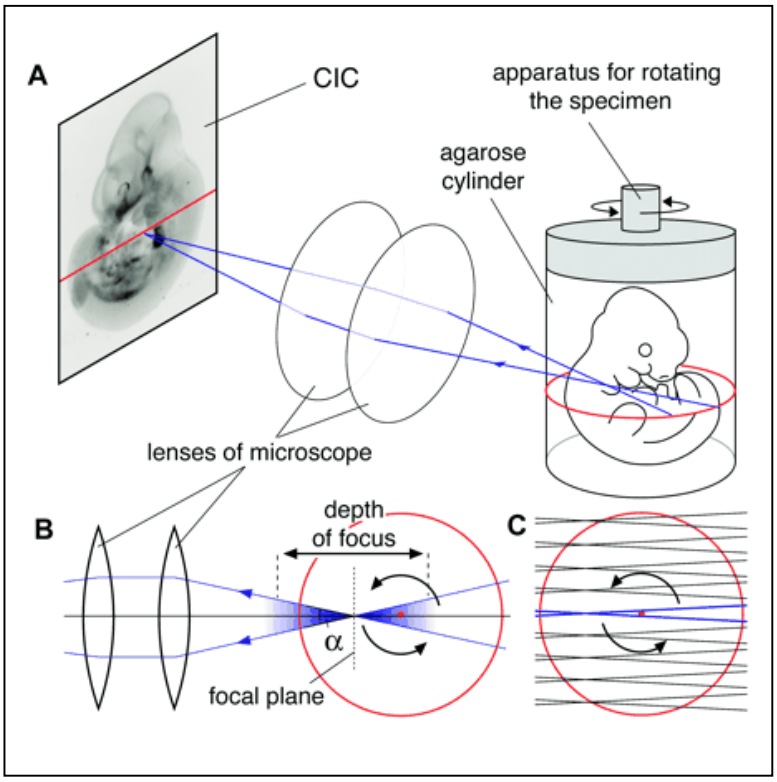


Figure 2.4 Schematic diagram showing the principles of OPT. CIC represents camera imaging chip

2.7.1 Tissue preparation for OPT

As none of the tissues used were freshly dissected, but had been embedded in paraffin wax (2.1), tissue samples were dewaxed using the following:

Solution	Time in solution	Solution refreshed during time course
Xylene	Overnight	3x
↓		
Absolute alchol	2hours	2x
↓		
74% OP IMS	2hours	2x
↓		
90% ethanol	2hours	2x
↓		
80% ethanol	2hours	2x
↓		
70% ethanol	2hours	2x
↓		
PBS		

2.7.2 Embedding & mounting tissue for OPT scanning

Tissue to be scanned requires to be mounted in 1% agarose. This was made by adding 1.5mg of agarose to 150ml of de-ionised water and heating in a microwave for approximately 2 minutes to homogenise the solution. The temperature was monitored until the agarose solution was 60°C. The solution was filtered through Whatman filter paper and the temperature further monitored until the solution was at 32°C, when it was poured into a petri dish placed on a cold plate at 4°C. The tissue was transferred from PBS, using tweezers, into the agrose and using forceps was suspended in the centre of the dish until the agarose had completely set around the specimen. The agarose around

the specimen was trimmed using a microtome blade and mounted, using superglue, upside down on to a specifically designed magnetic mount and allowed to set for 5-10 minutes. The mounted specimen was placed in a solution of methanol which was changed at least three times over the course of 2 days. One day prior to the sample being used in the OPT machine, the mounted tissue was transferred to BABB solution, which is 2 parts Benzyl Benzoate and 1 part benzyl alcohol. This solution clears the tissue specimen, making it transparent prior to scanning. The specimen was left in BABB overnight before being scanned.

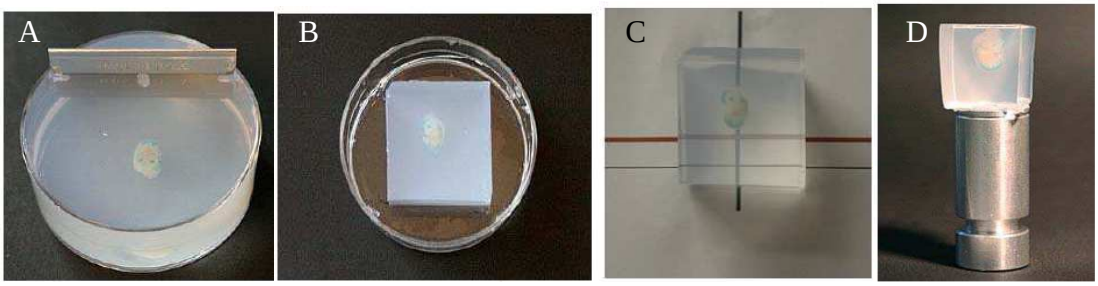


Figure 2.5 Process of embedding and mounting tissue for scanning. A: tissue in agarose being cut with blade. B: Tissue after being cut. C: correct positioning of tissue. D: Tissue mounted onto magnetic mount ready for BABB solution and then OPT imaging.

2.7.3 Scanning

The Bioptonics microscopy OPT Scanner 3001 was used to take 400 images of each sample, under both white light and UV light, under standard settings outlined in the user guide. The images were put together using the NRecon software, using standard settings.

2.8 Statistical analyses

All statistical analyses in this thesis were carried out using GraphPad Prism (version 5, GraphPad software Inc., SanDiego, CA). When appropriate, values are expressed as means ±SEM, and differences between means were analysed, for two groups, using an unpaired two tailed t-test. Three or more groups were analysed by either using a one way analysis of variance (ANOVA) followed by the Bonferroni post-hoc test (for 3 or more groups) or a two way analysis of variance (2-way ANOVA). Fisher's exact test was

used to calculate the values in the contingency table reported in chapter 3. If necessary (unequal variances, skewed distributions) values were logarithmically transformed prior to statistical analysis. Asterisks were used to indicate level of significance based on the following criteria: *P<0.05, **P<0.01 and ***P< 0.001.

2.9 Commonly used solutions

Bouin's Solution: purchased from Triangle Biomedical Sciences Ltd, Lancashire, UK.

Citrate Buffer: 42.02g citric acid monohydrate was added to 1.9L distilled water. Made up to 2lL and pH 6 using NaOH and used at 0.01M by diluting 1:10 in distilled water.

Eosin: 1% (w/v) eosin (15g) was diluted in 1200ml of distilled water. 1% (w/v) eosin (5g) was diluted in 500 ml methylated spirit. Both solutions were mixed together and filtered. 1ml formaldehyde was added to prevent bacterial growth.

Harris's haematoxylin 2.5g of haematoxylin was added to 25ml absolute alcohol and 50g aluminium potassium sulphate was added to 500ml distilled water. Both solutions were combined and boiled and 1.25g mercuric oxide added slowly. The solution was cooled on ice before being filtered. 4ml (per 100ml of haematoxylin) of glacial acetic acid was then added.

PBS: 1 tablet of PBS concentrate (Medicago, Sweden) in 1L of distilled water.

Scott's Tap Water: 10g potassium chloride and 100g magnesium sulphate were dissolved in 5L of tap water.

TBS: 60.6 Tris (Sigma) was added to 87.6 NaCl (Sigma) to 300 ml Hydrocholric acid (BDH) and adjusted to PH 7.4 using concentrated HCL.

3.1 Introduction

Masculinisation of males is set up by androgen exposure during the masculinisation programming window (MPW). Deficiency in androgen exposure in this window results in reduced size of all reproductive organs, increased risk of reproductive disorders and reduced anogenital distance (AGD) (Foster and Harris, 2005; MacLeod et al, 2010, Welsh et al, 2008). As outlined in section 1.1.6, it is unknown what determines the onset and closing of the MPW, and for example whether androgen exposure itself and/or the availability of androgen receptors is important in this regard. Moreover, as the level of androgen exposure within the MPW is all important for determining normal male reproductive development, a further question posed is what happens if there is additional (exogenous) androgen exposure and, for example, what happens if this exposure occurs before the MPW as opposed to during it? The primary objectives of the studies in the present chapter were to address these questions. Therefore, studies were undertaken to establish if androgen receptor mRNA and protein are expressed prior to the MPW or whether their ontogeny coincided with opening of the MPW. Following this, more detailed studies were undertaken to evaluate if masculinisation could be advanced or enhanced by treating pregnant rats with either 1 or 10 mg/kg/day dihydrotestosterone (DHT) prior to (early window, EW; e11.5-e14.5) or during the MPW (e15.5-e18.5), and then evaluating male (and female) offspring in fetal life (e18.5, e21.5), early puberty (day 25) or adulthood (~day 75).

Although there are some relevant studies in the literature (reviewed in Wolf et al, 2002; Welsh et al, 2008), these have not examined androgen administration *prior* to the MPW and have used testosterone formulations for treatment. For example, studies by Kawashima et al (1978) exposed rats to methyltestosterone, late in gestation, from e17.5-e20.5 and found that female offspring were masculinised in adulthood, demonstrating the some testosterone exposure can have some virilising effects even late in gestation. Furthermore, some studies exposed rats to testosterone on individual days of gestation to determine how the effect on female offspring correlated with exact day of

exposure. Even the earliest day of treatment for this study was e16.5 (Rhees et al, 1997). This study reported that exposure to testosterone on either e16.6, e17.5 or e18.5 caused a significant increase in female offspring AGD and treatments on any day between e16.5-e20.5 caused delays in vaginal opening. Other studies have found that female fetal exposure to testosterone can also cause (in addition to increased AGD) reduced number of areolas and nipples, cleft phallus and presence of prostate tissue (Wolf et al, 2002). This study used testosterone propionate from e14.5-e19.5 in rats, and in male offspring no effects of the testosterone exposure were found other than a reduction in glans penis weight. Indeed in the studies which really defined the MPW by Welsh et al (2008), testosterone was used as the choice of androgen exposure, and this exposure began at e15.5. Female offspring were reported to have developed prostates and seminal vesicles when exposed between e15.5-e19.5 but no masculinisation was reported when females were exposed between e19.5-e21.5 other than an increase in phallus length. No effects were reported in the male offspring after exposure to testosterone (Welsh et al, 2008). Thus there are no studies published that outline the effect of androgen exposure in the rat, in the fetal period, earlier than the MPW.

Testosterone can be readily converted by the aromatase enzyme to oestradiol, and aromatase is strongly expressed in the placenta during pregnancy. Moreover, if rats are exposed to oestradiol or other potent oestrogens during pregnancy they can also induce dystocia, fetal loss and growth restriction, much like testosterone treatment does. One major problem that emerged from the studies described that used testosterone treatment during pregnancy was complications with giving birth, essentially delayed birthing (dystocia). From descriptions in the various studies, this manifest as increased loss of pregnancies due to death in utero or cannibalisation, reduced litter size and growth restriction of the pups that were born (Fritz et al, 1984; McCoy and Shirley, 1992; Rosenberg and Sherman, 1974). For example, in the studies by Welsh et al (2008, 2010) using Wistar rats, these effects were so severe that the authors had to caesarian derive

pups from testosterone propionate (TP)-exposed dams and cross-foster them to other dams in order that offspring could be studied postnatally.

As fetal growth restriction and reduced litter size are changes that are potentially confounding in any study investigating aspects of fetal development, it was considered important to avoid these if it was possible. Therefore, in the present studies dihydrotestosterone (DHT), rather than testosterone propionate, was used for treatment, in the expectation that because it cannot be aromatised to oestradiol it would not cause the same adverse pregnancy effects as testosterone administration. Additionally, DHT is a more potent androgen than testosterone (~10-fold), because it binds to the androgen receptor with higher affinity and more tightly (i.e. it dissociates less readily form the receptor) than does testosterone (Grino et al, 1990; Toth and Zakar, 1982). DHT was also used to ensure that any effect induced could be clarified as an androgen response rather than an oestrogen (from conversion of testosterone to oestradiol) response.

3.2 Materials and Methods

3.2.1 Animals and treatments

Briefly, pregnant rats were treated with DHT, or corn oil during the MPW (e15.5-e18.5) or during the period immediately preceding the MPW (e11.5-e14.5), hereafter referred to as 'early window' (EW) (section 2.2). Treatments were administered daily by subcutaneous injection and in each time window. Initially 1mg/kg DHT was administered to pregnant dams but when no effect was seen in the females, this dose was increased to 10mg/kg.

Control and DHT-treated dams were either killed before term (e18.5, e21.5) or allowed to give birth, and resulting offspring then killed on either postnatal day (PND) 25 (= early puberty) or 75 (= adults). For fetal studies, pregnant dams were killed by inhalation of CO_2 followed by cervical dislocation, fetuses were removed, and placed in ice-cold PBS. E21.5 fetuses were weighed and AGD measured using digital callipers and the

fetuses then decapitated (see section 2.3). The genital tubercle, gonads and ducts were removed by microdissection from both e18.5 and e21.5 fetuses and imaged using a Leica MZ6 camera attached to a dissecting microscope fitted with cold lights. In studies designed to determine if AR was present in tissues prior to the MPW, control fetuses were recovered from pregnant dams at e11.5, e12.5, e13.5 and e14.5. The genital/lower abdominal area was dissected from each pup and either stored at -80°C for RNA extraction and PCR investigation or fixed in Bouin's fixative for 1 h before being transferred to 70% ethanol before being processed into paraffin wax blocks using standard procedures and an automatic tissue processor.

Postnatal animals were killed by inhalation of CO_2 followed by cervical dislocation. Bodyweight and AGD were measured before dissection of the phallus, gonads and ventral prostate (if present), which were weighed. The length of the phallus was measured by digital callipers. Tissue was fixed in Bouin's fixative for 4 hours before being transferred to 70% ethanol and embedded in paraffin wax as above.

3.2.2 RNA extraction and RT- PCR for androgen receptor (AR)

Briefly RNA was isolated from the genital area (perineum + genital tubercle) of control male and female pups at e11.5, e12.5, e13.5 and e14.5, and converted into cDNA (see section 2.6). The primers used for detection of *AR* exons 1 and 3 (forward AAGCAGGTAGCTCTGGGACA; reverse CGTTTCTGCTGGCACATAGA); revealed bands of 765 and 613 bp.

3.2.3 Immunohistochemistry for the androgen receptor (AR)

Briefly, immunohistochemical staining (section 2.5) for the AR was performed on sections of tissue from the lower abdomen/genital region of control animals at e11.5, e12.5, e13.5, e14.5 and e15.5. Table 3.1 outlines details of the AR antibody used.

Table 3.1 Details of AR antibody used in Chapter 3

Primary Antibody	Source	Retrieval	Species	Dilution
Androgen Receptor	Abcam, Cambridge	Citrate	Rabbit	1:500

3.3 Results

3.3.1 Effects of DHT exposure on the dams, gestational length and litter size

Treatment with either dose of DHT in either time window had no detectable effect on health of dams, gestational length or on fetus number (e18.5, e21.5) or litter size at birth. There was no significant difference in the weight of the dams on the last day of treatment with DHT in comparison to vehicle (Fig.3.1). Using a one way ANOVA followed by a bonferroni test, no significant differences were noted for number of pups per litter in each treatment groups and no dams were found to suffer from dystocia.

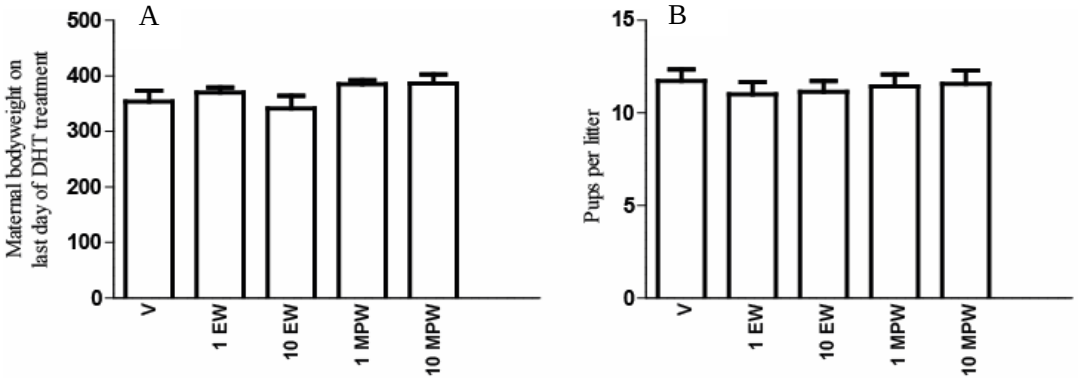
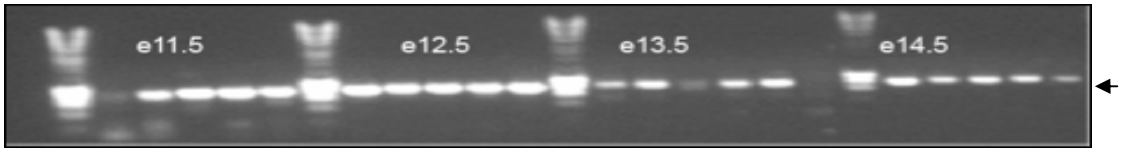


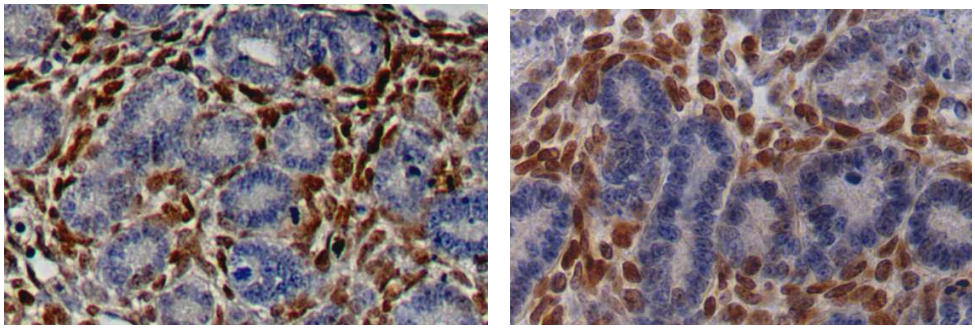
Figure 3.1 Effect of treatment with vehicle (V control) or dihydrotestosterone (DHT) at 1 or 10mg/kg/day before (EW: early window; e11.5-e14.5) or during the masculinisation programming window (MPW; e15.5-e18.5) on maternal bodyweight on the last day of treatment (A) and number of pups per litter (B). N=8 for each treatment group.

3.3.2 Androgen receptor (AR) expression

A



B



E13.5 E15.5

Figure 3.2 Evidence for expression of the androgen receptor before the masculinisation programming window (MPW), based on RT-PCR (arrow) on tissue extracts from the lower abdominal/genital area (A) and immunoexpression (B) in a tissue section from the mesonephric area of an e13.5 fetus (B). In A, each well corresponds to tissue from a different fetus and a product size of 765 bp. In B, a section from the mesonephric area at the onset of the MPW (e15.5) is shown for comparison.

RT-PCR of tissue extracts from the lower genital area showed detectable *AR* mRNA expression from e11.5 – e14.5 (Fig.3.2A), which is the period immediately preceding the MPW. As reproductive tissues are not well developed at most of these ages, assessment of whether AR protein was expressed immunohistochemically is problematical, but tissue from the region of the developing mesonephros clearly expressed the AR protein at e13.5 (Fig. 3.2B), suggesting that at least in some tissues the AR protein may be expressed prior to the MPW. A pragmatic conclusion from these studies was that further similar investigations were unlikely to be any more definitive, and that the tentative conclusion was that AR expression was unlikely to be the determining factor for opening of the MPW. Moreover, if AR was expressed prior to the MPW in relevant reproductive

tissues or progenitor tissues, then exposure to exogenous androgen (DHT) at this time would establish if these AR were functionally coupled in ways that would allow 'premature masculinisation'. Therefore, the logical next step was to investigate the effects of DHT exposure during these periods or during the MPW itself.

3.3.3 Effect of DHT exposure in utero on AGD in males and females

AGD measurement from e21.5 onwards provides an accurate read-out of androgen exposure within the MPW (Welsh et al, 2008; MacLeod et al, 2010). The effect of maternal treatment with 1 or 10mg/kg DHT before and during the MPW, on AGD in male and female offspring is shown in Fig. 3.3. As expected, AGD was significantly longer in control males at all ages than in females. In males, DHT exposure at either dose in either time window (i.e. before or during the MPW), had no significant effect on AGD. In females, AGD was significantly increased, compared with controls, at e21.5 after exposure to either 1 or 10mg DHT during the MPW, but this difference was not maintained postnatally (Fig. 3.3). In contrast, exposure of females to DHT at either dose during the EW (i.e. prior to the MPW) did not alter AGD at any age (Fig. 3.3). These results showed that androgen exposure prior to the MPW was without effect on AGD in fetuses of either sex.

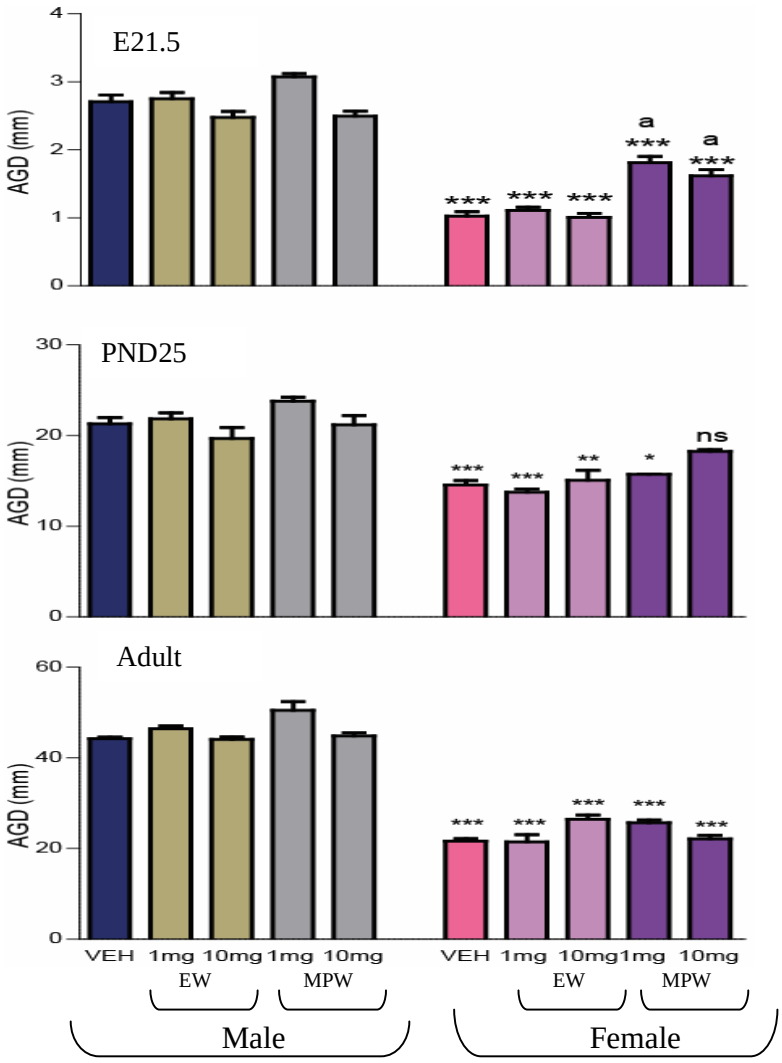


Figure 3.3 Effect of treatment with vehicle (VEH; control) or dihydrotestosterone (DHT) at 1 or 10mg/kg/day before (EW: early window; e11.5-e14.5) or during the masculinisation programming window (MPW; e15.5-e18.5) on anogenital distance (AGD) in male and female offspring in fetal life (top), early puberty (middle) and in adulthood (bottom). Values are the mean ± SEM for 4-24 animals per group from 3-6 different litters. *P<0.05, **P<0.01, ***P<0.001, in comparison with respective male control value. [a]P<0.001, in comparison with respective female control value. NS = not significantly different from male control value.

3.3.4 Effect of DHT exposure in utero on development of the Wolffian duct

Coiling of the Wolffian duct normally first appears at e19.5 in the male rat with full coiling developed by e21.5 (Welsh et al, 2007). Coiling is never observed at e18.5 in normal males, as confirmed in controls in the present studies (Fig. 3.4). Exposure to either 1 or 10mg DHT treatments prior to (EW) or during the MPW did not induce premature Wolffian duct coiling at e18.5 in males, nor did it exagerrate normal coiling evident at e21.5 (Fig. 3.4). In normal female rats the Wolffian duct has largely regressed by e18.5 (Welsh et al, 2009), but exposure to the 10mg dose of DHT during either the EW or the MPW resulted in stabilization of the Wolffian duct in 58% and 81% of females, respectively, at e21.5 (Fig. 3.4 and Table 3.2). In contrast, no retention of Wolffian ducts was observed in females exposed in either time window to the 1mg dose of DHT.

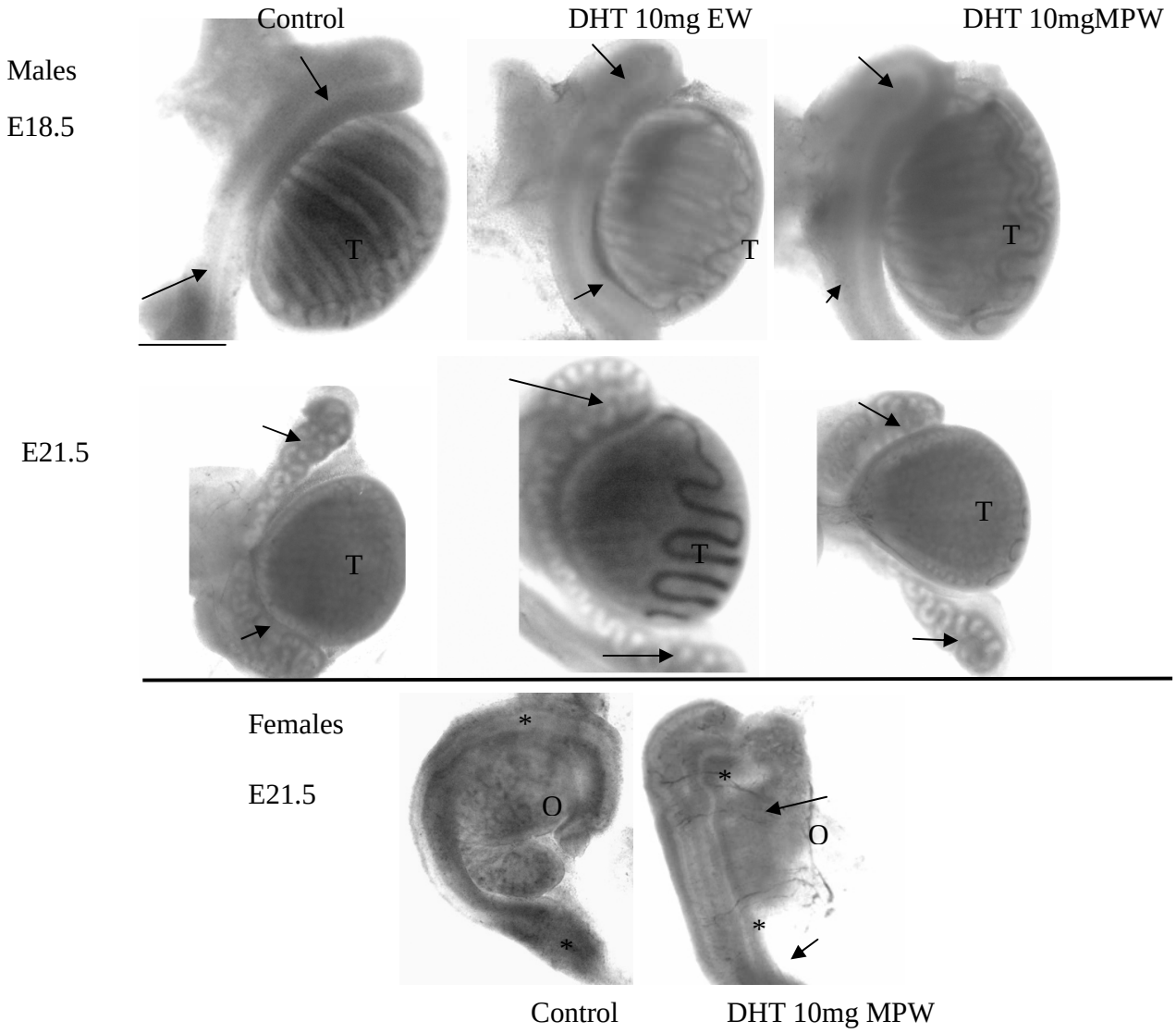


Figure 3.4 Effect of treatment with vehicle (Control) or dihydrotestosterone (DHT) at 1 or 10mg/kg/day before (EW: early window; e11.5-e14.5) or during the masculinisation programming window (MPW; e15.5-e18.5) on Wolffian duct development (arrows) in males (top two rows) and females (bottom row). Note that at e18.5 the Wolffian duct is a simple straight tube in males, whereas by e21.5 it has developed conspicuous coiling; DHT exposure in EW or MPW does not induce precocious coiling at e18.5 or affect it detectably at e21.5 (similar results for 1 mg DHT dose, not shown). In females, exposure to 10mg DHT, but not to 1mg (not shown), in the MPW and to a lesser extent in the EW (Table 3.2) induces stabilisation of the Wolffian duct so that it is evident at e21.5 whereas in controls it has regressed. T=testis, O=ovary, *=müllerian duct. The scale bar represents 200µm.

3.3.5 Effect of DHT exposure in utero on development of the ventral prostate (VP)

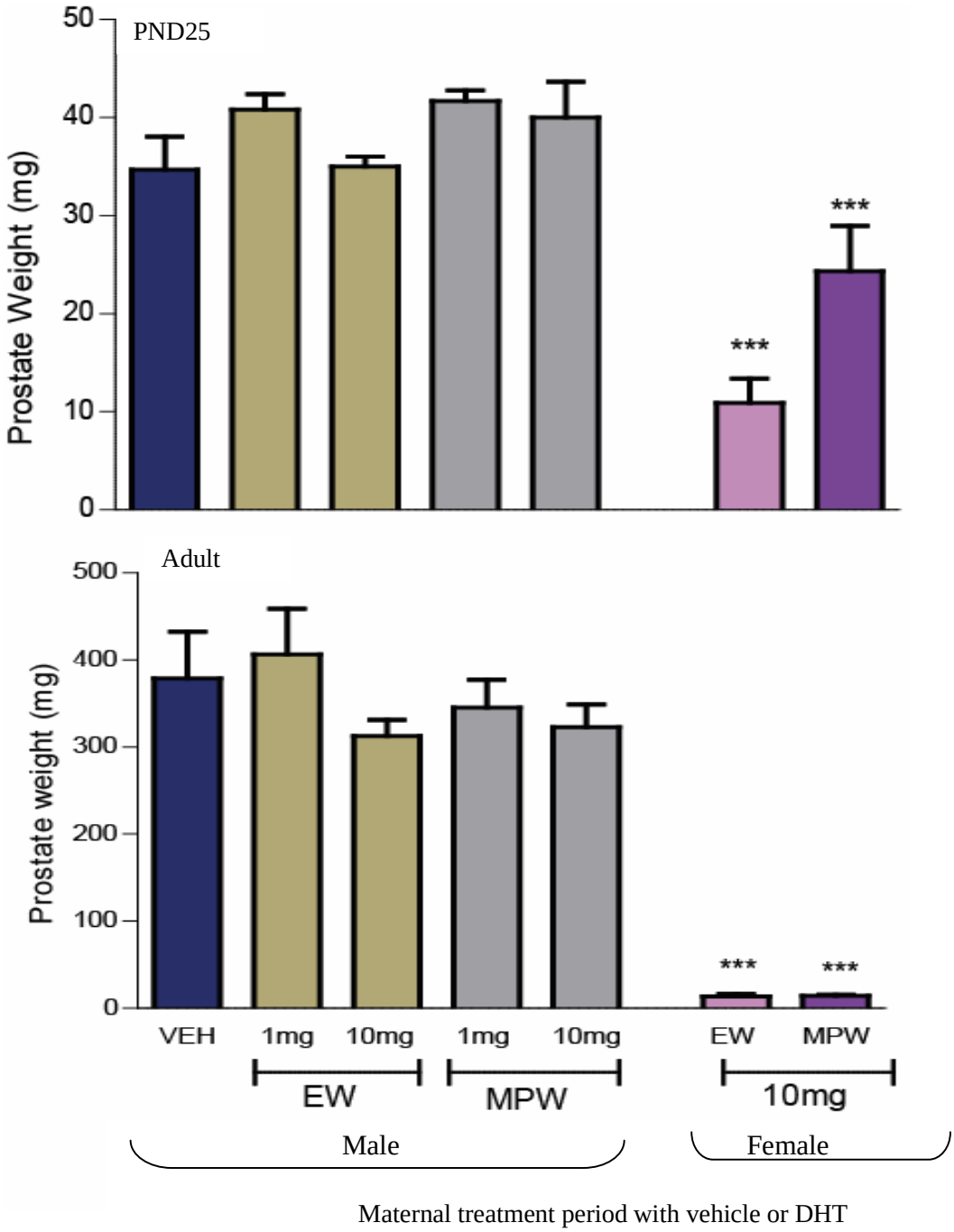


Figure 3.5 Effect of treatment with vehicle (VEH; Control) or dihydrotestosterone (DHT) at 1 or 10mg/kg/day before (EW: early window; e11.5-e14.5) or during the masculinisation programming window (MPW; e15.5-e18.5) on ventral prostate (VP) weight in early puberty (top) and in adulthood (bottom). Values are the mean ± SEM for 4-24 animals at each age from 3-6 different litters. ***p<0.001, in comparison with respective male control value. Note that in females, exposure to 1mg DHT in either EW or MPW did not result in VP formation and not all females exposed to 10mg DHT had a VP (see Table 3.2).

In utero exposure of males to either dose of DHT in either time window did not significantly alter VP weight at either PND25 or in adulthood (Fig. 3.5). In females, exposure to the 10mg DHT dose during the MPW induced a VP in all females when evaluated at PND25 or in adulthood, although the glands were always notably smaller than those found in normal males of the same age (Table 3.2; Fig. 3.5). A proportion of females exposed to 10mg DHT prior to the MPW (EW) also had some prostatic tissue but this was more variable than in females exposed during the MPW (e.g. only one lobe of a VP present). Females exposed to the 1mg dose of DHT in either time window did not show evidence of VP induction (Table 3.2).

Table 3.2. Effects of in utero DHT exposure on reproductive development in females

	Age	**Vehicle (control)[1]**	**e11.5-e14.5 (EW)**		**e15.5-e18.5 (MPW)**	
			DHT 1mg/kg	**DHT 10mg/kg**	**DHT 1mg/kg**	**DHT 10mg/kg**
Wolffian Duct present/absent	e21.5	0/20	0/20	14/10***	0/13	13/3***
Ventral prostate Present/absent	PND25	0/18	0/15	3/3**	0/4	5/0***
Ventral prostate Present/absent	Adult	0/20	0/10	10/4***	0/4	4/4**
Nipples Present/absent	PND25	18/0	20/0	0/6***	4/0	0/5***
Nipples Present/absent	Adult	20/0	10/0	0/14***	4/0	0/8***
Vaginal opening Present/absent	Adult	20/0	10/0	0/14***	4/0	0/8***
Uterine abnormalities	PND25	0/18	0/15	4/2**	0/4	0/5
Uterine abnormalities	Adult	0/20	0/20	4/10*	0/4	4/4**

[1]Females exposed to vehicle treatment in either the EW or MPW

*P<0.05, **P<0.01, ***P<0.001, in comparison with incidence in respective control group (Fisher's exact test)

3.3.6 Effect of DHT exposure in utero on development of the phallus

In utero exposure of males to either dose of DHT in either time window did not significantly alter penis length at either Pnd25 or in adulthood (Fig. 3.6).

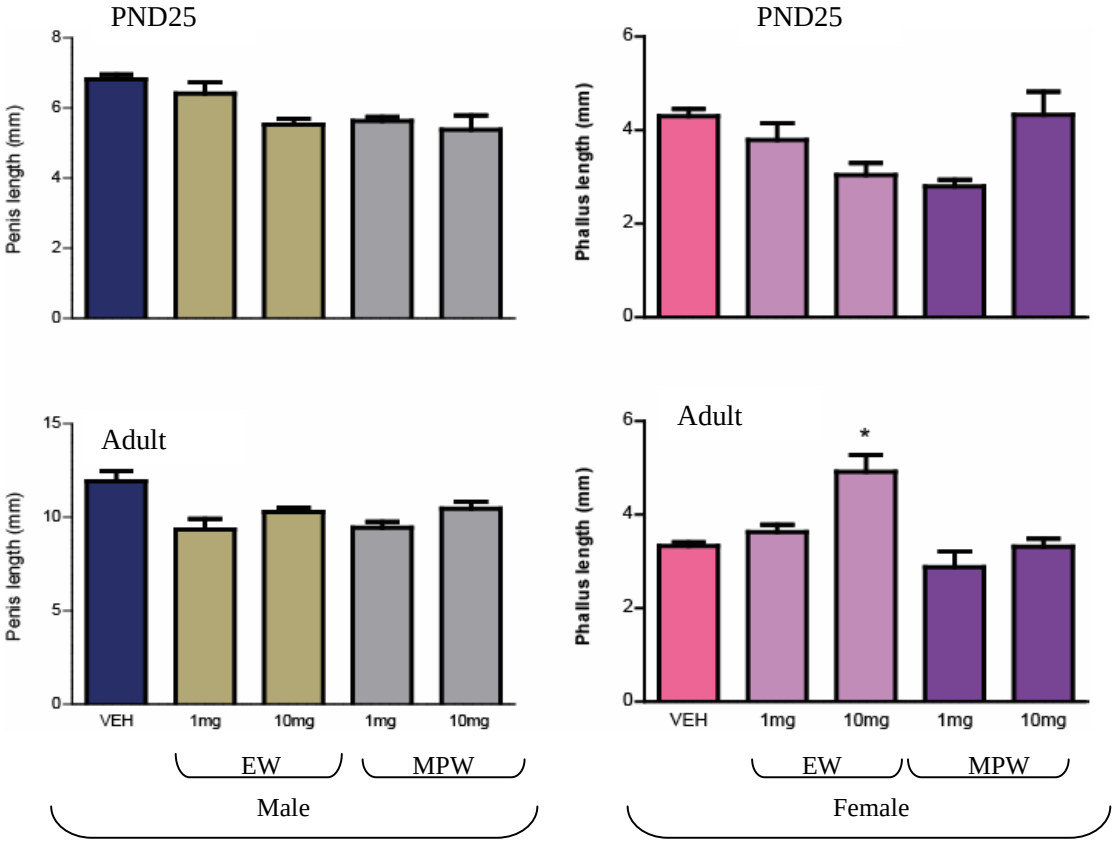


Figure 3.6 Effect of treatment with vehicle (VEH; Control) or dihydrotestosterone (DHT) at 1 or 10mg/kg/day before (EW: early window; e11.5-e14.5) or during the masculinisation programming window (MPW; e15.5-e18.5) on phallus length in early puberty (top) and in adulthood (bottom) in males and females. Note the different scales for male and female results. Values are the mean ± SEM for 4-24 animals at each age from 3-6 different litters. Results were analysed using a one way ANOVA. *P<0.05, in comparison with respective female control value.

However, surveyed as a whole, it appeared that mean penis length was slightly but consistently smaller in DHT-exposed males compared with controls (P=0.016 at PND25 and P=0.062 in adults by analysis of variance including data for either dose and either time window of exposure). Surprisingly, in females, there was little obvious impact of fetal DHT exposure on phallus length, except for a small significant increase in the 10 mg DHT EW group in adulthood (Fig. 3.6). However, this comparison is probably not accurate because the phallus is structurally indistinct in control females, thus making it difficult to dissect and accurately measure its length. In contrast, in most females at either PND25 or in adulthood that had been exposed in utero to 10mg DHT prior to the MPW, the phallus had a clear tubular structure as occurs in males, although much smaller in diameter; this change was not observed in most females exposed to 10mg DHT during the MPW.

3.3.7 Effect of DHT exposure in utero on development of the testis and ovaries

In utero exposure of males to either dose of DHT in either time window did not significantly alter gross morphology of the testis (not shown) or its weight at either e21.5, PND25 or in adulthood (Fig. 3.7). Ovarian weight in females exposed to DHT in utero tended to be smaller than in controls, but this only reached statistical significance for animals exposed to the 10mg DHT dose during the EW (Fig. 3.8).

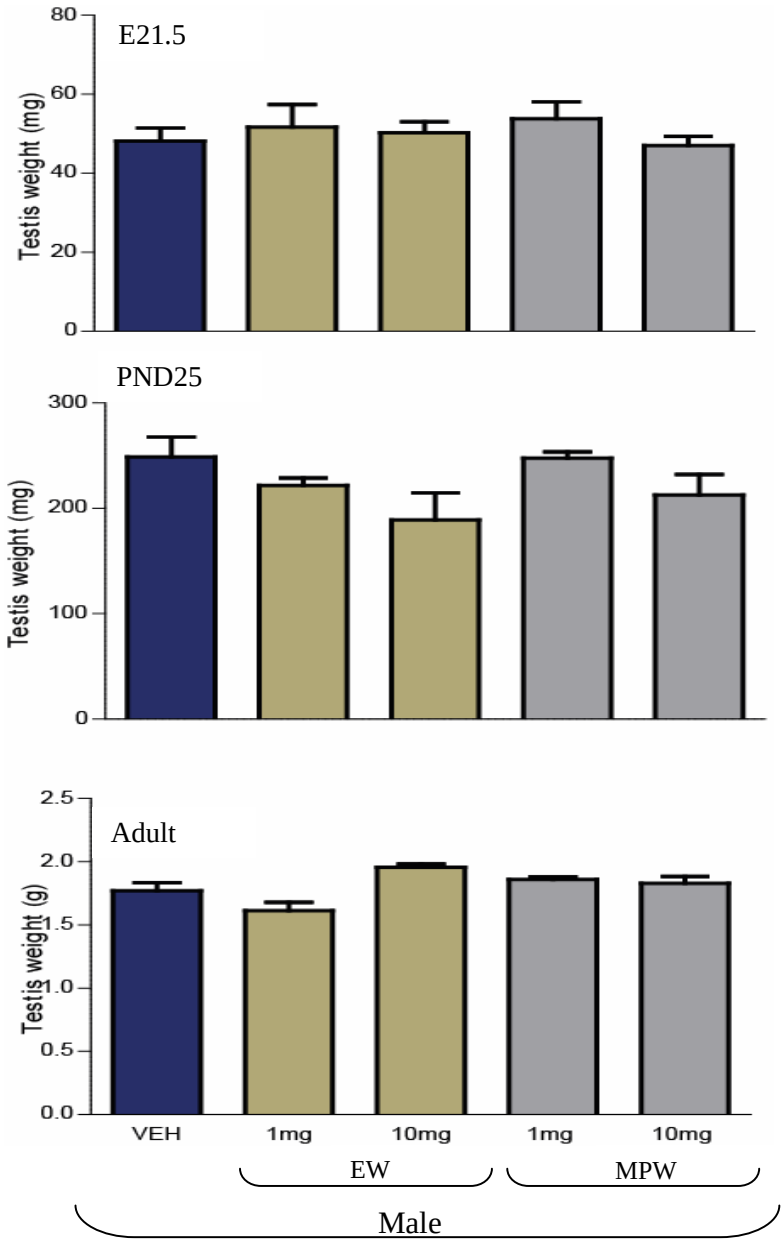


Figure 3.7 Effect of treatment with vehicle (VEH; Control) or dihydrotestosterone (DHT) at 1 or 10mg/kg/day before (EW: early window; e11.5-e14.5) or during the masculinisation programming window (MPW; e15.5-e18.5) on testis weight in fetal life (top), early puberty (middle) and in adulthood (bottom). Results were analysed using a one way ANOVA Values are the mean ± SEM for 5-24 animals at each age from 4-6 different litters.

3.3.8 Effect of DHT exposure in utero on development of other reproductvie tissues in females

Females exposed to 10 mg DHT in either time window lacked nipples at Pnd25 and in adulthood, whereas exposure to 1mg DHT had no effect (Table 3.2). Similarly, a vaginal opening was absent in all females exposed to 10mg DHT in either time window but was present in all females exposed to 1 mg DHT (Table 3.2). Fluid distension of the uterus (hydrometrocolpus) was evident in a proportion of DHT-exposed females that lacked a vaginal opening (Table 3.2), and in some affected individuals this accumulation was extensive, resulting in gross abdominal distension (Fig. 3.8); one animal had its uterus intertwined adhesively with the gut.

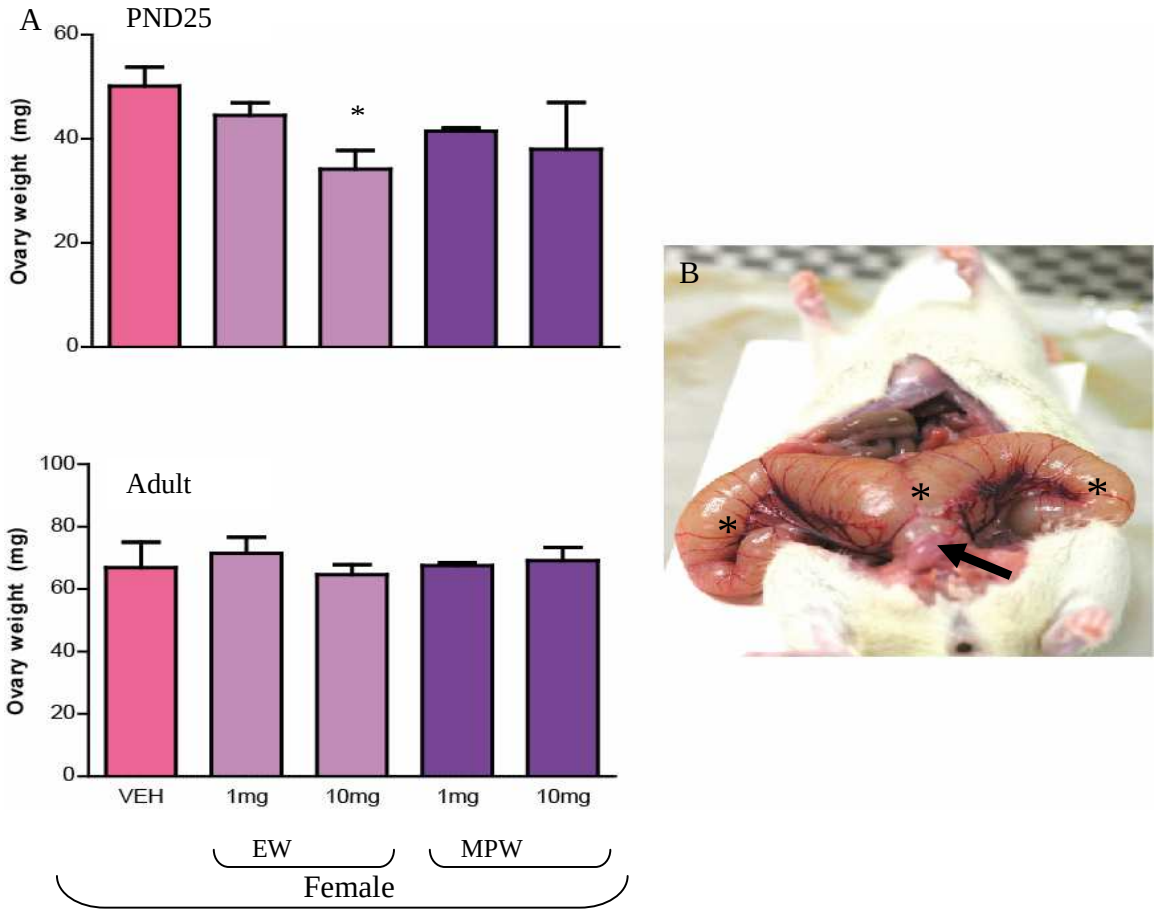


Figure 3.8 Effect of treatment with vehicle (VEH; Control) or dihydrotestosterone (DHT) at 1 or 10mg/kg/day before (EW: early window; e11.5-e14.5) or during the masculinisation programming window (MPW; e15.5-e18.5) on ovarian weight in early puberty (A, top) and in adulthood (A, bottom). Values are the mean ± SEM for 4-12 animals at each age from 3-5 different litters (N=2 litters for 1mg DHT MPW group). Results were analysed using a one way ANOVA *P<0.05, in comparison with respective control value. Panel B shows an example of gross distension of the uterus (asterisks), termed hydrometrocolpos, in a female rat exposed to 10mg DHT in the MPW (for incidence data see Table 1); arrow=bladder.

3.4 Discussion

The primary aim of the present studies was to establish if experimental exposure of the male rat fetus to a potent, non-aromatisable androgen (DHT), either during or prior to the MPW, was able to advance or enhance any aspect of male reproductive development. The results show unequivocally that DHT exposure of fetal males to a dose (10mg/kg/day) of DHT that is able to 'masculinise' females when they are exposed either prior to or during the MPW, has no detectable effect on male reproductive development. In particular, the results show that DHT exposure prior to the normal MPW cannot advance (timing of Wolffian duct differentiation) or enhance (testis, penis and VP size, AGD) male reproductive development. Furthermore, exposure of males to 'extra' androgen during the MPW, in the form of DHT, was similarly without any 'enhancing' effect. The results also indicate that sensitivity of the female reproductive tract to androgens may start earlier than the MPW.

The use of DHT for the present studies could be considered as non-physiological because the physiological androgen that circulates in fetal males is testosterone, whilst DHT is generally produced locally within androgen target tissues via the 5α-reductase enzyme (Imperato-McGinley & Zhu, 2002). However, previous studies that have used testosterone formulations for treatment of rats in pregnancy have all reported adverse pregnancy side-effects such as dystocia, fetal loss/growth restriction (Wolf et al, 2002; Welsh et al, 2008), which may result in confounding effects. In earlier studies by our own group using testosterone, they had to resort to caesarian delivery of pups and their cross-fostering to nursing mums to enable survival of pups for study of their postnatal development (Welsh et al, 2008, 2010). As oestrogen treatment of pregnant rats can result in similar pregnancy problems (Zimmerman et al, 1991), a logical conclusion is that the adverse pregnancy effects of maternal testosterone treatment result from its aromatisation. The present results support this interpretation by showing that administration of the non-aromatisable androgen, DHT, was without any adverse effect on pregnancy or timing of delivery. As oestrogens can exert important effects on male

reproductive development in rodents (Delbes et al, 2006; Goyal et al, 2007; Prins et al, 2007), the present studies also avoided any potential confounding from such effects. Therefore, the studies can be considered as providing a reasonably definitive assessment of the role and importance of androgens before and during the MPW in male reproductive development. The only caveat is that if some aspects of male reproductive programming during the MPW result from testosterone metabolism to oestradiol, then such effects will have been missed. However, as earlier findings involving the administration of testosterone during (and after) the MPW to rats did not report any 'enhancing' effects in male offspring (Wolf et al, 2002; Welsh et al, 2008, 2010), such effects seem unlikely.

A major goal of the present studies was to establish if programming of male reproductive development could be advanced by exposure to DHT prior to the MPW. The initial AR studies indicated that AR mRNA is expressed prior to the MPW and, at least in some tissues (meosnephros) the AR protein may be present prior to the MPW, as reported previously by others (Bentvelsen et al, 1995). Irrespective of this, DHT exposure prior to the MPW did not exert any detectable effect in males, and of most interest it did not advance coiling of the Wolffian duct, a change that occurs after completion of the MPW but which has been shown to be programmed within the MPW (Welsh et al, 2007). As reproductive organ size in DHT-exposed males was comparable to controls at all ages investigated, it is also concluded that reproductive development in males cannot be enhanced by DHT exposure prior to or during the MPW. This fits with previous conclusions from studies involving exogenous testosterone exposure during periods that included the MPW (but not earlier) (Wolf et al, 2002; Welsh et al, 2008, 2010); it also agrees with studies in rhesus monkeys involving fetal androgen exposure (Herman et al, 2000). This generates two further conclusions. First, that exposure to endogenous androgens during the normal MPW is sufficient to maximally program reproductive organ size. Second, that it is not the availability of androgens that 'opens' the MPW, an important conclusion as it suggests that other factors involved in AR

activation/signaling (e.g. co-regulators) may be important in delimiting androgen action within the MPW. These are the subject of investigation in chapter 4.

The most unexpected results from the present study were those in DHT-exposed female offspring. Although the 'masculinisation' changes that we report in females (stabilized Wolffian duct, VP induction, uterine abnormalities/hydrometrocolpos, lack of nipples, absent vaginal opening) have all been reported previously in female rats exposed to exogenous androgens during fetal life (reviewed and reported in Wolf et al, 2002; Welsh et al, 2008), the present studies are the first to have assessed the effect of androgen exposure only prior to the MPW. Whilst such exposure was without detectable effect in males, exposure of female offspring to 10mg/kg DHT prior to the MPW was only marginally different than exposure during the MPW in masculinising female offspring; in some respects, such as effects on the phallus, DHT exposure prior to the MPW may even have been more effective than during the MPW. These findings could simply mean that there has been 'spill-over' of DHT exposure from the early window of treatment (e11.5-e14.5) into the start of the MPW (e15.5-e18.5), a possibility that we cannot exclude as the DHT preparation used is relatively long-acting and was administered in oil so as to prolong absorption and exposure. On the other hand, exposure of females to 10mg/kg DHT prior to the MPW was without significant effect on AGD at e21.5, whereas some effect due to 'spill-over' would have been expected, as exposure to even 1mg/kg DHT during the MPW increased AGD at e21.5. This raises the possibility that androgen sensitivity of females may not be restricted to the MPW, as concluded previously (Welsh et al, 2008), but may be initiated somewhat earlier than this. If so, this suggests that androgen sensitivity of females is somewhat different to that of males. This would not be without precedent, as sensitivity of Wolffian duct degeneration to lack of androgens is different (occurs earlier) in females than in males, at least in rats and mice (Welsh et al, 2009). For other female-specific disorders, such as lack of nipples, lack of a vaginal opening and induction of hydrometrocolpos, there is not a literature on age-specific windows for induction, so it is feasible that the window of susceptibility is

wider than is the MPW in males. One unexpected, and unexplained, finding from the present studies was that the increase at e21.5 in female AGD induced by DHT-exposure during the MPW was no longer evident at PND25 and in adulthood. The present findings and those of others indicate that once AGD has been programmed by androgens in the MPW it is more or less a lifelong change. Other than the use of DHT, there is no other difference between the present and other studies that would readily explain this odd finding.

In conclusion, the studies reported in this chapter demonstrate that it is not the availability of androgens which initiates the MPW, and exposure of the male fetus to exogenous 'additional' androgens prior to or during the MPW does not advance or enhance masculinisation of the fetus, including ultimate reproductive organ size. Furthermore, the female reproductive tract was susceptible to virilisation by exogenous androgen exposure prior to, as well as during, the MPW, which to the best of my knowledge had not been reported before.

4.1 Introduction

The previous chapter demonstrated that the availability of androgens or AR were unlikely to be the factors regulating the MPW. There are various other possible pathways via which specific aspects of androgen action might be regulated within the MPW. Some of these possibilities include AR co-regulators, reviewed in section 1.7.1 and known to be vital in androgen action, and gene methylation which could silence or un-silence genes involved in the regulation of the MPW. There is a surprising lack of data characterizing AR co-regulators, methylation enzymes and general androgen biochemical associates in the fetal male reproductive tract and, considering the aims of this thesis, this warranted investigation.

AR co-regulators play an important role in androgen action and this is reviewed in detail in the literature review of this thesis. Over 300 nuclear receptor co-regulators have been identified. A large number of these have been characterized in relation to oestrogen or glucocorticoid receptors but less co-regulators have been identified to be AR specific or indeed regulated by AR and the overall picture is sketchy. It is well established that many co-regulators are fundamentally necessary in embryonic development and inactivating mutations of these can be embryonic lethal or cause disorders. However, characterization of key androgen co-regulators in the male reproductive tract, and furthermore their possible role in the MPW, has not been investigated. This was therefore an objective of this chapter.

To initially identify co-regulators which work with AR, GeneGo was employed. GeneGo is bioinformatics software which produces gene expression pathways. Kristine Belling a bio-informatics PhD student was very kind and carried out the studies using GeneGo to determine AR pathways. The co-regulators in these pathways were compared against a microarray study designed in our group and undertaken by a Finnish commercial enterprise using Affymetrix microarrays. The microarray was designed to identify potential androgen regulated genes, switched on in the GT during the MPW, by

comparing the genes expressed at e17.5 in rat control male GT (normal androgen action), control female GT (normal lack of androgen action in females), flutamide exposed male GT (blocked androgen action) and testosterone exposed female GT (excess androgen action that should mimic males). Genes which overlapped between GeneGo and the microarray outcome were investigated in the literature and those of interest were further investigated by Taqman-PCR. The genes identified by comparing the GeneGo pathways and the outcomes of the microarray study were *Brg1, Cbp, Fkbp5, Snurf, Transgelin* and *Vav3*. Of these, *Brg1* and *Cbp* had interesting patterns of expression during the MPW (see appendix 3 3).

Another co-regulator was studied in this chapter which was not found via the Genego pathway or the microarray. This was *Rwdd1*, a novel co-regulator which had been found to be androgen regulated in the adult thymus but again, for which there is currently no published data within the male reproductive tract (Kang et al 2008a, Kang et al 2008b).

Aside from androgen co-regulators, it was also important to consider other mechanisms of regulation and one key mechanism known to be vital in embryonic development is methylation (section 1.7.2). Methylation can cause silencing of normally active genes, or activate (demethylation) normally silent genes but ultimately leads to stable changes in gene expression (reviewed by Garcia-Carpizo et al, 2011). The AR gene encompasses an approximately 15-kb CpG island at the transcription start site and exon 1 (Jarrard et al, 1998) and most studies that have investigated AR methylation have focused on prostate cancer (Kinoshita et al, 2000; Sasaki et al, 2002; Takahashi et al, 2002). However, more recently, some studies have been published regarding the role of methylation in male fetal development. One study found that rat fetal exposure to vinclozin (anti-androgen) or to methyxychlor (an oestrogen) affected methylation patterns in the offspring resulting in decreased spermatogenic capacity and increased chances of infertility and the altered DNA methylation in the germ line was passed on for up to the four generations studied thereafter (Anway et al, 2005). However, these studies are somewhat

suspect as they have not so far proved to be repeatable (Inawaka et al, 2009). Furthermore, some studies have investigated DNMT3L, a regulator of methylation (section 1.7.2.1) and mouse knockout models have severe hypogonadism and azoospermia (Bourc'his et al, 2001). Another protein believed to be involved in methylation is BORIS (section 1.7.2.2) The expression of BORIS protein has only been found in the testis and it has been shown that disruption of DNA methylation can increase expression of BORIS and thus some scientists consider BORIS a marker for methylation in the testis. Taking into account that anti-androgens might affect methylation patterns in the fetus and that both DNMT3L, a methylation regulator and BORIS a methylation marker are important in the testis and also because another PhD project found both of these proteins to have changing expression in the male reproductive tract (M.S Jobbling submitted 2009), DNMT3L and BORIS were of key interest in the regulation of the MPW.

Table 4.1 shows the list of possible androgen biochemical associates which were targeted and investigated initially using Taqman-PCR. Of these, *Fkbp5*, *Snurf*, *transgelin* and *Vav3* did not show any obvious pattern of change in expression across the MPW, so were not selected for further study. (This is also summarised in appendix 3). In contrast, *Brg1*, *Cbp*, *Rwdd1*, *Dnmt3l* and *Boris* displayed interesting mRNA expression changes in relation to the MPW (at least in the GT) and were therefore investigated further. The aim of the studies in this chapter was to investigate genes which might be differentially expressed before, during, or after the MPW, and to at least provide preliminary evidence as to their possible implication in specifying the MPW or in modulating androgen action specifically within the MPW. The protein of the 3 co-regulators and 2 methylation regulators chosen for investigation, were also studied in testis. AR expression in the testis is well characterized at different time points, for example, in the SC AR is switched on between PND4-10 and more strongly thereafter. Consequently the testis provides a dynamic system in which to dissect the potential role and importance of the androgen biochemical associates.

4.2 Materials and Methods:

Two types of studies were done in this chapter, the first of these analyzed expression patterns across the MPW. The genes which had interesting expression patterns were then investigated in relation to androgen or oestrogen manipulation.

4.2.1 Animals and Treatments:

Briefly, pregnant rats were treated with flutamide, DBP or DES or appropriate vehicle during the MPW, (section 2.2). The genital tubercle, gonads and ducts were dissected from the resulting rat fetuses at e16.5, e17.5, e19.5 or e21.5, from both male and female pups. The penis and testis were dissected from PND25 and adult males. The tissue was fixed in Bouin's fixative and processed for immunohistochemical analysis or frozen at -80°C for RNA analysis

4.2.2 RNA analysis:

The mRNA level of co-regulators of interest was investigated using Taqman (section 2.6). Briefly, RNA was isolated from frozen tissue and converted into cDNA and quantitative RT-PCR performed. Time course experiments investigated the expression before, during, and after the MPW. The effect of treatments on co-regulator expression was investigated in the fetal life. The genes investigated and primers used are listed in Table 4.1.

Table 4.1 TaqMan primers and Universal probe library probes used in chapter 4 and why they were investigated

Gene	Forward Primer 5'→3'	Reverse Primer 3'→5'	UPL Probe Number:	Reasons for investigation	Interesting pattern during MPW found by Taqman-PCR
BORIS	GAA GAA AAA GAA AGA TGC GGT CT	GAG ATC CGG CTC AGC ATT T	82	Methylation enzyme of interest	Yes
BRG1	ACG GTG TCC CAG CTA GAT TC	GCA TCC GCA TGA ACA TAC TTC	20	Found in AR pathway GeneGo & Microarray	Yes
CBP/P300	GGC AAA GAG GAC CAG ACG	AAT CGG TGG GAA TTG ATG TC	22	Found in AR pathway GeneGo & Microarray	Yes
DNMT3L	GAG GGT GTG GAG CAA CAT TC	GCT CTT CCT TAG GGG TCA GG	41	Methylation enzyme of interest	Yes
FKBP5	CTC AAA CCC CAA TGA AGG AG	GCA GTC AAA CAC CCT TCC A	66	Found in AR pathway GeneGo & Microarray	No
RWDD1	ACA ACG CAA CGA GTT GGA G	GCT GGG TGG GTT TTC TGA TA	109	Novel & literature suggests AR regulation in thymus	Yes
SNURF	AAC AGC ACG TCC CAG AGA GT	AGA ATG CCG TGG GTA ACA GT	85	Found in AR pathway GeneGo & Microarray	No
Transgelin	AGT GTG GCC CTG ATG TGG	TCA CCA ACT TGC TCA GAA TCA	5	Found in AR pathway GeneGo & Microarray	No
VAV3	CCA TGG AGA AGT CGA ACC TG	TTC ACA TAT TGT GCC AAG TCC T	21	Found in AR pathway GeneGo & Microarray	No

4.2.3 Fluorescence immunohistochemical analysis

Fluorescence immunohistochemistry (section 2.5.2) was used to determine the co-localisation of AR with specific co-regulator proteins in the male reproductive tract, from control and treated animals. Briefly, staining was performed on sections of fetal and postnatal tissues. The various tissues examined in this chapter are summarized in Table 4.2. A minimum of 3 animals from 3 litters were investigated at each age and in each treatment group. The antibodies used are listed in Table 2.3.

Table 4.2 Details of tissues examined in chapter 4

Tissue	**Collection Ages**	**Treatments groups investigated (all treatments in MPW)**
Genital Tubercle	e16.5, e17.5, e19.5, e21.5	Control, Flutamide
Penis	PND25, PND90	Control, Flutamide
Testis	e21.5, PND4, PND10, PND25	Control, Flutamide, DES, DHT, Indomethacin, DBP
Wolffian Duct	e17.5, e19.5, e21.5	Control, Flutamide, DES, DHT, Indomethacin

4.3 Results

4.3.1 BRG1 expression in the male reproductive tract

BRG1 is a co-regulator involved in chromatin re-modelling and known to work with oestrogen receptor, glucocorticoid receptor and androgen receptor (reviewed in section 1.7.1.1.1). BRG1 expression has not been characterized in the male reproductive tract. To determine if *Brg1* was expressed in the developing genital tract at the mRNA level, Taqman-PCR was conducted.

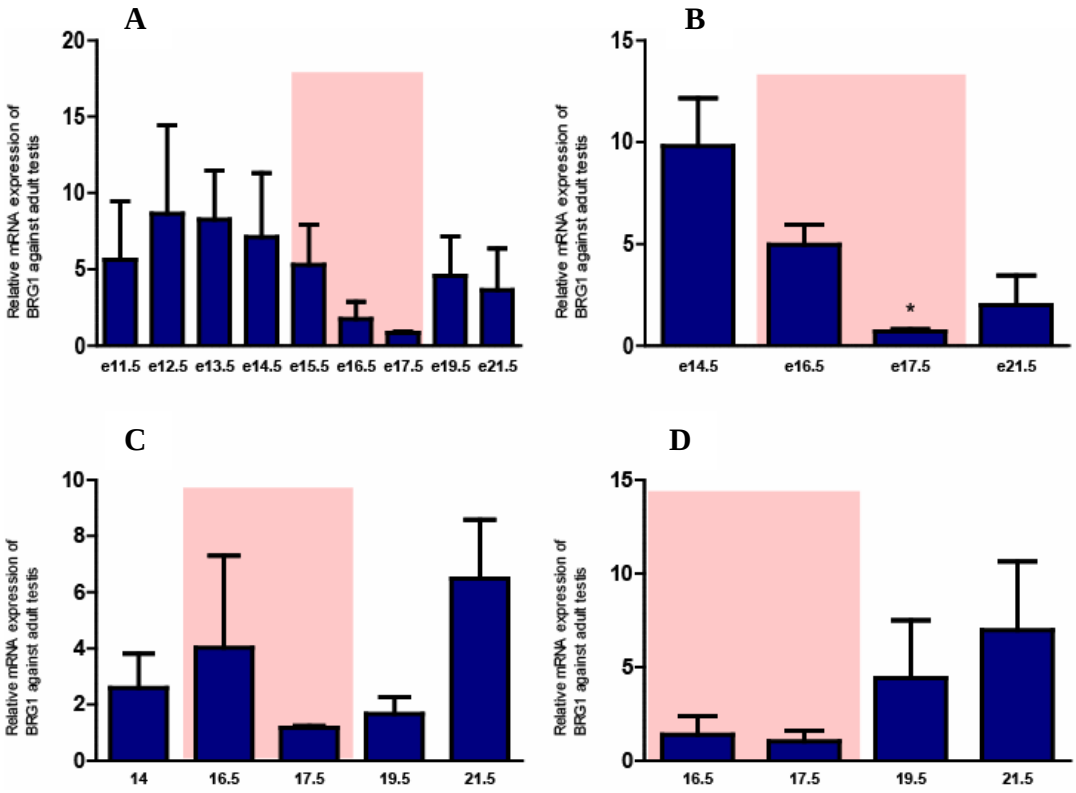


Figure 4.1 Quantitative analysis of *Brg1* mRNA levels in the lower abdomen/genital area (A), male GT (B), testis (C) and Wolffian duct (D) of control male rats. Using a one way ANOVA * P<0.01 in comparison with respective e14.5 value. Values are means ±SEM for 6 animals/group from a minimum of 3 litters. Pink shading depicts sampling ages within the MPW.

Brg1 mRNA expression decreased in the genital region and GT during the MPW, as highlighted in Fig. 4.1 (A, B). In the male genital tubercle, there was a significant decrease in expression of *Brg1* at e17.5 in comparison to e14.5, prior to the MPW. When

Brg1 mRNA was investigated in the testis and WD no significant differences in expression were found at any age, although, in both tissues expression appeared to increase after the MPW. BRG1 protein expression was investigated (using immunohistochemistry) in the genital tubercle (Fig.4.2) and testis (Fig. 4.3, 4.4 and 4.5).

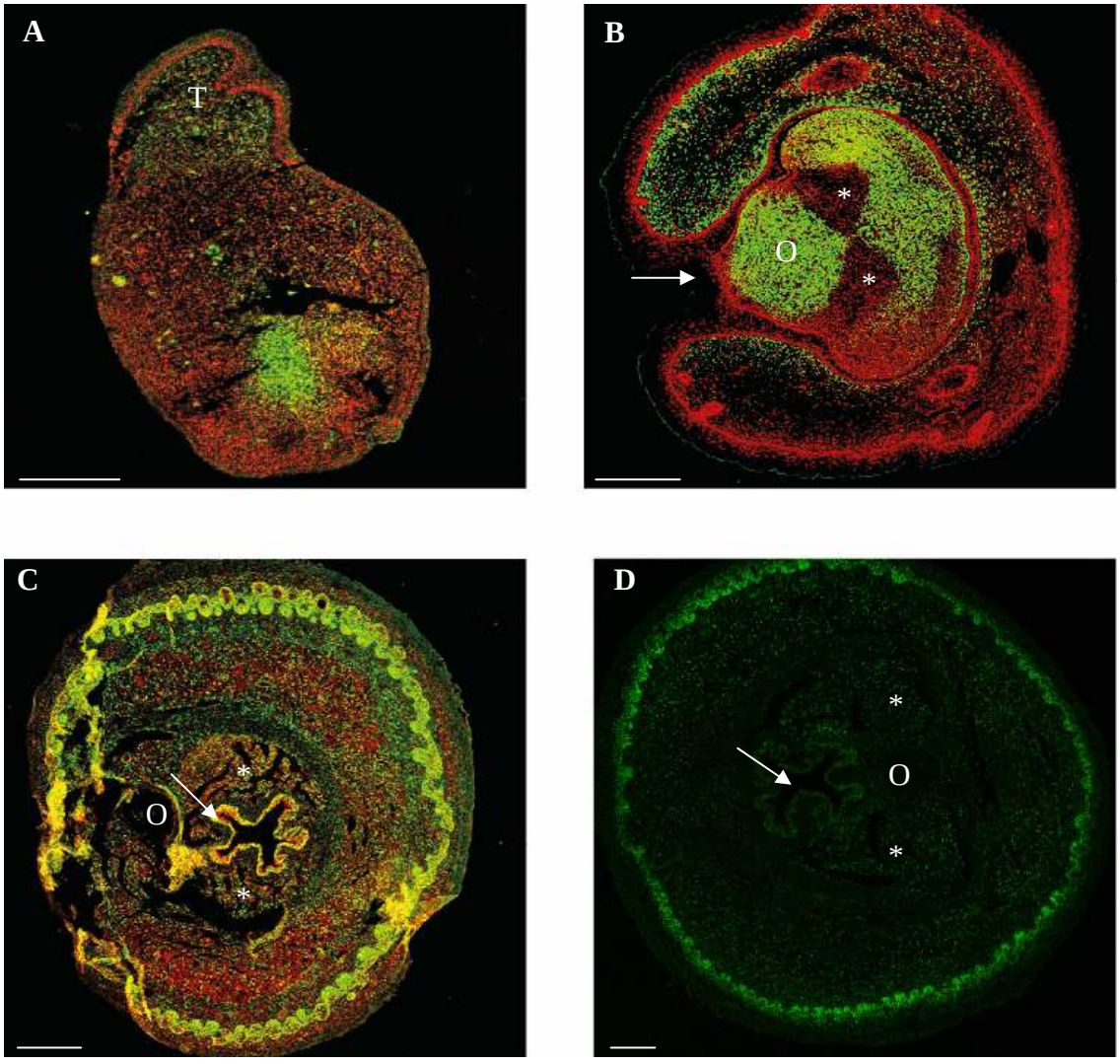


Figure 4.2 Representative photomicrographs of BRG1 expression (red) and AR expression (green) in control male GT at e17.5 (A), e21.5 (B) and PND25(C) T= the tip of the GT, O=Os penis; the uretheral area is indicated by an arrow and the corpus cavernosum by asterisks. Each scale bar (bottom left) represents 200µm. Panel D represents a negative control with no BRG1 antibody present. Note the more pronounced co-expression (yellow) of these two proteins at PND25 in comparison to earlier time points.

mRNA expression was lower in the GT during the MPW and increased thereafter. This did not appear to be reflected at the protein level as the e21.5 GT appeared to show the most intense BRG1 expression but this was not quantified. At PND25 in the penis, both AR and BRG1 mRNA expression had decreased, but in contrast to the fetal samples, strong co-localisation was seen, represented by the 'yellow' coloured cells (Fig. 4.2).

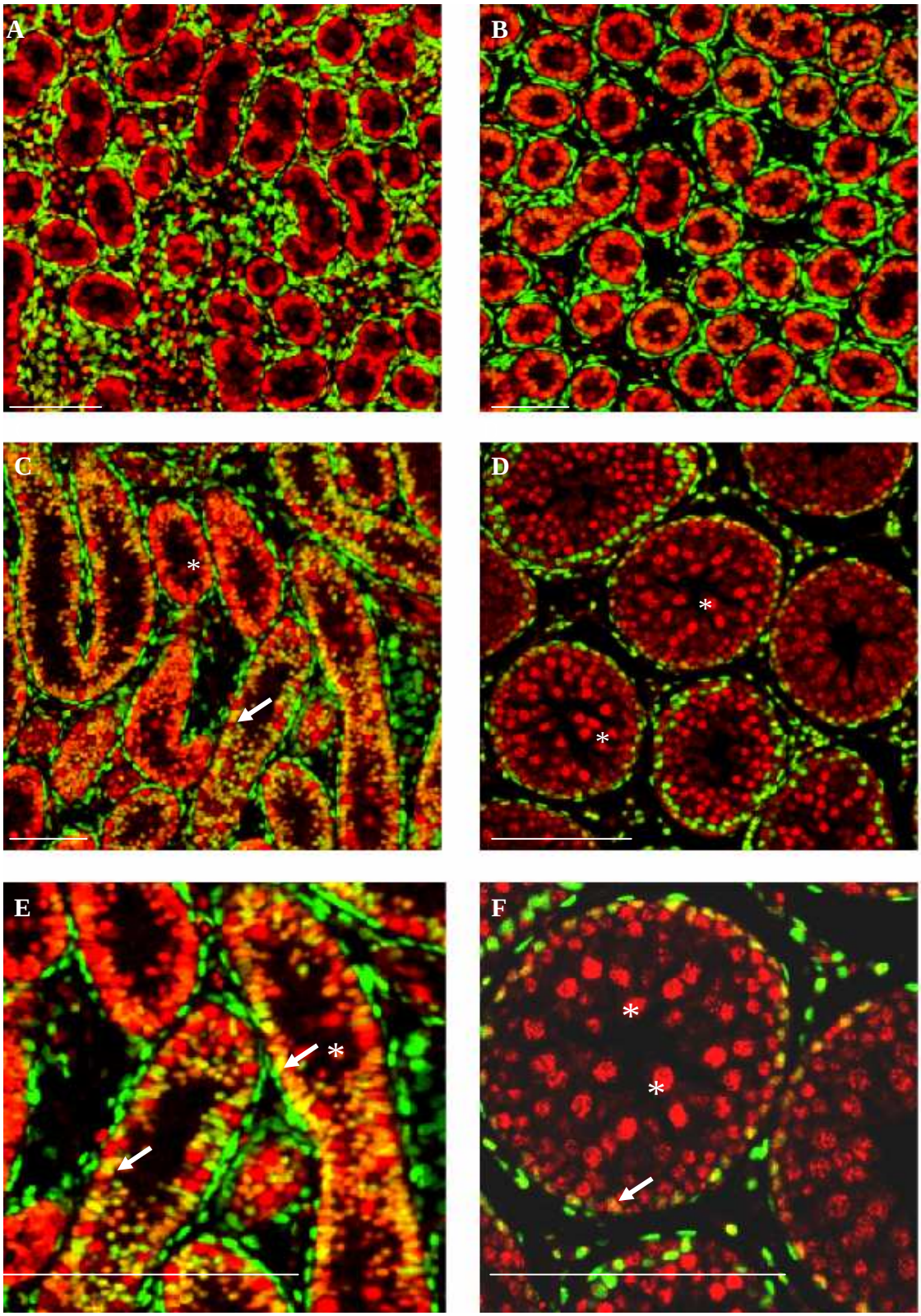


Figure 4.3 Representative photomicrographs of BRG1 expression (red) and AR expression (green) in testes from control rats at e21.5(A), PND4 (B), PND10 (C) and PND25(D). Co-localisation (yellow) is mainly apparent in Sertoli cells (indicated by the arrows), beginning at day 4 in some Sertoli cells and becoming 'stage-dependent' by PND10-25. Asterisks show BRG1 immunopositive germ cells. Scale bars represent 200µm.E and F depict increased magnification of C and D respectively.

The expression of BRG1 in the testis has not been reported previously. Using immunohistochemistry, nuclear BRG1 was found to be present in the testis at e21.5, PND4, PND10 and PND25 (Fig 4.3). At e21.5, BRG1 was expressed in the SC within the seminiferous tubules, perhaps weakly in some germ cells and also in some, but not all, cells within the interstitium. At PND4, BRG1 positive cells in the interstitium were infrequent but SC continued to express BRG1, as did some germ cells. Interestingly, as AR expression switched on at PND4 within some SC, co-localisation with BRG1 first became evident. This co-localization became far more pronounced at PND10, but also became highly variable between seminiferous tubules and this trend became even more marked at PND25. It is possible that this variation reflects 'stage-dependency', but this remains to be investigated. At PND10 and PND25 expression of BRG1 in GCs, especially in spermatocytes, also became much more obvious, although as GCs are AR negative, no co-localisation was seen.

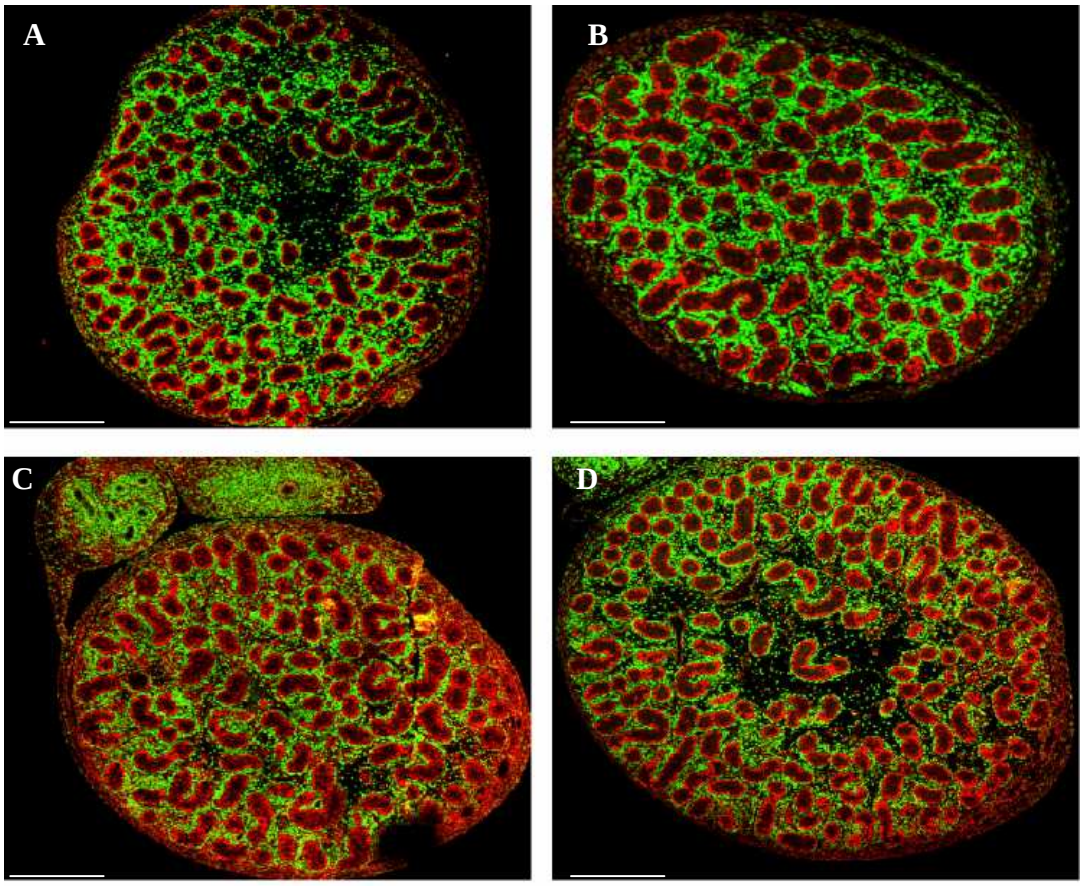


Figure 4.4 Representative photomicrographs of BRG1 expression (red) and AR expression (green) in e21.5 testes from animals exposed to DBP (A), DES (B), flutamide (C) and vehicle (D) during the MPW. Note the virtual absence of co-localisation (yellow) and lack of any obvious treatment effects (A, B and C) on patterns of expression in comparison to control (D). Scale bar represents 200µm.

4.3.1.1 Effects of fetal exposures during the MPW on BRG1 expression

Immunohistochemistry was used to determine if altered androgen/oestrogen action in the testis by exposure to DBP, DES, or flutamide during the MPW, would affect BRG1 expression. In the e21.5 testis, where nuclear BRG1 expression was found mainly in the SC as well as in occasional interstitial cells in controls, a similar pattern of staining was found in testes from fetuses which had been exposed to DBP, DES, or flutamide, with no obvious treatment effects (Fig 4.4).

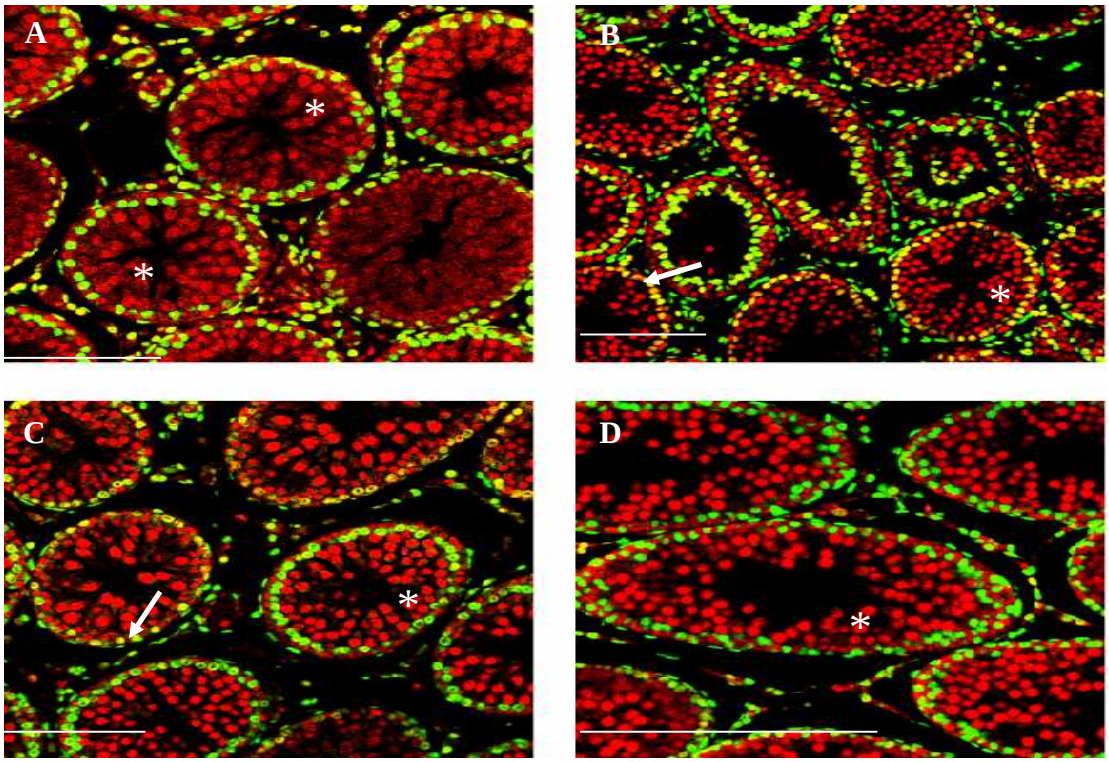


Figure 4.5 Representative photomicrographs of BRG1 expression (red) and AR expression (green) in PND25 testes from animals exposed during the MPW to DBP (A), DES (B), flutamide (C) or vehicle(D). Note the prominent expression of BRG1 in germ cells (asterisks) and the co-localisation of AR and BRG1 (yellow) in some but not all Sertoli cells (arrows). The scale bar represent 200µm.

In the PND25 testis, there was prominent nuclear BRG1 expression in SC and some interstitial cells and a similar pattern of staining was found in testes from animals which had been exposed in the MPW to DBP, DES, flutamide or vehicle (Fig.4.5). As expected, AR was prominently expressed in SC, peritubular myoid cells and some interstitial cells. Co-localisation of AR and BRG1 was evident in some, but not all, SC and in occasional interstitial cells. There was no obvious treatment-related change in the pattern or intensity of expression of either AR or BRG1, alone or together.

4.3.2 CBP expression in the male reproductive tract

CBP is a co-regulator involved in histone modification and is a well established co-regulator that modulates transcription in many different pathways (reviewed in section 1.7.1.2.1). CBP is well known to work in collaboration with BRG1 but the expression of CBP in the developing male reproductive tract has not been reported in the literature. To determine if *Cbp* was expressed in the developing genital tract at the mRNA level and whether this varied before, during and after the MPW, Taqman-PCR was conducted.

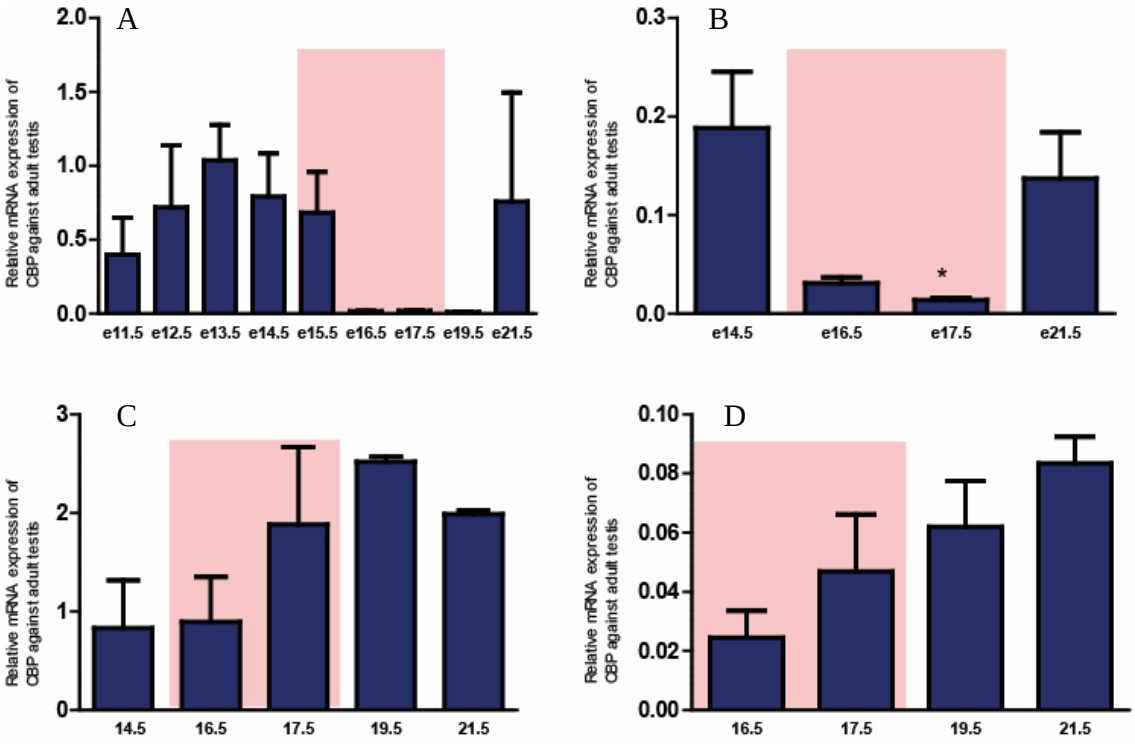


Figure 4.6 Quantitative analysis of *Cbp* mRNA level in the lower abdominal/genital region (A), GT (B), testis (C) and Wolffian duct (D) of control male rats. Using a one way ANOVA * P<0.01 in comparison to e14.5. Values are means ±SEM for 6 animals/group from a minimum of 3 litters. Pink shading depicts sampling ages within the MPW.

Cbp mRNA decreased in expression during the MPW in the lower abdomen of rats when compared with ages before and after the MPW, as highlighted in Fig.4.6 (A). In the male genital tubercle, there was also a significant decrease in expression of *Cbp* at e17.5 in comparison to e14.5 GT, prior to the MPW, with higher expression again after the MPW at e21.5, although the latter increase was not statistically significant (Fig. 4.6B). In contrast, *Cbp* mRNA expression in the testis and WD did not exhibit any significant differences in expression in or outside of the MPW. CBP protein expression was investigated using immunohistochemistry in the genital tubercle (Fig. 4.7) and testis (Fig.4.8, 4.9 and 4.10) to see if these mRNA findings could be confirmed at the protein level and also to investigate the relationship of CBP expression to that of AR.

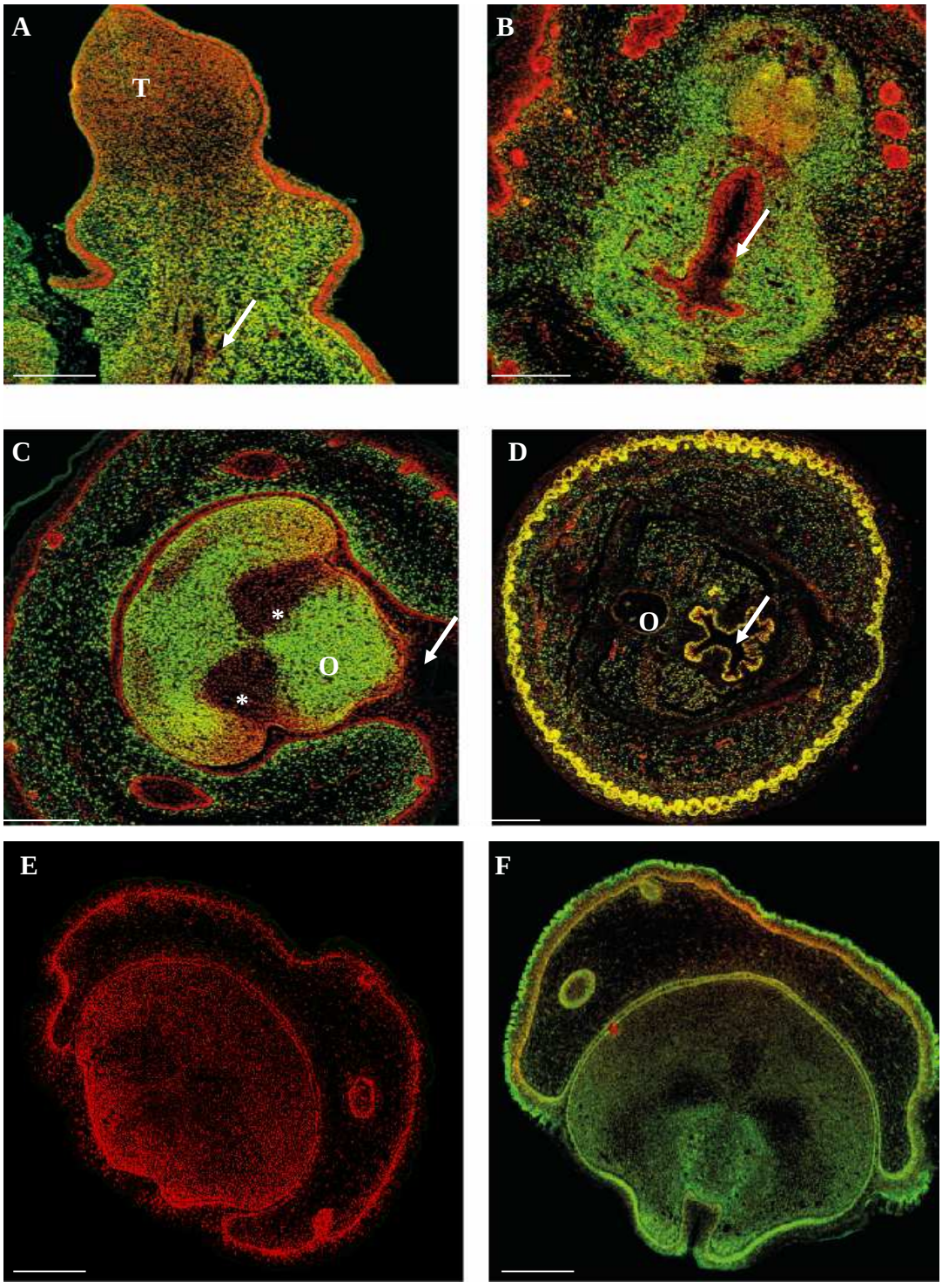


Figure 4.7 Representative photomicrographs of CBP expression (red) and AR expression (green) in control male GT at e17.5 (A), female GT at e19.5 (B), male GT at e21.5 (C) and PND25 penis (D). T= the tip of the GT, O=OS penis, the urethral area is indicated with an arrow and corpus cavernosum with asterisks. Co-localisation of CBP and AR shows as yellow. Negative control for AR (E) and CBP (F) in e21.5 male GT. Scale bar represents 200µm.

Similar to the results found for BRG1, nuclear CBP expression was found in the GT. Although at e17.5 it was not possible to obtain a transverses section, only a vertical one and therefore it was unclear exactly which cells CBP was expressed in at this age. However, it can be seen that at e17.5, CBP expression was near the tip of the GT and much of this was not co-localised with AR expression. In the female GT at e19.5, CBP was found only in the urothelial cells and blood vessels and staining was specific to these areas; it seems likely that a similar expression pattern would be found in the e17.5 male GT if a transverse section had been obtained. In the male GT at e21.5, CBP was not expressed in the urothelial cells and at PND25, in the penis, AR was still widely expressed whereas CBP was much more restircted in expression, but wherever it was expressed, it mainly co-localised strongly with AR in the urethral epithelium and in the outer epidermis and prepuce (Fig.4.7).

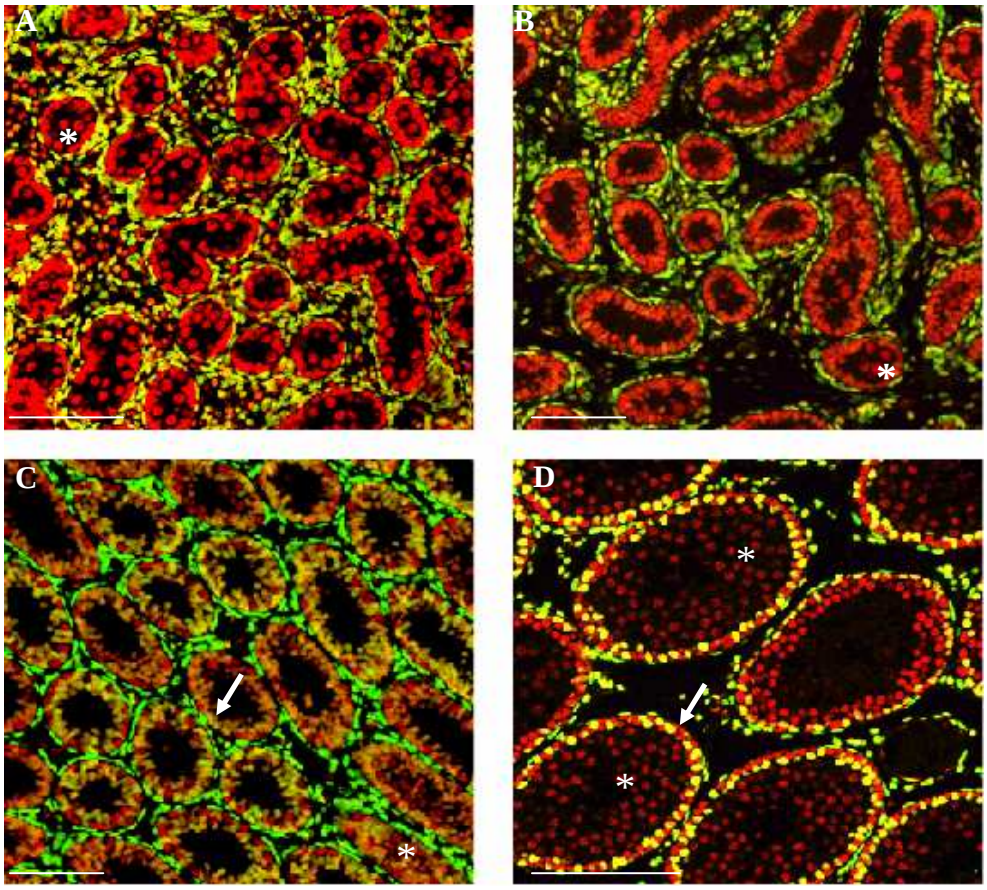


Figure 4.8 Representative photomicrographs of CBP expression (red) and AR expression (green) in control rat testes at e21.5 (A), PND4 (B), PND10 (C) and PND25 (D).Note that CBP is only co-expressed with AR in SC (arrows) from around day 10, and that this showed some evidence for 'stage-dependence'. Note, in contrast that CBP co-localized with AR in the interstitial cells extensively at e21.5 with a progressive reduction thereafter up to PND10. Note also that CBP was expressed in some, but not all, germ cells (*) at all ages. Scale bar represents 200µm

The expression of CBP in the testis has never been reported. Using immunohistochemistry, nuclear CBP was found to be present in the testis at e21.5, PND4, PND10 and PND25. At e21.5, CBP was expressed in the SC and some germ cells within the seminiferous tubules and also strongly within cells of the interstitum. At PND4, CBP expression in interstitial cells was much less pronounced than at e21.5, but SCs continued to express CBP strongly. CBP only co-expressed with AR in SC from around day 10, and this co-expression demonstrated some 'stage-dependence'. However, in contrast CBP co-localized with AR in the interstitial cells extensively at e21.5 with a progressive reduction thereafter up to PND10. CBP was also found to be expressed in some germ cells at each age investigated (Fig.4.8).

4.3.2.1 Effects of fetal exposures during the MPW on CBP expression

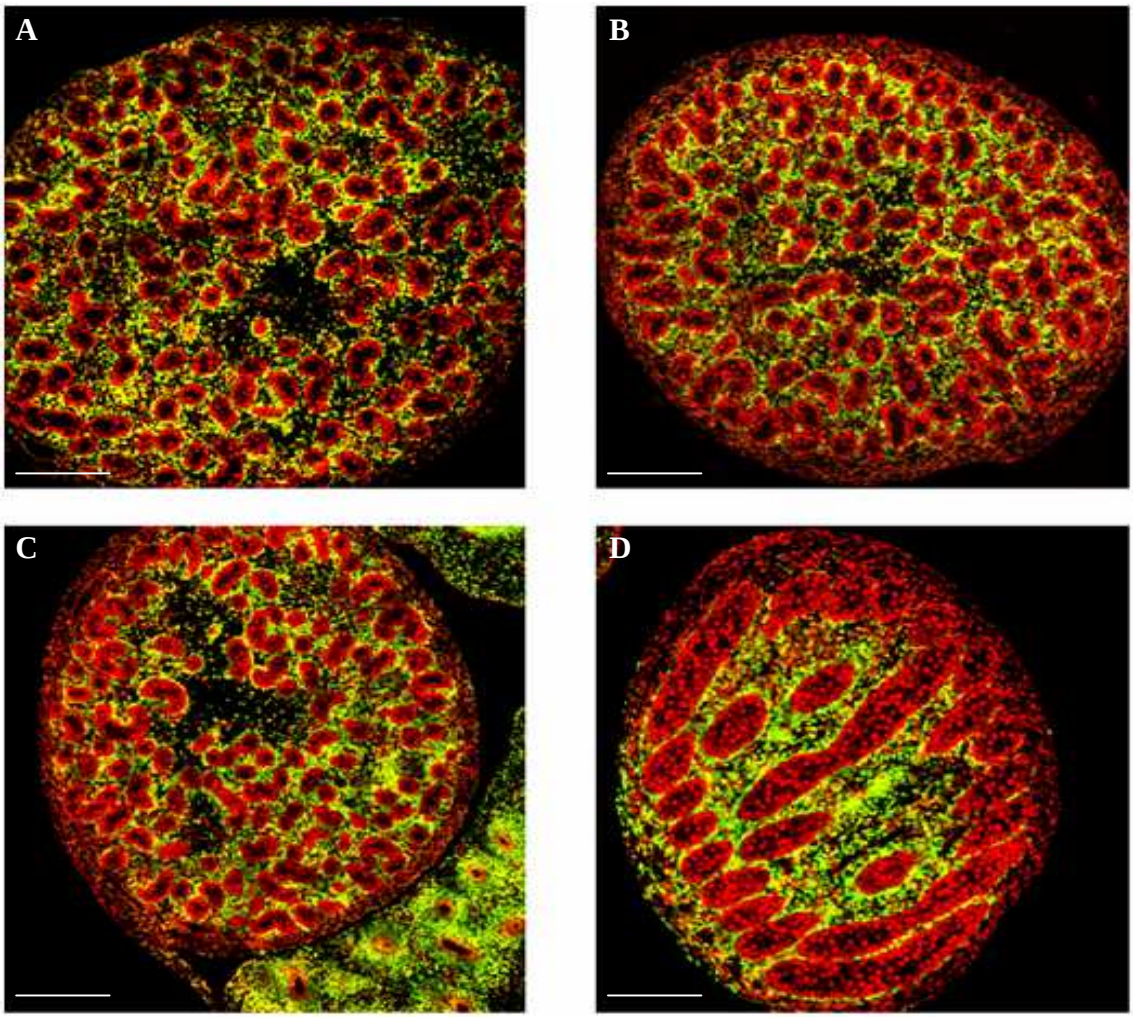


Figure 4.9 Representative photomicrographs of CBP expression (red) and AR expression (green) in e21.5 testes from fetuses exposed to DBP (A), DES (B), flutamide (C) or vehicle (D) during the MPW. Note the similar pattern of expression in the different treatment groups and that co-localisation of CBP and AR (yellow) is restricted to some interstitial cells. Scale bar represents 200µm.

Immunohistochemistry was used to determine if possible disrupted androgen action in the testis by exposure to either DBP, DES, flutamide or vehicle, during the MPW affected subsequent CBP expression. In the e21.5 testis, nuclear CBP was found in SC and some germ cells as well as in the interstitium in control tissue and co-localisation of CBP with AR was only evident in interstitial cells. A similar pattern of staining was

found in testes from animals which had been exposed to DBP, DES, flutamide or vehicle (Fig.4.9).

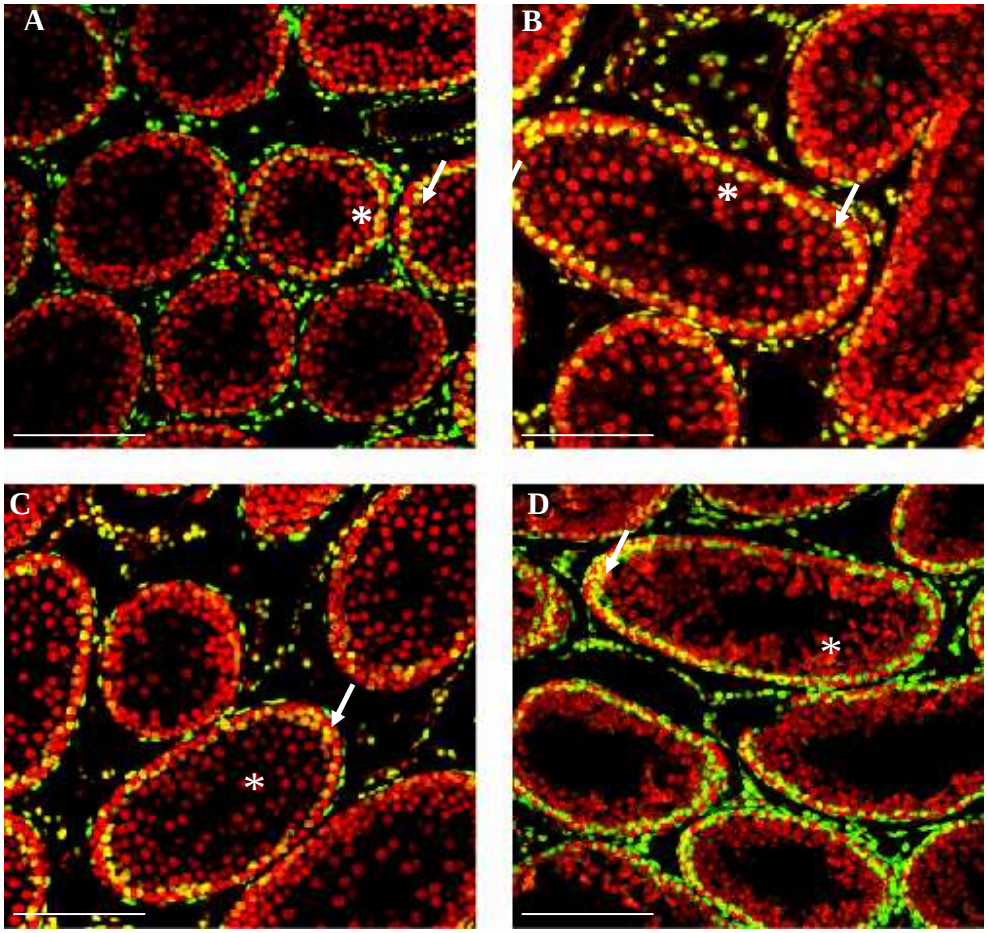
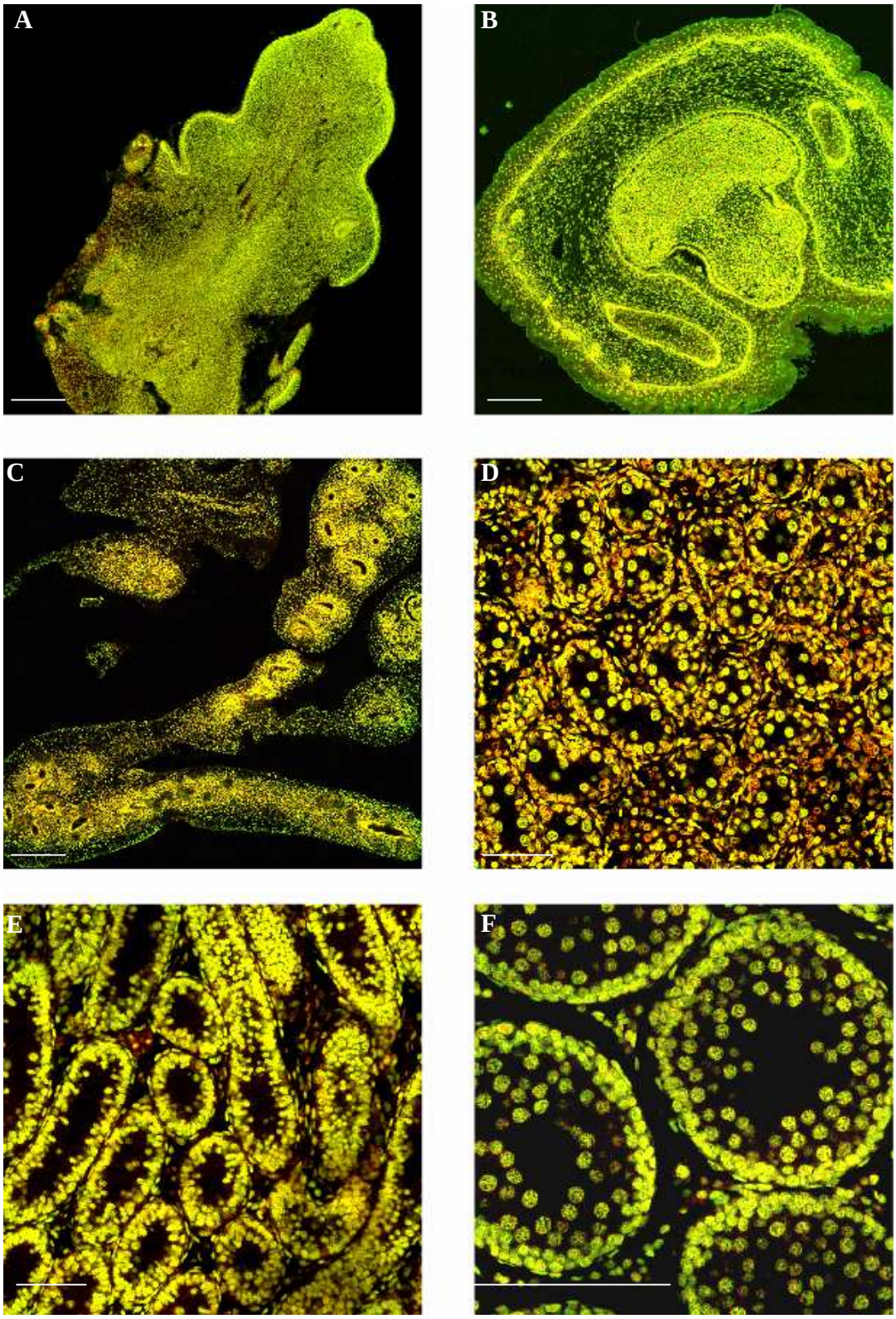


Figure 4.10 Representative photomicrographs of CBP expression (red) and AR expression (green) in PND25 testes from animals exposed to DBP (A), DES (B), flutamide (C) or vehicle (D) during the MPW. Note that AR and CBP co-localise in SC (yellow) but this varies between seminiferous tubules ('stage dependent') and does not appear to be treatment modulated. Scale bars represent 200µm.

At PND25 in control testes, nuclear CBP expression was found in SC, most GCs and in occasional interstitial cells. Clear co-localisation could be seen with AR and CBP in the SC but this varied between seminiferous tubules which suggested this co-localisation was stage dependent. A similar pattern of co-expression was found in animals exposed to DBP, DES, or flutamide, and thus these did not appear to affect AR or CBP expression or co-localisation. (Fig.4.10).

4.3.2.2 Co-expression of BRG1 and CBP

It has been reported that BRG1 and CBP work in collaboration in many cell types in their roles to co-regulate transcription. In order to determine if both co-regulators were expressed in the same cell types in the male reproductive tract, double immunohistochemistry was used. The genital tubercle, WD and testis were examined and it was found that BRG1 and CBP were expressed together in every cell type in each of these tissues irrespective of age. One was never obviously expressed without the other (Fig 4.11).



Fiure 4.11 Representative photomicrographs of BRG1 expression (red) and CBP expression (green) in male control e17.5 GT (A), e21.5 GT (B), e21.5 WD (C) e21.5 testis (D) PND 10 testis (E) and PND25 testis (F). Note that for the most part, BRG1 and CBP are co-expressed (yellow), irrespective of age or tissue investigated. Scale bar represents 200µm.

4.3.3 RWDD1 expression in the male reproductive tract

There is some evidence which suggests that RWDD11 can co-regulate the AR and this may be important in the thymus. RWDD1 is a very newly discovered co-regulator and its expression patterns in the developing reproductive tract are completely unknown. *Rwdd1* expression was first investigated in the GT using Taqman RT-PCR and then using immunohistochemistry, to determine i) if its expression changed in relation to the MPW, ii) if its expression was altered by flutamide and iii) if this co-regulator co-localised with AR in the cells of the testis or GT.

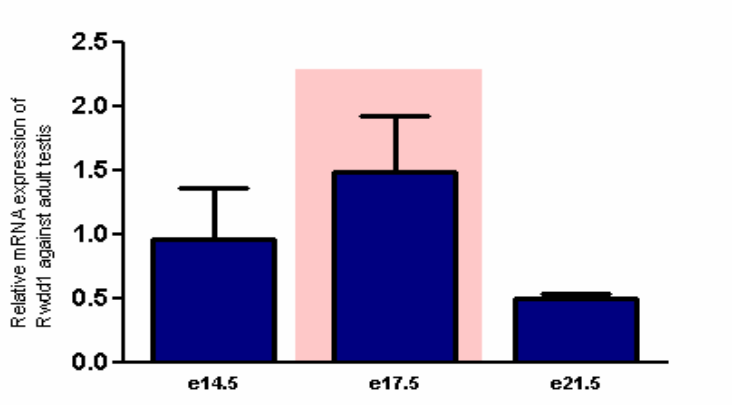


Figure 4.12 Quantitative analysis of *Rwdd1* mRNA level in control male rat GT at e14.5, e17.5 and e21.5. No significance was found using a one way ANOVA. Values are means ±SEM for 6 animals/group from a minimum of 3 litters. Pink shading depicts sampling age that lies within the MPW.

In the male control GT, expression of *Rwdd1* was non-significantly higher at an age during the MPW in comparison to ages before or after this time point (Fig 4.12).

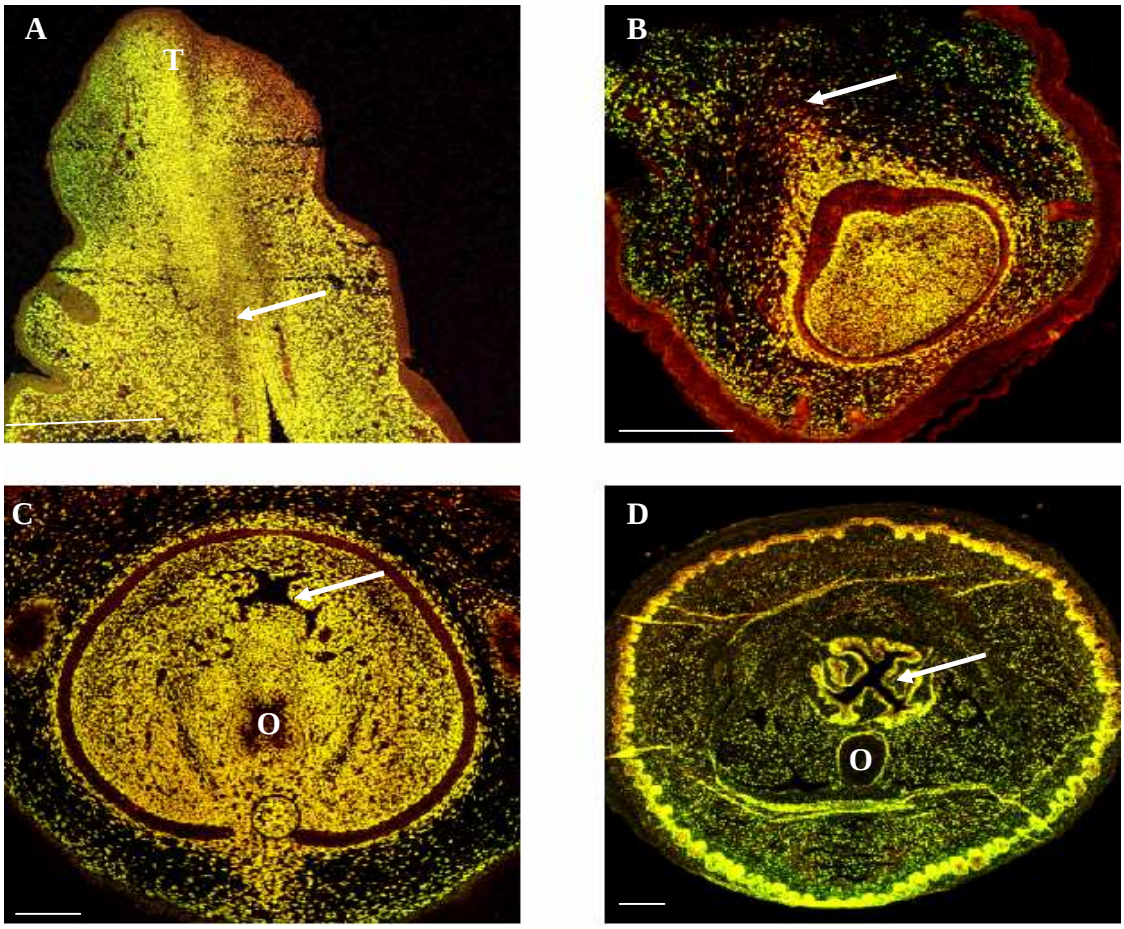


Figure 4.13 Representative photomicrographs of RWDD1 expression (red) and AR expression (green) in control male GT at e17.5 (A) female GT at e19.5 (B) male GT at e21.5 (C) and PND25 penis (D). Note the widespread co-localization of AR and Rwdd1 (yellow) in the developing GT and the more restricted co-localization at PND25. Arrows indicate the urethral area and o=os penis. Scale bar shows 200µm.

Immunohistochemistry was used to investigate if RWDD1 was present at the protein level in the GT and if it might be expressed in the same cells as AR. RWDD1 was found to co-localise with AR in most cell types that expressed AR in the developing GT whereas co-expression was more restricted in the differentiated PND25 penis. In the female GT at e19.5 there was still substantial co-expression of RWDD1 and AR, but this was more restricted than in the male GT at 17.5 and e21.5 (Fig 4.13). As the co-localisation of RWDD1 and AR in the male GT and penis was rather striking, it was of

interest to determine the effect of blocking androgen action using flutamide on *Rwdd1* expression.

4.3.3.1 Effect of flutamide exposure in the MPW on *Rwdd1* expression

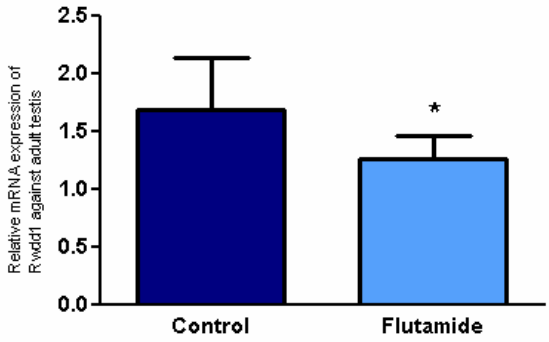


Figure 4.14 Effect of flutamide exposure in the MPW on *Rwdd1* mRNA level in e17.5 male GT. Using a unpaired, two tailed t-test *P<0.05 in comparison with respective control. Values are means ±SEM for 9 animals/group from a minimum of 2 litters.

To determine if perhaps *Rwdd1* might be androgen regulated in the GT as it is suggested to be in the thymus, *Rwdd1* mRNA expression was determined in GT from vehicle and flutamide exposed fetuses during the MPW. A significant decrease in *Rwdd1* expression was found in e17.5 GT from flutamide exposed males compared with controls (Fig 4.14).

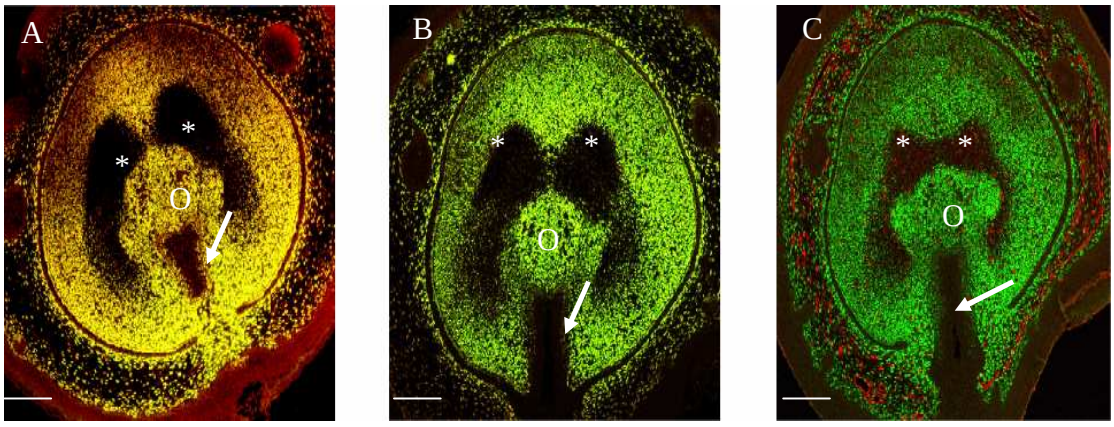


Figure 4.15 Representative photomicrographs of RWDD1 expression (red) and AR expression (green) in the male GT at e21.5 after exposure in the MPW to vehicle (A) or flutamide (B and C). Note that whilst flutamide exposure does not affect AR expression, it profoundly inhibits RWDD1 expression so that there is minimal co-localisation (yellow). Arrows show the urethral area whilst asterisks indicate the corpora cavernosa and o=os penis. Scale bar represent 200µm.

Immunohistochemistry was used to determine if the significant difference seen in *Rwdd1* mRNA expression in GT from control and flutamide exposed fetuses (Fig 4.14) was reflected at the protein level. As it was not possible to obtain representative and comparable e17.5 GT sections to do this, e21.5 GT sections were used. RWDD1 protein expression was profoundly reduced in e21.5 GT obtained from fetuses that had been exposed to flutamide during the MPW. This was found for four different fetuses from 2 different litters, although the degree of protein suppression was somewhat variably affected (Fig 4.15).

RWDD1 expression in the testis

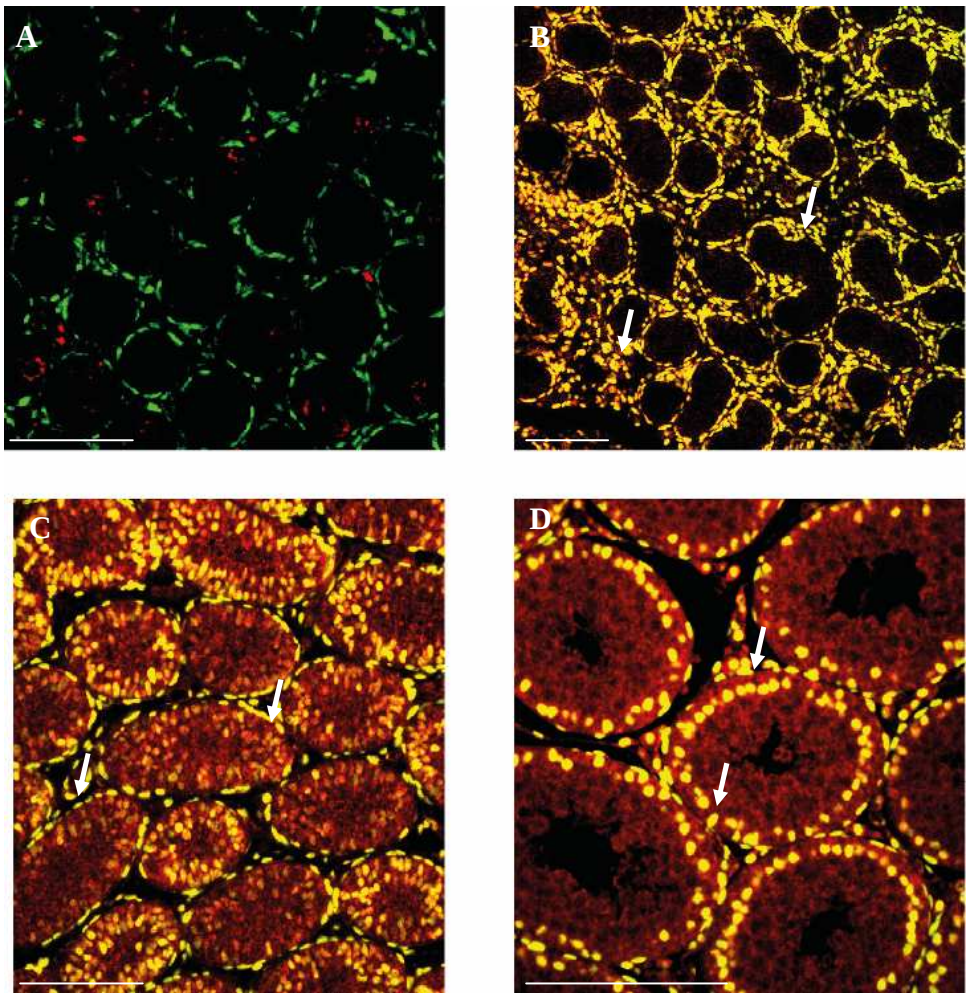


Figure 4.16 Representative photomicrographs of RWDD1 expression (red) and AR expression (green) in control rat testes at e21.5 (B) PND10 (C) and PND25 (D). Negative control can be seen in panel A with no Rwdd1 antibody. Co-localization is indicated by arrows and was pronounced in Sertoli cells at Pnd10-25 (absent at e21.5) and in some interstitial cells at all ages. Scale bar represents 200µm.

The expression of RWDD1 in the testis has never been reported and given the interesting results reported in Fig 4.15, RWDD1 expression was investigated in the testis. Nuclear RWDD1 was found to be present in the testis at e21.5, PND10 and PND25 (Fig. 4.16). At e21.5, RWDD1 was expressed in peritubular myoid cells and cells within the interstitium and in all of these cells it co-localised with AR. At PND10, co-localization of RWDD1 with AR was found in SC and co-localisation was also still evident in peritubular myoid and some interstitial cells, and this pattern was maintained and became even clearer by PND25 (Fig. 4.16). At PND10 and PND25, Rwdd1 expression was also apparent in most germ cells, although it is unclear whether or not this is specific. Unfortunately further studies of RWDD1 protein expression were not possible because subsequent batches of the antibody obtained from the supplier no longer worked. This also meant that more definitive studies on the GT, and of flutamide effects, were not feasible at the protein level.

4.3.5 BORIS expression in the male reproductive tract

In the context of investigating regulation of the MPW, one possibility which merited investigation was methylation enzymes. Preliminary studies were carried out on a methylation regulator, DNMT3L and a methylation marker, BORIS. Both methylation associates were investigated initially using Taqman-PCR followed by immunohistochemistry.

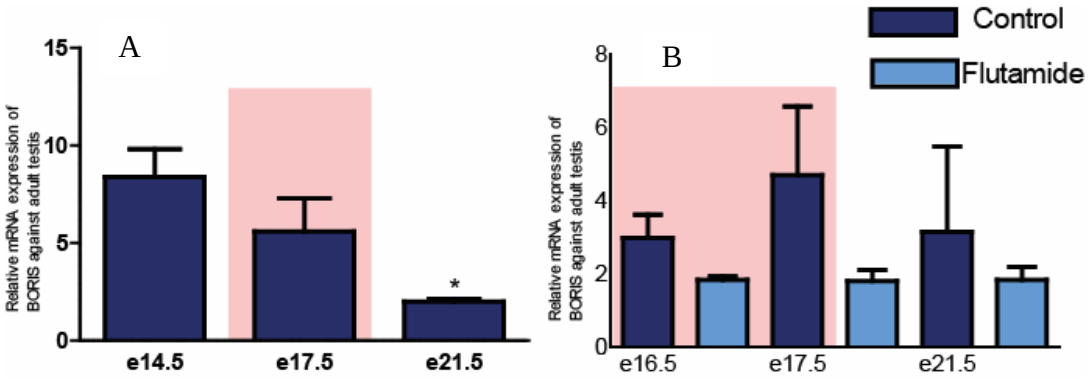


Figure 4.17 Quantitative analysis of *Boris* mRNA level in the male GT at different fetal ages (A) and after vehicle or flutamide exposure in the MPW (B).Using a one way ANOVA * P<0.05 in comparison with e14.5 GT. Values are means ±SEM for 3-6 animals/group from a minimum of 2 litters. Pink shading indicates sampling ages that lie within the MPW.

Boris mRNA expression in the male control GT was found to be highest prior to the MPW at e14.5, was marginally lower during the MPW at e17.5 and then decreased significantly by e21.5 (Fig. 4.17 A). Taqman-PCR was used to determine if blocking androgen action in fetuses by exposure to flutamide would affect *Boris* expression. The GT of these fetuses had a lower expression of *Boris* in comparison to control male GT at each age studied but the differences were not significant (Fig 4.17 B)

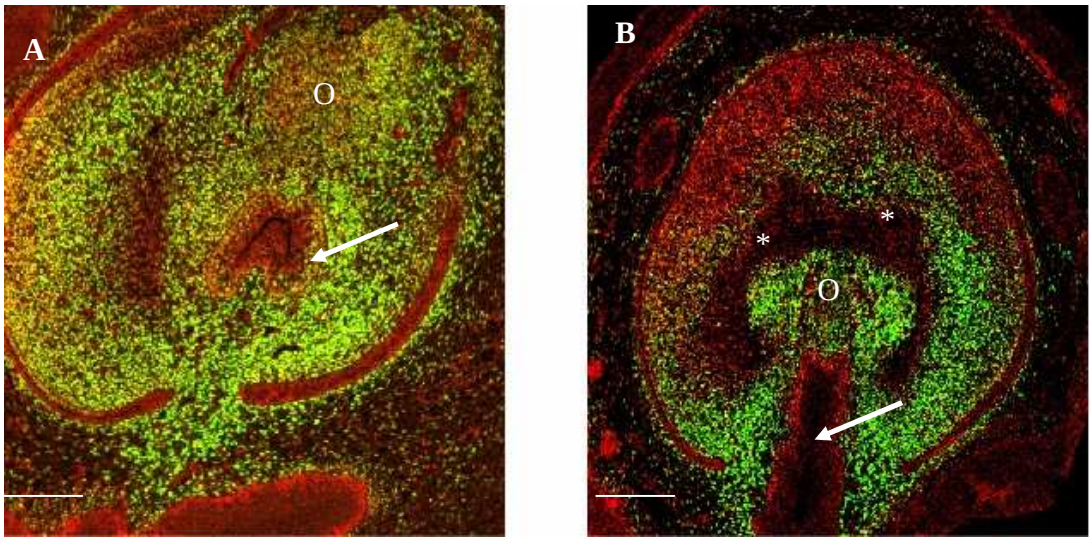


Figure 4.18 Representative photomicrographs of BORIS expression (red) and AR expression (green) in control male GT at e21.5 (A) and in a GT from a flutamide exposed fetus (B). Note that although considerable co-localization occurs (yellow), parting some areas AR and BORIS expression appear not to coincide. Scale bar represent 200µm. O=Os penis; the uretheral area is indicated by an arrow and the corpus cavernosum by an asterisks.

Immunohistochemistry was used to determine if the (non-significant) decrease in *BORIS* mRNA expression in GT from flutamide-exposed fetuses was incidental or whether it was also seen at the protein level. At e21.5, in the male GT, BORIS protein expression did appear to be less extensively expressed and there appeared to be larger areas in which AR was expressed without coincident BORIS expression, as found in the GT control. However, this is at best a tentative conclusion.

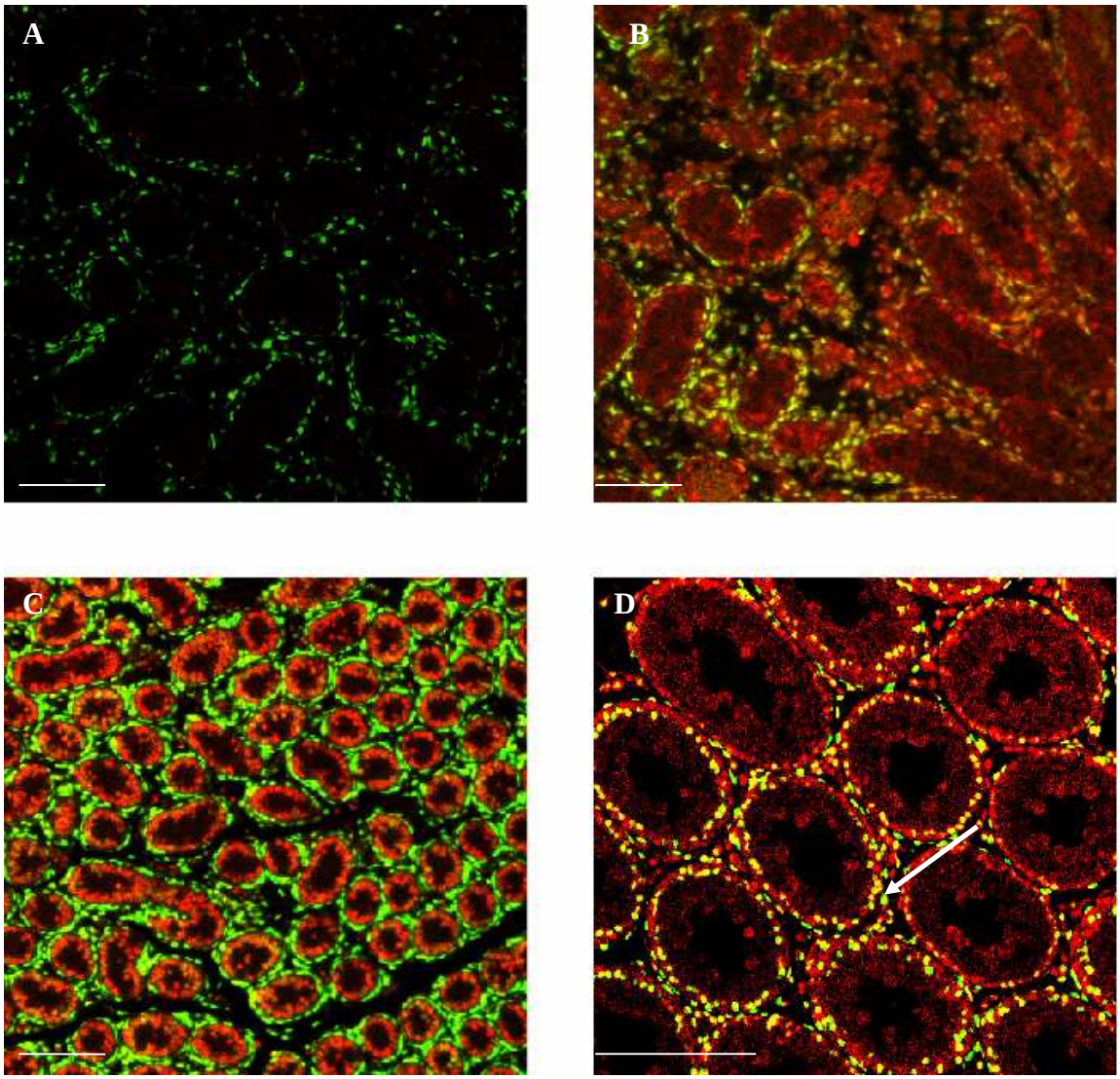


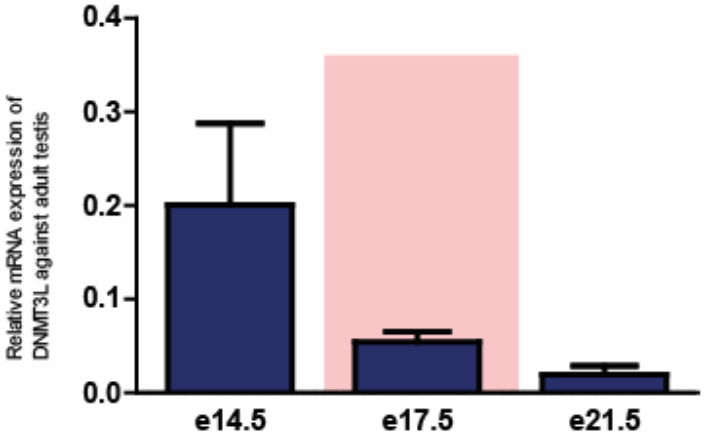
Figure 4.19 Representative photomicrographs of BORIS expression (red) and AR expression (green) in control male testis at e21.5 (B) PND4 (C) and PND25(D). BORIS expression is absent or minimal at e21.5 but is expressed in the SC at PND4 but not in the interstitial cells. At PND25, expression of BORIS can be seen in SC, some germ cells, PTM cells and cells of the interstitium. Co-localisation of AR and BORIS (yellow; arrows) was evident in peritubular cells, some Sertoli cells and some interstitial cells. Scale bars represent 200µm. The negative control for BORIS antibody is shown in panel A

Using immunohistochemistry, BORIS was detected at e21.5 in control male testis, in the SC, GC, interstitial cells and PTM cells and some co-localisation with AR could be seen in he PTM and interstitial cells (Fig. 4.19). Nuclear BORIS expression was detected in

PND4 testis in the SC and in some GC and some cells of the interstitium. By PND25, BORIS was clearly expressed in the interstitium, also co-localised with AR in the SCs as well as in the PTM cells.

4.3.6 DNMT3L expression in the male reproductive tract

DNMT3L is a more recently discovered methylation enzyme and some preliminary studies have reported it may be involved in AR methylation and was therefore a strong candidate for present investigation. *Dnmt3l* message expression was investigated using Taqman-RT-PCR and the preliminary protein investigations done using immunohistochemistry.



Fiure 4.20 Quantitative analysis of *Dnmt3l* mRNA levels in male GT. No significant differences were found using a one way ANOVA. Values are means ±SEM for 4-6 animals/group from a minimum of 2 litters. Pink shading shows sampling age within the MPW.

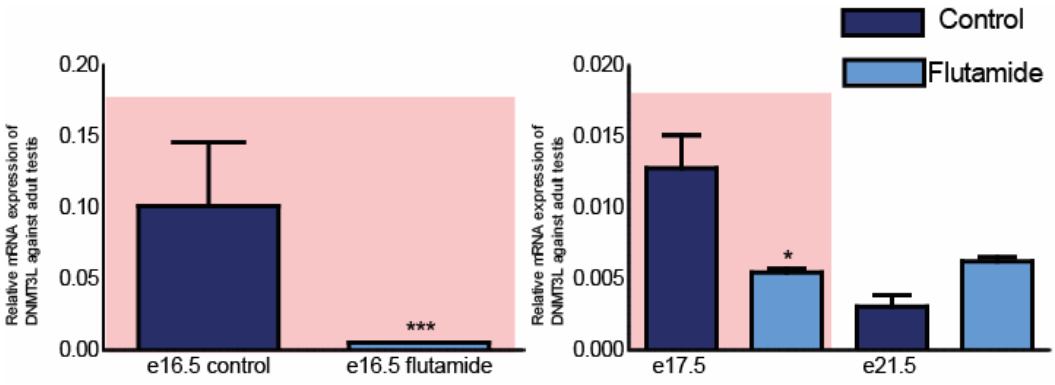


Figure 4.21 Quantitative analysis of *Dnmt3l* mRNA levels in GT from vehicle (control) and flutamide exposed fetuses. Using a one way ANOVA *P<0.005, ***P<0.0001 in comparison with respective control. GT. Values are means ±SEM for 5 animals/group from a minimum of 2 litters. Pink shading shows sampling age within the MPW.

Similar to *Boris* expression (Fig 4.17), *Dnmt3l* expression in the normal male GT decreased through fetal development with expression being highest at e14.5 and lower at e17.5 and e21.5, although these differences were not significant, probably because of the low N number. To determine if blocking androgen action in the MPW affected *Dnmt3l* expression, GT from animals exposed to flutamide during the MPW were compared with age matched vehicle-exposed animals. During the MPW, at e16.5 and e17.5, there was a statistically significant decrease in *Dnmt3l* mRNA expression in GT from flutamide exposed males in comparison to respective control GT. This decrease was not evident after the MPW at e21.5 (Fig. 4.21). Interpretation of this flutamide effect is difficult, not least because the direction of effect is opposite from what might be deduced from the age related change in *Dnmt3l* expression (Fig. 4.20). In the latter situation, *Dnmt3l* expression is lower in the MPW than beforehand, so if this lowering is androgen-regulated, then blockade of this by flutamide should increase DNMT3L expression in the GT during the MPW, but the opposite was found. On balance, this perhaps makes it more likely that the non-significant but higher expression of *DNMT3L* at e14.5 is not accurate, and in this regard it is noteworthy that values for *DNMT3L* mRNA expression at this age were extremely variable.

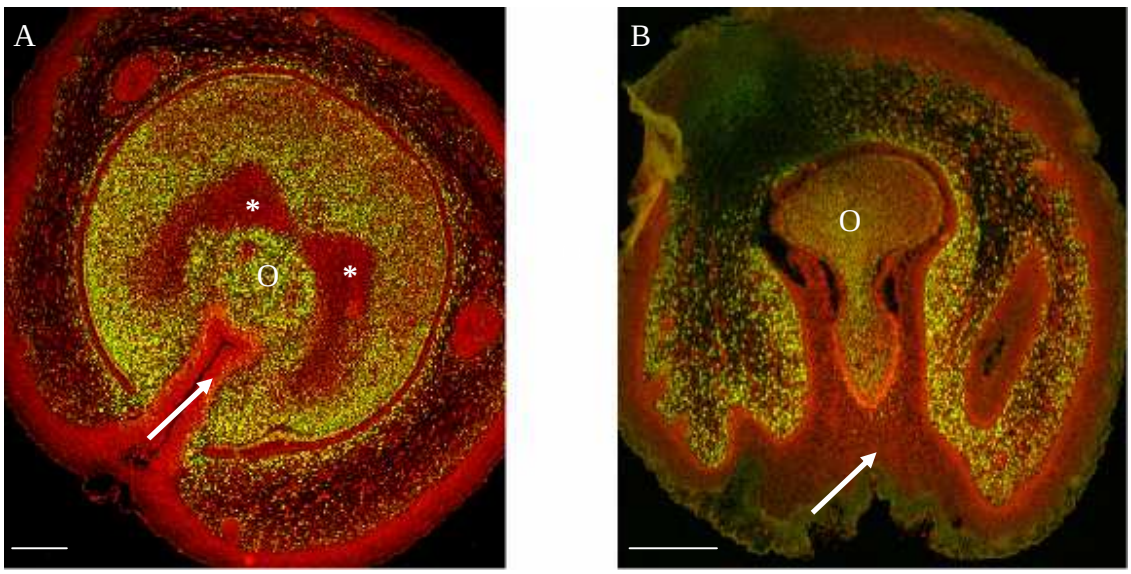


Figure 4.22 Representative photomicrographs of DNMT3L expression (red) and AR expression (green) in male GT at e21.5 exposed during the MPW to vehicle (A) or flutamide (B). Scale bars represent 200µm. O=Os penis; the uretheral area is indicated by an arrow and the corpus cavernosum by asterisks.

Using immunohistochemistry, DNMT3L protein expression was investigated in the male GT at e21.5. At e21.5, in the control male GT, DNMT3L was found to be expressed in most cells types and co-localised with AR, in addition, DNMT3L was also found in the cells in which AR are not expressed, mainly the corpus cavernousum and blood vessels. Taqman-PCR results suggested that DNMT3L expression decreases in flutamide exposed GT in comparison to control GT but only at e16.5 and e17.5. Unfortunately it was not possible to determine the DNMT3L protein expression at these ages, however in the e21.5 GT of fetuses exposed to flutamide, DNMT3L protein expression was found in the cells around the urethra and in the blood vessels and expression was not markedly different (Fig.4.22).

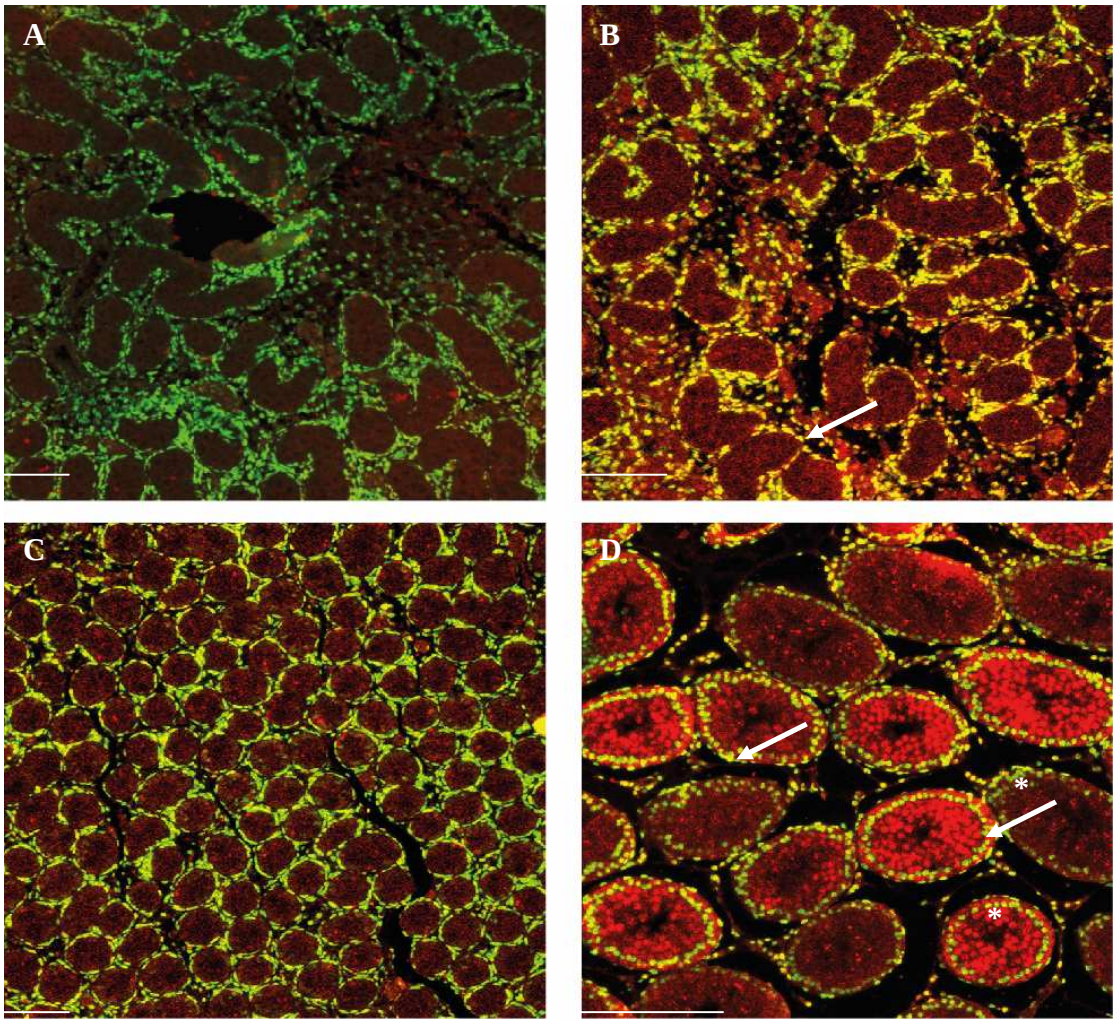


Figure 4.23 Representative photomicrographs of DNMT3L expression (red) and AR expression (green) in control testis at e21.5 (A, B) PND4 (C) and PND25 (D). The arrows indicate co-localisation of AR and DNMT3L. A shows a negative control illustrating absence of DNMT3L antibody. Note that DNMT3L is expressed in peritubular myoid and some interstitial cells at e21.5 where it co-localizes with AR. A similar picture was evident at PND4 and PND25, except that DNMT3L was also expressed in some germ cells. Scale bar represents 200µm.

DNMT3L protein expression was investigated in the testis. DNTM3L co-localised with AR in peritubular myoid cells and some interstitial cells in e21.5 control testes. Some non-specific staining could be seen in the seminiferous tubules at this age and also at PND4. However in PND4 testes DNMT3L expression in peritubular myoid cells and interstitial cells was less prominent than at e21.5, although there was still co-localisation

with AR. At PND25, DNMT3L co-localised with AR in the SCs, peritubular myoid and some interstitial cells and stage specific DNMT3L expression was also evident in some GCs.

The protein expression patterns of BORIS and DNMT3L in the testis called for further investigation. It was of importance to determine if disrupting androgen action by flutamide, DBP or DES in the testes affected DNMT3L expression, however both of these antibodies ran out around the same time. Upon re-ordering, it was not possible to get the DNMT3L antibody originally used (rabbit polyclonal) and a chicken polyclonal was ordered as this was the only one available. This antibody had been used to detect protein by western blotting and not imuunohistochemistry and despite attempts on the bench and on the bond machine, the chicken polyclonal DNMT3L antibody would not work on the rat testis using immunohistochemistry. Although the BORIS antibody ordered was of the same specificity as the previous, it also did not work well. Whilst writing this thesis, the rabbit monoclonal antibody for DNMT3L from Abcam has become available again and this will be used for future experiments.

The tables below summarize the cell types in the testis within which the 3 androgen co-regulators and 2 methylation regulators were found. Highlighted cells outline when and where these regulators were found to co-localise with AR.

Table 4.3 Summary of 3 co-regulator and 2 methylation regulator protein expression in cells types of the testis, found by immunohistochemistry. Co-localisation with AR highlighted in green

BRG1			
	e21	**PND4**	**PND25**
SC	Yes	Yes	Yes
GC	Yes	Yes	Yes
Interstitial Cells	Yes	Yes	Yes
PTM	No	No	No

CBP			
	e21	**PND4**	**PND25**
SC	Yes	Yes	Yes
GC	Yes	Yes	Yes
Interstitial Cells	Yes	Yes	Yes
PTM	Yes	No	Yes

Rwdd1			
	e21	**PND10**	**PND25**
SC	No	Yes	Yes
GC	No	Yes	Yes
Interstitial Cells	Yes	Yes	Yes
PTM	Yes	Yes	Yes

BORIS			
	e21	**PND4**	**PND25**
SC	Yes	Yes	Yes
GC	Yes	Yes	Yes
Interstitial Cells	Yes	Yes	Yes
PTM	Yes	No	Yes

DNMT3L			
	e21	**PND4**	**PND25**
SC	No	No	Yes
GC	No	Yes	Yes
Interstitial Cells	Yes	No	Yes
PTM	Yes	Yes	Yes

4.4 Discussion

The studies in this chapter aimed to investigate a range of factors that might potentially modulate androgen action during the MPW or be modulated by androgens during the MPW. The starting point to narrow down the vast number of androgen biochemical associates reported was to use bioinformatics software; Genego. This identified gene expression pathways involved with AR. These pathways were compared to the results of the microarray study done by our group, (see section 6.1). Six androgen co-regulators were found to overlap between pathways identified using Genego and genes identified in the microarray namely*; Brg1, Cbp, Fkbp5, Snurf, Transgelin* and *Vav3*. The expression of each of these co-regulators was investigated using Taqman-RT-PCR in the genital area of fetuses from e11.5-e21.5. The mRNA expression patterns of *Cbp* and *Brg1* were interesting and both were found to be decreased in expression during the MPW. Furthermore, it is well established that these two co-regulators can work in conjunction (Direnzo et al, 2000; Naidu et al, 2009). It was surprising that neither BRG1 nor CBP had been characterized in the male GT or in the testis before and consequently both were investigated using immunohistochemistry.

Both BRG1 and CBP were found to be expressed in the genital tubercle. Due to difficulties in gaining the correct orientation of GT at e17.5 and younger ages, it was not possible to detect with certainty if protein levels of BRG1 and CBP were decreased during the MPW, in comparison to before and after the MPW as suggested by the Taqman RT-PCR results. However, in the e21.5 GT, it was clear that BRG1 and CBP were expressed but did not co-localise with AR. Both of these co-regulators were found to be expressed in the blood vessels, corpus cavernosum and urothelial cells. In the testis, both BRG1 and CBP were expressed in interstitial cells throughout development from e21.5 to PND25, it is interesting that BRG1 was found to be expressed in a population of cells of the interstitium which do not express AR and no co-localisation was found between AR and BRG1 in the interstitial cells at any age. In contrast, CBP was found to be in cells of the interstitium which do express AR and co-localisation

between CBP and AR was found in the interstitial cells of the testis throughout development at e21.5, PND4 and PND25. The investigations of the co-expression of BRG1 and CBP demonstrates that CBP and BRG1 are co-localised in some cells of the interstitium, thus there is a population of cells in the testis which express CBP and AR and not BRG1 and there is separate population of interstitial cells which express CBP and BRG1 but not AR. Further studies could use immuohistichemistry to determine what these populations are, for example fetal Leydig cells or progenitor adult Leydig cells. Both BRG1 and CBP were found to be expressed in the SC and co-localised with AR when AR was switched on at by PND10. Although the co-localisation of CBP with AR was much clearer than BRG1 with AR. Both CBP and BRG1 were found to be expressed in the GCs at all ages investigated. It has been reported that both BRG1 and CBP knockout models are embryonic lethal, however some studies have conducted targeted knockouts to determine role of each of these in a particular tissue type, for example, BRG1 was knocked out of the lung and this potentiated lung cancer (Glaros et al, 2008). A targeted knockout of either CBP or BRG1 in the testis might provide some very interesting answers as to roles these proteins may play in the SC and GCs. Another useful study would be to determine the exact stages of spermatogenesis which correlates with CBP and BRG1 expression. Exposure to DES, DBP or flutamide during the MPW did not affect the expression of BRG1 or CBP et e21.5 or at PND25 but further experiments on flutamide exposed GT and also characterization with the female reproductive tract are called for to determine if BRG1 or CBP might be androgen regulated.

In the GT and testis, RWDD1 was found to co-localise with AR. Furthermore a significant decrease in *Rwdd1* expression was found at the mRNA and protein level in GT from animals which had been exposed to flutamide during the MPW and consequently had disrupted androgen action. Many further studies need to be conducted to determine if RWDD1 is androgen regulated throughout the development of the GT, for example looking at younger ages. Also the effect of other anti-androgenic chemicals

as well as oestrogens on the expression of RWDD1 should be investigated. In addition, RWDD1 should be characterized in the ducts, in the testis and also within the female reproductive tract to determine if RWDD1 is specific to the male GT and reproductive tract. It would also be interesting to investigate RWDD1 expression in the tissue of AR knock out mice. No knockout of RWDD1 has yet been reported. In the testis, RWDD1 was found to co-localise with AR in some interstitial cells as well as the PTM cells at all ages investigated. Of interest also was that RWDD1 could not be seen in the e21.5 SC but was found in the postnatal period at PND4 and PND25, co-localizing with AR, once it has switched on. Although some staining was seen in the GC it was non-specific. To the best of my knowledge these are the first reporting's of RWDD1 in the GT and in the testis and these findings show that RWDD1 is expressed, in the tissue investigated at least, in all cell types that express AR. Furthermore, the effect of flutamide exposure on RWDD1 expression in the GT calls for urgent further experiments. As mentioned above, these should start with full characterization in the male and female reproductive tract and then compare with various androgen disrupting treatments.

It is widely accepted that methylation is vital in embryonic development (Shoeck et al, 2011; Woroniecki et al, 2011) and some methyl transferase enzymes are known to be involved in AR gene regulation (Gaughan et al, 2010; Metzger et al, 2005) (Section 1.7.2). For example some studies have shown that hypermethylation of AR can occur in prostate cancer (Jarrad et al, 1998; Kinoshita et al, 2000) and a more recent study has shown that in hypospadias patients, the penile foreskin has decreased expression of AR and increased expression of DNMT3A (a key methylation enzyme) in comparison to control patients, suggesting that hypermethylation of the AR maybe involved in hypospadias (Vottero et al, 2011). Although altered methylation of specific genes could provide an explanation for aspects of the MPW (e.g. silencing after the MPW of genes that are androgen-regulated within the MPW), this first requires identification of genes that are androgen-regulated in the MPW (see chapter 6). In the absence of such information, the approach taken was to investigate whether expression of genes involved

in methylation regulation might either change during the MPW or be androgen-regulated during this period. BORIS and DNMT3L (indicators of methylation activity) were chosen for study initially, with the aim to possibly study further the expression of other DNMT enzymes at a later stage.

CTCF is well established in the literature as an 11-zinc finger protein involved in gene regulation and forms methylation sensitive insulators which regulate X-chromosome inactivation and expression of imprinted genes (Loukinov et al, 2002). CTCF is expressed in the nucleus of most somatic cells. BORIS shares a similar structure to CTCF but has been defined in the literature as testis specific because it has been found in the cytoplasm of spermatocytes (Loukinov et al, 2002). BORIS expression is thought to be negatively regulated by DNA methylation, p53 and CTCF (Renaud et al, 2007). Considering BORIS expression is thought to be negatively regulated by methylation in the testis, and in addition BORIS expression has never been reported in the GT, this was investigated in this chapter. These studies found that BORIS is expressed in the GT at both the mRNA and protein level. Taqman-PCR results suggested that *Boris* expression decreases in the developing GT and a significant decrease was seen after the MPW at e21.5 in comparison to before the MPW at e14.5. Unfortunately, due to tissue orientation issue, it was not possible to determine if this change in expression of *Boris* was true at the protein level, however immunohistochemistry did show that the BORIS protein is expressed in the GT at e21.5 in the urothelial cells, the os penis, the corpus caversoum and the blood vessel, this expression was similar in the GT of male fetuses that had been exposed to flutamide during the MPW. In the testis, BORIS was found to be expressed in the interstitial cell, the SCs, the GCs and the PTM, although expression was not found in the latter at PND4. Furthermore, expression of BORIS in the GC (at the three ages investigated) was non-specific and some literature does state that BORIS expression in the GC is cytoplasmic and not nuclear. BORIS is classified at a cancer testis antigen (CTA) because its expression is found in human neoplasma and not in normal tissue other than the testis (Villella et al, 2005). The studies in this chapter

however have found BORIS to be expressed in the GT and also in other cell types other than the GC in the testis, namely SC and cells in the interstitium. These studies however are preliminary and require strengthening by further experiments, which would firstly include determining BORIS expression before and during the MPW as well as after the MPW. Also it would be useful to use immunohistochemistry and markers of various cells types to characterize which population of cells in the interstitium BORIS is expressed in. Recently, the outcomes of a BORIS knockout mouse model were reported (Suzuki et al, 2010). These studies reported that BORIS knock out mice were fertile, but had small testis in comparison to controls and also had defects in spermatogenesis. This study investigated the changes in expression of certain genes in the testis of BORIS knockout models but there was no characterization of BORIS expression in the cell types of control testis other than GCs, and this is important to understand why a deficiency in BORIS expression might affect spermatogenesis.

DNMT3L is a member of the DNMT family of enzymes involved in methylation. DNMT3L has no methyl transferase catalytic site but is thought to regulate methylation through histone deactylase activity. DNMT3L knock out mice have severe hypogonadism and are azoospermic, however this study did not report if any effect was seen in the penis, for example, hypospadias. The studies in this chapter found *Dnmt3l* mRNA was expressed in the GT before, during and after the MPW and although no significant difference in expression was found, the results suggest a possible decrease in *Dnmt3l* expression as the GT develops. A significant decrease in *Dnmt3l* expression was seen in the GT of fetuses exposed to flutamide at e16.5 and e17.6 (both time points fall in the MPW). At the protein level, DNMT3L was found to be expressed in the GT at e21.5 in control males and the GT of males exposed to flutamide during the MPW and although staining of DNMT3L looked less pronounced in the GT of flutamide exposed fetuses, this was not quantified. In the testis, DNMT3L was expressed in some cells in the interstitium and the PTM cells at e21.5 and co-localised with AR in these cells. DNMT3L was not found to be expressed in SC until PND25 and at this age, DNMT3L

and AR co-localised. Interestingly DNMT3L was only found to be expressed in the GCs in the postnatal period at PND4 and PND25, the expression of DNMT3L appears to be stage specific. The characterization of DNMT3L in relation to AR in the testis has not been previously reported and given the phenotype of the DNMT3L knockout model, this information is important to understand the mechanisms by which DNMT3L works in the testis. The DNMT3L results reported in this chapter are very preliminary and require further investigation; firstly within the developing control male reproductive tract and female reproductive tract, but also further studies are called for on the effect of flutamide on DNMT3L protein expression in the GT before and during the MPW.

This requirement for GT tissue before and during the MPW is not just restricted to the further experiments called for in DNMT3L protein expression investigation, but also for the other 4 androgen biochemical associates investigated in this chapter. The lack of protein expression data in the GT before and during the MPW is the major weakness of the studies reported in this chapter and this issue should be resolved to investigate the 3 androgen co-regulators and 2 methylation regulators in correlation to the regulation of the MPW.

In these studies, the testis provided a method to investigate the expression of the 5 regulators in relation to well documented AR expression. Each of the 5 regulators was found to co-localize with AR in the SC by the time AR expression was fully switched on at PND25 and the importance of this co-localization requires further investigation. One of the major set-backs with the studies in this chapter was that when 3 working antibodies were replaced, namely Rwdd1, BORIS and DNMT3L, frustratingly, it was not possible to determine if disrupting androgen action would affect expression of these regulators in the testis at various ages, which is important information particularly as RWDD1 expression appears to be an androgen regulated in the GT and DNMT3L knockout mice are azoospermic.

In conclusion, the mRNA expression of 9 genes was investigated before, during and after the MPW and, each 5 of these displayed interesting patterns of expression during the MPW in the GT. It could not be verified at this stage, if these patterns of expression, during the MPW, were reflected at the protein level. The approach taken therefore was to investigate expression of these proteins in relation to AR expression, with emphasis on studies in situations of dynamic changes in AR expression and or androgen action, namely in the fetal to postnatal testis transition and in the GT and testis from animals exposed to the AR antagonist, flutamide, in the MPW. Whilst these studies have not clearly resolved the involvement of the AR biochemical associates in the MPW, they have raised a number of interesting findings and questions. For example, the differential co-expression of BRG1 and CBP with AR in interstitial cells versus SC in the developing testis suggests that different co-activators may be used by these cell types and or that androgen action is fundamentally different. Further studies in this area are likely to provide useful new insights into the role(s) and regulation of androgen action during development in the male reproductive system.

5.1 Introduction

Prostaglandins (PGs) are involved in many different systems of the developing embryo. There is conflicting data about the effects of blocking PGs using indomethacin, a COX enzyme inhibitor, in utero and this is reviewed in sections 1.3.1.4.1 and 1.4.3. The conflict mainly arises from differences in species used, doses administered, route of administration and treatment time in gestation. Nonetheless, there is substantial evidence that prostaglandins are important in the production of testosterone (Kubota et al, 2011; Sawada et al, 1998; Wang et al, 2003) and some studies suggest that obstructing PG pathways using indomethacin can affect masculinisation of the male mouse embryo (Gupta and Goldman, 1986; Gupta, 1989; Gupta and Bentlekewski, 1992). This suggests that correct androgen action may be related to correct PG action. However these studies, which were conducted in the late 1980's, have never been repeated or built on despite the important finding that PGs may be vital in androgen action.

The main aim of the studies in this chapter was to determine if PGs are essential for androgen action in the MPW and for consequent male reproductive tract programming which occurs during this time, in the rat. This was investigated by using indomethacin to block PG synthesis during the MPW and to examine the outcomes in fetuses, neonates, pubertal animals and adult animals.

One of the aims of these thesis studies is to gain insight into factors which may be involved in the 'opening' of the MPW. To determine if PGs are involved in this regulation and also to understand if PGs are essential prior to the MPW for later masculinisation, indomethacin was administered to some animals prior to the MPW from e11.5- e14.5 (the early window, EW) and endpoints noted in fetal, pubertal and adult animals.

5.2 Materials and Methods

5.2.1 Animals and treatments

Briefly, pregnant rats were treated with indomethacin or appropriate vehicle during the MPW (e15.5-e18.5) or during the EW (e11.5-e14.5) (section 2.2). Initially 8 animals were treated with 2mg/kg indomethacin by oral gavage during the MPW. However, these dams became extremely ill with a 75% mortality rate, see Table 5.1. Therefore a further 6 animals were treated with 1mg/kg of indomethacin, administered using subcutaneous injection, during the MPW. Although there were still some complications, sufficient data was collected to address the study aims. A further 4 litters were exposed in the EW to indomethacin with less complications. AGD and bodyweight was measured in male and female pups at e21.5 prior to the GT, gonads and ducts being dissected, in animals treated in the MPW or in the EW. Postnatal measurements were taken at PND8 (MPW exposed animals only), PND25 and PND75 (section 2.3.3). Collected tissue was fixed in Bouins and processed for possible immunohistochemical analysis.

GT were also dissected and collected from control fetuses at e14.5, e16.5, e17.5 and e21.5 and frozen at -80°C for RNA analysis (section 2.6) to determine if the COX1 and COX2 enzymes necessary to make PGs are present in the GT and if this expression varies with age.

Table 5.1 Details of litters, treatment windows, doses and complications of indomethacin exposure in animal experiments in chapter 5

Dam No	Treatment window / dose	Route of administration	Gestational period	Fate
1	MPW / 2mg/kg	Gavage	21.5	Culled at e21.5
2	MPW / 2mg/kg	Gavage	20	Found dead
3	MPW / 2mg/kg	Gavage	20	Found dead
4	MPW / 2mg/kg	Gavage	19	culled at e19.5
5	MPW / 2mg/kg	Gavage	19	Found dead
6	MPW / 2mg/kg	Gavage	20	Found dead
7	MPW / 2mg/kg	Gavage	21	Found dead
8	MPW / 2mg/kg	Gavage	21	Found dead
9	MPW / 1mg/kg	Subcutaneous Injection	21	culled at e21. only 4 pups
10	MPW / 1mg/kg	Subcutaneous Injection	21	PND8, 25 & 75
11	MPW / 1mg/kg	Subcutaneous Injection	24	Culled due to no birth
12	MPW / 1mg/kg	Subcutaneous Injection	22	PND8, 25 & 75
13	MPW / 1mg/kg	Subcutaneous Injection	21	PND8, 25 & 75
14	MPW / 1mg/kg	Subcutaneous Injection	25	culled due to no birth
15	EW / 1mg/kg	Subcutaneous Injection	21	Culled at e21.5
16	EW / 1mg/kg	Subcutaneous Injection	21	Culled at e21.5
17	EW / 1mg/kg	Subcutaneous Injection	21	PND25 & 75
18	EW / 1mg/kg	Subcutaneous Injection	21	PND25 & 75

5.2.2 RNA analysis:

The mRNA level of *COX* enzymes in control male GT was investigated using Taqman (section 2.6). Briefly, RNA was isolated from frozen tissue and converted into cDNA and quantitative RT-PCR performed. Expression was studied before during and after the MPW. The genes investigated and primers used are listed in Table 5.2.

Table 5.2 Taqman primers and Universal probe library probes used in chapter 5

Gene	**Forward Primer 5'→3'**	**Reverse Primer 3'→5'**	**UPL Probe Number:**
COX1	GCT CTT CAA GGA TGG GAA ACT	TTC TAC GGA AGG TGG GTA CAA	42
COX2	CTA CAC CAG GGC CCT TCC	TCC AGA ACT TCT TTT GAA TCA AGG	5

5.2.3 Immunohistochemical (IHC) investigations

Briefly, IHC staining (section 2.5.1) was performed on processed and sectioned fetal GT, gonads and ducts to determine the presence of the COX2 protein.

Table 5.3 Details of COX2 antibody used in Chapter 5

Primary Antibody	**Source**	**Retrieval**	**Species**	**Dilution**
COX2	Gift from Henry Jabbour	Citrate	Goat	1:50

5.3 Results

5.3.1 Expression of COX enzymes

To determine if the COX enzymes are present in the male GT, *COX* mRNA levels were investigated in the developing GT using Taqman (Fig. 5.1) and the COX2 protein investigated using immunohistochemistry (Fig 5.2).

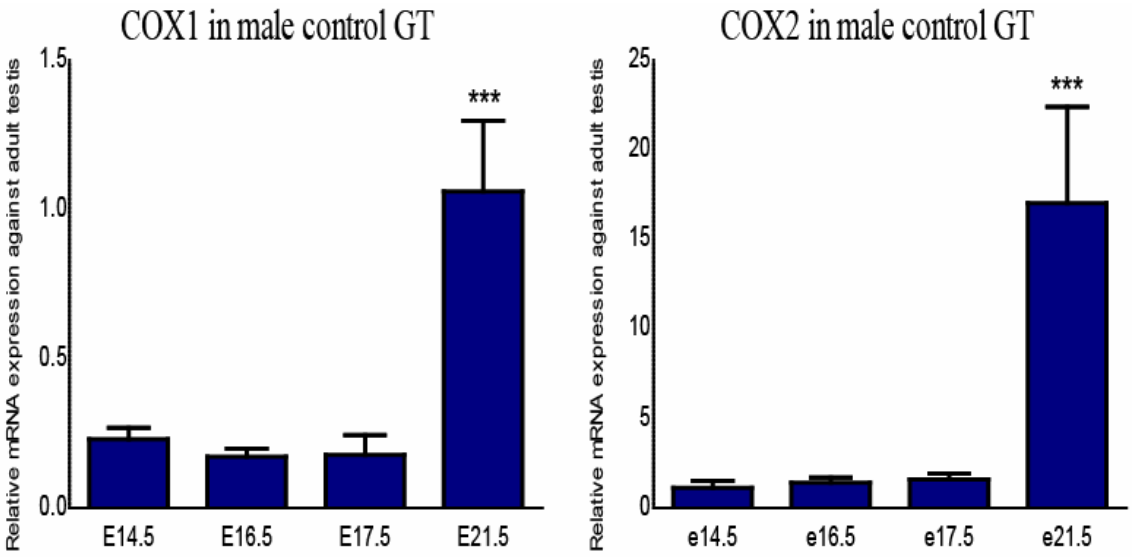


Figure.5.1 Quantitative analysis of *COX1* (A) and *COX2* (B) mRNA levels in the developing male GT. Using one way ANOVA*** P<0.0001, in comparison to each younger age. Values are means ±SEM for 7-14 animals/group from a minimum of 3 litters.

Similar to findings in the literature, relative mRNA *COX1* was expressed at a much lower level in the male GT than relative *COX2* mRNA. The relative mRNA of each enzyme showed a similar pattern, with consistent levels of expression throughout development from e14.5 until just before parturition when levels were found to be significantly increased. The COX2 protein was further examined using IHC.

The fetal GT at e17.5 was difficult to embed in a consistent orientation. IHC could not be conducted on an e17.5 male GT which would be of a precise plane within the tissue and comparable to e21.5 GT.

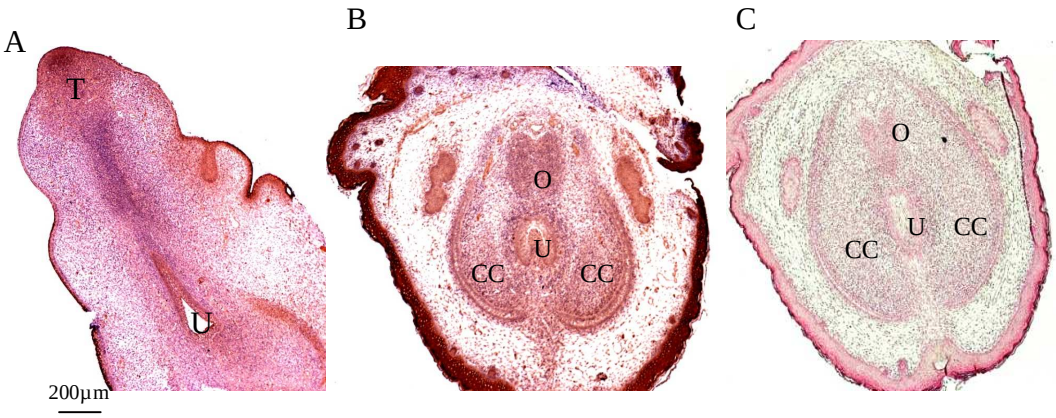


Figure 5.2 Representative photomicrographs of COX2 staining (brown) in the control male GT at e17.5 (A) and e21.5 (B). An e21.5 negative control is shown in panel C. T = the tip of the GT, U = urothelial cells, O = os penis, CC = corpus cavernosum.

COX2 IHC staining was positive at e17.5 and e21.5 in the male GT. The exact location could not be identified at e17.5, but suggested greater COX2 expression around the tip of the GT. At e21.5 the COX2 protein was nuclear and was expressed in the OS penis, the urothelial cells and around the blood vessels (Fig 5.2).

5.3.2 Effect of indomethacin administration during the MPW

Initially 8 animals were treated with 2mk/kg of indomethacin by gavage during the MPW to block PG synthesis. Due to complications with the mothers, these fetuses were not used for these studies. All animals thereafter were treated with 1mg/kg of indomethacin administered subcutaneously either during the MPW or during the EW.

5.3.3 Effect of Indomethacin exposure on number of pups per litter

Some literature reports that exposure to indomethacin causes an increased rate of resorption of pups (O'Grady et al, 1972;). In order to determine if this was true in rats treated with 1mg/kg during the MPW, number of pups per litter was recorded.

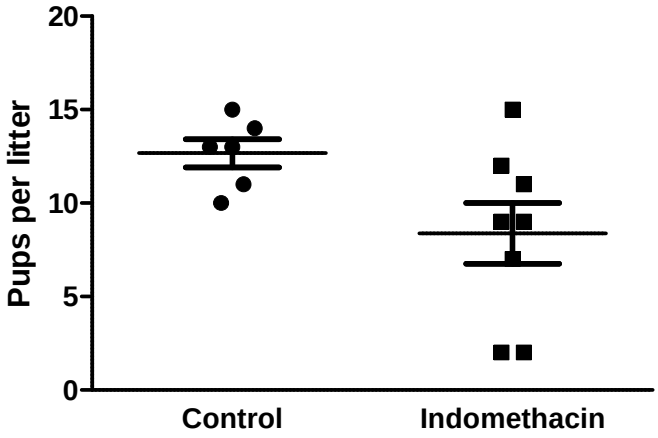


Figure 5.3 Number of pups per litter in vehicle exposed and indomethacin exposed animals

Using an unpaired T-test, no significant difference was found in the number of pups per litter between vehicle exposed and indomethacin exposed animals. However Fig 5.3 shows a large degree of variability in the number of pups per litter from animals treated with indomethacin with some evidence of a decrease in two litters, so it is possible that with an increase in animal numbers a decrease may have been found.

5.3.4 Effects of indomethacin exposure on males

To identify if exposing animals to indomethacin during the MPW was affecting androgen action, Bodyweight, AGD and testis weight were measured at e21.5, PND8, PND25 and PND75 and penis sized was measured at PND25 and 75.

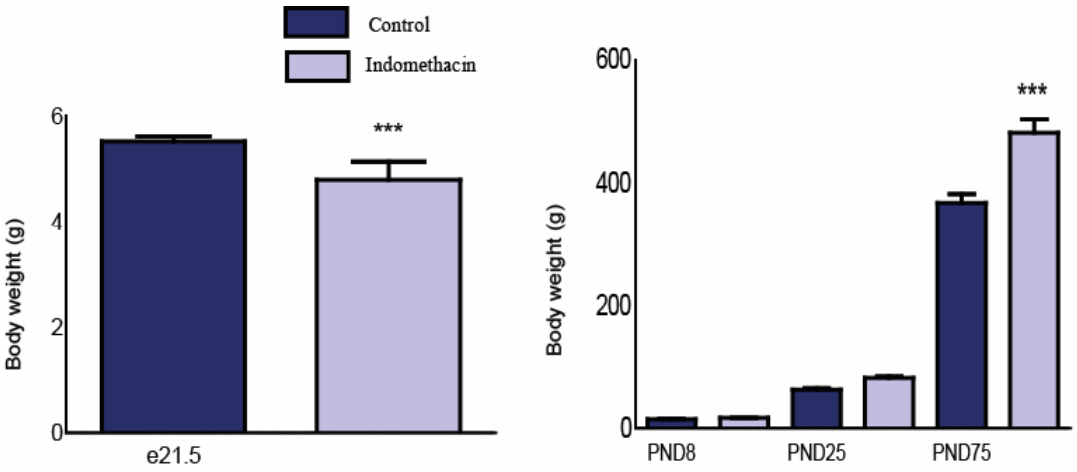


Figure 5.4 Control male body weight compared with animals exposed to 1mg/kg indomethacin during the MPW. *** P<0.0001. Values are means ±SEM for 6-11 animals/group from a minimum of 2 litters.

A significant reduction in bodyweight was found at e21.5 in males exposed to indomethacin. However by adulthood, there was a significant increase in bodyweight of indomethacin exposed males in comparison to respective controls. These opposite results could be due to the rather small numbers of litters or animals and or a high variability of bodyweight at any age. However, the results for females in Fig.5.10 suggest that in utero indomethacin exposure does decrease bodyweight in the fetal period. This is discussed in more detail in section 5.4.

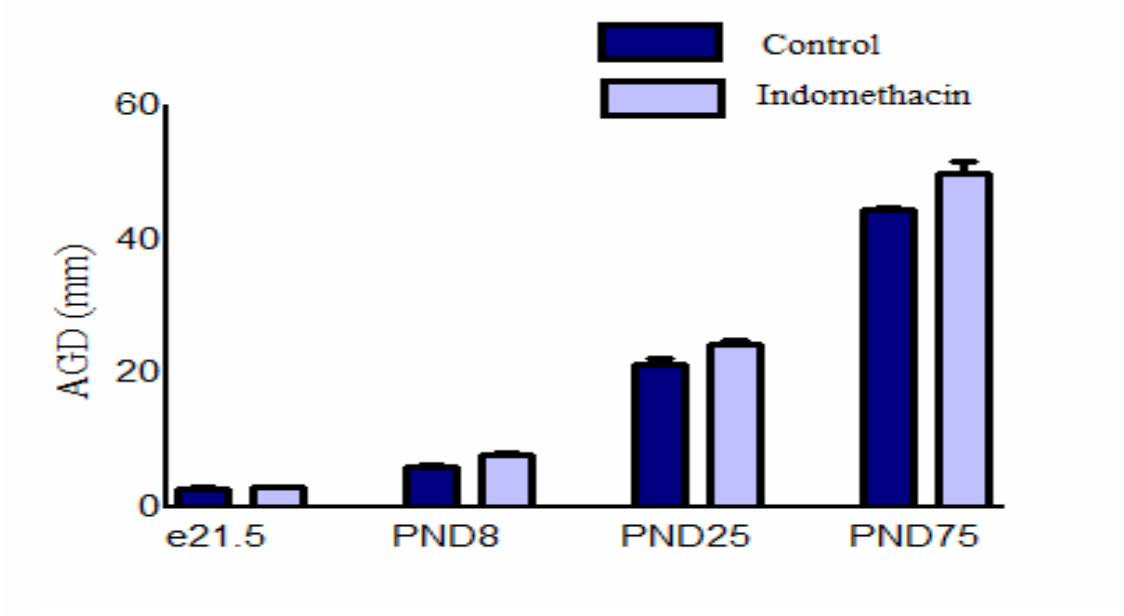


Figure 5.5 AGD at different ages in control males compared against males exposed to 1mg/kg of indomethacin during the MPW. Values are means ±SEM for 6-11 animals/group from a minimum of 2 litters at each age.

There was no significant difference in the AGD of males exposed to indomethacin compared with respective control males. This was true in the fetal period at e21.5, in the neonatal period at PND8, at puberty (PND25) and in adult animals.

To determine if indomethacin treatment during the MPW affected the testis, animals were examined for cryptorchidism at PND25 and adulthood. Testes were dissected, their weight measured and their gross cellular composition investigated. No cryptorchidism was found in any animals exposed to indomethacin or vehicle.

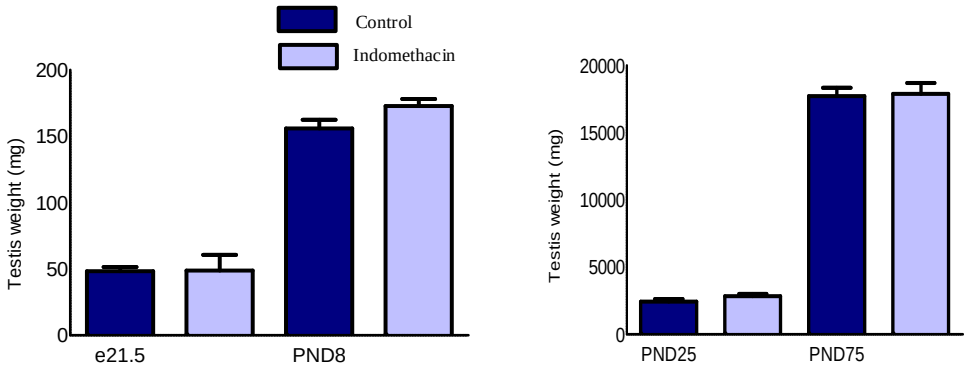


Figure 5.6 Testis weights of control animals compared to animals exposed to 1mg/kg indomethacin during the MPW. Values are means ±SEM for 6-11 animals/group from a minimum of 2 litters.

Testis weights were not significantly different between control animals and animals exposed to indomethacin in either fetal or postnatal life (Fig 5.6). In order to determine if there was any gross difference in cellular composition, testes were immunostained using VASA, a marker of germ cells.

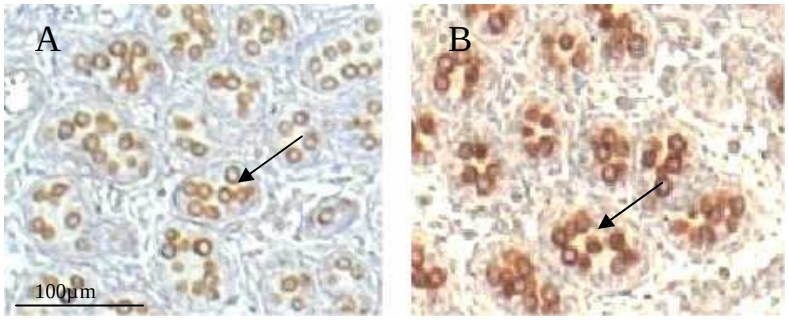


Figure 5.7 Representative photomicrographs showing immunostaining for VASA (brown) in e21.5 testis from a control (A) and an indomethacin exposed (B) animal.

However, no gross difference in apparent germ cell numbers or distribution was found when comparing control animals with animals exposed to indomethacin, although germ cell numbers were not counted using stereology.

The postnatal penis was examined for the presence of hypospadias, and its length and weight measured. No hypospadias was found in any of the males exposed to indomethacin or vehicle.

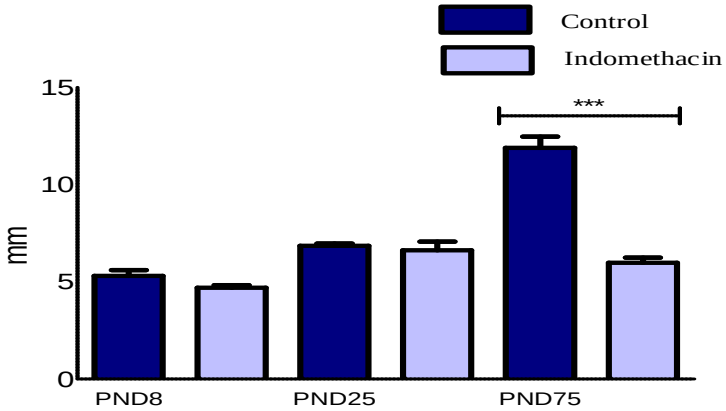


Figure 5.8 Penis length in rats exposed to either vehicle or 1 mg/kg indomethacin during the MPW. Usng a one way ANOVA *** P<0.0001, in comparison with respective control. Values are means ±SEM for 4-9 animals/group from a minimum of 2 litters

No significant difference was found in the penis length of animals exposed to indomethacin in comparison to control animals at PND8 and PND25. However, by adulthood a significant decrease in penile length was seen in animals that were exposed to indomethacin, although this was based only on 4 animals from 2 litters.

5.3.5 Effects of indomethacin exposure on females

To determine if blocking prostaglandins, using indomethacin affected the development of females, AGD, bodyweight, and ovary weight were examined.

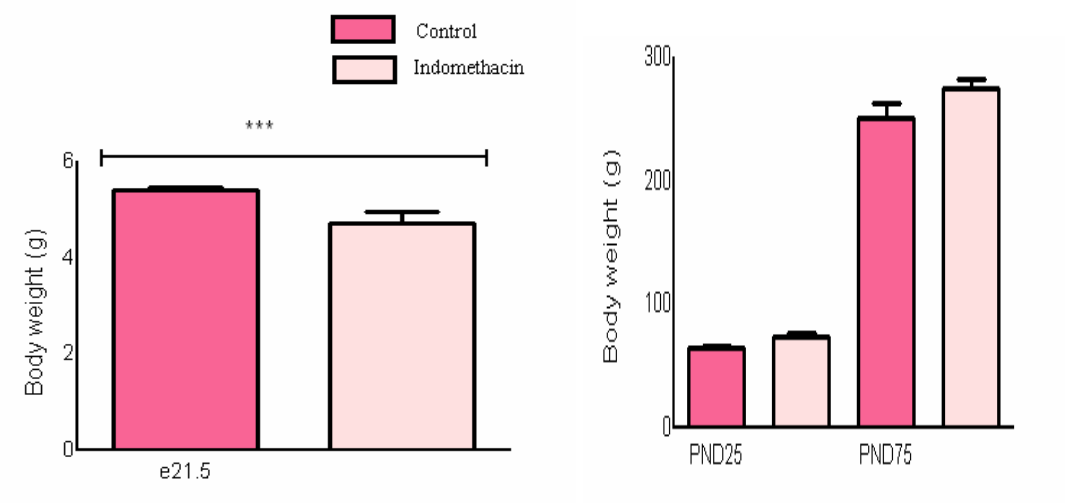


Figure 5.9 Female bodyweight in rats whose mothers were treated with vehicle or indomethacin during the MPW. *** P<0.0001, in comparison with respective control. Values are means ±SEM for 3-14 animals/group from a minimum of 2 litters.

Similar to results for e21.5 males, there was a small but significant decrease in bodyweight of females exposed to indomethacin at e21.5. No significant difference in bodyweight was seen at puberty or in adulthood (Fig 5.9).

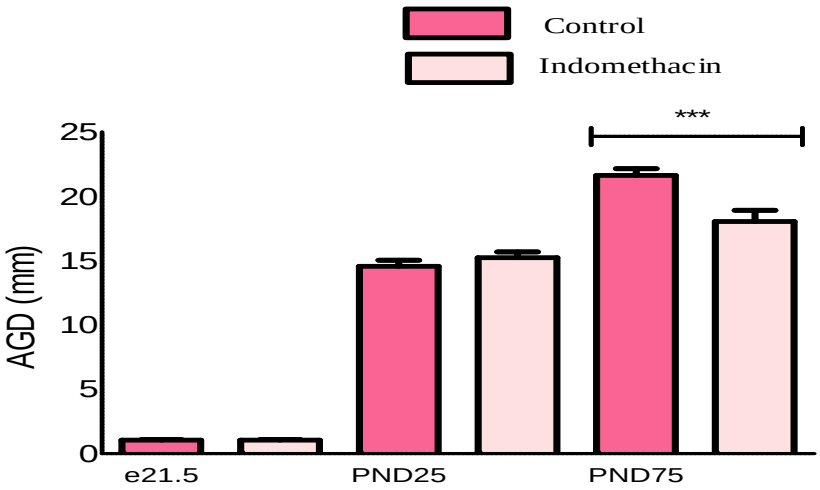


Figure 5.10 AGD at different ages in control females compared with females exposed to 1mg/kg of indomethacin during the MPW. *** P<0.0001, in comparison with respective control. Values are means ±SEM for 3-14 animals/group from a minimum of 2 litters.

At e21.5 and PND25 there was no significant difference in AGD between control females and those exposed to indomethacin during the MPW (Fig 5.10). In adulthood, the AGD of indomethacin exposed females was significantly reduced in comparison to animals exposed to vehicle.

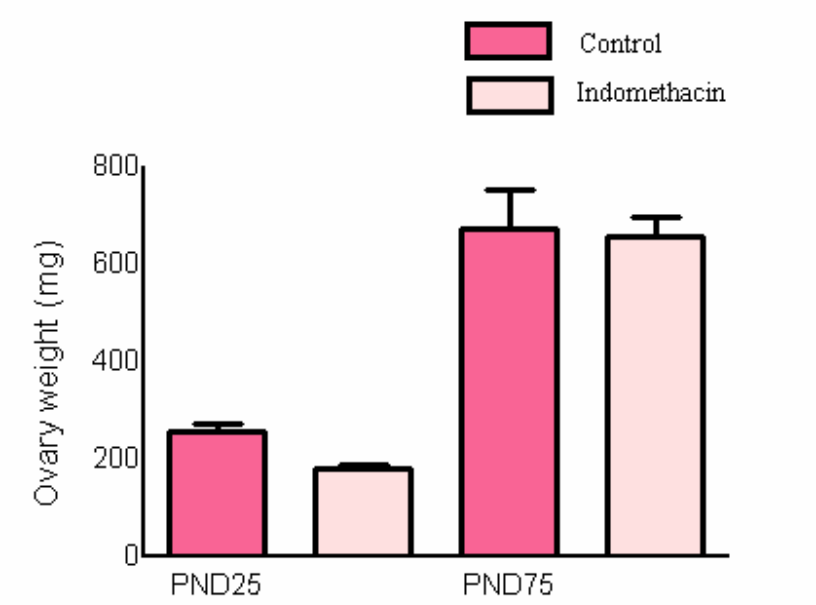


Figure 5.11 Ovary weight of female rats exposed to either vehicle or 1mg/kg indomethacin during the MPW. Values are means ± SEM for 3-14 animals from a minimum of 2 litters. Using an un-paired t-test, no significant difference was found in ovary weight at PND25 or at PND75 between control and indomethacin exposed females

No significant difference was seen in the weight of ovaries of females exposed to indomethacin during the MPW in comparison to respective controls. However, when dissecting the adult ovaries, it became evident that each female exposed to indomethacin in utero had a slightly different position of ovaries in comparison to control females. In control animals, the ovaries are usually found at the distal end of the uterine horns, near the kidneys, at the edge of the abdominal cavity. However in adult females exposed to indomethacin, the ovaries in each animal studied were present at the end of the distal end of the uterus but were more 'centrally located' in the abdomen, often with the uterus intertwined with other organs (Fig. 5.12). The weights (Fig 5.11) and gross apprearance of the ovaries appeared normal but the location within the body was not. In order to determine if these females were fertile, timed matings were set up using three adult animals. Of the three animals, one did not plug at all and the two which did gave birth

late. The first of these had an extended gestation period of 1 day and gave birth at e23.5 and the other had an extended gestation time of 3 days and gave birth at e25.5.

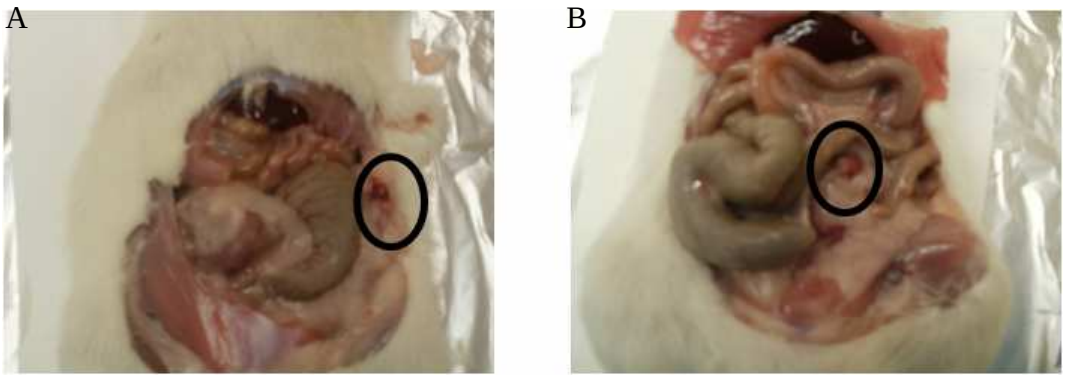


Figure 5.12 Position of right ovary (circled) in control female adult (A) and indomethacin exposed female adult (B)

5.3.6 Effect of indomethacin exposure prior to the MPW

To determine if PGs play a role in regulation of the MPW or are important in the opening of the MPW, pregnant dams were administered 1mg/kg indomethacin from e11.5-e14.5 and pups examined at either e21.5 or postnatally. As androgens are produced from e14.5 by the fetal testis, it was unlikely that blocking PGs prior to the MPW would have any androgen related effect in males, and so only 2 litters of indomethacin exposed animals were examined at each age in this set of experiments.

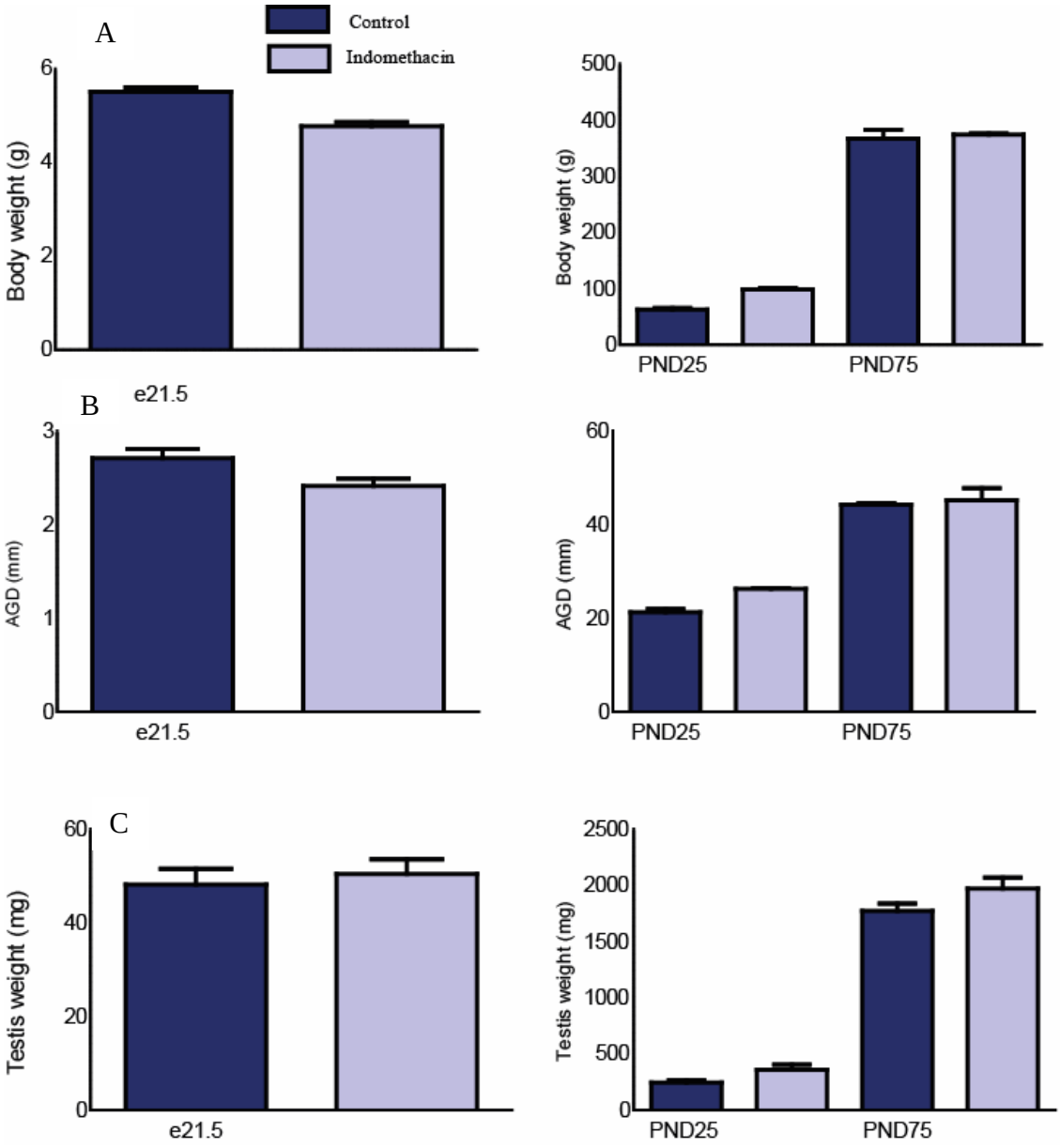


Figure 5.13 Bodyweight (A), AGD (B) and testis weight (C) of males whose mothers were treated with vehicle or 1mg/kg indomethacin during the EW. Values are means ±SEM for 2-6 animals/group from 2 litters. Using un-paired t-tests, no significant differences were found between control and indomethacin exposed males.

Only minor changes in AGD, PND25 testis and body weight were seen in animals exposed to indomethacin, however, none of these changes were significantly different

from controls and was based on small animal numbers. Nevertheless, the results suggest that blocking PG synthesis prior to the MPW does not have any major effects on masculinisation.

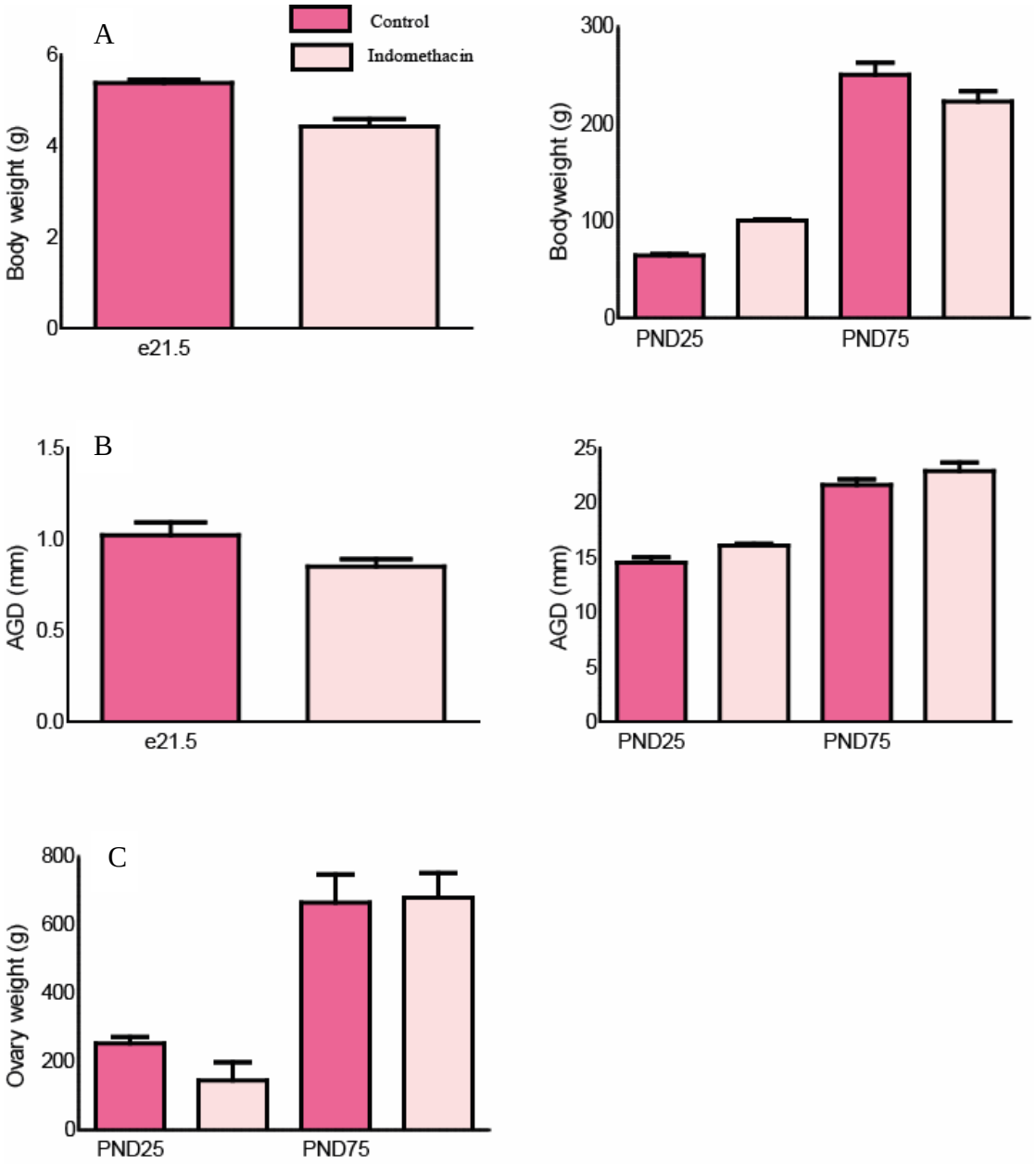


Figure 5.14 Bodyweight (A), AGD (B) and ovary weight (C) in females whose mothers were treated with vehicle or 1mg/kg indomethacin during the EW. Values are means ±SEM for 2-6 animals/group from 2 litters. Using un-paired t-tests no significant differences were found between control and indomethacin exposed females.

5.4 Discussion

The aim of the studies in this chapter was to determine if prostaglandins play a role in androgen programming of the male fetus which occurs during the MPW, and if blocking PGs during this time (e15.5-e18.5) would affect virilisation as suggested by studies in mice published in the 1980's (Gupta and Goldman, 1986; Gupta, 1989; Gupta and Bentlekewski). A secondary aim was to determine if PGs might be involved in regulation of the MPW, in particular the 'opening' of the window. Thus, PG synthesis was blocked in a small number of animals, in the period prior to the MPW, namely e11.5-e14.5.

The primary investigation in these studies set out to determine if the enzymes required to make PGs were present in the GT. *COX1* and *COX2* are isoenzymes and each is expressed on different chromosomes in the human and also in the rat (Ogawa et al, 2001; Taketa; 1998). COX1 and COX2 proteins have 60% homology and their structure can be divided into three domains; an N-terminal EGF domain, a membrane binding domain and a catalytic site, and the latter contains the cyclooxygenase active site (Kurumbail et al, 1996). The binding site in COX2 for NSAIDs is reported to be larger than the binding site in COX1. COX1 is present in most tissues to produce prostaglandins which are important in regulating cell function whereas COX2 in postnatal life is expressed mainly in response to acute inflammation (Kurumbail et al, 1996). In fetal life however, COX2 is more highly expressed than COX1, particularly in the testis but also in the reproductive tract as a whole (Kirschenbaum et al, 1999; Schell et al, 2007). Fitting with the literature the studies reported in section 5.3.1 found *COX1* and *COX2* to be expressed in the male GT, and *COX2* was expressed at higher levels than *COX1* (Fig.5.1). Protein immunohistochemistry demonstrated the COX2 protein is present at both e17.5 and e21.5 in the developing male GT, most abundantly at e21.5, suggesting that PG synthesis and action could be important in the male GT. This fitted in also with analysis of mRNA expression which showed much higher levels of *COX2* mRNA in the GT at e21.5 than at earlier fetal ages.

Indomethacin is well established as a compound which blocks the COX enzymes non-selectively and this inhibits prostaglandin synthesis (Dannhardt and Kiefer, 2001). Indomethacin binds deep into the cyclooxygenase active site of both COX1 and COX2 proteins.

The literature on the fetal effects of indomethacin are conflicting, with variation in effects reported according to species, doses and timing of administration (section 1.6.1.4.1 and section 1.7.3). In the late 1980's Gupta reported that indomethacin at various doses affected masculinisation of male mice. Furthermore, it was reported that this effect was seen when PGs were inhibited during 'the critical period for differentiation', what we now term the MPW. Indomethacin was chosen as the choice of PG synthesis inhibitor for the experiments in this chapter in order to determine if the results reported by Gupta could be repeated in the rat.

A number of studies have been conducted using indomethacin in rodents, with large differences reported in the dose administered whereby some studies used 1mg/kg whereas others used up to 20mg/kg. For example, when investigating the effect of indomethacin on implantation sites, one study administered 3mg/kg twice daily to rats and reported a 77% decrease in implantation sites (Poyser, 1999). In addition, it has been reported that pregnant rats treated with 5mg/kg indomethacin had similar effects and treatment with 10mg/kg caused severe gastrointestinal bleeding and animal death (Sookvanichsilp and Pulbutr, 2002). However both of these studies administered indomethacin early in gestation from gestational days 3-5. In contrast, Randall et al (1987) exposed mice to either 10mg/kg or 20mg/kg indomethacin by subcutaneous injection and (on gestational day 10 alone) and found a slight effect on fetal body weight but otherwise development was normal despite toxic effects found in the dams. Of interest, one study tried three different routes of indomethacin administration (oral, subcutaneous and intramuscular) to pregnant mice from days 9-15 of gestation, with a dose of 7.5mg/kg and reported no teratogenic effects (Kalter et al, 1973). However when

Kusangi et al administered the same dose (7.5mg/kg) to mice from days 7-15 of gestation, using oral gavage, fetuses were reported to have fused ribs, vertebral abnormalities and skeletal defects (Kusangi et al, 1971). In rabbits, 8mg/kg or 16mg/kg (per day) of indomethacin was administered from day of mating until birth and although these studies found an increased resorption of developing embryos, no defects were found in the surviving embryos (O'Grady et al, 1972). Thus, it appears that the effect of indomethacin on fetuses depends on the species used, the dose administered, the route of administration and also the length of exposure. However, even allowing for variation in these parameters it appears that there is a degree of inconsistency in results obtained.

For the studies reported in this chapter, an initial dose of 2mg/kg was administered by oral gavage. This was chosen as an initial dose for the rats as it was twice as high as the dose administered by Gupta to the smaller mice, and according to the literature this was the higher of 'low doses' that have been used. Gavage was chosen as the method of administration to mimic the oral exposure route in humans. However, dams treated orally with 2mg/kg indomethacin became very ill and some had difficulty giving birth. Upon investigation, these dams had severe intra-gastric bleeding an accepted side affect of non-steroidal anti-inflammatory drugs such as indomethacin. It was decided to lower the dose to 1mg/kg and change the route of administration to subcutaneous injection. This also mimicked the dose and route used in most studies by Gupta in mice.

The bodyweight of both male and female fetuses was reduced at e21.5 in animals exposed to indomethacin in comparison to respective controls. Conversely, the bodyweight of indomethacin exposed adult males was increased significantly in comparison to respective controls. In females however no significant difference was seen in bodyweight at any age after fetal life. This suggests that the litters exposed to indomethacin have a smaller body weight prenatally but this is corrected during development postnatally. Furthermore, the decrease in bodyweight seen at e21.5 was not associated with any other defect or disorder and, in particular, the reproductive tract of

the males was normally developed. It is well established in the literature that intrauterine growth restriction can result in compensatory overgrowth postnatally, especially in males, and consequently an increase in bodyweight/obesity in adulthood, so it is possible that this may have occurred also in indomethacin-exposed males (Desai et al, 2004).

When animals were exposed to 1mg/kg of indomethacin during the MPW, there was very little effect on the development of the male reproductive tract. AGD was unaffected at any age, in comparison to control males. This suggests that androgen action was not disrupted during the MPW. Furthermore, testis weight at each age examined and apparent germ cell numbers at e21.5 were similar between treated and control animals, demonstrating that indomethacin exposure did not induce any gross effect on the testis prenatally or postnatally.

Male penis length at PND8 and PND25 was unaffected by indomethacin exposure in the MPW, however, by adulthood there was a significant decrease in penis length in animals exposed to indomethacin. This is an odd finding as penis length is determined during the MPW and to reach the programmed length, requires postnatal testosterone action, including during puberty (Boas et al, 2006; Van den Driesche et al, 2011). The lack of difference seen at PND8 and PND25 compared to the difference in adulthood suggests there may have been a deficit in testosterone during later puberty as the AGD results suggest that androgen action in the MPW was not affected (Foster and Harris, 2005; Welsh et al, 2008; Macleod et al, 2010). However, it should be noted that only 4 animals from 2 litters were examined in this group and perhaps future studies with an increase in animal number may alter these findings, although the reduction in penile length noted here was not trivial.

Indomethacin exposure during the MPW had small but significant effects in adult females although, as these were based on small animal numbers, it is unclear if they are real or incidental effects. Although no difference was observed in the AGD of control

versus exposed female animals, at e21.5 or at PND25 there was a significant decrease in AGD in indomethacin-exposed females in adulthood in comparison to respective controls. This finding is unexplained and may simply reflect low animal numbers. The one finding that seems to represent a real effect was the mal-positioning of ovaries in indomethacin exposed females in adulthood. What determines ovarian position, other than their site of origin and their attachment to the abdominal wall via the cranial suspensory ligament, is unclear, so it is not possible to suggest how the ovarian mis-location would have occurred, nor whether it would be of any functional consequence.

The studies reported in this chapter were primarily designed to repeat the studies of Gupta et al, which suggested that PGs were important for masculinisation in mice and that blocking PGs by indomethacin exposure in mice, during the critical period of the MPW caused male reproductive tract abnormalities. In contrast, in the investigations for this thesis, exposing rats to 1mg/kg indomethacin during the MPW or in the period prior to the MPW, did not cause any significant impairment of masculinsation in males. No hypospadias or cryptorchidism was noted for any of the male animals and there was no difference in AGD at any age investigated, suggesting that androgen action during the MPW was not affected. Testis weight was also comparable to controls. The only isolated effect found was with regard to reduced penis length in adulthood in indomethacin exposed animals. However, assuming that this effect is real (see above), it must reflect a deficiency in late pubertal or adult testosterone exposure as no effect on this parameter were found at earlier ages. This might indicate, for example, that development or function of adult Leydig cells was somehow altered by fetal indomethacin exposure, but the absence of any change in adult testis weight speaks against this. An alternative would be a defect in growth potential of the penis itself or, for example, in its ability to generate DHT locally for growth/size maintenance. However, as no defects in penis size were found at earlier ages, it is difficult to envisage how such an age-selective effect would occur. Overall, the results, especially those showing unaltered AGD, points to no impairment of androgen action in the MPW as a result of indomethacin exposure.

It is important to consider that transplacental transfer of indomethacin cannot be accurately determined. Some studies suggest that indomethacin only transfers to the fetus just before parturition and that exposure prior to this does not cross the placenta (Aselton et al, 1985; Klein et al, 1981; Traeger et al, 1973). Given the results of this chapter, it is difficult to determine if fetuses were actually exposed to the indomethacin, although bodyweight was affected; however this could have been because of a placental or maternal effect. It is obvious that the indomethacin affected the dams due to their illness and difficulty in giving birth, but just how much of the drug crossed the placenta to expose the fetus was not determined. On the other hand, the resorption or variability in the number of pups from dams treated with indomethacin and the ovary displacement in adult females from this group suggests that sufficient indomethacin did cross the placenta. Previous studies have determined the exact difference in PG expression after indomethacin exposure using enzyme immunoassays (Tanaka et al, 2002), and radioimmunoassays (Shahbazian et al, 2001), but these experiments were in the animal (rat in both instances) being treated with the indomethacin directly rather than determining fetal exposure. Attempts have been made to understand the level of indomethacin crossing the placenta, and it is thought that early in gestation placental transfer is minimal and this increases closer to parturition in rats (Klein et al, 1981) and in humans (Aselton et al, 1985) Although there are publications outlining how indomethacin works and the various effects noted (or absent) in fetuses, not a great deal of advance has been made in quantifying the level of PGs which are inhibited in the fetus by indomethacin exposure. One method of doing this is by radioimmnoassyas (Jaffe et al, 1973; Oki et al, 1974; Thomas et al, 1978), however, some PGs have a very short half life and the accuracy of this method remains to be determined.

One hypothesis which might explain the difference in results reported here and those by Gupta is differences in species. Gupta used mice as the rodent model and the experiments reported here used rats. It may also be important that Gupta did not investigate the effects of indomethacin directly. Rather, masculinisation was disrupted

using exposure to oestradiol 17-β or cyproterone acetate and arachidonic acid was then administered which, rescued masculinisation. It was this rescuing action that was then blocked using indomethacin (Gupta and Goldman, 1986). In the second study, Gupta characterized two different PGs, namely PGE2 and 6-keto-PGF1 in the fetal genital tracts of males and females. The study reported that both males and females had equal levels of expression of these PGs at the indifferent stage, however through development, both PGs increased in expression in the male reproductive tract and decreased in expression in the female reproductive tract. Gupta reported that fetal exposure to testosterone caused an increase in expression of PGE2 and 6-keto-PGF1 in the female reproductive tracts whereas exposure to cyproterone acetate (an anti-androgen, but also has progestational activity) decreased the expression of these PGs in the male reproductive tract. Moreover Gupta reported that exposure of fetuses to indomethacin inhibited PGE2 and 6-keto-PGF1 in the reproductive tract (Gupta, 1989). In the final study by Gupta, Wolffian ducts were put into culture and their testosterone dependent differentiation was blocked using indomethacin (Gupta and Bentlekewski, 1992). Thus perhaps another explanation for the differences in the results reported in this chapter and the results by Gupta may be due to experimental protocols. Perhaps indomethacin can only block masculinisation which has been rescued by arachidonic acid rather than block androgen mediated masculinisation in the absence of arachidonic acid. In addition, cyproterone acetate can also have progestagenic effects and so some of the effects reported by Gupta could have been due to blocking an oestrogen effect, which could decrease testosterone levels and perhaps even AR levels.

In conclusion, exposing fetal rats to 1mg/kg of indomethacin prior to or during the MPW did not affect masculinisation of male pups. A significant decrease in prenatal bodyweight was observed in these animals although it is difficult to be certain that this was due to the indomethacin as opposed to reflecting variability between litters. Furthermore, the F1 generation of females exposed to indomethacin displayed some symptoms of difficulty in plugging and of the two which did, both were late in

parturition. The effects of PG inhibitors on pregnant females, in both rodents and human, are well reported in the literature as well as some of the effects on growth and development of the F1 generation. However, the effects on fecundity and possible difficulties in giving birth in the F1 generation are less well documented and merit further investigation.

6.1 Introduction

Hypospadias is a congenital defect of the penis which consists of an abnormal urethral opening, sometimes associated with abnormal prepuce and ventral penile curvature. Hypospadias involves abnormal location of the urethral meatus which, in relatively mild cases, involves the urethral opening being located on the glans of the penis, in moderate cases on the upper half of the penile shaft and in severe cases at the base of the penis in the perineal region. One cause of hypospadias is when androgen action is subnormal within the masculinisation programming window as reviewed in section 1.1.1.

The androgen dependent molecular mechanisms involved in penis development and hypospadias are poorly understood, if at all (section 1.9.3). The first aim of the studies in this chapter was to study the anatomy of the developing rat genital tubercle (GT) in male and female rats through to postnatal day 25, when an adult type penis is present except that growth is incomplete and preputial separation has not occurred. The second aim was to investigate genes expressed in the GT during the MPW that might be androgen regulated, and to establish if they might be involved in hypospadias. The initial step of understanding the anatomy of the GT was undertaken so as be able to assess the cellular localization of expression of any androgen-regulated genes which would allow a link to specific structures and events in GT development.

The basic approach used in the studies described was to block androgen action in the MPW and to investigate changes in gene expression in the GT by microarray. This study was undertaken in our group by Dr David MacLeod and the microarrays were undertaken using Affymetrix. It was designed to identify potential androgen regulated genes in the MPW, by comparing the genes expressed at e17.5 in rat control (vehicle exposed) male GT (normal androgen action), control female GT (normal ‘absence’ of androgen action in females), flutamide exposed male GT (blocked androgen action) and testosterone exposed female GT (excess androgen action). Several genes were identified to be potentially regulated by androgens (Table 6.1). One such gene was Asporin, which

is a member of the small leucine-rich repeat proteoglycan (SLRP) family but with a unique stretch of aspartate residues (Lorenzo et al 2001). Expression of ASPORIN has been found in various human tissues including aorta, uterus, heart and liver and also in mouse tissues (Lorenzo et al, 2001). Asporin has been shown to bind to TGF-β and to inhibit chondrogenesis, whereas supra-normal expression of Asporin has been linked to oesteoathritis (Nakajima et el 2007). Recent studies have shown that Asporin has the ability to inhibit the activation of BMP receptors (Tomoeda et al 2008) as well as being involved in collagen mineralization (Kalamajski et al 2009). The microarray results indicated that *Asporin* was negatively regulated by androgens.

An alternative to the microarray approach was to investigate candidate genes, which other studies have suggested might be androgen regulated in males. Examples of such genes include *Slit2* and *Robo1*. Studies within another collaborative group in our Unit had suggested that *Slit2* and *Robo1* may be regulated by androgens in the prostate during development and indeed some studies had been published outlining the effect of *Slit2* and *Robo1* expression in prostate cancer cells induced in rats (Latil et al, 2003). In vertebrates, three *slit (Slit1, 2, 3)* and four *Robo (Robo1, 2, 3, 4)* genes have been identified. SLIT is the ligand for the ROBO receptor and it has been shown that the Slit/Robo pathway is involved in tissue growth, development and remodeling. Three main roles have been well documented for Slit/Robo which are i) conserved role in guiding axons during assembly of the nervous system (Andrews et al, 2007; Dickson et al 2002 Hinck et al, 2004), ii) regulating chemotaxis (Prasad et al, 2007; Wu et al, 2001), and iii) suppressing tumourogenesis by promoting apoptosis and inhibiting cell migration (Fujiwara et al 2006). Such roles would be relevant to urethral fold closure during penis differentiation, as this involves substantial cell migration, and thus it was hypothesized that androgen regulation of *Robo1*, and or *Slit2* in the GT might be important in, or after, the MPW. In summary, the aim of the studies in this chapter was to gain an understanding of the anatomy of the developing GT and to investigate the potential androgen regulation of the three aforementioned genes during and after the

MPW. Although the GT was the primary tissue of interest, it was also important to establish if any identified mechanisms of androgen action within the GT might be common to other male (or female) reproductive tract tissues. Therefore, expression of the three genes of interest was also investigated in the testes, ovaries, Wolffian and Müllerian ducts.

6.2 Materials and Methods

6.2.1 Animals and treatments

Briefly, pregnant rats were treated with flutamide, TP, DHT or DES or appropriate vehicle during the MPW (section 2.2). The genital tubercle was dissected from rat fetuses at e16.5, e17.5, e19.5 or e21.5, from both male and female pups. The GT was fixed in Bouins and processed for immunohistochemical analysis or frozen at -80°C for RNA analysis.

6.2.2 Anatomical analysis

Due to the nature of the GT tissue and its differing cellular composition throughout vertical planes, the most popular method of studying this tissue is to take serial sections from the base through to the tip of the GT. Immunohistochemistry is usually conducted on every 2-3 sections. This technique had not been established within our group prior to this PhD and optimizing the correct orientation of tissue during embedding was vital for identification of tissue regions and thus correct interpretation of results. This proved challenging and was best achieved by using a green biomarker (section 2.3.2) to mark the tip of the GT which was then embedded in paraffin wax in a vertical manner.

The anatomy of the GT and flutamide effects were initially identified by simply using H&E staining (section 2.9).

6.2.3 RNA Analysis

Briefly, RNA was isolated from frozen GT and converted into cDNA and Taqman quantitative RT-PCR performed to establish quantitative mRNA levels. The genes investigated and primers used are listed in Table 6.1. The expectation was that if any of the candidates were important in androgen regulated GT development, then expression would switch on during and / or after the MPW and this pattern would be disrupted by flutamide in males and induced in the GTs of females exposed to testosterone.

Table 6.1 List of primers and probes used in Taqman-PCR analysis in chapter 6

Gene	**Forward Primer 5'→3'**	**Reverse Primer 3'→5'**	**UPL Probe Number:**
Asporin	AAG AAA ACC TCT CTT CTG ACA AGG	GCA CAC AGC CAA AAG CAG TA	56
ROBO1	GCA GAG AGG CCT ACA CAG ATG	CAC TGG GCG ATT TTA TAG CAG	10
SLIT2	GCC ATT CAG GAC TTC ACC TG	CGA AGG ACA ACG GGA GAG	108

6.2.4 Optical Projection Tomography (OPT)

In order to image the GT as a whole, optical projection tomography (section 2.7) was carried out on e21.5 control male GT. This software is attached on a CD, with instructions (appendix 3).

6.3 Results

6.3.1 Anatomy of the GT and the postnatal day 25 penis

In order to understand the anatomy of the rat penis under normal conditions, two approaches were taken. The first of these was to serial section a male control PND25 penis and stain using haematoxylin and eosin (H&E) (section 2.9) and image. The second approach used Optical Projection Tomography (OPT) to gain insight into the 3-dimensional internal structure of the e21.5 GT (Fig.6.2).

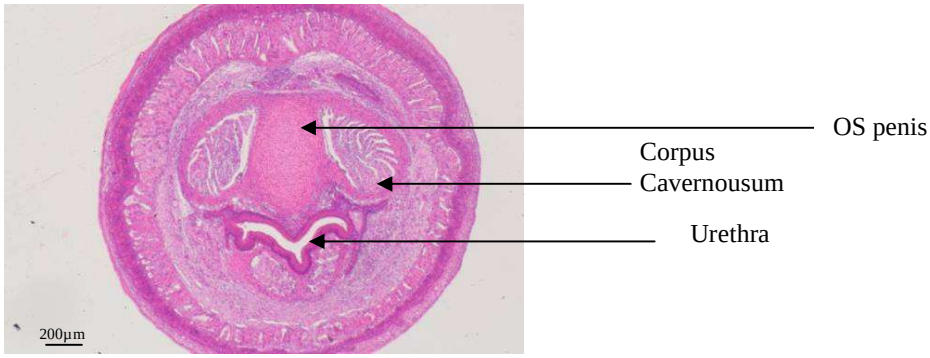


Figure 6.1 H&E stain of control male rat PND25 penis-higher power image from Fig.6.3 to clearly distinguish OS penis, corpus cavernosum and urethra.

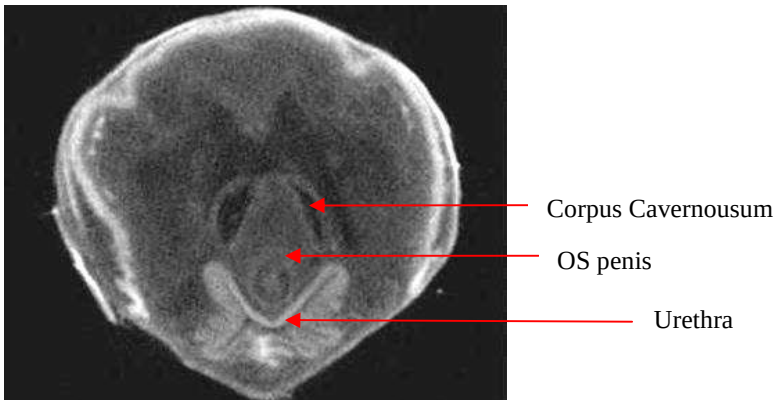


Figure 6.2 Still OPT image of control e21.5 male rat GT. Urethra, corpus cavernousum and OS penis are clearly visible

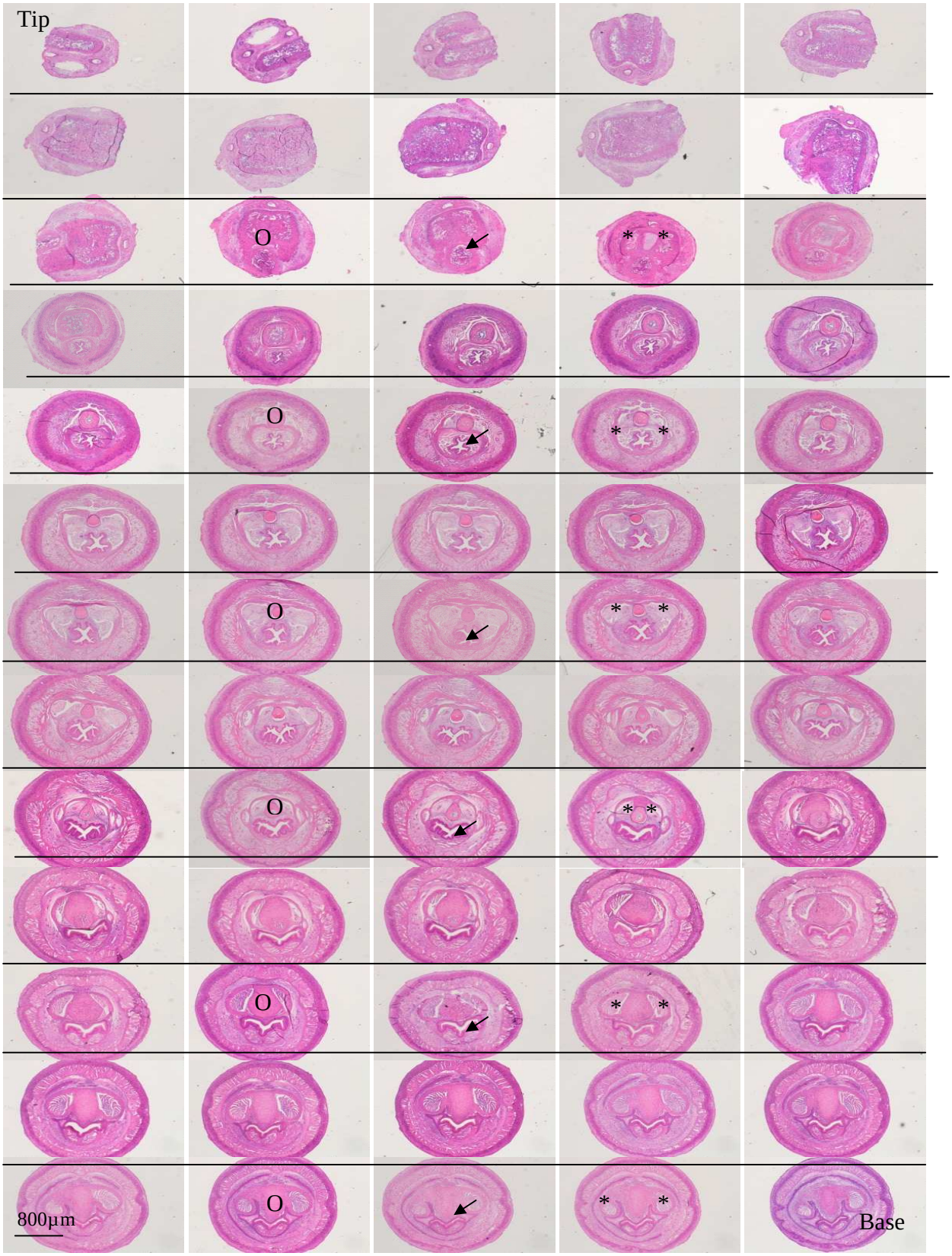


Figure 6.3 Representative serial sections from the tip to the base of a PND25 control male penis. For guidance, section order is by rows, 'O' represent the OS penis, the urethra is indicated by an arrow and the corpus cavernousum is marked by asterisks in some of the sections.

Serial sectioning and H&E showed the various internal structures of the fully formed penis, namely the OS penis, urethra, corpus cavernosum and how each structure varied in morphology depending on their specific location between distal and proximal regions (Fig 6.1 and 6.3). This information was supported by the OPT 3D reconstruction, a still image of which (Fig 6.2) also clearly shows how the various key structures within the developing penis. The complete imaging movie and associated software are attached in a CD (appendix 3).

6.3.2 Effect of flutamide exposure on gross morphology of the penis

To visualize the impact that androgen action had on structural development of the penis, pregnant animals were treated with various doses of flutamide during the MPW and the fetuses or offspring then studied. The dose response in Fig 6.4 illustrates the increasing severity of hypospadias corresponding with the increasing dose of flutamide administered.

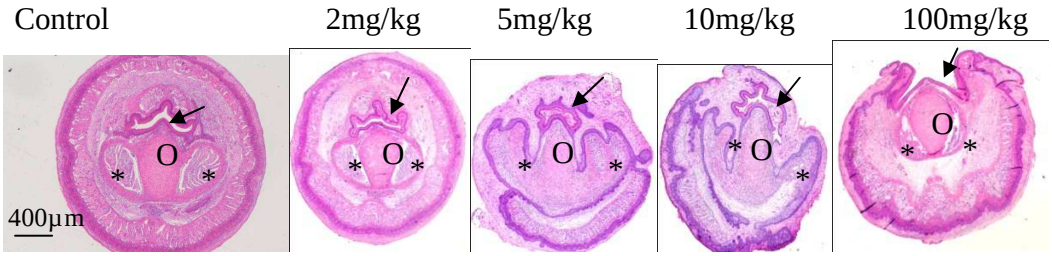


Figure 6.4 Representative penile cross sections at PND25 from animals exposed to various doses of flutamide during the MPW. O=OS penis, the arrow indicates the the urethra and *= the bilateral corpora cavernosa. Note that as the dose of flutamide increases, the more superficial the urethra becomes, such that after exposure to 100mg/kg flutamide the urethra lies open at the surface of the penis (=hypospadias).

Flutamide exposure in the MPW induces hypospadias in a dose responsive manner (Welsh et al, 2008, 2010). When exposed in the MPW to 2mg/kg flutamide, no obvious defects of the penis were evident at PND25 but exposure to higher flutamide doses resulted in progressively worse disruption of normal penile anatomy and in particular the

failure of complete urethral closure (i.e. hypospadias) but also the OS penis and corpora cavernosa became malformed, appearing as a single rather than a bilateral structure (Fig. 6.4).

6.3.3 Investigation of gene expression in the GT and the effects of flutamide

6.3.3.1 Expression of *Asporin*

In order to identify, with as much certainty as possible, androgen regulated genes in the GT four treatment groups were chosen and GTs collected on e17.5; the groups were control male, flutamide exposed male, control female and testosterone exposed female. Three GTs were pooled from three separate litters and the three pools from each treatment group then run on the microarray. E17.5 was chosen for study because it is towards the end of the MPW and should therefore capture any androgen-regulated change in gene expression that is specific to the MPW. The microarray results (affymetrix) found the following pattern of *Asporin* expression in rat GT at e17.5:

Table 6.2 Results of affymetrix microarray results for *Asporin* expression in rat e17.5 GT. N=9 from 3 different litters.

GT comparison	**Asporin expression –fold change**
e17.5 Flutamide males vs e17.5 control males	+1.74
e17.5 control females vs e17.5 control males	+1.78
e17.5 flutamide males vs e17.5 testosterone females	+2.10
e17.5 Testosterone females vs e17.5 control females	-2.15

Each of the comparisons of the four treatment groups investigated strongly indicated that *Asporin* may be negatively regulated by androgens in the GT during the MPW, and for each comparison the comparison was statistically significant ($p<0.05$). To validate the microarray results, and quantify the change in expression of *Asporin*, Taqman PCR was conducted on the following GT tissues at e17.5; vehicle exposed male, flutamide

exposed male, vehicle exposed female and testosterone exposed female. All animals were exposed during the MPW and were from a separate study to those used for the microarray analysis to ensure independent verification.

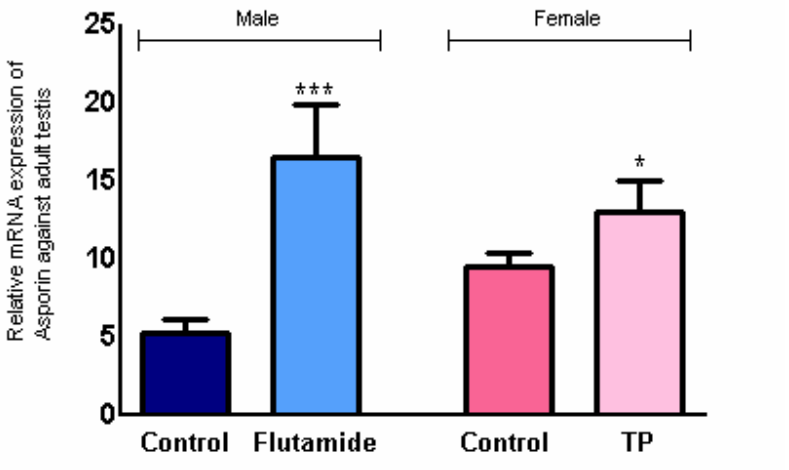


Figure 6.5 Quantitative analysis of *Asporin* mRNA level in e17.5 rat GT. Usnig a one way ANOVA* P<0.01 in comparison with respective female control. *** P<0.001 in comparison to respective male control Values are means ±SEM for 12 animals/group from a minimum of 4 litters.

Taqman confirmed 3 out of the 4 microarray results for e17.5 GT expression of *Asporin*. In males exposed to flutamide from e15.5-e16.5 (MPW), *Asporin* expression was significantly increased in the GT at e17.5 in comparison to the vehicle exposed males. E17.5 control female GTs also expressed *Asporin* at a higher level than did control male GTs, although this increase was not statistically significant. Interestingly, the results showed that GTs from females which had been exposed to testosterone during the MPW, had a significantly increased expression of asporin in comparison to vehicle exposed females at e17.5. This was in complete contrast to the results from the microarray, which showed that GTs from females which had been exposed to testosterone during the MPW had decreased *Asporin* expression in comparison to control females.

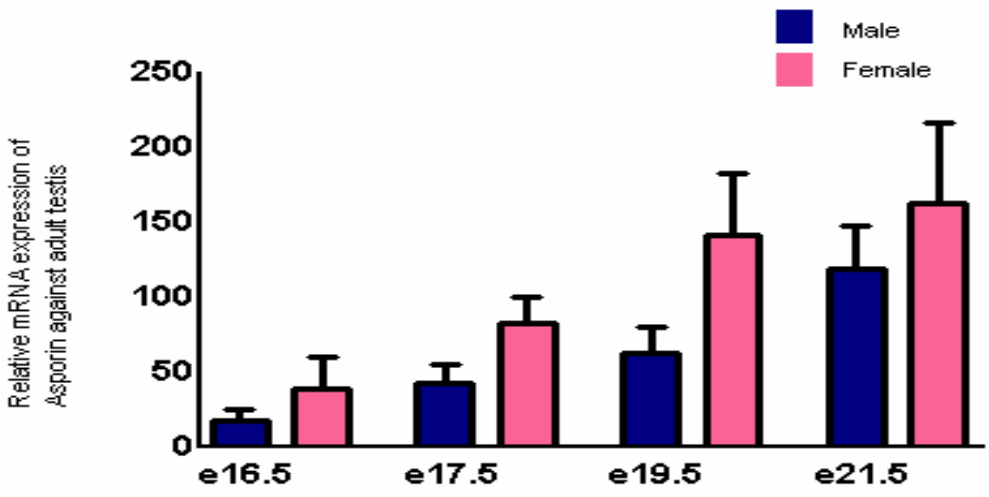


Figure 6.6 Quantitative analysis of *Asporin* mRNA level in the developing male and female GT according to fetal age. Values are means ±SEM for 6-10 animals/group from a minimum of 4 litters. Using a two way ANOVA a significant difference of Asporin expression was observed among the four ages ($P < 0.01$) and between the two sexes ($P<0.05$). Bonferroni post-hoc tests showed no sex differences at any of the individual ages

In order to determine if the difference in *Asporin* expression between control males and females changed during and after the MPW, Taqman analyses were conducted on GT from vehicle exposed (during the MPW) males and females at e16.5, e17.5, e19.5 and e21.5 (Fig. 6.6). *Asporin* expression was consistently higher in the female GT than in the males throughout development. Although none of the results were individually significantly different, a significant overall difference was found between the two sexes as well as a significant difference between the ages. An increase in tissue size may explain the increase in *Asporin* expression through the developing GT.

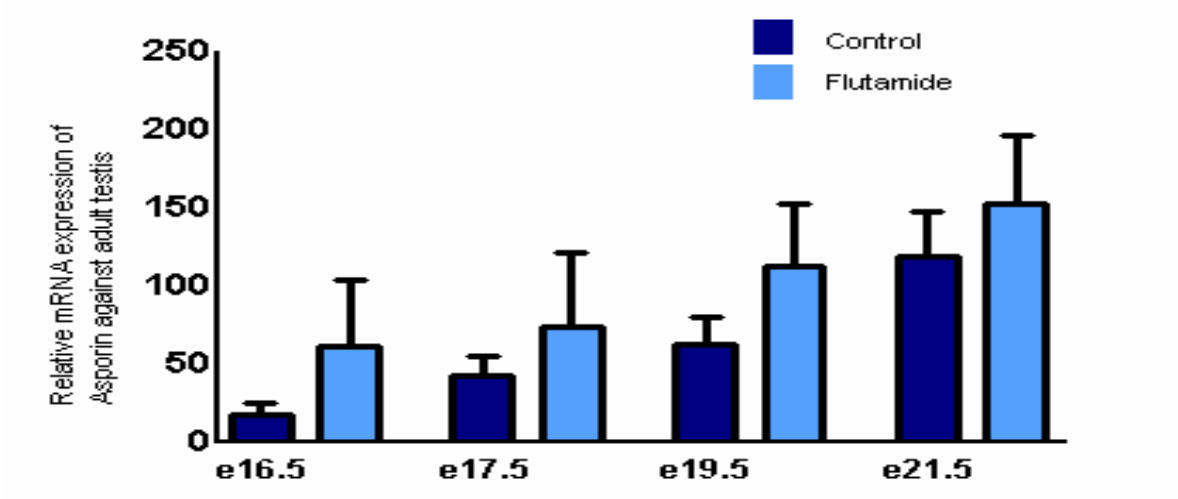


Figure 6.7 Quantitative analysis of *Asporin* mRNA level in the male GT from fetuses exposed to either vehicle or flutamide during the MPW. Values are means ±SEM for 8-10 animals/group from a minimum of 4 litters. At no age were values for control and flutamide significantly different. Using a two way ANOVA a significant difference of Asporin expression was observed among the four ages ($P<0.05$) but no significant difference was found between control and flutamide exposed tissue.

The initial microarray validation experiment (Fig. 6.5) showed that *Asporin* expression increased in the e17.5 male GT when androgen action was blocked during the MPW using flutamide, in comparison to vehicle exposed males, when androgen action is normal. A similar trend of expression was found at e16.5 and also after the MPW at e19.5 and e21.5, and using a two way ANOVA no significant difference in *Asporin* expression was found between control and flutamide exposed fetuses but there was a significant difference among ages, overall, which was probably due to an increase in tissue size.

Possible role for oestrogens

As described above, there was considerable variability and even conflict in some of the results on *Asporin* mRNA expression after fetal exposure to flutamide and especially exposure to TP. In considering potential explanations, it was recognized that variable contribution from oestrogens or from disturbance of the androgen-oestrogen balance

could potentially play a role, especially as the GT is an established oestrogen target (Kalfa et al, 2008; Yucel et al, 2003). For example, as TP can be aromatized to oestradiol, this could have exerted effects that were being interpreted as 'androgenic'. Even the effects of flutamide could conceivably result from disturbance of the normal androgen-oestrogen balance, because by blocking androgen but not oestrogen action, this balance is automatically altered. To address some of these possibilities, pregnant rats were therefore dosed during the MPW with either DES (a potent, synthetic oestrogen) or with DHT, which is a more potent androgen than testosterone but which cannot be aromatized to oestradiol.

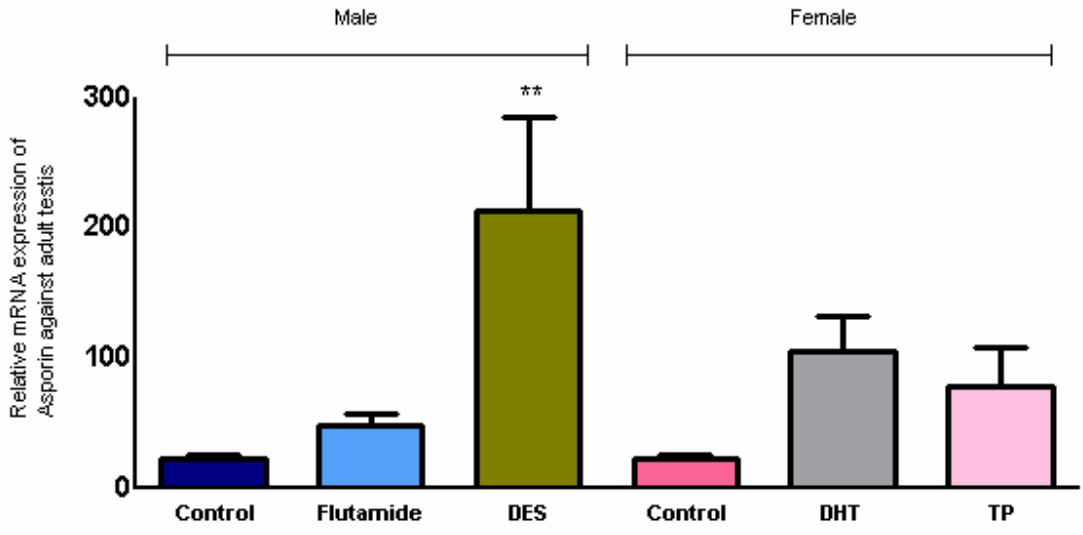


Figure 6.8 Quantitative analysis of *Asporin* mRNA level in the e17.5 GT after exposure of fetuses during the MPW to flutamide, DES, TP or DHT. Usnig a one way ANOVA **P<0.0025 in comparison to control male, flutamide male, control female, DHT and TP female. Values are means ±SEM for 6-8 animals/group from a minimum of 3 litters.

Results shown in Fig 6.8 show that *Asporin* expression in the male GT increased dramatically in DES-exposed fetuses. This increase in expression was significant when compared against the control male GT value, but was also significantly higher ($P<0.05$) than and the values for flutamide exposed males. Although at face value the increase in expression of asporin in GTs from DES exposed male fetuses suggests that oestrogens

can regulate asporin expression, it may also be related to distortion of the androgen:oestrogen balance, which can be fundamentally important in males (see section 1.5). DES treatment also reduces testosterone production by the fetal rat testis (Haavisto et al, 2003), which means that it will severely distort the endogenous androgen:oestrogen balance by both lowering androgen action at the same time as elevating oestrogen action. In contrast, although flutamide reduces androgen action it will not elevate oestrogen levels, so that there is a smaller distortion of the androgen:oestrogen balance than in the case of DES. This is speculation, but would perhaps reconcile the flutamide and DES results in males.

As found in the earlier study (Fig. 6.5), TP exposure of females increased asporin expression in the GT, as also did DHT (Fig. 6.8), suggesting that the effect is androgen-mediated as opposed to alteration of the androgen:oestrogen balance, although both treatments will increase the androgen:oestrogen balance, if this is important in females. Irrespective of the explanation the fact that *Asporin* expression changes in the same direction in females exposed to androgens as in males in which androgen action is decreased, points to a fundamental difference (opposite) in response between the sexes as far as the GT is concerned.

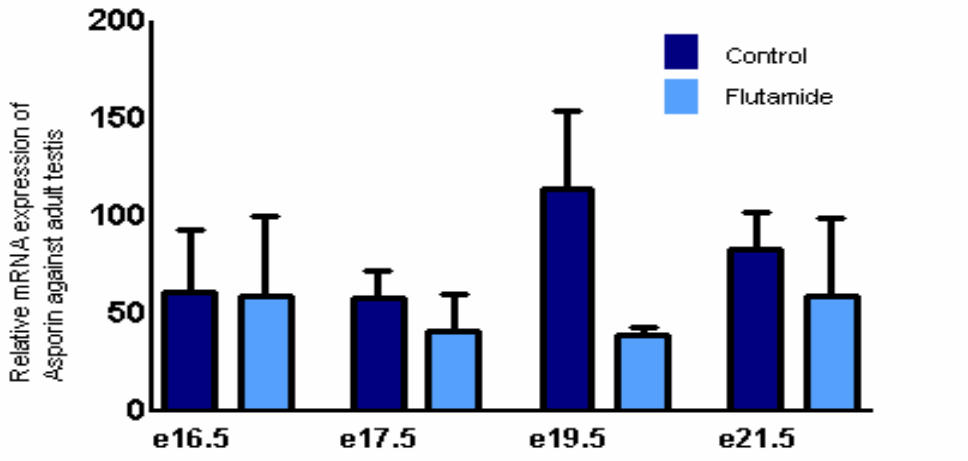


Figure 6.9 Quantitative analysis of *Asporin* mRNA levels, at various fetal ages in the WD of males exposed to either vehicle or flutamide during the MPW. Values are means ±SEM for 3-5

animals/group from a minimum of 3 litters. Using a one way ANOVA, no significant difference was found between GTs of control fetuses in comparison to those of flutamide exposed animals.

In order to determine if the treatment-related changes in expression of *Asporin* found in the GT was common to other male (or female) reproductive tract tissues, the expression of *Asporin* was also investigated in the testes, ovaries, Wolffian and Müllerian ducts. No pattern emerged which suggested that *Asporin* expression may be negatively regulated by androgens from any of these tissues. For example, the expression of *Asporin* was similar between control fetuses and flutamide exposed fetuses in the testis and WD and no significant difference were found between the treatment groups or ages, an example of which is shown in Fig. 6.9, but other results are not presented.

6.3.3.2 Investigation of *SLIT2* expression in the GT

The slit2/robo1 pathway was suggested to be androgen regulated in the prostate. Therefore Taqman-PCR was used to determine if either this ligand *(Slit2)* or its receptor *(Robo1)* are expressed in the rat GT. As the tissue for the *Asporin* analysis had already been collected and appropriate cDNA available, it was decided to use the same approach to investigate the expression of *Slit2* and *Robo1*.

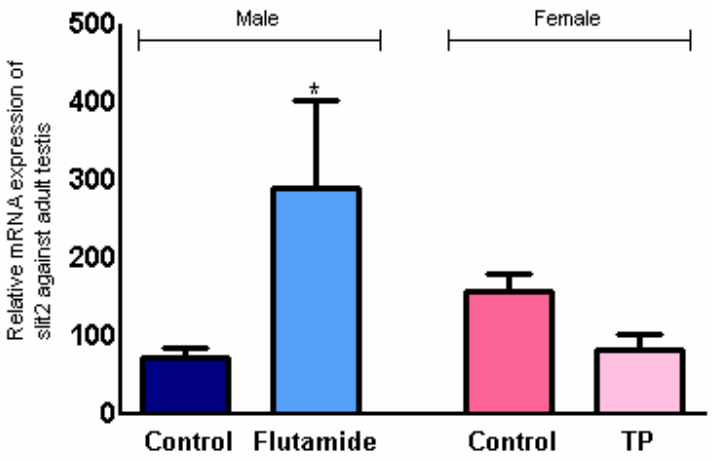


Figure 6.10 Quantitative analysis of *Slit2* mRNA level in e17.5 rat GT after exposure to vehicle, flutamide, or TP during the MPW.Using a one way ANOVA * P<0.01 in comparison with respective male control. Values are means ±SEM for 12 animals/group from a minimum of 4 litters.

Expression of *Slit2* increased in e17.5 male GT from fetuses that had been exposed to flutamide during the MPW, in comparison to the expression of *Slit2* in GT from vehicle-exposed e17.5 males. Furthermore, e17.5 control female GT also expressed slit2 at a higher mean level than did the male control tissue, although this difference was not statistically significant (Fig. 6.10). Mean *Slit2* expression was also decreased in the GT of female fetuses exposed to testosterone during the MPW, in comparison to control females, although again this difference was not statistically significant. Nevertheless, viewed overall, the results suggested that *Slit2* might be negatively regulated by androgens. To investigate *Slit2* expression further in the MPW and later, Taqman-PCR was conducted on the GT through development from e16.5-e21.5, in both control male and female fetuses.

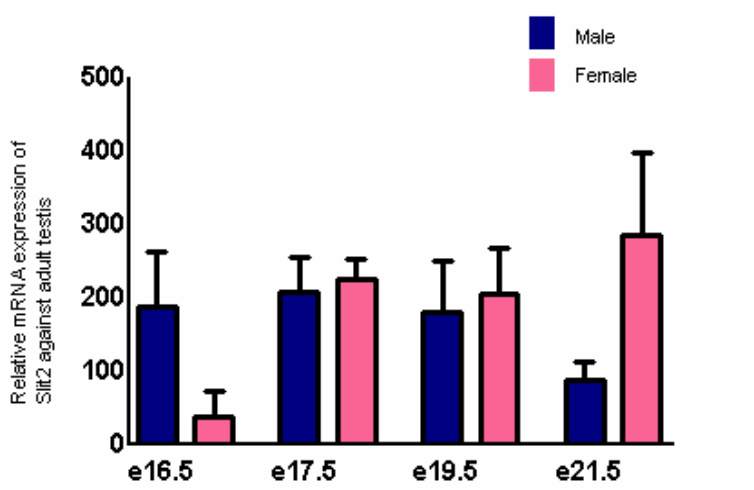


Figure 6.11 Quantitative analysis of *Slit2* mRNA expression at various fetal ages in the developing male and female GT. Using a one way ANOVA no significant difference was found between male and female GT expression of *Slit2*. Values are means ±SEM for 6-10 animals/group from a minimum of 4 litters.

Although this analysis showed some possible differences between *Slit2* expression in male and female GTs, these differences were inconsistent in direction and none was statistically significant (Fig. 6.11). It was therefore concluded that a simple male-female

comparison does not support the notion that androgens negatively regulate *Slit2*. To test this conclusion more directly, an age course study was undertaken comparing *Slit2* expression in GT from control males and males exposed to flutamide during the MPW (Fig. 6.12).

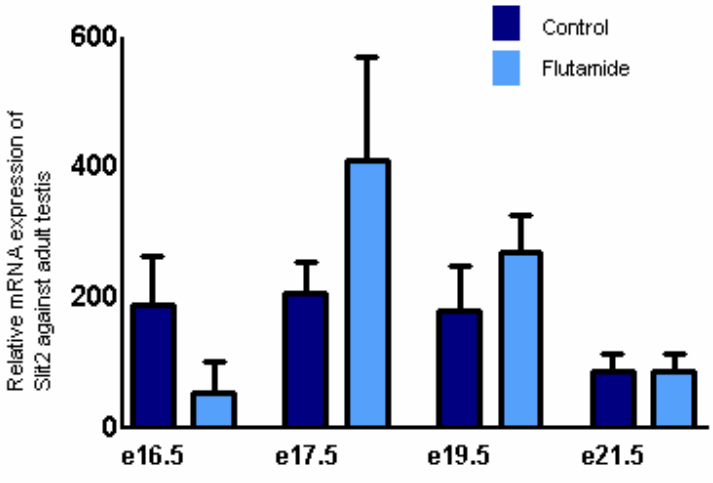


Figure 6.12 Time course quantitative analysis of *Slit2* mRNA level in the male GT form vehicle exposed controls and males exposed to flutamide during the MPW. Values are means ±SEM for 8-10 animals/group from a minimum of 4 litters. Using a one way ANOVA no significant differences were found between control male GTs and those exposed to flutamide

Similar to what was found in the original study (Fig. 6.10) an increase in the expression of *Slit2* was found in the e17.5 GT of male fetuses exposed to flutamide, but this increase was not statistically significant (Fig. 6.12). Additionally, no significant differences were found in the expression of *Slit2* between control and flutamide groups at other ages, namely at e16.5, e19.5 and e21.5 (Fig.6.12). These results therefore seem to suggest that *Slit2* is not androgen-regulated in the GT during or after the MPW. However, bearing in mind the thoughts about importance of the androgen:oestrogen balance discussed above for asporin expression, the effect of fetal exposure to flutamide, DHT, DES or testosterone, during the MPW on GT expression of *Slit2* at e17.5 was investigated (Fig. 6.13).

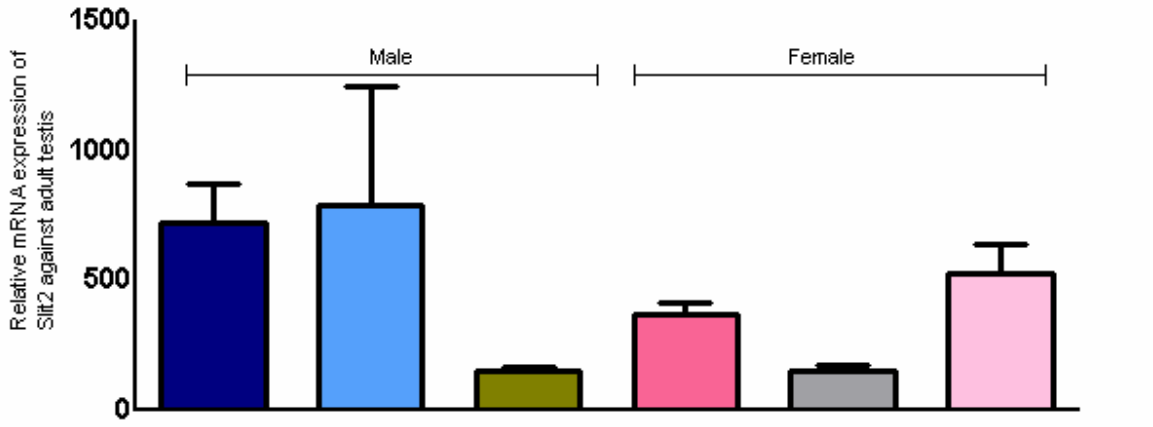


Figure 6.13 Quantitative analysis of *Slit2*mRNA levels in the e17.5 GT after exposure of fetuses during the MPW to flutamide, DES, DHT or TP. Usig a one way ANOVA, no significant difference was found between the treatment groups. Values are means ±SEM for 6-8 animals/group from a minimum of 3 litters.

This analysis showed a number of differences in mean *Slit2* expression levels between males and females ± exposure to flutamide, DES, TP or DHT, but none of the differences were statistically significant, again not supporting the notion that *Slit2* might be negatively regulated by androgens in either males or females, and with insufficient data to conclude that oestrogens might negatively regulate *Slit2* expression in the male GT (Fig.6.13). Overall, perhaps the most reasonable conclusion is that *Slit2* expression is naturally quite variable and that the one original results pointing to androgen regulation (Fig. 6.10) was simply a chance effect.

6.3.3.3 Investigation of *Robo1* expression in the GT

In parallel to determining the expression of *Slit2* and possible androgen regulation in the GT, expression of the gene for the *Slit2* receptor, *Robo1*, was also investigated using Taqman-PCR. The same samples and method of investigation were used as described for asporin and *Slit2*.

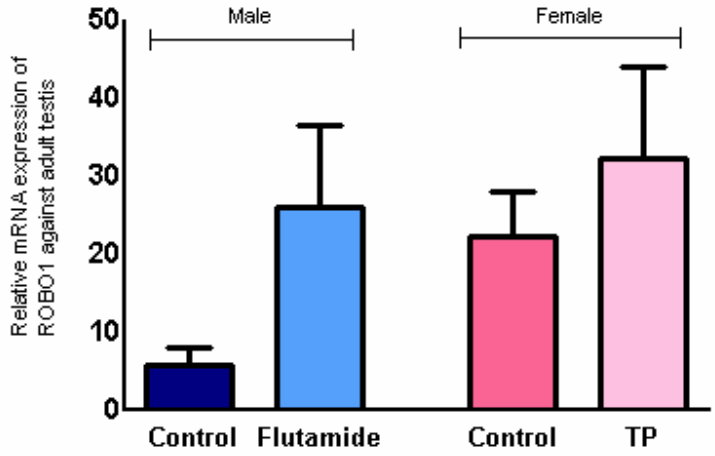


Figure 6.14 Quantitative analysis of *Robo1* mRNA level in e17.5 rat GT. Values are means ±SEM for 12 animals/group from a minimum of 4 litters. Using a one way ANOVA there were no statistically significant differences between any of the groups.

Fig. 6.14 shows the Taqman-PCR results for the expression of *Robo1* in e17.5 GT. There were no significant differences in *Robo1* expression between male and female GT and no significant impact of flutamide or TP exposure, although there were suggestive trends. For example, in males when androgens were blocked in the MPW using flutamide, an increase in mean *Robo1* expression was found in the e17.5 male GT in comparison to e17.5 control male GT. Another trend was an increase in expression of *Robo1* in the GT of female fetuses exposed to testosterone during the MPW in comparison to control females and males. In order to follow this up, *Robo1* expression was investigated in control male and female GT at e16.5, e17.5 and after the MPW at e19.5 and e21.5.

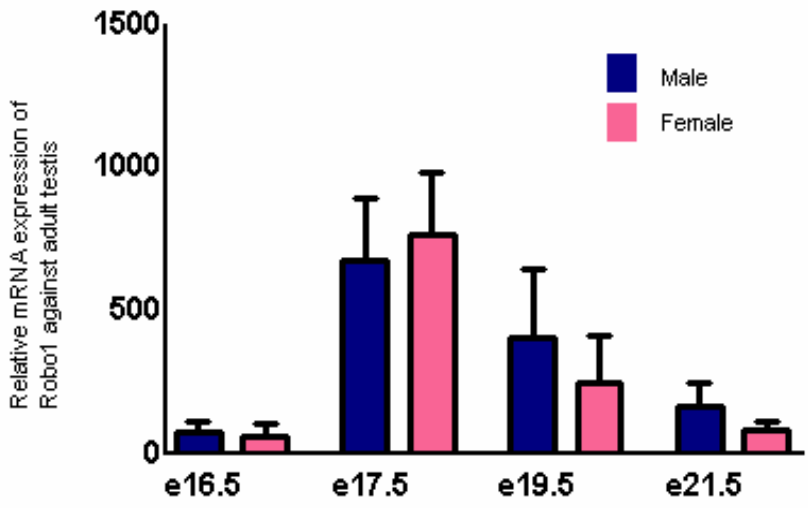


Figure 6.15 Time course quantitative analysis of *Robo1* mRNA level in the developing male and female GT. Values are means ±SEM for 6-10 animals/group from a minimum of 4 litters. Using a one way ANOVA there were no significant differences between males and female at any age.

Taqman-PCR showed that *Robo1* was expressed at similar levels between control male and control female GTs throughout fetal development, and this expression was at its highest during the MPW at e17.5 (Fig. 6.15). In order to completely rule out the possibility that *Robo1* was androgen regulated, Taqman-PCR was conducted on GT from fetuses across the fetal age range after exposure to flutamide during the MPW and compared to the GT of control fetuses. In addition, the effect of DES and DHT exposure on *Robo1* expression were investigated.

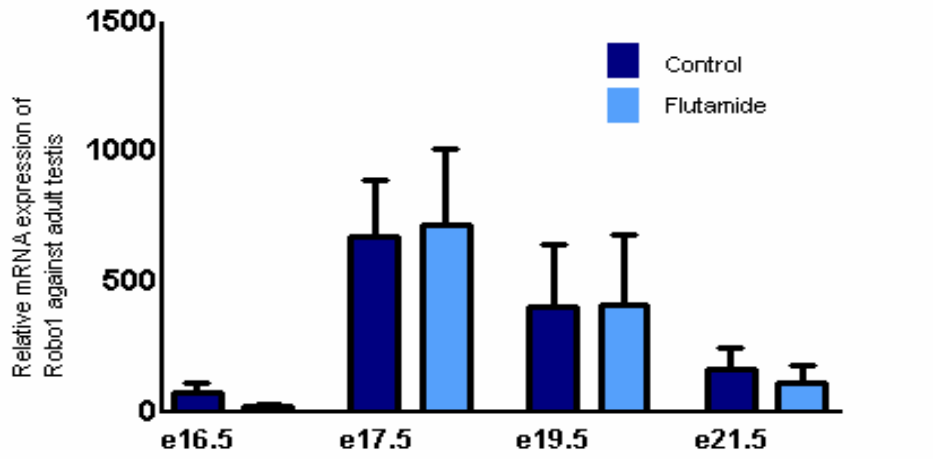


Figure 6.16 Time course quantitative analysis of *Robo1* mRNA level in the male GT from control males and those exposed to flutamide during the MPW. Values are means ±SEM for 8-10 animals/group from a minimum of 4 litters. Usnig a one way ANOVA was no significant effect of flutamide exposure at any age.

An earlier experiment (Fig.6.15) that found a suggestive upward change in *Robo1* expression in e17.5 male GT after exposure to flutamide, was not confirmed in the time series set of experiments (Fig.6.16); in fact *Robo1* was found to be expressed at similar levels in the GT between control and flutamide exposed fetuses at all ages, although the expression was highest at e17.5 during the MPW.

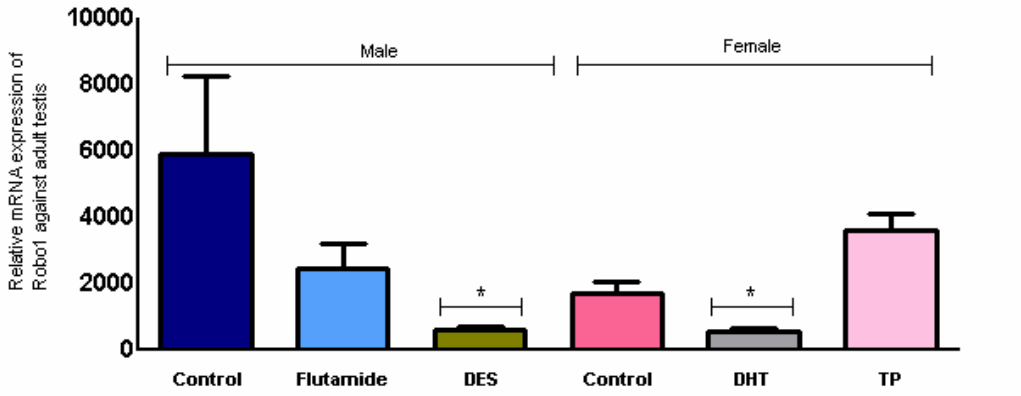


Figure 6.17 Quantitative analysis of *Robo1* mRNA levels in the e17.5 GT after exposure of fetuses during the MPW to flutamide, DES, DHT or TP. Using a one way ANOVA *P<0.05 in comparison to control male GT. Values are means ±SEM for 6-8 animals/group from a minimum of 3 litters.

The results for *Robo1* expression reported in Fig. 6.14, Fig. 6.15 and Fig. 6.16 were inconsistent and variable. However as *Robo1* is the receptor for *Slit2,* it was of interest to determine if patterns of *Robo1* expression in the GT were similar to that of *Slit2*. Taqman-PCR was used to investigate *Robo1* expression in e17.5 from fetuses which had been exposed to vehicle, flutamide, DES, DHT or testosterone during the MPW (Fig. 6.17). Consistent with what was found for *Slit2* expression patterns (Fig. 6.13), DES exposure of males lowered *Robo1* expression whereas DHT exposure lowered *Robo1* expression in female GTs (Fig. 6.17). It seems unlikely that *Robo1* is androgen or oestrogen regulated and as postulated with the asporin results, the possibility arises that disruption of the androgen:oestrogen balance may affect *Robo1* expression, however overall patterns of expression of *Robo1* in the various treatment groups was not repeatable and it seems likely that *Robo1* expression in the GT is highly variable.

6.4 Discussion

Originally, there were three main aims to the studies in this chapter; 1) to understand the anatomy of the developing genital tubercle and postnatal penis, 2) to investigate if three candidate genes might be androgen regulated during and after the MPW in the GT and 3) to use the understanding of the anatomy of the developing GT to asses cellular localization of any androgen regulated genes. These investigations were designed to better understand the specific structures and events in GT development during and after the MPW and to gain insight into possible androgen regulated genes within the MPW which might be linked to the origins of hypospadias.

Two methods were used to study the anatomy of the GT; serial sectioning and OPT. Combined, these provided insight into the key structures, namely the urethra, OS penis, corpus cavernosum, and how these structures developed within the GT over time. Embedding of the GT at any age before e19.5 proved to be problematic. The GT is very small during the fetal period and it was a difficult task to dissect and embed this tissue in a consistent orientation to provide reproducible results in relation to same plane of transverse section and orientation. The aim was to embed the tissue 'tip' down in a vertical manner to allow serial sectioning from base to tip. However, even if the GT was at a slight angle, the sections were not then consistent with regards to plane of section through the tissue, and this was potentially exacerbated by flutamide treatment which can result in curvature of the GT/penis. Furthermore, during the MPW, the GT is not as elongated as it is after the MPW, and it was difficult to identify the tip and base from the almost circular structure that is the GT at e17.5 or before. Consequently, a new approach was taken, and this was to mark the 'tip' of the GT during micro-dissection using a green biomarker. This approach provided good results for e19.5 and e21.5 GT; however it was still difficult to embed the smaller GTs from younger age animals and in a consistent orientation. This was also encountered when trying to embed tissue for OPT analysis. It was possible to embed GTs from e21.5 onwards but prior to this the orientation was difficult and only provided ambiguous results. OPT of e21.5 GT

however provided an elegant method to observe the internal morphological changes in structure of the intact GT from the base to the tip, and to compare against individual cut sections to identify the exact plane of section being investigated.
Animals were exposed to various doses of flutamide during the MPW to determine the effects of blocking androgen action on the developing GT. Increasing doses of flutamide clearly correlated with increasing severity of hypospadias such that closure of the urethra was completely inhibited in the GT of males exposed to 100mg/kg flutamide but to a lesser (more anterior) and more variable extent at lower doses.

Three genes were chosen for investigation of their expression in the GT during the MPW. *Asporin* was chosen from the genes identified through the microarray study which strongly suggested asporin to be negatively regulated, in the GT, at e17.5 at least. *Slit2* and *Robo1* (ligand and receptor respectively) were chosen as these two genes were also suggested to be negatively regulated by androgens in the prostate in work done by another Unit group. The published roles of each of the three chosen genes, and pathways they are involved in could be relevant to the development of the GT. Therefore their expression was studied primarily in GT at e17.5 during the MPW, and then extended to time points after the MPW. Gene expression was studied in GT of fetuses from four different 'androgen' settings; 1) in the GTs of male fetuses exposed to vehicle, in which androgen action is considered normal, 2) in the GT of male fetuses that were exposed to flutamide, in which androgen action is blocked 3) in the GT of female fetuses that been exposed to vehicle and normal androgen action is considered absent/ minimal and 4) in the GT of female fetuses that had been exposed to testosterone during the MPW and in which androgen action is 'enhanced'. It was not possible to validate the microarray results for the GTs from female fetuses exposed to testosterone during the MPW and this raised the possibility that testosterone exposure in the female fetuses, may be being aromatized to oestradiol and the changes in asporin expression might be an oestrogen response rather than an androgen response. Therefore it was decided to expose animals

to DES or DHT during the MPW to further elucidate if the observed changes in expression might be due to androgen or oestrogen regulation.

The microarray results for *Asporin* expression in e17.5 GT were validated using Taqman RT-PCR. The validation experiments used a different set of GTs to the samples sent for the microarray and also increased the 'n' numbers in comparison to the microarray. Three of the four results from the microarray were validated and these experiments showed the GT of control female fetuses expressed asporin at a higher level than control male fetuses, thus in the absence of 'normal androgen action' asporin expression increases. Furthermore, the GT of male fetuses that had been exposed to flutamide during the MPW, had a higher level of *Asporin* expression in comparison to control male GT. This result also suggested that in the absence of androgen action, *Asporin* expression increase in the GT. RT-PCR was used to determine if both of these results, which suggested that *Asporin* expression is negatively regulated by androgens, was also found at other ages in the developing GT. Indeed, a significant trend was found whereby female control GTs expressed *Asporin* at a higher level than male control GTs, at e16.5, e17.5, e19.5 and e21.5. In addition, a significant difference was also was also found in GTs of male fetuses that had been exposed to flutamide during the MPW which expressed *Asporin* at higher level than control male GT at e16.5, e17.5, e19.5 and e21.5. Thus it could be hypothesized that *Asporin* expression was negatively regulated by androgens, although the degree of androgen regulation was perhaps modest and somewhat inconsistent. In further studies, the GT from male fetuses that had been exposed to DES expressed *Asporin* at a significantly higher level than in any other treatment group investigated and this result might suggest that oestrogens can positively regulate asporin expression. This might explain the TP results from the validation experiments, whereby TP might be aromatized to oestradiol and then exert an effect. However, if this theory were true, it would not explain why the GT from female fetuses exposed to DHT also had an increased expression of *Asporin* in comparison to male and female control GTs but also to male GTs which had been exposed to flutamide, unless

there is an inherent difference between male and female GTs. Another possible explanation is that, in each of the treatment groups, the androgen-oestrogen balance was disturbed and in each treatment group when this balance is disturbed, asporin expression increases in comparison to control male and female GTs. Although these results looked potentially interesting, the studies were hindered by the lack of a good working antibody for ASPORIN in the rat, which would have established if asporin protein is expressed in the GT, if similar effects were seen in the various treatment groups and what cells and structures within the GT expressed asporin.

The first Taqman-PCR experiment that investigated *Slit2* mRNA expression suggested that it might be negatively regulated by androgens, as the GT of male fetuses exposed to flutamide, in which androgen action is blocked, expressed *Slit2* at a significantly higher level than did control male GT. Furthermore, when exposed to androgens, in the form of TP, female GT had a decreased expression of *Slit2* in comparison to female controls (i.e. changing in a 'male' direction). In addition, control female GTs expressed asporin at a higher level than did control male GTs. Thus, this expression pattern was investigated at different ages in the developing GT. However, *Slit2* expression was not found to be significantly different in the GTs of control male and female fetuses at e16.5, e17.5, e19.5 and e21.5. Furthermore, the difference in *Slit2* expression in the male and female control GTs, found at e17.5 in the previous experiment, was not repeatable. Lastly, *Slit2* expression at e16.5, e17.5, e19.5 and e21.5 in the GT of male fetuses exposed to either flutamide or vehicle, found that *Slit2* was expressed at similar levels in the control male GT throughout development from e16.5-e21.5, and although small increases in *Asporin* expression were found after flutamide exposure, these were only evident at e17.5 and e19.5 and were not statistically significant. Further investigation of *Slit2* expression in male and female fetuses exposed to DES or DHT did not provide any more conclusive insight. Thus, viewing results from the various separate experiments overall, it appeared that *Slit2* expression was highly variable (at e17.5) in expression and was not obviously androgen-regulated.

A similar scenario to *Slit2* was found for results for *Robo1*, the receptor for *Slit2*. The initial experiment investigating *Robo1* expression suggested that disturbance of the androgen-oestrogen balance in GTs from fetuses, which had been exposed to flutamide or TP, increased *Robo1* expression in comparison to control male GT. However further studies in this age group and also at e16.5, 19.5 and e21.5 found that these results were not repeatable and the latter experiments suggested *Robo1* mRNA was expressed at similar levels in male and female GTs and little difference was found in *Slit2* expression of the GT of fetuses exposed to flutamide in comparison to control males, at each age investigated. The final experiment investigating *Robo1* mRNA expression found a significant decrease in the GT of male fetuses exposed to DES and in female fetuses exposed to DHT in comparison to control male. This finding could potentially have been interpreted as reflecting disturbance of the normal androgen:oestrogen balance. However, the fact that these results contradicted the findings of the initial *Robo1* experiment, in which flutamide exposure in the male or TP exposure in the female increased *Robo1* expression in comparison to control males, suggested that results were simply variable and inconsistent. Thus similar to its ligand, *Robo1* might have variable expression in the GT and these experiments suggest the mRNA of either *Slit2* or *Robo1* are not androgen or oestrogen regulated. Similar to *Asporin*, further studies of *Slit2* and *Robo1* were hindered by the lack of a working antibody (which would work on rat GT) for immunohistochemical protein analysis.

To conclude, the difficulty of investigating a tissue which has different cellular compositions at different planes could be addressed to some extent, by orientating the embedded tissue and serial sectioning. This, combined with OPT, which visualized the tissue in its entirety, provide good methods for studying the anatomy of the GT. However, to then utilize the former approach would require good working antibodies to localize the proteins of interest to specific cell types and to investigate the impact of androgen or oestrogen modulation. This was not possible for any of the three proteins studied in this chapter, so the approach used was restricted to investigation of gene

expression. Three genes were studied to determine if they are regulated by androgen action in the GT. Although some initial results indicated that *Asporin*, *Slit2* and *Robo1* might be regulated by androgens, the Taqman-PCR results proved variable and not always repeatable. Studies in this chapter have proved that although Taqman PCR may give indications of mRNA expression patterns, to really understand the events within a tissue such as GT, in which molecular mechanisms not completely established, good working antibodies are an absolute requirement. Furthermore, it was very disappointing that even when microarray results all pointed in one direction, it was still be impossible to confirm consistently.

7. Final discussion

The general aims of this thesis were to investigate what regulates the MPW, to identify pathways regulated by androgens during the MPW that are involved in the development of the male reproductive tract, and through these to further knowledge of the origins of TDS. To date, the studies published on the MPW have focused on the importance of this time frame, demonstrating that disrupted androgen action, be it through phthalates, oestrogens or anti-androgenic compounds, can induce TDS disorders (Clark et al, 1990; Foster and Harris, 2005; MacLeod et al, 2009; McIntyre et al, 2001; Welsh et al, 2008). Such studies have highlighted that the programming which androgens direct during the MPW is unique to fetal development and does not occur at any other time in life. Deficits in androgen action during this time cannot be compensated for later in life (Welsh et al, 2010; van den Driesche et al, 2011). However no studies have addressed the question of what makes the MPW unique, what regulates it and indeed what pathways are activated by androgens within it.

In order to investigate these gaps in our knowledge, the obvious starting point was to investigate if androgens themselves and the availability of the AR might be involved in opening of the MPW, which would be logical as the natural onset of testosterone production coincides with the onset of the MPW (chapter 3). In addition, it was recognized that androgens do not exert their effect alone (Heemers and Tindall, 2007; Heinlein and Chang, 2002) and require various biochemical associates and the expression of some of these were also investigated (chapter 4). Some studies published almost 20 years ago had defined a time frame called the ‘critical period of development’ and outlined the effects of disrupted androgen action during this time, well before the ‘MPW’ became a common definition (Gupta and Goldman, 1986; Gupta, 1989; Gupta and Bentlejewski, 1992). These studies highlighted the importance of prostaglandins during the MPW and yet no further investigations have been published either supporting or contradicting this. Furthermore, given the various roles PGs play in embryonic development, and their roles which have been defined in the testis, it would be

understandable if PGs were indeed vital during the MPW and this was investigated for the first time in the rat (chapter 5). Finally, it was hypothesized that the molecular mechanisms regulated by androgens during the MPW might be unique to any other time frame. To gain insight into genes which might be regulated during this time, a microarray study was conducted. This thesis investigated one of the genes from the microarray study as well as two further candidate genes, each of which was hypothesized to be regulated by androgens in the GT (chapter 6).

A majority of the studies which have been published investigating androgen action have used testosterone formulations as the choice of androgen. Such studies have also reported the testosterone can cause adverse pregnancy effects such as dystocia and fetal growth restriction (Wolf et al, 2002; Welsh et al, 2008, 2010). Furthermore testosterone can be converted to oestrodial by aromatase which can have effects on male reproductive development (Goyal et al, 2007) but also means it is not always clear if an effect seen is due to an androgen effect or an oestrogen effect. Some, however, may deliberate that using testosterone formulations provides a more realistic physiology as testosterone *is* the common androgen produced and acts as a precursor for both oestrogens and DHT in males. In the studies reported in chapter 3, to ensure any effect seen was that of androgen action, DHT was used; a more potent androgen which cannot be aromatized to oestradiol and which, as results in chapter 3 showed, does not cause adverse pregnancy effects.

It was almost considered a given that the onset of testosterone production controls the 'opening' of the MPW and consequently one of the questions which arises from this theory is what might be closing the MPW as it is widely accepted that androgens and AR are present after the MPW. A second hypothesis which arose was that if the availability of androgens is the limiting factor in the on-set of the MPW, then would providing androgens earlier than this, initiate the MPW early, or advance or enhance

masculinisation? Furthermore, would providing 'extra' (exogenous) androgens during the MPW also advance or enhance masculinisation?

The studies reported in chapter 3 found that AR mRNA was present prior to the MPW, and the immounohistochemical studies suggested that AR protein is also available prior to the MPW thus the availability of AR does not appear to be a factor in regulating opening of the MPW. Therefore initial studies of this thesis exposed fetuses to DHT prior to (e11.5-e14.5) and during the MPW (e15.5-e18.5). One of the more surprising results of these experiments was the effects found on the female offspring. Virilisation of females by exposure to testosterone during the fetal period is well known and well documented (Rhees et al,1997; Welsh et al, 2008 Wolf et al, 2002). Primarily such studies used testosterone during the MPW. No studies have been published on the effects of androgens on females prior to the MPW. An important finding of this thesis is that females exposed to DHT prior to the MPW are virilised similar to females exposed to DHT during the MPW. In fact, certain tissues, such as the female phallus, were more susceptible to masculinisation when exposed to DHT prior to the MPW. These findings suggest the females window of sensitivity to androgens is not restricted to the MPW and may be wider than the MPW in males. This may be useful knowledge for scientists investigating female reproductive tract development if the time frame of susceptibility to androgen perturbations in the female development could be better defined.

Exposing male fetuses to DHT prior to or during the MPW did not advance or enhance masculinisation or cause any detectable positive effects in male offspring. No early coiling of the WD was detectable, and the tissues of the reproductive tract known to be programmed by androgens during the MPW were comparable to vehicle exposed animals. Four conclusions can be drawn from these experiments. First, DHT exposure prior to the MPW does not exert any detectable effects in the male offspring. Second, masculinisation is not enhanced by the early exposure to DHT. Third, endogenous androgens produced in the fetus are normally sufficient to maximally program

reproductive organ size. Fourth, it is not the availability of androgens that 'opens' the MPW and other factors must be involved.

Other factors which are known to work with androgens are androgen co-regulators. The possibility of the pathways of androgen and co-regulators which might be involved in the MPW are vast but to narrow this down, a logical approach was taken to compare bioinformatics software of AR pathways with results of a microarray study designed to specifically recognize androgen regulated genes, in the GT, during the MPW. Two co-regulators found by this method showed interesting patterns of mRNA expression around the MPW, *BRG1* and *CBP*. Both of these co-regulators are known to work together to alter chromatin organization and to induce histone modifications, and both were expressed in the GT and within the SC, GC and interstitial cells in the fetal and postnatal period. Furthermore, both of these co-regulators co-localised with AR in the SC at PND25. BRG1 and CBP expression did not change in the testis of fetuses which had been exposed to DBP, DES or flutamide, suggesting that expression of the genes themselves is not androgen regulated. However, further studies should focus on determining the protein level of expression of CBP and BRG1 in the GT before and during the window to determine if the mRNA expression changes that were found when comparing before, during and after the MPW, are also seen at the protein level. One of the weaknesses throughout the studies in this thesis was the inability to determine protein expression levels before and during the MPW accurately in the GT. If this issue could be resolved, it would facilitate evaluation of the involvement of potential factors in regulation of the MPW. However, aside from regulation of the MPW, BRG1 and CBP expression in the testis and their co-localisation with AR warrants further investigation to determine what role (if any) these proteins might play in the testis in modulating AR activation and thus androgen action. This would be a crucial question for SC, as these display late onset AR expression and androgen-sensitivity and poorly understood 'stage-dependent' changes in function in adulthood (Sharpe, 2005). A targeted knock-out of

either of these genes in SC would be most informative, and could be achieved by use of the *Amh-Cre* mouse line and generation of floxed *BRG1* or *CBP* lines.

RWDD1 expression does appear to be androgen regulated and RWDD1 protein expression was substantially reduced in the GT at e21.5 at least when androgen action in the MPW was blocked using flutamide. It would be interesting to determine if this effect is seen in e17.5 GT and if possible in earlier GT as well, especially as *Rwdd1* mRNA expression did not change greatly across the MPW. Nevertheless, as RWDD1 showed impressive co-localisation with AR in the GT and testis, it is of considerable interest for follow-up studies. The effect of exposure to other androgen disrupting chemicals (which cause hypospadias) on RWDD1 expression would be useful to compare with the flutamide results. Detailed characterization of RWDD1 throughout the male reproductive tract through to adulthood in relation to AR expression/androgen action should be a first step, as well as comparing expression patterns between males and females. Such studies require a working antibody, due to the cellular complexity of the tissues and the fact that not all cell types express the AR. The fact that in the present studies, flutamide exposure had an apparently much greater effect on RWDD1 protein expression than on mRNA expression reinforces this view. Unfortunately, it appears that current batches of the RWDD1 antibody do not work.

In addition to androgen co-regulators, other methods of regulation of the MPW were also considered and one of these is methylation. The preliminary investigations conducted on the methylation regulators *BORIS* and *DNMT3L* have demonstrated interesting decreases in mRNA expression during the MPW. DNMT3L is a member of the DNMT3 enzymes which catalyze methylation reactions. Despite being a member of this family, however, DNMT3L does not itself catalyse methylation reactions (reviewed by Garcia-Carpizo et al, 2011), but is thought to regulate methylation through histone deacetylase activity (Aapola et al, 2002). DNMT3L expression was significantly decreased in the GT of fetuses exposed to flutamide during the MPW in comparison to

controls. A recent study has found that the methylation enzyme DNMT3A is increased in expression in the foreskin of hypospadias patients in comparison to control males (Vottero et al, 2011) and consequently in future studies it would be important to determine the expression of DNMT3L protein in flutamide exposed GT throughout development and also in the postnatal penis.

Targeted disruption of DNMT3L in the testis has been reported to cause hypogonadism and azoospermia (Bourc'his et al, 2001). Further studies of the testis in these mice reported the semineferous tubules to only contain SCs and some leptotene-like cells. The GCs were reported to be arrested and and died around the meitoc stage (Hata et al, 2006). This study did not report any other effect on masculinisation. Microarray expression profiling found particular sex linked genes to be down regulated, such as *Sox3, Pern, Ott* each of which is gonad specific but it is unclear exactly what this means (Hata et al, 2006). However the relationship between expression of DNMT3L and AR in control testis has not yet been reported. The studies reported in chapter 4 found that DNMT3L was expressed in cells of the interstitium at e21.5 and PND25 but not at PND4. Further studies should determine what cells of the interstitium DNMT3L is being expressed in, using Leydig cell-specific markers. Moreover, at PND25, DNMT3L was expressed in a stage specific manner in the spermatocytes and it would be interesting to characterize what steps of meiotic development this coincides with, as the DNMT3L knockouts show a failure of GC to complete meiosis. The latter findings led the authors in question to conclude that it was an underlying problem with gene methylation in spermatocytes that caused this GC loss. However, as the present findings show that DNMT3L is co-expressed with AR in the SC, it is possible that deletion of DNMT3L perturbs normal androgen action in SC, and it is this that is responsible for the GC loss, especially as SC-selective knockout of the AR (SCARKO mice) leads to a similar demise of GC during meiosis (de Gendt et al, 2003). Experiments to distinguish between these possibilities may assist our understanding of the mechanisms used by DNMT3L and why it's absence causes azoospermia.

BORIS, an 11 zinc finger protein, has been shown to play a role in chromatin remodeling as well as being considered to have pro-proliferative characteristics (Nguyen et al, 1996). BORIS expression has only been found in the testis and studies suggest that DNA methylation acts as a negative regulator of BORIS expression, thus the BORIS gene activity is methylation controlled (Kelnova et al, 2002; Woloszynska-Read et al, 2007). The studies in chapter 4 found that BORIS was expressed in the GT as well as in the testis. The expression of BORIS in the GT has not been reported before and its role in the GT is unknown and calls for further investigation. Taqman-PCR suggested that *BORIS* expression is decreased during the MPW and in future studies it would be interesting to determine if this is reflected at the protein level. The expression of BORIS protein was found in the developing testis at e21.5, PND4 and PND25, in interstitial cells, SC and GCs and at e21.5 and PND25 in the PTM cells also. A recent study reported a *BORIS* knockout mouse model to be fertile but have small testes and impaired spermatogenesis, in comparison to controls (Suzuki et al, 2010); Through microarray analysis of the testis of the BORIS knockout model, this group identified the down regulation of a transcript termed cerebroside sulfotransferase (CST), known to be involved in meiosis. Furthermore, the study reported BORIS can bind to and activate the CST promoter. This study concluded BORIS to have a critical role in transcriptional regulation of CST important for spermatogeneisis (Suzuki et al, 2010). Thus recent progress has been made in gaining insight into the mechanisms which BORIS might play in the testis, however many there are still many questions. Firstly, it is surprising that the expression of BORIS with AR in control testis has not been previously fully characterized. This characterization of co-expression of BORIS and AR in SC and in other somatic cells could be due to a functional relationship, which may result in deficient androgen action when BORIS is knocked out which could then causes GC problems as a consequence. Methylation is known to be important in GC, but it seems when a methylation-associated gene or protein is found to be expressed in the GCs level of AR activity in relation to this is not always investigated and yet this activity may explain (or contribute to) results of knockout and give dramatic new insight into

androgen action in the testis and elsewhere. As suggested for BRG1 or CBP, a targeted knockout of either *BORIS* or *DNMT3L* in SC (for example via *Amh-Cre* system*)* would provide more precise information of the roles these genes play in the testis.

Prostaglandins are known to be involved in various cellular processes in the body and indeed during the embryonic period (Challis et al, 2000; Lala, 1989, Norwitz et al, 2001). Indomethacin is well established as a non-selective COX enzyme inhibitor and consequently blocks PG synthesis (Norton, 1997). Reports that blocking PGs during the MPW, using indomethacin, in the mouse could affect masculinisation were published over 20 years ago (Gupta and Goldman 1986; Gupta, 1989) yet the role of PGs in the MPW has never been fully defined or even further investigated. Studies in this thesis used the highest concentration of indomethacin possible, in the rat, which allowed for dams to avoid adverse effects and parturition to occur around about on time. Even at the dose used (1mg/kg) some adverse effects were noted, such as on fetal growth and perhaps litter size. Of interest the F1 female generation had misplaced ovaries and there was some suggestion of difficulties in estrus cycles and thereafter with parturition. This suggests the indomethacin was crossing the placenta and fetuses were being exposed, to some level at least. Despite this, no affect was seen on AGD or on any measured aspect of the reproductive tract of males exposed to indomethacin. A decrease was found in penile length in adult rats that had been exposed to indomethacin during the MPW, but this was based on small numbers of animals. Moreover, even if correct, it did not point to impaired androgen action in the MPW, as penis size was normal at PND25 and AGD and the remainder of reproductive tract development was normal in these animals. However, the N number for these animals would need to be increased to determine whether or not the effect on adult penis size is real, and such studies should also investigate circulating hormone levels (notably testosterone) and the expression of 5α-reductase in the penis to see if changes in either of these might explain the findings.

Thus one of the important conclusions of this thesis is that exposure of rats to indomethacin during the MPW, and possible blocking of PGs, does not affect masculinisation of the male reproductive tract, at least in the rat. The preliminary studies to determine if PGs are involved prior to the MPW were also negative, suggesting that PGs are unlikely to be a major player in regulation of the MPW. The studies reported in this chapter could be strengthened by determining the level of prostaglandins in control fetuses and comparing this against prostaglandin production in fetuses after exposure to indomethacin. Radioimmunoassays for PGs have been established for many years (Jaffe et al, 1973; Oki et al, 1974; Thomas et al, 1978) and could therefore be used for this purpose. However, as prostaglandins have a relatively short half life and act over short distances in an autocrine or paracrine manner (Hull et al, 2004), such studies may not be straightforward.

Development of the male GT is an androgen-dependent process and when androgen action is deficient during the MPW, hypospadias can occur (Baskin et al, 2001;Yamada et al, 2003). Consequently it was hypothesized that studying the genes within the GT, under normal circumstances and when androgen action is blocked, might provide insight into androgen pathways functioning in the MPW, but more specifically, pathways which might be involved in hypospadias. Of the three genes studied, *Asporin* provided the most convincing results, whereby it showed a trend towards higher expression in control female GT than control male GT, throughout development. Similarly a trend was seen in the GT of flutamide exposed fetuses, which expressed *Asporin* at higher levels than control males throughout development. It was not clear if *Asporin* was either androgen or oestrogen regulated, however, as results tended not to be entirely consistent. In attempting to reconcile the various datasets, it was postulated that if the androgen-oestrogen balance was disturbed, *Asporin* expression increased. Whilst this remains a possibility, to take the idea further would require determination of the androgen-oestrogen balance and to correlate changes observed with those in *Asporin* expression. As the androgen-oestrogen balance would need to be measured either in blood (difficult

in the fetus because of small volumes, and probably impractical in the MPW for this reason) or in the GT itself, which would again pose problems based on small tissue amounts available. A major limiting factor in studying Asporin, is the absence of an antibody that works on rodent tissue. Overall, the conclusion was that asporin did not appear of sufficient interest to warrant further studies with any priority.

It was more difficult to obtain convincing results for *Slit2* and *Robo1* expression in the GT. Initial experiments indicated that *Slit2* might be negatively regulated by androgens, but upon repetition and increasing time points and treatment groups, the expression of *Slit2* and actually *Robo1* as well, appeared more variable. This could be due to the gene having variable expression or another possibility which must be considered was noise level within the experiment and one possibility to address this might be an increase in PCR cycles. However in order to fully understand the expression of *Asporin*, *Robo1* and *Slit2* in the development of the GT requires an antibody which worked in the rat and unfortunately we could not obtain such antibodies.

In summary, this thesis aimed to investigate the regulation of the MPW and androgen events which take place during and to some extent before the MPW. It was shown (for the first time) that AR is present before the MPW and availability of androgens does not regulate the MPW. Furthermore, providing excess androgens either prior to, or during the MPW does not advance or enhance masculinisation. Another novel finding was that the female reproductive tract is susceptible to perturbations by androgen exposure prior to the MPW and thus virilisation of the female reproductive tract by excess androgen exposure is not limited to the MPW. Androgen co-regulators and methylation regulators were investigated, originally to determine if certain pathways might be involved in i) the regulation of the MPW or ii) events within the MPW. Although this was not achieved, preliminary characterization studies of 3 androgen co-regulators and 2 methylation enzymes in the reproductive tract was reported. It is surprising that so many known androgen-co regulators have not been characterized in the male reproductive tract, both in fetal and postnatal life. For the first time, this thesis reports that BRG1 and CBP

change in expression in cell types in the developing testis and co-localize with AR in what appears to be a stage dependent manner. Furthermore, the preliminary studies in this thesis suggest that RWDD1 expression is androgen regulated and future studies should determine if it is involved in the pathway to hypospadias. This study can also conclude that exposure to indomethacin during the MPW, in the rat, does not affect masculinisation. Although this study did not determine if PGs synthesis was blocked in the fetus by indomethacin and to what extent, the adverse outcomes reported in the female offspring suggested that fetuses were exposed and this did not affect androgen action during the MPW. Finally, this thesis found that investigating the development of the male penile anatomy is not an easy task! However using serial sectioning and OPT provided a good method for understanding the development of the rat GT at least from e19.5 onwards. Furthermore, in order to investigate pathways which are involved in the GT and in hypospadias almost certainly requires working antibodies for immunohistochemistry, as without such tools it is difficult to draw firm conclusions. Protein expression and localization in the different planes of the GT is an absolute requirement for the understanding of the pathways involved in the development and disorders of the GT.

The studies in this thesis have shown that certain entities are not involved in regulation of the MPW. However, it still remains to be determined what is regulating the MPW and what is unique about this time frame. Although preliminary, the results from the study of androgen co-regulators look promising and, irrespective of their role or not in the MPW, further studies may lead to a better understanding of the importance of co-regulators and their interaction with AR in modulating androgen action in the male reproductive tract. This may also lead to specific pathways which are important in TDS and the MPW as suggested by the RWDD1 results found. In addition, investigating methylation activities before during and after the MPW also looks to be an important angle for future studies into regulation of the MPW, although this would be greatly facilitated if methylation of specific genes was being investigated. One of the areas of interest for this thesis was

genes involved in GT development and hypospadias. Hypospadias is a disorder which involves both the environment as well as genetics (Kalfa et al, 2011). In order to investigate the molecular mechanisms of androgen action in the development of the GT during the MPW, which might be disrupted in the hypospadias disorder, the use of microarrays as a starting point may not be the most promising approach.

Aapola, U., I. Liiv, and P. Peterson, *Imprinting regulator DNMT3L is a transcriptional repressor associated with histone deacetylase activity.* Nucleic Acids Res, 2002. **30**(16): p. 3602-8.

Aarnisalo, P., J.J. Palvimo, and O.A. Janne, *CREB-binding protein in androgen receptor-mediated signaling.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2122-7.

Abbott, D.H., et al., *Androgen excess fetal programming of female reproduction: a developmental aetiology for polycystic ovary syndrome?* Hum Reprod Update, 2005. **11**(4): p. 357-74.

Abbott, D.H., et al., *Fetal programming of adrenal androgen excess: lessons from a nonhuman primate model of polycystic ovary syndrome.* Endocr Dev, 2008. **13**: p. 145-58.

Abel, M.H., et al., *The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction.* Endocrinology, 2000. **141**(5): p. 1795-803.

Achermann, J.C., et al., *A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans.* Nat Genet, 1999. **22**(2): p. 125-6.

Achermann, J.C., et al., *Gonadal determination and adrenal development are regulated by the orphan nuclear receptor steroidogenic factor-1, in a dose-dependent manner.* J Clin Endocrinol Metab, 2002. **87**(4): p. 1829-33.

Ahmed, S.F., et al., *Phenotypic features, androgen receptor binding, and mutational analysis in 278 clinical cases reported as androgen insensitivity syndrome.* J Clin Endocrinol Metab, 2000. **85**(2): p. 658-65.

Akarasereenont, P., et al., *Involvement of tyrosine kinase in the induction of cyclo-oxygenase and nitric oxide synthase by endotoxin in cultured cells.* Br J Pharmacol, 1994. **113**(4): p. 1522-8.

Akiyama, H., et al., *Essential role of Sox9 in the pathway that controls formation of cardiac valves and septa.* Proc Natl Acad Sci U S A, 2004. **101**(17): p. 6502-7.

Alfsen, A., *[Study of the Testosterone-Serum Albumin Bond and the Action of Heptane on the Serum Albumin Molecule].* C R Trav Lab Carlsberg, 1963. **33**: p. 415-31.

Almind, G.J., et al., *11p Microdeletion including WT1 but not PAX6, presenting with cataract, mental retardation, genital abnormalities and seizures: a case report.* Mol Cytogenet, 2009. **2**: p. 6.

Andrews, W.D., M. Barber, and J.G. Parnavelas, *Slit-Robo interactions during cortical development.* J Anat, 2007. **211**(2): p. 188-98.

Anway, M.D., et al., *Epigenetic transgenerational actions of endocrine disruptors and male fertility.* Science, 2005. **308**(5727): p. 1466-9.

Arakane, F., et al., *The mechanism of action of steroidogenic acute regulatory protein (StAR). StAR acts on the outside of mitochondria to stimulate steroidogenesis.* J Biol Chem, 1998. **273**(26): p. 16339-45.

Archambeault, D.R., et al., *Epithelial-mesenchymal crosstalk in Wolffian duct and fetal testis cord development.* Genesis, 2009. **47**(1): p. 40-8.

Arishima, K., et al., *Onset of the constrictive effect of indomethacin on the ductus arteriosus in fetal rats.* Acta Anat (Basel), 1991. **142**(3): p. 231-5.

Aselton, P., et al., *First-trimester drug use and congenital disorders.* Obstet Gynecol, 1985. **65**(4): p. 451-5.

Aubert, M.L., et al., *Ontogeny of hypothalamic luteinizing hormone-releasing hormone (GnRH) and pituitary GnRH receptors in fetal and neonatal rats.* Endocrinology, 1985. **116**(4): p. 1565-76.

Auyeung, B., et al., *Fetal testosterone predicts sexually differentiated childhood behavior in girls and in boys.* Psychol Sci, 2009. **20**(2): p. 144-8.

Bacino, C., *ROR2-Related Robinow Syndrome*. In: Pagon RA, Bird TD, Dolan CR, Stephens K, editors. GeneReviews [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2005 Jul 28

Baerts, W., et al., *Cerebral lesions in preterm infants after tocolytic indomethacin.* Dev Med Child Neurol, 1990. **32**(10): p. 910-8.

Baker, J., et al., *Effects of an Igf1 gene null mutation on mouse reproduction.* Mol Endocrinol, 1996. **10**(7): p. 903-18.

Barbaux, S., et al., *Donor splice-site mutations in WT1 are responsible for Frasier syndrome.* Nat Genet, 1997. **17**(4): p. 467-70.

Barclay, D.L., *Congenital diethylstilbestrol-associated vaginal/cervical adenosis (DES babies).* J Ark Med Soc, 1979. **75**(12): p. 451-2.

Bardoni, B., et al., *A dosage sensitive locus at chromosome Xp21 is involved in male to female sex reversal.* Nat Genet, 1994. **7**(4): p. 497-501.

Barnes, R.B., et al., *Ovarian hyperandrogynism as a result of congenital adrenal virilizing disorders: evidence for perinatal masculinization of neuroendocrine function in women.* J Clin Endocrinol Metab, 1994. **79**(5): p. 1328-33.

Barrionuevo, F., et al., *Homozygous inactivation of Sox9 causes complete XY sex reversal in mice.* Biol Reprod, 2006. **74**(1): p. 195-201.

Barsoum, I. and H.H. Yao, *The road to maleness: from testis to Wolffian duct.* Trends Endocrinol Metab, 2006. **17**(6): p. 223-8.

Barteczko, K.J. and M.I. Jacob, *The testicular descent in human. Origin, development and fate of the gubernaculum Hunteri, processus vaginalis peritonei, and gonadal ligaments.* Adv Anat Embryol Cell Biol, 2000. **156**: p. III-X, 1-98.

Baskin, L.S., *Hypospadias and Genital Development* (Advances in Experimental Medicine and Biology) **545**, Springer (2004)

Baskin, L.S., J.W. Duckett, and T.F. Lue, *Penile curvature.* Urology, 1996. **48**(3): p. 347-56.

Baskin, L.S., et al., *Urethral seam formation and hypospadias.* Cell Tissue Res, 2001. **305**(3): p. 379-87.

Baskin, L.S., K. Himes, and T. Colborn, *Hypospadias and endocrine disruption: is there a connection?* Environ Health Perspect, 2001. **109**(11): p. 1175-83.

Bateman, D.N., *NSAIDs: time to re-evaluate gut toxicity.* Lancet, 1994. **343**(8905): p. 1051-2.

Beato, M. and J. Klug, *Steroid hormone receptors: an update.* Hum Reprod Update, 2000. **6**(3): p. 225-36.

Bell, J.B., et al., *Pathways for androgen biosynthesis from [7 alpha-3H] pregnenolone and [4-14C]progesterone by rat testis interstitium in vitro.* Biochim Biophys Acta, 1968. **164**(2): p. 412-20.

Bennetts, H.W., E.J. Underwood, and F.L. Shier, *A specific breeding problem of sheep on subterranean clover pastures in Western Australia.* Aust Vet J, 1946. **22**: p. 2-12.

Bentvelsen, F.M., et al., *Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat.* Mol Cell Endocrinol, 1995. **113**(2): p. 245-53.

Bentvelsen, F.M., et al., *Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat.* Mol Cell Endocrinol, 1995. **113**(2): p. 245-53.

Bentvelsen, F.M., et al., *The androgen receptor of the urogenital tract of the fetal rat is regulated by androgen.* Mol Cell Endocrinol, 1994. **105**(1): p. 21-6.

Bergstrom, D.E., et al., *Related function of mouse SOX3, SOX9, and SRY HMG domains assayed by male sex determination.* Genesis, 2000. **28**(3-4): p. 111-24.

Berman, D.M., H. Tian, and D.W. Russell, *Expression and regulation of steroid 5 alpha-reductase in the urogenital tract of the fetal rat.* Mol Endocrinol, 1995. **9**(11): p. 1561-70.

Berta, P., et al., *Genetic evidence equating SRY and the testis-determining factor.* Nature, 1990. **348**(6300): p. 448-50.

Betney, R. and I.J. McEwan, *Role of conserved hydrophobic amino acids in androgen receptor AF-1 function.* J Mol Endocrinol, 2003. **31**(3): p. 427-39.

Biancardi, M.F., et al., *Testosterone promotes an anabolic increase in the rat female prostate (Skene's paraurethral gland) which acquires a male ventral prostate phenotype.* Anat Rec (Hoboken). **293**(12): p. 2163-75.

Birk, O.S., et al., *The LIM homeobox gene Lhx9 is essential for mouse gonad formation.* Nature, 2000. **403**(6772): p. 909-13.

Blobel, G.A., *CREB-binding protein and p300: molecular integrators of hematopoietic transcription.* Blood, 2000. **95**(3): p. 745-55.

Boas, M., et al., *Postnatal penile length and growth rate correlate to serum testosterone levels: a longitudinal study of 1962 normal boys.* Eur J Endocrinol, 2006. **154**(1): p. 125-9.

Boisen, K.A., et al., *Hypospadias in a cohort of 1072 Danish newborn boys: prevalence and relationship to placental weight, anthropometrical measurements at birth, and reproductive hormone levels at three months of age.* J Clin Endocrinol Metab, 2005. **90**(7): p. 4041-6.

Boisen, K.A., et al., *Difference in prevalence of congenital cryptorchidism in infants between two Nordic countries.* Lancet, 2004. **363**(9417): p. 1264-9.

Bose, J., L. Grotewold, and U. Ruther, *Pallister-Hall syndrome phenotype in mice mutant for Gli3.* Hum Mol Genet, 2002. **11**(9): p. 1129-35.

Boukari, K., et al., *Lack of androgen receptor expression in Sertoli cells accounts for the absence of anti-Mullerian hormone repression during early human testis development.* J Clin Endocrinol Metab, 2009. **94**(5): p. 1818-25.

Bourc'his, D., et al., *Dnmt3L and the establishment of maternal genomic imprints.* Science, 2001. **294**(5551): p. 2536-9.

Bray, F., et al., *Trends in testicular cancer incidence and mortality in 22 European countries: continuing increases in incidence and declines in mortality.* Int J Cancer, 2006. **118**(12): p. 3099-111.

Bremner, W.J., et al., *Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens.* Endocrinology, 1994. **135**(3): p. 1227-34.

Brennan, J. and B. Capel, *One tissue, two fates: molecular genetic events that underlie testis versus ovary development.* Nat Rev Genet, 2004. **5**(7): p. 509-21.

Brennan, J., J. Karl, and B. Capel, *Divergent vascular mechanisms downstream of Sry establish the arterial system in the XY gonad.* Dev Biol, 2002. **244**(2): p. 418-28.

Brennan, J., C. Tilmann, and B. Capel, *Pdgfr-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad.* Genes Dev, 2003. **17**(6): p. 800-10.

Brinkmann, A.O., *Molecular basis of androgen insensitivity.* Mol Cell Endocrinol, 2001. **179**(1-2): p. 105-9.

Britten, R.J. and E.H. Davidson, *Gene regulation for higher cells: a theory.* Science, 1969. **165**(891): p. 349-57.

Bromer, J.G., et al., *Hypermethylation of homeobox A10 by in utero diethylstilbestrol exposure: an epigenetic mechanism for altered developmental programming.* Endocrinology, 2009. **150**(7): p. 3376-82.

Brunetti, L., et al., *Cafeteria diet increases prostaglandin E2 levels in rat prostate, kidney and testis.* Int J Immunopathol Pharmacol. **23**(4): p. 1073-8.

Butler, C.M., et al., *Developmental expression of the androgen receptor during virilization of the urogenital system of a marsupial.* Biol Reprod, 1998. **59**(4): p. 725-32.

Cancilla, B., et al., *Regulation of prostate branching morphogenesis by activin A and follistatin.* Dev Biol, 2001. **237**(1): p. 145-58.

Canto, P., et al., *A heterozygous mutation in the desert hedgehog gene in patients with mixed gonadal dysgenesis.* Mol Hum Reprod, 2005. **11**(11): p. 833-6.

Cao, G., et al., *Developmental and hormonal regulation of murine scavenger receptor, class B, type 1.* Mol Endocrinol, 1999. **13**(9): p. 1460-73.

Carlson, B.M., *Human embryology and developmental biology* . 2004, Chapter 16 ’Urogenitral system’, Mosby, (St. Louis, Mo), Third edition

Carlsen, E., et al., *Evidence for decreasing quality of semen during past 50 years.* BMJ, 1992. **305**(6854): p. 609-13.

Carmichael, S.L., et al., *Maternal corticosteroid use and hypospadias.* J Pediatr, 2009. **155**(1): p. 39-44, 44 e1.

Carr, B.R., et al., *Regulation of human fetal testicular secretion of testosterone: low-density lipoprotein-cholesterol and cholesterol synthesized de novo as steroid precursor.* Am J Obstet Gynecol, 1983. **146**(3): p. 241-7.

Cassady, G., et al., *A randomized, controlled trial of very early prophylactic ligation of the ductus arteriosus in babies who weighed 1000 g or less at birth.* N Engl J Med, 1989. **320**(23): p. 1511-6.

Catti, M., et al., *Management of severe hypospadias.* Indian J Urol, 2008. **24**(2): p. 233-40.

Cederroth, C.R., et al., *Genetic programs that regulate testicular and ovarian development.* Mol Cell Endocrinol, 2007. **265-266**: p. 3-9.

Cederroth, C.R., et al., *Estrogen receptor alpha is a major contributor to estrogen-mediated fetal testis dysgenesis and cryptorchidism.* Endocrinology, 2007. **148**(11): p. 5507-19.

Centenera, M.M., et al., *The contribution of different androgen receptor domains to receptor dimerization and signaling.* Mol Endocrinol, 2008. **22**(11): p. 2373-82.

Chakravarti, D., et al., *A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity.* Cell, 1999. **96**(3): p. 393-403.

Challis, J.R.G., et al., *Endocrine and paracrine regulation of birth at term and preterm.* Endocr Rev, 2000. **21**(5): p. 514-50.

Chassot, A.A., et al., *Activation of beta-catenin signaling by Rspo1 controls differentiation of the mammalian ovary.* Hum Mol Genet, 2008. **17**(9): p. 1264-77.

Chedin, F., M.R. Lieber, and C.L. Hsieh, *The DNA methyltransferase-like protein DNMT3L stimulates de novo methylation by Dnmt3a.* Proc Natl Acad Sci U S A, 2002. **99**(26): p. 16916-21.

Chen, S. and D.F. Smith, *Hop as an adaptor in the heat shock protein 70 (Hsp70) and hsp90 chaperone machinery.* J Biol Chem, 1998. **273**(52): p. 35194-200.

83. Chen, Y.C. and P.V. Woolley, Jr., *Genetic studies on hypospadias in males.* J Med Genet, 1971. **8**(2): p. 153-9.

Cheng, J., S.C. Watkins, and W.H. Walker, *Testosterone activates mitogen-activated protein kinase via Src kinase and the epidermal growth factor receptor in sertoli cells.* Endocrinology, 2007. **148**(5): p. 2066-74.

Chrivia, J.C., et al., *Phosphorylated CREB binds specifically to the nuclear protein CBP.* Nature, 1993. **365**(6449): p. 855-9.

Chung, L.W. and G.R. Cunha, *Stromal-epithelial interactions: II. Regulation of prostatic growth by embryonic urogenital sinus mesenchyme.* Prostate, 1983. **4**(5): p. 503-11.

Clark, R.L., et al., *Critical developmental periods for effects on male rat genitalia induced by finasteride, a 5 alpha-reductase inhibitor.* Toxicol Appl Pharmacol, 1993. **119**(1): p. 34-40.

Clark, R.L., et al., *External genitalia abnormalities in male rats exposed in utero to finasteride, a 5 alpha-reductase inhibitor.* Teratology, 1990. **42**(1): p. 91-100.

Cleutjens, K.B., et al., *An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter.* Mol Endocrinol, 1997. **11**(2): p. 148-61.

Coffey, J.C., et al., *Steroid metabolism by testicular homogenates of the Stanley-Gumbreck pseudohermaphrodite male rat. I. Increased formation of androsterone and androstanediol.* Steroids, 1972. **19**(3): p. 433-54.

Cohn, M.J., *Development of the external genitalia: conserved and divergent mechanisms of appendage patterning.* Dev Dyn. **240**(5): p. 1108-15.

92. Cohn, M.J., et al., *Fibroblast growth factors induce additional limb development from the flank of chick embryos.* Cell, 1995. **80**(5): p. 739-46.

Conde, I., et al., *DAX-1 expression in human breast cancer: comparison with estrogen receptors ER-alpha, ER-beta and androgen receptor status.* Breast Cancer Res, 2004. **6**(3): p. R140-8.

Conte, D., et al., *Aspirin inhibits androgen response to chorionic gonadotropin in humans.* Am J Physiol, 1999. **277**(6 Pt 1): p. E1032-7.

Cooke, P.S., P. Young, and G.R. Cunha, *Androgen receptor expression in developing male reproductive organs.* Endocrinology, 1991. **128**(6): p. 2867-73.

Cool, J., et al., *Peritubular myoid cells are not the migrating population required for testis cord formation in the XY gonad.* Sex Dev, 2008. **2**(3): p. 128-33.

Cunha, G.R., et al., *Neonatal seminal vesicle mesenchyme induces a new morphological and functional phenotype in the epithelia of adult ureter and ductus deferens.* Development, 1991. **111**(1): p. 145-58.

Dadras, S.S., et al., *Inhibition of 5alpha-reductase in rat prostate reveals differential regulation of androgen-response gene expression by testosterone and*

Dai, Y., et al., *Prohibitin and the SWI/SNF ATPase subunit BRG1 are required for effective androgen antagonist-mediated transcriptional repression of androgen receptor-regulated genes.* Carcinogenesis, 2008. **29**(9): p. 1725-33.

Dannhardt, G. and W. Kiefer, *Cyclooxygenase inhibitors--current status and future prospects.* Eur J Med Chem, 2001. **36**(2): p. 109-26.

D'Arcy, V., et al., *BORIS, a paralogue of the transcription factor, CTCF, is aberrantly expressed in breast tumours.* Br J Cancer, 2008. **98**(3): p. 571-9.

De Felici, M., et al., *Experimental approaches to the study of primordial germ cell lineage and proliferation.* Hum Reprod Update, 2004. **10**(3): p. 197-206.

De Gendt, K., et al., *A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis.* Proc Natl Acad Sci U S A, 2004. **101**(5): p. 1327-32.

De Gendt, K. and G. Verhoeven, *Tissue- and cell-specific functions of the androgen receptor revealed through conditional knockout models in mice.* Mol Cell Endocrinol, 2011 August 18, article in press

Delbes, G., C. Levacher, and R. Habert, *Estrogen effects on fetal and neonatal testicular development.* Reproduction, 2006. **132**(4): p. 527-38.

Deplus, R., et al., *Dnmt3L is a transcriptional repressor that recruits histone deacetylase.* Nucleic Acids Res, 2002. **30**(17): p. 3831-8.

Desai, M., et al., *Programmed obesity in intrauterine growth-restricted newborns: modulation by newborn nutrition.* Am J Physiol Regul Integr Comp Physiol, 2005. **288**(1): p. R91-6.

DiRenzo, J., et al., *BRG-1 is recruited to estrogen-responsive promoters and cooperates with factors involved in histone acetylation.* Mol Cell Biol, 2000. **20**(20): p. 7541-9.

Domenice, S., et al., *Mutations in the SRY, DAX1, SF1 and WNT4 genes in Brazilian sex-reversed patients.* Braz J Med Biol Res, 2004. **37**(1): p. 145-50.

Donahoe, P.K., et al., *Mullerian inhibiting substance in human testes after birth.* J Pediatr Surg, 1977. **12**(3): p. 323-30.

Donjacour, A.A. and G.R. Cunha, *Assessment of prostatic protein secretion in tissue recombinants made of urogenital sinus mesenchyme and urothelium from normal or androgen-insensitive mice.* Endocrinology, 1993. **132**(6): p. 2342-50.

Dronadula, N., et al., *Involvement of cAMP-response element binding protein-1 in arachidonic acid-induced vascular smooth muscle cell motility.* J Lipid Res, 2006. **47**(4): p. 767-77.

Dufort, I., et al., *Characteristics of a highly labile human type 5 17beta-hydroxysteroid dehydrogenase.* Endocrinology, 1999. **140**(2): p. 568-74.

Dumesic, D.A., R.D. Schramm, and D.H. Abbott, *Early origins of polycystic ovary syndrome.* Reprod Fertil Dev, 2005. **17**(3): p. 349-60.

Dunaif, A., *Insulin resistance and the polycystic ovary syndrome: mechanism and implications for pathogenesis.* Endocr Rev, 1997. **18**(6): p. 774-800.

Eckner, R., et al., *Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor.* Genes Dev, 1994. **8**(8): p. 869-84.

Eder, I.E., et al., *Molecular biology of the androgen receptor: from molecular understanding to the clinic.* Eur Urol, 2001. **40**(3): p. 241-51.

Eisenberg, M.L., et al., *The relationship between anogenital distance, fatherhood, and fertility in adult men.* PLoS One. **6**(5): p. e18973.

Emmen, J.M., et al., *Involvement of insulin-like factor 3 (Insl3) in diethylstilbestrol-induced cryptorchidism.* Endocrinology, 2000. **141**(2): p. 846-9.

Fan, C.Y., et al., *The type I Hsp40 zinc finger-like region is required for Hsp70 to capture non-native polypeptides from Ydj1.* J Biol Chem, 2005. **280**(1): p. 695-702.

Felzien, L.K., et al., *Specificity of cyclin E-Cdk2, TFIIB, and E1A interactions with a common domain of the p300 coactivator.* Mol Cell Biol, 1999. **19**(6): p. 4241-6.

Fisher, J.S., *Environmental anti-androgens and male reproductive health: focus on phthalates and testicular dysgenesis syndrome.* Reproduction, 2004. **127**(3): p. 305-15.

Fliss, A.E., et al., *Domain requirements of DnaJ-like (Hsp40) molecular chaperones in the activation of a steroid hormone receptor.* J Biol Chem, 1999. **274**(48): p. 34045-52.

Fluck, C.E., W.L. Miller, and R.J. Auchus, *The 17, 20-lyase activity of cytochrome p450c17 from human fetal testis favors the delta5 steroidogenic pathway.* J Clin Endocrinol Metab, 2003. **88**(8): p. 3762-6.

Foradori, C.D., M.J. Weiser, and R.J. Handa, *Non-genomic actions of androgens.* Front Neuroendocrinol, 2008. **29**(2): p. 169-81.

Forest, M.G. and A.M. Cathiard, *Pattern of plasma testosterone and delta4-androstenedione in normal newborns: Evidence for testicular activity at birth.* J Clin Endocrinol Metab, 1975. **41**(5): p. 977-80.

Foster, P.M., *Disruption of reproductive development in male rat offspring following in utero exposure to phthalate esters.* Int J Androl, 2006. **29**(1): p. 140-7; discussion 181-5.

Foster, P.M. and M.W. Harris, *Changes in androgen-mediated reproductive development in male rat offspring following exposure to a single oral dose of flutamide at different gestational ages.* Toxicol Sci, 2005. **85**(2): p. 1024-32.

Fowler, W.C., Jr. and D.A. Edelman, *In utero exposure to DES. Evaluation and followup of 199 women.* Obstet Gynecol, 1978. **51**(4): p. 459-63.

Fraga, M.F., *Genetic and epigenetic regulation of aging.* Curr Opin Immunol, 2009. **21**(4): p. 446-53.

Freiman, R.N. and R. Tjian, *Regulating the regulators: lysine modifications make their mark.* Cell, 2003. **112**(1): p. 11-7.

Fritz, H., K. Giese, and H.P. Suter, *Prenatal and postnatal development of rats following the maternal treatment with testosterone during the late period of embryogenesis.* Arzneimittelforschung, 1984. **34**(7): p. 780-2.

Fronsdal, K., et al., *CREB binding protein is a coactivator for the androgen receptor and mediates cross-talk with AP-1*. J Biol Chem, 1998. **273**(48): p. 31853-9.

Gakunga, P., et al., *Hyaluronan is a prerequisite for ductal branching morphogenesis.* Development, 1997. **124**(20): p. 3987-97.

Gao, S., et al., *Androgen receptor and prostate apoptosis response factor-4 target the c-FLIP gene to determine survival and apoptosis in the prostate gland.* J Mol Endocrinol, 2006. **36**(3): p. 463-83.

Garcia-Carpizo, V., et al., *The growing role of gene methylation on endocrine function.* J Mol Endocrinol. **47**(2): p. R75-89.

Garcia-Ortiz, J.E., et al., *Foxl2 functions in sex determination and histogenesis throughout mouse ovary development.* BMC Dev Biol, 2009. **9**: p. 36.

Gardiner-Garden, M. and M. Frommer, *CpG islands in vertebrate genomes.* J Mol Biol, 1987. **196**(2): p. 261-82.

Gaughan, L., et al., *Regulation of androgen receptor and histone deacetylase 1 by Mdm2-mediated ubiquitylation.* Nucleic Acids Res, 2005. **33**(1): p. 13-26.

Geissler, W.M., et al., *Male pseudohermaphroditism caused by mutations of testicular 17 beta-hydroxysteroid dehydrogenase 3.* Nat Genet, 1994. **7**(1): p. 34-9.

Gelmann, E.P., *Molecular biology of the androgen receptor.* J Clin Oncol, 2002. **20**(13): p. 3001-15.

George, F.W., et al., *Studies on the regulation of the onset of steroid hormone biosynthesis in fetal rabbit gonads.* Endocrinology, 1979. **105**(5): p. 1100-6.

Gessler, M., et al., *Homozygous inactivation of WT1 in a Wilms' tumor associated with the WAGR syndrome.* Genes Chromosomes Cancer, 1993. **7**(3): p. 131-6.

Giguere, V., *Orphan nuclear receptors: from gene to function.* Endocr Rev, 1999. **20**(5): p. 689-725.

Glaros, S., et al., *Targeted knockout of BRG1 potentiates lung cancer development.* Cancer Res, 2008. **68**(10): p. 3689-96.

Goldblatt, M.W., *A depressor substance in seminal fluid.* Chem Ind, 1933: p. 1056-1057.

Goodman, R.H. and S. Smolik, *CBP/p300 in cell growth, transformation, and development.* Genes Dev, 2000. **14**(13): p. 1553-77.

Goulis, D.G., et al., *Serum inhibin-B and follicle stimulating hormone as predictors of the presence of sperm in testicular fine needle aspirate in men with azoospermia.* Hormones (Athens), 2008. **7**(2): p. 140-7.

Goyal, H.O., et al., *Role of estrogen in induction of penile dysmorphogenesis: a review.* Reproduction, 2007. **134**(2): p. 199-208.

Grande, M., et al., *Tissue concentrations of tissue polypeptide antigen (TPA) and prostatic specific antigen (PSA) in 42 patients with prostatic carcinoma.* Prostate, 2000. **45**(4): p. 299-303.

Gray, L.E., Jr., et al., *Perinatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat.* Toxicol Sci, 2000. **58**(2): p. 350-65.

Gray, L.E., et al., *Effects of environmental antiandrogens on reproductive development in experimental animals.* Hum Reprod Update, 2001. **7**(3): p. 248-64.

Gray, L.E., Jr., et al., *Adverse effects of environmental antiandrogens and androgens on reproductive development in mammals.* Int J Androl, 2006. **29**(1): p. 96-104; discussion 105-8.

Gray, L.E., Jr., et al., *Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat.* Toxicol Ind Health, 1999. **15**(1-2): p. 94-118.

Griffin, D.K., et al., *Transcriptional profiling of luteinizing hormone receptor-deficient mice before and after testosterone treatment provides insight into the hormonal control of postnatal testicular development and Leydig cell differentiation.* Biol Reprod. 2010, **82**(6): p. 1139-50.

Grino, P.B., J.E. Griffin, and J.D. Wilson, *Testosterone at high concentrations interacts with the human androgen receptor similarly to dihydrotestosterone.* Endocrinology, 1990. **126**(2): p. 1165-72.

Grinspon, R.P. and R.A. Rey, *Anti-mullerian hormone and sertoli cell function in paediatric male hypogonadism.* Horm Res Paediatr. 2010, **73**(2): p. 81-92.

Grishkovskaya, I., et al., *Crystal structure of human sex hormone-binding globulin: steroid transport by a laminin G-like domain.* EMBO J, 2000. **19**(4): p. 504-12.

Gubbay, J., et al., *A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes.* Nature, 1990. **346**(6281): p. 245-50.

Gupta, C., *The role of prostaglandins in masculine differentiation: modulation of prostaglandin levels in the differentiating genital tract of the fetal mouse.* Endocrinology, 1989. **124**(1): p. 129-33.

Gupta, C. and C.A. Bentlejewski, *Role of prostaglandins in the testosterone-dependent wolffian duct differentiation of the fetal mouse.* Biol Reprod, 1992. **47**(6): p. 1151-60.

Gupta, C. and A.S. Goldman, *The arachidonic acid cascade is involved in the masculinizing action of testosterone on embryonic external genitalia in mice.* Proc Natl Acad Sci U S A, 1986. **83**(12): p. 4346-9.

Gustafson, D.R., M.J. Wen, and B.M. Koppanati, *Androgen receptor gene repeats and indices of obesity in older adults.* Int J Obes Relat Metab Disord, 2003. **27**(1): p. 75-81.

Haavisto, T., et al., *Prenatal testosterone and luteinizing hormone levels in male rats exposed during pregnancy to 2,3,7,8-tetrachlorodibenzo-p-dioxin and diethylstilbestrol.* Mol Cell Endocrinol, 2001. **178**(1-2): p. 169-79.

Haavisto, T.E., et al., *Effects of 4-tert-octylphenol, 4-tert-butylphenol, and diethylstilbestrol on prenatal testosterone surge in the rat.* Reprod Toxicol, 2003. **17**(5): p. 593-605.

Habert, R. and R. Picon, *Control of testicular steroidogenesis in foetal rat: effect of decapitation on testosterone and plasma luteinizing hormone-like activity.* Acta Endocrinol (Copenh), 1982. **99**(3): p. 466-73.

Hallmark, N., et al., *Effects of monobutyl and di(n-butyl) phthalate in vitro on steroidogenesis and Leydig cell aggregation in fetal testis explants from the rat: comparison with effects in vivo in the fetal rat and neonatal marmoset and in vitro in the human.* Environ Health Perspect, 2007. **115**(3): p. 390-6.

Hammar, M. and F. Petersson, *Testosterone production in vitro in human testicular tissue.* Andrologia, 1986. **18**(2): p. 196-200.

Han, G., et al., *Hormone status selects for spontaneous somatic androgen receptor variants that demonstrate specific ligand and cofactor dependent activities in autochthonous prostate cancer.* J Biol Chem, 2001. **276**(14): p. 11204-13.

Hannema, S.E. and I.A. Hughes, *Regulation of Wolffian duct development.* Horm Res, 2007. **67**(3): p. 142-51.

Haraguchi, R., et al., *Molecular analysis of external genitalia formation: the role of fibroblast growth factor (Fgf) genes during genital tubercle formation.* Development, 2000. **127**(11): p. 2471-9.

Hardell, L., et al., *Concentrations of polychlorinated biphenyls in blood and the risk for testicular cancer.* Int J Androl, 2004. **27**(5): p. 282-90.

Hardy, M.P., et al., *Stress hormone and male reproductive function.* Cell Tissue Res, 2005. **322**(1): p. 147-53.

Hase, T., et al., *Cyclooxygenase-1 and -2 in human testicular tumours.* Eur J Cancer, 2003. **39**(14): p. 2043-9.

Hashimoto, R., *Development of the human Mullerian duct in the sexually undifferentiated stage.* Anat Rec A Discov Mol Cell Evol Biol, 2003. **272**(2): p. 514-9.

Hashimoto, R., *Development of the human Mullerian duct in the sexually undifferentiated stage.* Anat Rec A Discov Mol Cell Evol Biol, 2003. **272**(2): p. 514-9.

Hata, K., et al., *Meiotic and epigenetic aberrations in Dnmt3L-deficient male germ cells.* Mol Reprod Dev, 2006. **73**(1): p. 116-22.

Hawcroft, G., et al., *Indomethacin induces differential expression of beta-catenin, gamma-catenin and T-cell factor target genes in human colorectal cancer cells.* Carcinogenesis, 2002. **23**(1): p. 107-14.

Hayes, J.J. and J.C. Hansen, *Nucleosomes and the chromatin fiber.* Curr Opin Genet Dev, 2001. **11**(2): p. 124-9.

Heathcott, R.W., et al., *A review of the phenotypic variation due to the Denys-Drash syndrome-associated germline WT1 mutation R362X.* Hum Mutat, 2002. **19**(4): p. 462.

Heemers, H.V. and D.J. Tindall, *Androgen receptor (AR) coregulators: a diversity of functions converging on and regulating the AR transcriptional complex.* Endocr Rev, 2007. **28**(7): p. 778-808.

Heinlein, C.A. and C. Chang, *Androgen receptor (AR) coregulators: an overview.* Endocr Rev, 2002. **23**(2): p. 175-200.

Herbst, A.L., *Clear cell adenocarcinoma and the current status of DES-exposed females.* Cancer, 1981. **48**(2 Suppl): p. 484-8.

Herbst, A.L., H. Ulfelder, and D.C. Poskanzer, *Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women.* N Engl J Med, 1971. **284**(15): p. 878-81.

Herman, R.A., et al., *Timing of prenatal androgen exposure: anatomical and endocrine effects on juvenile male and female rhesus monkeys.* Horm Behav, 2000. **38**(1): p. 52-66.

Hess, R.A., *Estrogen in the adult male reproductive tract: a review.* Reprod Biol Endocrinol, 2003. **1**: p. 52.

Hess, R.A., et al., *A role for oestrogens in the male reproductive system.* Nature, 1997. **390**(6659): p. 509-12.

Heudorf, U., V. Mersch-Sundermann, and J. Angerer, *Phthalates: toxicology and exposure.* Int J Hyg Environ Health, 2007. **210**(5): p. 623-34.

Hinck, L., *The versatile roles of "axon guidance" cues in tissue morphogenesis.* Dev Cell, 2004. **7**(6): p. 783-93.

Hiort, O. and P.M. Holterhus, *The molecular basis of male sexual differentiation.* Eur J Endocrinol, 2000. **142**(2): p. 101-10.

Hirshfield, A.N., *Development of follicles in the mammalian ovary.* Int Rev Cytol, 1991. **124**: p. 43-101.

Hong, C.Y., et al., *Modulation of androgen receptor transactivation by the SWI3-related gene product (SRG3) in multiple ways.* Mol Cell Biol, 2005. **25**(12): p. 4841-52.

Horrobin, D.F., et al., *Regulation of cytoplasmic calcium: interactions between prostaglandins, prostacyclin, thromboxane A2, zinc, copper and taurine.* Can J Neurol Sci, 1978. **5**(1): p. 93-6.

Hotchkiss, A.K., et al., *A mixture of the "antiandrogens" linuron and butyl benzyl phthalate alters sexual differentiation of the male rat in a cumulative fashion.* Biol Reprod, 2004. **71**(6): p. 1852-61.

Hotchkiss, A.K., et al., *In utero exposure to an AR antagonist plus an inhibitor of fetal testosterone synthesis induces cumulative effects on F1 male rats.* Reprod Toxicol, 2010, **30**(2): p. 261-70.

Hsieh, M.H., et al., *Associations among hypospadias, cryptorchidism, anogenital distance, and endocrine disruption.* Curr Urol Rep, 2008. **9**(2): p. 137-42.

Huang, P.C., et al., *Association between prenatal exposure to phthalates and the health of newborns.* Environ Int, 2009. **35**(1): p. 14-20.

Hughes, I.A. and C.L. Acerini, *Factors controlling testis descent.* Eur J Endocrinol, 2008. **159 Suppl 1**: p. S75-82.

Huhtaniemi, I.T. and K. Aittomaki, *Mutations of follicle-stimulating hormone and its receptor: effects on gonadal function.* Eur J Endocrinol, 1998. **138**(5): p. 473-81.

Hull, M.A., S.C. Ko, and G. Hawcroft, *Prostaglandin EP receptors: targets for treatment and prevention of colorectal cancer?* Mol Cancer Ther, 2004. **3**(8): p. 1031-9.

Hutson, J.M., S. Hasthorpe, and C.F. Heyns, *Anatomical and functional aspects of testicular descent and cryptorchidism.* Endocr Rev, 1997. **18**(2): p. 259-80.

Ikeda, Y., et al., *Steroidogenic factor 1 and Dax-1 colocalize in multiple cell lineages: potential links in endocrine development.* Mol Endocrinol, 1996. **10**(10): p. 1261-72.

Imperato-McGinley, J., et al., *Nipple differentiation in fetal male rats treated with an inhibitor of the enzyme 5 alpha-reductase: definition of a selective role for dihydrotestosterone.* Endocrinology, 1986. **118**(1): p. 132-7.

Imperato-McGinley, J. and Y.S. Zhu, *Androgens and male physiology the syndrome of 5alpha-reductase-2 deficiency.* Mol Cell Endocrinol, 2002. **198**(1-2): p. 51-9.

Inawaka, K., et al., *Maternal exposure to anti-androgenic compounds, vinclozolin, flutamide and procymidone, has no effects on spermatogenesis and DNA methylation in male rats of subsequent generations.* Toxicol Appl Pharmacol, 2009. **237**(2): p. 178-87.

Ivell, R. and S. Hartung, *The molecular basis of cryptorchidism.* Mol Hum Reprod, 2003. **9**(4): p. 175-81.

Jaaskelainen, J., *Molecular biology of androgen insensitivity.* Mol Cell Endocrinol.2011, article in press

Jaffe, B.M., H.R. Behrman, and C.W. Parker, *Radioimmunoassay measurement of prostaglandins E, A, and F in human plasma.* J Clin Invest, 1973. **52**(2): p. 398-405.

Jager, R.J., et al., *A human XY female with a frame shift mutation in the candidate testis-determining gene SRY.* Nature, 1990. **348**(6300): p. 452-4.

Jakob, S. and R. Lovell-Badge, *Sex determination and the control of Sox9 expression in mammals.* FEBS J, 2011 **278**(7): p. 1002-9.

Jakobsen, L.P., et al., *Pierre Robin sequence may be caused by dysregulation of SOX9 and KCNJ2.* J Med Genet, 2007. **44**(6): p. 381-6.

Jarrard, D.F., et al., *Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells.* Cancer Res, 1998. **58**(23): p. 5310-4.

Jeays-Ward, K., et al., *Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad.* Development, 2003. **130**(16): p. 3663-70.

Jegou, B., *The saga of sperm count decrease in humans and wild and farm animals. The Male Gamete: From Basic Knowledge to Clinical Applications*, 1999: p. 445–454.

. Jensen, T.K., et al., *Time trends in waiting time to pregnancy among Danish twins.* Hum Reprod, 2005. **20**(4): p. 955-64.

Jirasek, J.E., *Genital ducts and external genitalia: development and anomalies.* Birth Defects Orig Artic Ser, 1971. **7**(6): p. 131-9.

Jirsova, Z. and Z. Vernerova, *The differentiation and involution of the mullerian duct in the rat.* Funct Dev Morphol, 1993. **3**(2): p. 105-9.

Joffe, M., *What has happened to human fertility?* Hum Reprod. 2010, **25**(2): p. 295-307.

Joffe, M., *Time trends in biological fertility in Britain.* Lancet, 2000. **355**(9219): p. 1961-5.

Jordan, B.K., et al., *Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans.* Am J Hum Genet, 2001. **68**(5): p. 1102-9.

Jordan, B.K., et al., *Up-regulation of WNT-4 signaling and dosage-sensitive sex reversal in humans.* Am J Hum Genet, 2001. **68**(5): p. 1102-9.

Jorgensen, N., et al., *Regional differences in semen quality in Europe.* Hum Reprod, 2001. **16**(5): p. 1012-9.

Josso, N., et al., *AMH and AMH receptor defects in persistent Mullerian duct syndrome.* Hum Reprod Update, 2005. **11**(4): p. 351-6.

Kadam, S. and B.M. Emerson, *Transcriptional specificity of human SWI/SNF BRG1 and BRM chromatin remodeling complexes.* Mol Cell, 2003. **11**(2): p. 377-89.

Kaiser, U.B., et al., *A mechanism for the differential regulation of gonadotropin subunit gene expression by gonadotropin-releasing hormone.* Proc Natl Acad Sci U S A, 1995. **92**(26): p. 12280-4.

Kalaitzoglou, G. and M.I. New, *Congenital adrenal hyperplasia. Molecular insights learned from patients.* Receptor, 1993. **3**(3): p. 211-22.

Kalfa, N., et al., *Hypospadias: interactions between environment and genetics.* Mol Cell Endocrinol. 2011, **335**(2): p. 89-95.

Kalter, H., *Nontetratology of indomethacin.* Teratology, 1973, 7:a19.

Kang, N., et al., *Rwdd1, a thymus aging related molecule, is a new member of the intrinsically unstructured protein family.* Cell Mol Immunol, 2008. **5**(5): p. 333-9.

Kang, N., et al., *Identification and characterization of a novel thymus aging related protein Rwdd1.* Cell Mol Immunol, 2008. **5**(4): p. 279-85.

Kashanian, M., S. Bahasadri, and B. Zolali, *Comparison of the efficacy and adverse effects of nifedipine and indomethacin for the treatment of preterm labor.* Int J Gynaecol Obstet. 2011. **113**(3): p. 192-5.

Kashimada, K., Svingen, T., Feng, CW., Pelosi, E., Bagheri-fam, S, Harley, V., Schlessinger, D., Bowles, J., and Koopman, P., *Antagonistic regulation of Cyp26b1 by transcription factors SOX9/SF1 and FOXL2 during gonadal development in mice,* FASEB J, 2011 **25**: p3561-3569

Katz, Z., et al., *Absence of teratogenicity of indomethacin in ovarian hyperstimulation syndrome.* Int J Fertil, 1984. **29**(3): p. 186-8.

Kawashima, K., et al., *Virilizing effect of methyltestosterone on female descendants in the rat.* Endocrinol Jpn, 1978. **25**(1): p. 1-6.

Kemppainen, J.A., et al., *Androgen receptor phosphorylation, turnover, nuclear transport, and transcriptional activation. Specificity for steroids and antihormones.* J Biol Chem, 1992. **267**(2): p. 968-74.

Khan, O.Y., et al., *Multifunction steroid receptor coactivator, E6-associated protein, is involved in development of the prostate gland.* Mol Endocrinol, 2006. **20**(3): p. 544-59.

Khavari, P.A., et al., *BRG1 contains a conserved domain of the SWI2/SNF2 family necessary for normal mitotic growth and transcription.* Nature, 1993. **366**(6451): p. 170-4.

Kimmins, S. and T.H. MacRae, *Maturation of steroid receptors: an example of functional cooperation among molecular chaperones and their associated proteins.* Cell Stress Chaperones, 2000. **5**(2): p. 76-86.

Kinoshita, H., et al., *Methylation of the androgen receptor minimal promoter silences transcription in human prostate cancer.* Cancer Res, 2000. **60**(13): p. 3623-30.

Kirschenbaum, A., et al., *Immunohistochemical localization of cyclooxygenase-1 and cyclooxygenase-2 in the human fetal and adult male reproductive tracts.* J Clin Endocrinol Metab, 2000. **85**(9): p. 3436-41.

Kitamura, K., et al., *Mutation of ARX causes abnormal development of forebrain and testes in mice and X-linked lissencephaly with abnormal genitalia in humans.* Nat Genet, 2002. **32**(3): p. 359-69.

Klamt, B., et al., *Frasier syndrome is caused by defective alternative splicing of WT1 leading to an altered ratio of WT1 +/-KTS splice isoforms.* Hum Mol Genet, 1998. **7**(4): p. 709-14.

Klein, K.L., et al., *Indomethacin--placental transfer, cytotoxicity, and teratology in the rat.* Am J Obstet Gynecol, 1981. **141**(4): p. 448-52.

Klenova, E.M., et al., *The novel BORIS + CTCF gene family is uniquely involved in the epigenetics of normal biology and cancer.* Semin Cancer Biol, 2002. **12**(5): p. 399-414.

Klonisch, T., P.A. Fowler, and S. Hombach-Klonisch, *Molecular and genetic regulation of testis descent and external genitalia development.* Dev Biol, 2004. **270**(1): p. 1-18.

Konkle, A.T. and M.M. McCarthy, *Developmental time course of estradiol, testosterone, and dihydrotestosterone levels in discrete regions of male and female rat brain.* Endocrinology. 2011, **152**(1): p. 223-35.

Koopman, P., et al., *Male development of chromosomally female mice transgenic for Sry.* Nature, 1991. **351**(6322): p. 117-21.

Korach, K.S., et al., *Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes.* Recent Prog Horm Res, 1996. **51**: p. 159-86; discussion 186-8.

Kousteni, S., et al., *Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity.* Cell, 2001. **104**(5): p. 719-30.

Krasna, I.H. and H. Kim, *Indomethacin administration after temporary ischemia causes bowel necrosis in mice.* J Pediatr Surg, 1992. **27**(7): p. 805-7.

Krongrad, A., et al., *Androgen increases androgen receptor protein while decreasing receptor mRNA in LNCaP cells.* Mol Cell Endocrinol, 1991. **76**(1-3): p. 79-88.

Kubota, H., et al., *Cyclooxygenase-2 protects germ cells against spermatogenesis disturbance in experimental cryptorchidism model mice.* J Androl. 2010, **32**(1): p. 77-85.

Kung, A.L., et al., *Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP.* Genes Dev, 2000. **14**(3): p. 272-7.

Kurumbail, R.G., et al., *Structural basis for selective inhibition of cyclooxygenase-2 by anti-inflammatory agents.* Nature, 1996. **384**(6610): p. 644-8.

Kurzrock, E.A., et al., *Epithelial-mesenchymal interactions in development of the mouse fetal genital tubercle.* Cells Tissues Organs, 1999. **164**(3): p. 125-30.

Kwan, T.K., et al., *Steroid profiling in the study of rat testicular steroidogenesis.* Biochim Biophys Acta, 1988. **962**(2): p. 214-9.

Kwok, C., et al., *Mutations in SOX9, the gene responsible for Campomelic dysplasia and autosomal sex reversal.* Am J Hum Genet, 1995. **57**(5): p. 1028-36.

Laier, P., et al., *Mechanisms of action underlying the antiandrogenic effects of the fungicide prochloraz.* Toxicol Appl Pharmacol, 2006. **213**(2): p. 160-71.

Lala, P.K., *Similarities between immunoregulation in pregnancy and in malignancy: the role of prostaglandin E2.* Am J Reprod Immunol, 1989. **20**(4): p. 147-52.

Lambrot, R., et al., *Use of organ culture to study the human fetal testis development: effect of retinoic acid.* J Clin Endocrinol Metab, 2006. **91**(7): p. 2696-703.

Lambrot, R., et al., *Phthalates impair germ cell development in the human fetal testis in vitro without change in testosterone production.* Environ Health Perspect, 2009. **117**(1): p. 32-7.

Landschulz, K.T., et al., *Regulation of scavenger receptor, class B, type I, a high density lipoprotein receptor, in liver and steroidogenic tissues of the rat.* J Clin Invest, 1996. **98**(4): p. 984-95.

Latil, A., et al., *Quantification of expression of netrins, slits and their receptors in human prostate tumors.* Int J Cancer, 2003. **103**(3): p. 306-15.

Latronico, A.C., et al., *Brief report: testicular and ovarian resistance to luteinizing hormone caused by inactivating mutations of the luteinizing hormone-receptor gene.* N Engl J Med, 1996. **334**(8): p. 507-12.

Lavery, D.N. and I.J. McEwan, *The human androgen receptor AF1 transactivation domain: interactions with transcription factor IIF and molten-globule-like structural characteristics.* Biochem Soc Trans, 2006. **34**(Pt 6): p. 1054-7.

Lee, T.I. and R.A. Young, *Transcription of eukaryotic protein-coding genes.* Annu Rev Genet, 2000. **34**: p. 77-137.

Lee, W.J., et al., *Regulation of androgen receptor expression at the onset of functional overload in rat plantaris muscle.* Am J Physiol Regul Integr Comp Physiol, 2003. **285**(5): p. R1076-85.

Lee, Y.S., et al., *Phenotypic variability in 17beta-hydroxysteroid dehydrogenase-3 deficiency and diagnostic pitfalls.* Clin Endocrinol (Oxf), 2007. **67**(1): p. 20-8.

Lehmann, J.M., et al., *Peroxisome proliferator-activated receptors alpha and gamma are activated by indomethacin and other non-steroidal anti-inflammatory drugs.* J Biol Chem, 1997. **272**(6): p. 3406-10.

Li, J., E. Willingham, and L.S. Baskin, *Gene expression profiles in mouse urethral development.* BJU Int, 2006. **98**(4): p. 880-5.

Lin, C., et al., *Temporal and spatial dissection of Shh signaling in genital tubercle development.* Development, 2009. **136**(23): p. 3959-67.

Link, K.A., et al., *BAF57 governs androgen receptor action and androgen-dependent proliferation through SWI/SNF.* Mol Cell Biol, 2005. **25**(6): p. 2200-15.

Little, M.H., et al., *Evidence that WT1 mutations in Denys-Drash syndrome patients may act in a dominant-negative fashion.* Hum Mol Genet, 1993. **2**(3): p. 259-64.

Livera, G., et al., *Multiple effects of retinoids on the development of Sertoli, germ, and Leydig cells of fetal and neonatal rat testis in culture.* Biol Reprod, 2000. **62**(5): p. 1303-14.

Loffler, K.A. and P. Koopman, *Charting the course of ovarian development in vertebrates.* Int J Dev Biol, 2002. **46**(4): p. 503-10.

Logan, I.R., et al., *Human PIRH2 enhances androgen receptor signaling through inhibition of histone deacetylase 1 and is overexpressed in prostate cancer.* Mol Cell Biol, 2006. **26**(17): p. 6502-10.

Lopez-Teijon, M., M. Elbaile, and J.G. Alvarez, *Geographical differences in semen quality in a population of young healthy volunteers from the different regions of Spain.* Andrologia, 2008. **40**(5): p. 318-28.

Lorenzo, P., et al., *Identification and characterization of asporin. a novel member of the leucine-rich repeat protein family closely related to decorin and biglycan.* J Biol Chem, 2001. **276**(15): p. 12201-11.

Lottrup, G., et al., *Possible impact of phthalates on infant reproductive health.* Int J Androl, 2006. **29**(1): p. 172-80; discussion 181-5.

Loukinov, D.I., et al., *BORIS, a novel male germ-line-specific protein associated with epigenetic reprogramming events, shares the same 11-zinc-finger domain with CTCF, the insulator protein involved in reading imprinting marks in the soma.* Proc Natl Acad Sci U S A, 2002. **99**(10): p. 6806-11.

Lovell-Badge, R. and E. Robertson, *XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy.* Development, 1990. **109**(3): p. 635-46.

Lubahn, D.B., et al., *Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene.* Proc Natl Acad Sci U S A, 1993. **90**(23): p. 11162-6.

Luccio-Camelo, D.C. and G.S. Prins, *Disruption of androgen receptor signaling in males by environmental chemicals.* J Steroid Biochem Mol Biol. 2011, doi: .10.1016/j.jsbmb.2011.04.004

Luke, M.C. and D.S. Coffey, *Human androgen receptor binding to the androgen response element of prostate specific antigen.* J Androl, 1994. **15**(1): p. 41-51.

Luo, X., Y. Ikeda, and K.L. Parker, *A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation.* Cell, 1994. **77**(4): p. 481-90.

Luttge, W.G., *Dihydrotestosterone, androstenedione, testosterone: Comparative effectiveness in masculinizing and defeminizing reproductive systems in male and female rats* Hormones and Behavior 1970. **1**(4): p. 265-281.

Ma, Z.Q., et al., *Cdc25B functions as a novel coactivator for the steroid receptors.* Mol Cell Biol, 2001. **21**(23): p. 8056-67.

Macleod, D.J., et al., *Androgen action in the masculinization programming window and development of male reproductive organs.* Int J Androl. 2009 **33**(2): p. 279-87.

Maimoun, L., et al., *Phenotypical, biological, and molecular heterogeneity of 5alpha-reductase deficiency: an extensive international experience of 55 patients.* J Clin Endocrinol Metab. 2011 **96**(2): p. 296-307.

Majdic, G., M.R. Millar, and P.T. Saunders, *Immunolocalisation of androgen receptor to interstitial cells in fetal rat testes and to mesenchymal and epithelial cells of associated ducts.* J Endocrinol, 1995. **147**(2): p. 285-93.

Marshall, T.W., et al., *Differential requirement of SWI/SNF for androgen receptor activity.* J Biol Chem, 2003. **278**(33): p. 30605-13.

Mascaro, C., et al., *Contribution of steroidogenic factor 1 to the regulation of cholesterol synthesis.* Biochem J, 2000. **350 Pt 3**: p. 785-90.

Matzuk, M.M. and D.J. Lamb, *The biology of infertility: research advances and clinical challenges.* Nat Med, 2008. **14**(11): p. 1197-213.

McCoy, S.J. and B.A. Shirley, *Effects of prenatal administration of testosterone and cortisone on the reproductive system of the female rat.* Life Sci, 1992. **50**(9): p. 621-8.

McEwan, I.J., *Molecular mechanisms of androgen receptor-mediated gene regulation: structure-function analysis of the AF-1 domain.* Endocr Relat Cancer, 2004. **11**(2): p. 281-93.

McEwan, I.J. and J. Gustafsson, *Interaction of the human androgen receptor transactivation function with the general transcription factor TFIIF.* Proc Natl Acad Sci U S A, 1997. **94**(16): p. 8485-90.

McGlynn, K.A., et al., *Risk factors for cryptorchism among populations at differing risks of testicular cancer.* Int J Epidemiol, 2006. **35**(3): p. 787-95.

McIntyre, B.S., N.J. Barlow, and P.M. Foster, *Androgen-mediated development in male rat offspring exposed to flutamide in utero: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues.* Toxicol Sci, 2001. **62**(2): p. 236-49.

McIntyre, B.S., et al., *Effects of in utero exposure to linuron on androgen-dependent reproductive development in the male Crl:CD(SD)BR rat.* Toxicol Appl Pharmacol, 2000. **167**(2): p. 87-99.

McKee, R.H., et al., *An assessment of the potential developmental and reproductive toxicity of di-isoheptyl phthalate in rodents.* Reprod Toxicol, 2006. **21**(3): p. 241-52.

McKenna, N.J., R.B. Lanz, and B.W. O'Malley, *Nuclear receptor coregulators: cellular and molecular biology.* Endocr Rev, 1999. **20**(3): p. 321-44.

McKinnell, C., et al., *Suppression of androgen action and the induction of gross abnormalities of the reproductive tract in male rats treated neonatally with diethylstilbestrol.* J Androl, 2001. **22**(2): p. 323-38.

Meeks, J.J. and E.M. Schaeffer, *Genetic regulation of prostate development.* J Androl. 2010, **32**(3): p. 210-7.

Meisel, R.L., *The physiology of male sexual behavior. In: The physiology of reproduction.* 1994. **2**: p. 3-105.

Mendiola, J., et al., *Shorter anogenital distance predicts poorer semen quality in young men in Rochester, New York.* Environ Health Perspect. 2011 **119**(7): p. 958-63.

Mendonca, B.B., et al., *46,XY DSD due to impaired androgen production.* Best Pract Res Clin Endocrinol Metab. 2010 **24**(2): p. 243-62.

Merke, D.P. and S.R. Bornstein, *Congenital adrenal hyperplasia.* Lancet, 2005. **365**(9477): p. 2125-36.

Metzger, E., et al., *LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription.* Nature, 2005. **437**(7057): p. 436-9.

Miles, H.L., et al., *The role of androgens in fetal growth: observational study in two genetic models of disordered androgen signalling.* Arch Dis Child Fetal Neonatal Ed. 2010, **95**(6): p. F435-8.

Miller, W.L., *Molecular biology of steroid hormone synthesis.* Endocr Rev, 1988. **9**(3): p. 295-318.

Miller, W.L., *Congenital lipoid adrenal hyperplasia: the human gene knockout for the steroidogenic acute regulatory protein.* J Mol Endocrinol, 1997. **19**(3): p. 227-40.

Miller, W.L., *Androgen biosynthesis from cholesterol to DHEA.* Mol Cell Endocrinol, 2002. **198**(1-2): p. 7-14.

Miller, W.L., *Disorders of androgen synthesis--from cholesterol to dehydroepiandrosterone.* Med Princ Pract, 2005. **14 Suppl 1**: p. 58-68.

Mills, I.G., et al., *Huntingtin interacting protein 1 modulates the transcriptional activity of nuclear hormone receptors.* J Cell Biol, 2005. **170**(2): p. 191-200.

Min, H., et al., *Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to Drosophila branchless.* Genes Dev, 1998. **12**(20): p. 3156-61.

Mitchell, J.A., et al., *Selectivity of nonsteroidal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase.* Proc Natl Acad Sci U S A, 1993. **90**(24): p. 11693-7.

Miyagawa, S., et al., *The role of sonic hedgehog-Gli2 pathway in the masculinization of external genitalia.* Endocrinology. 2011 **152**(7): p. 2894-903.

Miyamoto, N., et al., *Defects of urogenital development in mice lacking Emx2.* Development, 1997. **124**(9): p. 1653-64.

Moise, K.J., Jr., et al., *Placental transfer of indomethacin in the human pregnancy.* Am J Obstet Gynecol, 1990. **162**(2): p. 549-54.

Momose, Y., D.W. Goh, and J.M. Hutson, *Calcitonin gene-related peptide stimulates motility of the gubernaculum via cyclic adenosine monophosphate.* J Urol, 1993. **150**(2 Pt 2): p. 571-3.

Monroy, M.A., et al., *SNF2-related CBP activator protein (SRCAP) functions as a coactivator of steroid receptor-mediated transcription through synergistic interactions with CARM-1 and GRIP-1.* Mol Endocrinol, 2003. **17**(12): p. 2519-28.

Montero, J.A., et al., *Expression of Sox8, Sox9 and Sox10 in the developing valves and autonomic nerves of the embryonic heart.* Mech Dev, 2002. **118**(1-2): p. 199-202.

Moore, K.l., The Developing Human: Clinically Oriented Embryology, 8th Edition , chapter 12, The urogenital system, Elsevier Health Sciences, 2007

Moraga, P.F., M.N. Llanos, and A.M. Ronco, *Arachidonic acid release from rat Leydig cells depends on the presence of luteinizing hormone/human chorionic gonadotrophin receptors.* J Endocrinol, 1997. **154**(2): p. 201-9.

Morgan, E.A., et al., *Loss of Bmp7 and Fgf8 signaling in Hoxa13-mutant mice causes hypospadia.* Development, 2003. **130**(14): p. 3095-109.

Mortlock, D.P. and J.W. Innis, *Mutation of HOXA13 in hand-foot-genital syndrome.* Nat Genet, 1997. **15**(2): p. 179-80.

Murakami, R. and T. Mizuno, *Proximal-distal sequence of development of the skeletal tissues in the penis of rat and the inductive effect of epithelium.* J Embryol Exp Morphol, 1986. **92**: p. 133-43.

Murashima, A., et al., *Essential roles of androgen signaling in Wolffian duct stabilization and epididymal cell differentiation.* Endocrinology. 2011, **152**(4): p. 1640-51.

Nachtigal, M.W., et al., *Wilms' tumor 1 and Dax-1 modulate the orphan nuclear receptor SF-1 in sex-specific gene expression.* Cell, 1998. **93**(3): p. 445-54.

Naidu, S.R., et al., *The SWI/SNF chromatin remodeling subunit BRG1 is a critical regulator of p53 necessary for proliferation of malignant cells.* Oncogene, 2009. **28**(27): p. 2492-501.

Narumi, Y., et al., *Genital abnormalities in Pallister-Hall syndrome: Report of two patients and review of the literature.* Am J Med Genet A.2010 **152A**(12): p. 3143-7.

Nawaz, Z. and B.W. O'Malley, *Urban renewal in the nucleus: is protein turnover by proteasomes absolutely required for nuclear receptor-regulated transcription?* Mol Endocrinol, 2004. **18**(3): p. 493-9.

Nazareth, L.V. and N.L. Weigel, *Activation of the human androgen receptor through a protein kinase A signaling pathway.* J Biol Chem, 1996. **271**(33): p. 19900-7.

Nef, S. and J.D. Vassalli, *Complementary pathways in mammalian female sex determination.* J Biol, 2009. **8**(8): p. 74.

Nguyen, A.P., A. Chandorkar, and C. Gupta, *The role of growth hormone in fetal mouse reproductive tract differentiation.* Endocrinology, 1996. **137**(9): p. 3659-66.

Nguyen, P., et al., *CTCFL/BORIS is a methylation-independent DNA-binding protein that preferentially binds to the paternal H19 differentially methylated region.* Cancer Res, 2008. **68**(14): p. 5546-51.

Nishimura, K., et al., *Modulation of androgen receptor transactivation by gelsolin: a newly identified androgen receptor coregulator.* Cancer Res, 2003. **63**(16): p. 4888-94.

Niu, Y., et al., *Tissue prostate-specific antigen facilitates refractory prostate tumor progression via enhancing ARA70-regulated androgen receptor transactivation.* Cancer Res, 2008. **68**(17): p. 7110-9.

Norton, M.E., *Teratogen update: fetal effects of indomethacin administration during pregnancy.* Teratology, 1997. **56**(4): p. 282-92.

Norton, M.E., et al., *Neonatal complications after the administration of indomethacin for preterm labor.* N Engl J Med, 1993. **329**(22): p. 1602-7.

Norwitz, E.R., D.J. Schust, and S.J. Fisher, *Implantation and the survival of early pregnancy.* N Engl J Med, 2001. **345**(19): p. 1400-8.

Novy, M.J., *Effects of indomethacin on labor, fetal oxygenation, and fetal development in rhesus monkeys.* Adv Prostaglandin Thromboxane Res, 1978. **4**: p. 285-300.

Ogawa, T., et al., *Developmental changes in cyclooxygenase mRNA expression in the kidney of rats.* Pediatr Nephrol, 2001. **16**(8): p. 618-22.

O'Grady, J.P., et al., *The effects of an inhibitor of prostaglandin synthesis (indomethacin) on ovulation, pregnancy, and pseudopregnancy in the rabbit.* Prostaglandins, 1972. **1**(2): p. 97-106.

O'Hara, L., et al., *Androgen receptor expression in the caput epididymal epithelium is essential for development of the initial segment and epididymal spermatozoa transit.* Endocrinology. 2011, **152**(2): p. 718-29.

Oki, S., et al., *Radioimmunoassays of prostaglandin F-2-alpha and prostaglandin F-2-alpha-main urinary metabolite with prostaglandin-125-I-tyrosine methylester amide.* Prostaglandins, 1974. **6**(2): p. 137-48.

Onoe, Y., et al., *Expression of estrogen receptor beta in rat bone.* Endocrinology, 1997. **138**(10): p. 4509-12.

Ooi, S.K., et al., *DNMT3L connects unmethylated lysine 4 of histone H3 to de novo methylation of DNA.* Nature, 2007. **448**(7154): p. 714-7.

Orban, P.C., D. Chui, and J.D. Marth, *Tissue- and site-specific DNA recombination in transgenic mice.* Proc Natl Acad Sci U S A, 1992. **89**(15): p. 6861-5.

O'Shaughnessy, P.J., et al., *Localization of 17beta-hydroxysteroid dehydrogenase/17-ketosteroid reductase isoform expression in the developing mouse testis--androstenedione is the major androgen secreted by fetal/neonatal leydig cells.* Endocrinology, 2000. **141**(7): p. 2631-7.

Ostby, J., et al., *The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro.* Toxicol Ind Health, 1999. **15**(1-2): p. 80-93.

Osterlund, K.L., R.J. Handa, and R.J. Gonzales, *Dihydrotestosterone alters cyclooxygenase-2 levels in human coronary artery smooth muscle cells.* Am J Physiol Endocrinol Metab. 2010, **298**(4): p. E838-45.

Ottolenghi, C., et al., *Loss of Wnt4 and Foxl2 leads to female-to-male sex reversal extending to germ cells.* Hum Mol Genet, 2007. **16**(23): p. 2795-804.

Owoyele, B.V., O.T. Adenekan, and A.O. Soladoye, *Effects of honey on inflammation and nitric oxide production in Wistar rats.* Journal of Chinese Integrative Medicine: 2011; **9**(4): 447-452

Pakarinen, P., et al., *Pituitary hormones are not required for sexual differentiation of male mice: phenotype of the T/ebp/Nkx2.1 null mutant mice.* Endocrinology, 2002. **143**(11): p. 4477-82.

Panet-Raymond, V., et al., *Interactions between androgen and estrogen receptors and the effects on their transactivational properties.* Mol Cell Endocrinol, 2000. **167**(1-2): p. 139-50.

Park, S.Y., et al., *Nuclear receptors Sf1 and Dax1 function cooperatively to mediate somatic cell differentiation during testis development.* Development, 2005. **132**(10): p. 2415-23.

Parks, L.G., et al., *The plasticizer diethylhexyl phthalate induces malformations by decreasing fetal testosterone synthesis during sexual differentiation in the male rat.* Toxicol Sci, 2000. **58**(2): p. 339-49.

Patrao, M.T., E.J. Silva, and M.C. Avellar, *Androgens and the male reproductive tract: an overview of classical roles and current perspectives.* Arq Bras Endocrinol Metabol, 2009. **53**(8): p. 934-45.

Paulozzi, L.J., *International trends in rates of hypospadias and cryptorchidism.* Environ Health Perspect, 1999. **107**(4): p. 297-302.

Payne, A.H. and D.B. Hales, *Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones.* Endocr Rev, 2004. **25**(6): p. 947-70.

Payne, A.H. and G.L. Youngblood, *Regulation of expression of steroidogenic enzymes in Leydig cells.* Biol Reprod, 1995. **52**(2): p. 217-25.

Peets, E.A., M.F. Henson, and R. Neri, *On the mechanism of the anti-androgenic action of flutamide (alpha-alpha-alpha-trifluoro-2-methyl-4'-nitro-m-propionotoluidide) in the rat.* Endocrinology, 1974. **94**(2): p. 532-40.

Pelletier, G., C. Labrie, and F. Labrie, *Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs.* J Endocrinol, 2000. **165**(2): p. 359-70.

Pelletier, G., et al., *Immunocytochemical localization of type 5 17beta-hydroxysteroid dehydrogenase in human reproductive tissues.* J Histochem Cytochem, 1999. **47**(6): p. 731-8.

Penning, T.M., *Molecular endocrinology of hydroxysteroid dehydrogenases.* Endocr Rev, 1997. **18**(3): p. 281-305.

Perissi, V., et al., *Factor-specific modulation of CREB-binding protein acetyltransferase activity.* Proc Natl Acad Sci U S A, 1999. **96**(7): p. 3652-7.

Perovic, S., M. Djordjevic, and N. Djakovic, *Natural erection induced by prostaglandin-E1 in the diagnosis and treatment of congenital penile anomalies.* Br J Urol, 1997. **79**(1): p. 43-6.

Peters, F.T., et al., *Screening for and validated quantification of amphetamines and of amphetamine- and piperazine-derived designer drugs in human blood plasma by gas chromatography/mass spectrometry.* J Mass Spectrom, 2003. **38**(6): p. 659-76.

Peterziel, H., et al., *Rapid signalling by androgen receptor in prostate cancer cells.* Oncogene, 1999. **18**(46): p. 6322-9.

Petre-Draviam, C.E., et al., *Specificity of cyclin D1 for androgen receptor regulation.* Cancer Res, 2003. **63**(16): p. 4903-13.

Podlasek, C.A., D. Duboule, and W. Bushman, *Male accessory sex organ morphogenesis is altered by loss of function of Hoxd-13.* Dev Dyn, 1997. **208**(4): p. 454-65.

Pont, A., et al., *Ketoconazole blocks testosterone synthesis.* Arch Intern Med, 1982. **142**(12): p. 2137-40.

Poukka, H., et al., *Coregulator small nuclear RING finger protein (SNURF) enhances Sp1- and steroid receptor-mediated transcription by different mechanisms.* J Biol Chem, 2000. **275**(1): p. 571-9.

Poyner, D.R., et al., *International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors.* Pharmacol Rev, 2002. **54**(2): p. 233-46.

Poyser, N.L., *A comparison of the effects of indomethacin and NS-398 (a selective prostaglandin H synthase-2 inhibitor) on implantation in the rat.* Prostaglandins Leukot Essent Fatty Acids, 1999. **61**(5): p. 297-301.

Prasad, A., et al., *Slit-2/Robo-1 modulates the CXCL12/CXCR4-induced chemotaxis of T cells.* J Leukoc Biol, 2007. **82**(3): p. 465-76.

Prins, G.S., et al., *Developmental estrogen exposures predispose to prostate carcinogenesis with aging.* Reprod Toxicol, 2007. **23**(3): p. 374-82.

Pu, Y., L. Huang, and G.S. Prins, *Sonic hedgehog-patched Gli signaling in the developing rat prostate gland: lobe-specific suppression by neonatal estrogens reduces ductal growth and branching.* Dev Biol, 2004. **273**(2): p. 257-75.

Qi, W., et al., *A novel function of caspase-8 in the regulation of androgen-receptor-driven gene expression.* EMBO J, 2007. **26**(1): p. 65-75.

Quigley, C.A., et al., *Androgen receptor defects: historical, clinical, and molecular perspectives.* Endocr Rev, 1995. **16**(3): p. 271-321.

Rajpert-De Meyts, E., et al., *Developmental arrest of germ cells in the pathogenesis of germ cell neoplasia.* APMIS, 1998. **106**(1): p. 198-204; discussion 204-6.

Rajpert-De Meyts, E., M. Kvist, and N.E. Skakkebaek, *Heterogeneity of expression of immunohistochemical tumour markers in testicular carcinoma in situ: pathogenetic relevance.* Virchows Arch, 1996. **428**(3): p. 133-9.

Randall, C.L., R.F. Anton, and H.C. Becker, *Effect of indomethacin on alcohol-induced morphological anomalies in mice.* Life Sci, 1987. **41**(3): p. 361-9.

Randazzo, F.M., et al., *brg1: a putative murine homologue of the Drosophila brahma gene, a homeotic gene regulator.* Dev Biol, 1994. **161**(1): p. 229-42.

Rasmussen, K.K., H. Vilmann, and M. Juhl, *Os penis of the rat. V. The distal cartilage process.* Acta Anat (Basel), 1986. **125**(3): p. 208-12.

Ray, M.R., et al., *Cyclin G-associated kinase: a novel androgen receptor-interacting transcriptional coactivator that is overexpressed in hormone refractory prostate cancer.* Int J Cancer, 2006. **118**(5): p. 1108-19.

Rees, I., et al., *The E3 ubiquitin ligase CHIP binds the androgen receptor in a phosphorylation-dependent manner.* Biochim Biophys Acta, 2006. **1764**(6): p. 1073-9.

Reese, J., et al., *Coordinated regulation of fetal and maternal prostaglandins directs successful birth and postnatal adaptation in the mouse.* Proc Natl Acad Sci U S A, 2000. **97**(17): p. 9759-64.

Reginensi, A., et al., *SOX9 controls epithelial branching by activating RET effector genes during kidney development.* Hum Mol Genet, 2011 **20**(6): p. 1143-53.

Renaud, S., et al., *Expression of the CTCF-paralogous cancer-testis gene, brother of the regulator of imprinted sites (BORIS), is regulated by three alternative promoters modulated by CpG methylation and by CTCF and p53 transcription factors.* Nucleic Acids Res, 2007. **35**(21): p. 7372-88.

Reutens, A.T., et al., *Cyclin D1 binds the androgen receptor and regulates hormone-dependent signaling in a p300/CBP-associated factor (P/CAF)-dependent manner.* Mol Endocrinol, 2001. **15**(5): p. 797-811.

Rey, R., et al., *Testicular steroidogenesis in the Cebus monkey throughout postnatal development.* Biol Reprod, 1995. **52**(5): p. 997-1002.

Rey, R., et al., *Anti-mullerian hormone in children with androgen insensitivity.* J Clin Endocrinol Metab, 1994. **79**(4): p. 960-4.

Rey, R. and J.Y. Picard, *Embryology and endocrinology of genital development.* Baillieres Clin Endocrinol Metab, 1998. **12**(1): p. 17-33.

Rey, R.A., et al., *Evaluation of gonadal function in 107 intersex patients by means of serum antimullerian hormone measurement.* J Clin Endocrinol Metab, 1999. **84**(2): p. 627-31.

Rey, R.A. and R.P. Grinspon, *Normal male sexual differentiation and aetiology of disorders of sex development.* Best Pract Res Clin Endocrinol Metab. 2011, **25**(2): p. 221-38.

Rey, R.A., et al., *Ontogeny of the androgen receptor expression in the fetal and postnatal testis: its relevance on Sertoli cell maturation and the onset of adult spermatogenesis.* Microsc Res Tech, 2009. **72**(11): p. 787-95.

Reyes, J.C., et al., *Altered control of cellular proliferation in the absence of mammalian brahma (SNF2alpha).* EMBO J, 1998. **17**(23): p. 6979-91.

Rhees, R.W., et al., *Effects of prenatal testosterone on sexual behavior, reproductive morphology and LH secretion in the female rat.* Dev Neurosci, 1997. **19**(5): p. 430-7.

Rivas, A., et al., *Induction of reproductive tract developmental abnormalities in the male rat by lowering androgen production or action in combination with a low dose of diethylstilbestrol: evidence for importance of the androgen-estrogen balance.* Endocrinology, 2002. **143**(12): p. 4797-808.

Roberts, L.M., J.A. Visser, and H.A. Ingraham, *Involvement of a matrix metalloproteinase in MIS-induced cell death during urogenital development.* Development, 2002. **129**(6): p. 1487-96.

Rodeck, C.H., et al., *Testosterone levels in midtrimester maternal and fetal plasma and amniotic fluid.* Prenat Diagn, 1985. **5**(3): p. 175-81.

Roeder, R.G., *The role of general initiation factors in transcription by RNA polymerase II.* Trends Biochem Sci, 1996. **21**(9): p. 327-35.

Romanelli, F., et al., *Arachidonic acid and its metabolites effects on testosterone production by rat Leydig cells.* J Endocrinol Invest, 1995. **18**(3): p. 186-93.

Rosenberg, K.M. and G.F. Sherman, *Testosterone induced pup-killing behavior in the ovariectomized female rat.* Physiol Behav, 1974. **13**(5): p. 697-9.

Rosenfeld, M.G., et al., *Production of a novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing.* Nature, 1983. **304**(5922): p. 129-35.

Rosenfield, R.L., *Ovarian and adrenal function in polycystic ovary syndrome.* Endocrinol Metab Clin North Am, 1999. **28**(2): p. 265-93.

Rouleau, N., et al., *Novel ATPase of SNF2-like protein family interacts with androgen receptor and modulates androgen-dependent transcription.* Mol Biol Cell, 2002. **13**(6): p. 2106-19.

Sadovsky, Y. and C. Dorn, *Function of steroidogenic factor 1 during development and differentiation of the reproductive system.* Rev Reprod, 2000. **5**(3): p. 136-42.

Sajjad, Y., *Development of the genital ducts and external genitalia in the early human embryo.* J Obstet Gynaecol Res. **36**(5): p. 929-37.

Sajjad, Y., et al., *Immunohistochemical localization of androgen receptors in the urogenital tracts of human embryos.* Reproduction, 2004. **128**(3): p. 331-9.

Sajjad, Y., et al., *Expression of androgen receptors in upper human fetal reproductive tract.* Hum Reprod, 2004. **19**(7): p. 1659-65.

Salazar-Martinez, E., et al., *Anogenital distance in human male and female newborns: a descriptive, cross-sectional study.* Environ Health, 2004. **3**(1): p. 8.

Santos, F.C., et al., *Testosterone stimulates growth and secretory activity of the female prostate in the adult gerbil (Meriones unguiculatus).* Biol Reprod, 2006. **75**(3): p. 370-9.

Sasaki, M., et al., *Methylation and inactivation of estrogen, progesterone, and androgen receptors in prostate cancer.* J Natl Cancer Inst, 2002. **94**(5): p. 384-90.

Sauer, B., *Functional expression of the cre-lox site-specific recombination system in the yeast Saccharomyces cerevisiae.* Mol Cell Biol, 1987. **7**(6): p. 2087-96.

Sauer, B. and N. Henderson, *Site-specific DNA recombination in mammalian cells by the Cre recombinase of bacteriophage P1.* Proc Natl Acad Sci U S A, 1988. **85**(14): p. 5166-70.

Saunders, P.T., et al., *Expression of oestrogen receptor beta (ER beta) in multiple rat tissues visualised by immunohistochemistry.* J Endocrinol, 1997. **154**(3): p. R13-6.

Sawada, T., *Effects of Oxytocin and Prostaglandin $F_{2\alpha}$ on Androgen Production of Adult Rat Testis In Vivo.* Prostaglandins and other lipid mediators, 1998. **55**(2-3): p. 121-126.

Schell, C., et al., *A prostaglandin D2 system in the human testis.* Fertil Steril, 2007. **88**(1): p. 233-6.

Schlondorff, D., *Renal complications of nonsteroidal anti-inflammatory drugs.* Kidney Int, 1993. **44**(3): p. 643-53.

Schrager, S. and B.E. Potter, *Diethylstilbestrol exposure.* Am Fam Physician, 2004. **69**(10): p. 2395-400.

Scott, H.M., et al., *Relationship between androgen action in the "male programming window," fetal sertoli cell number, and adult testis size in the rat.* Endocrinology, 2008. **149**(10): p. 5280-7.

Scott, H.M., J.I. Mason, and R.M. Sharpe, *Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds.* Endocr Rev, 2009. **30**(7): p. 883-925.

Seifert, A.W., B.D. Harfe, and M.J. Cohn, *Cell lineage analysis demonstrates an endodermal origin of the distal urethra and perineum.* Dev Biol, 2008. **318**(1): p. 143-52.

Seifert, A.W., et al., *Sonic hedgehog controls growth of external genitalia by regulating cell cycle kinetics.* Nat Commun. 2010 **1**: p. 23.

Shahbazian, A., et al., *Disturbance of peristalsis in the guinea-pig isolated small intestine by indomethacin, but not cyclo-oxygenase isoform-selective inhibitors.* Br J Pharmacol, 2001. **132**(6): p. 1299-309.

Sharpe, G.L., K.S. Larsson, and B. Thalme, *Studies on closure of the ductus arteriosus. XII. In utero effect of indomethacin and sodium salicylate in rats and rabbits.* Prostaglandins, 1975. **9**(4): p. 585-96.

Sharpe, R.M., *The roles of oestrogen in the male.* Trends Endocrinol Metab, 1998. **9**(9): p. 371-7.

Sharpe, R.M., *Hormones and testis development and the possible adverse effects of environmental chemicals.* Toxicol Lett, 2001. **120**(1-3): p. 221-32.

Sharpe, R.M., *The 'oestrogen hypothesis'- where do we stand now?* Int J Androl, 2003. **26**(1): p. 2-15.

Sharpe, R.M., *Sertoli Cell Endocrinology and Signal Transduction: Androgen Regulation.* 2005: p. 199-216.

Sharpe, R.M., *Pathways of endocrine disruption during male sexual differentiation and masculinization.* Best Pract Res Clin Endocrinol Metab, 2006. **20**(1): p. 91-110.

Sharpe, R.M., *"Additional" effects of phthalate mixtures on fetal testosterone production.* Toxicol Sci, 2008. **105**(1): p. 1-4.

Sharpe, R.M., et al., *Testosterone and spermatogenesis. Identification of stage-specific, androgen-regulated proteins secreted by adult rat seminiferous tubules.* J Androl, 1992. **13**(2): p. 172-84.

Sharpe, R.M. and N.E. Skakkebaek, *Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract?* Lancet, 1993. **341**(8857): p. 1392-5.

Sharpe, R.M. and N.E. Skakkebaek, *Testicular dysgenesis syndrome: mechanistic insights and potential new downstream effects.* Fertil Steril, 2008. **89**(2 Suppl): p. e33-8.

Sheflin, L., et al., *Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels.* Biochem Biophys Res Commun, 2000. **276**(1): p. 144-50.

Shen, W.H., et al., *Nuclear receptor steroidogenic factor 1 regulates the mullerian inhibiting substance gene: a link to the sex determination cascade.* Cell, 1994. **77**(5): p. 651-61.

Shiama, N., *The p300/CBP family: integrating signals with transcription factors and chromatin.* Trends Cell Biol, 1997. **7**(6): p. 230-6.

Shinoda, K., et al., *Developmental defects of the ventromedial hypothalamic nucleus and pituitary gonadotroph in the Ftz-F1 disrupted mice.* Dev Dyn, 1995. **204**(1): p. 22-9.

Shock, L.S., et al., *DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria.* Proc Natl Acad Sci U S A. 2011 **108**(9): p. 3630-5.

Shore, L., et al., *Effect of hCG injection on prostaglandin E concentrations in ram seminal plasma.* Prostaglandins Other Lipid Mediat, 2003. **70**(3-4): p. 291-301.

Simard, J., et al., *Characteristics of interaction of the antiandrogen flutamide with the androgen receptor in various target tissues.* Mol Cell Endocrinol, 1986. **44**(3): p. 261-70.

Simard, J., et al., *Molecular basis of congenital adrenal hyperplasia due to 3 beta-hydroxysteroid dehydrogenase deficiency.* Mol Endocrinol, 1993. **7**(5): p. 716-28.

Simmons, D., et al., *Sex differences in umbilical cord serum levels of inhibin, testosterone, oestradiol, dehydroepiandrosterone sulphate, and sex hormone-binding globulin in human term neonates.* Biol Neonate, 1994. **65**(5): p. 287-94.

Skakkebaek, N.E., E. Rajpert-De Meyts, and K.M. Main, *Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects.* Hum Reprod, 2001. **16**(5): p. 972-8.

Slob, A.K., et al., *Prenatal testosterone propionate and postnatal ovarian activity in the rat.* Acta Endocrinol (Copenh), 1983. **103**(3): p. 420-7.

Sookvanichsilp, N. and P. Pulbutr, *Anti-implantation effects of indomethacin and celecoxib in rats.* Contraception, 2002. **65**(5): p. 373-8.

Springer, D., et al., *Identification of cytochrome p450 enzymes involved in the metabolism of 4'-methyl-alpha-pyrrolidinopropiophenone, a novel scheduled designer drug, in human liver microsomes.* Drug Metab Dispos, 2003. **31**(8): p. 979-82.

Staack, A., et al., *Mouse urogenital development: a practical approach.* Differentiation, 2003. **71**(7): p. 402-13.

Staack, A., et al., *Quantitation of apoptotic activity following castration in human prostatic tissue in vivo.* Prostate, 2003. **54**(3): p. 212-9.

Staack, R.F., J. Fehn, and H.H. Maurer, *New designer drug p-methoxymethamphetamine: studies on its metabolism and toxicological detection in urine using gas chromatography-mass spectrometry.* J Chromatogr B Analyt Technol Biomed Life Sci, 2003. **789**(1): p. 27-41.

Staack, R.F., G. Fritschi, and H.H. Maurer, *New designer drug 1-(3-trifluoromethylphenyl) piperazine (TFMPP): gas chromatography/mass spectrometry and liquid chromatography/mass spectrometry studies on its phase I and II metabolism and on its toxicological detection in rat urine.* J Mass Spectrom, 2003. **38**(9): p. 971-81.

Staack, R.F. and H.H. Maurer, *Piperazine-derived designer drug 1-(3-chlorophenyl)piperazine (mCPP): GC-MS studies on its metabolism and its toxicological detection in rat urine including analytical differentiation from its precursor drugs trazodone and nefazodone.* J Anal Toxicol, 2003. **27**(8): p. 560-8.

Staack, R.F. and H.H. Maurer, *Toxicological detection of the new designer drug 1-(4-methoxyphenyl)piperazine and its metabolites in urine and differentiation from an intake of structurally related medicaments using gas chromatography-mass spectrometry.* J Chromatogr B Analyt Technol Biomed Life Sci, 2003. **798**(2): p. 333-42.

Stein, R.W., et al., *Analysis of E1A-mediated growth regulation functions: binding of the 300-kilodalton cellular product correlates with E1A enhancer repression function and DNA synthesis-inducing activity.* J Virol, 1990. **64**(9): p. 4421-7.

Steketee, K., et al., *Amino acids 3-13 and amino acids in and flanking the 23FxxLF27 motif modulate the interaction between the N-terminal and ligand-binding domain of the androgen receptor.* Eur J Biochem, 2002. **269**(23): p. 5780-91.

Stocco, D.M., *Tracking the role of a star in the sky of the new millennium.* Mol Endocrinol, 2001. **15**(8): p. 1245-54.

Stocco, D.M. and B.J. Clark, *Regulation of the acute production of steroids in steroidogenic cells.* Endocr Rev, 1996. **17**(3): p. 221-44.

Suetake, I., et al., *DNMT3L stimulates the DNA methylation activity of Dnmt3a and Dnmt3b through a direct interaction.* J Biol Chem, 2004. **279**(26): p. 27816-23.

Sufrin, G. and D.S. Coffey, *Flutamide. Mechanism of action of a new nonsteroidal antiandrogen.* Invest Urol, 1976. **13**(6): p. 429-34.

Sultan, C., et al., *Disorders linked to insufficient androgen action in male children.* Hum Reprod Update, 2001. **7**(3): p. 314-22.

Sun, A., et al., *Aberrant expression of SWI/SNF catalytic subunits BRG1/BRM is associated with tumor development and increased invasiveness in prostate cancers.* Prostate, 2007. **67**(2): p. 203-13.

Supowit, S.C., H. Zhao, and D.J. DiPette, *Nerve growth factor enhances calcitonin gene-related peptide expression in the spontaneously hypertensive rat.* Hypertension, 2001. **37**(2 Part 2): p. 728-32.

Suzuki, T., et al., *Expression of a testis-specific form of Gal3st1 (CST), a gene essential for spermatogenesis, is regulated by the CTCF paralogous gene BORIS.* Mol Cell Biol. 2010 **30**(10): p. 2473-84.

Swain, A. and R. Lovell-Badge, *Mammalian sex determination: a molecular drama.* Genes Dev, 1999. **13**(7): p. 755-67.

Swain, A., et al., *Mouse Dax1 expression is consistent with a role in sex determination as well as in adrenal and hypothalamus function.* Nat Genet, 1996. **12**(4): p. 404-9.

Swan, S.H., *Environmental phthalate exposure in relation to reproductive outcomes and other health endpoints in humans.* Environ Res, 2008. **108**(2): p. 177-84.

Swan, S.H., E.P. Elkin, and L. Fenster, *The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996.* Environ Health Perspect, 2000. **108**(10): p. 961-6.

Swan, S.H., et al., *Decrease in anogenital distance among male infants with prenatal phthalate exposure.* Environ Health Perspect, 2005. **113**(8): p. 1056-61.

Swanson, H.E. and J.J. Werff ten Bosch, *The "early-androgen" syndrome; effects of pre-natal testosterone propionate.* Acta Endocrinol (Copenh), 1965. **50**(3): p. 379-90.

Takagi, S., et al., *Sex differences in fetal gonadotropins and androgens.* J Steroid Biochem, 1977. **8**(5): p. 609-20.

Takahashi, S., et al., *DNA methylation in the androgen receptor gene promoter region in rat prostate cancers.* Prostate, 2002. **52**(1): p. 82-8.

Takane, K.K., F.W. George, and J.D. Wilson, *Androgen receptor of rat penis is down-regulated by androgen.* Am J Physiol, 1990. **258**(1 Pt 1): p. E46-50.

Taketo, M.M., *Cyclooxygenase-2 inhibitors in tumorigenesis (part I).* J Natl Cancer Inst, 1998. **90**(20): p. 1529-36.

Tan, J.A., et al., *The rat androgen receptor: primary structure, autoregulation of its messenger ribonucleic acid, and immunocytochemical localization of the receptor protein.* Mol Endocrinol, 1988. **2**(12): p. 1276-85.

Tanaka, A., et al., *Role of cyclooxygenase (COX)-1 and COX-2 inhibition in nonsteroidal anti-inflammatory drug-induced intestinal damage in rats: relation to various pathogenic events.* J Pharmacol Exp Ther, 2002. **303**(3): p. 1248-54.

Tang, H., et al., *Notch signaling maintains Leydig progenitor cells in the mouse testis.* Development, 2008. **135**(22): p. 3745-53.

Terada, M., et al., *Calcitonin gene-related peptide receptors in the gubernaculum of normal rat and 2 models of cryptorchidism.* J Urol, 1994. **152**(2 Pt 2): p. 759-62.

Tevosian, S.G., et al., *Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2.* Development, 2002. **129**(19): p. 4627-34.

Thiele, B., et al., *Complete androgen insensitivity caused by a new frameshift deletion of two base pairs in exon 1 of the human androgen receptor gene.* J Clin Endocrinol Metab, 1999. **84**(5): p. 1751-3.

Thieulant, M.L., et al., *[Increasing of the 5-alpha-steroid reductase activity of purified cell nuclei from the pituitary gland of castrate male rats]*. C R Acad Sci Hebd Seances Acad Sci D, 1974. **278**(9): p. 1281-4.

Thomas, C.M., et al., *Radioimmunoassays for prostaglandins. I. Technical validation of prostaglandin F2alpha measurements in human plasma using sephadex G-25 gelfiltration.* Prostaglandins, 1978. **15**(5): p. 839-47.

Thompson, C.J., S.M. Ross, and K.W. Gaido, *Di(n-butyl) phthalate impairs cholesterol transport and steroidogenesis in the fetal rat testis through a rapid and reversible mechanism.* Endocrinology, 2004. **145**(3): p. 1227-37.

Thompson, S.T., et al., *Transverse testicular ectopia in a man with persistent Mullerian duct syndrome.* Arch Pathol Lab Med, 1994. **118**(7): p. 752-5.

Thomson, A.A. and G.R. Cunha, *Prostatic growth and development are regulated by FGF10.* Development, 1999. **126**(16): p. 3693-701.

Thornton, J., J.L. Zehr, and M.D. Loose, *Effects of prenatal androgens on rhesus monkeys: a model system to explore the organizational hypothesis in primates.* Horm Behav, 2009. **55**(5): p. 633-45.

Tilley, W.D., et al., *Characterization and expression of a cDNA encoding the human androgen receptor.* Proc Natl Acad Sci U S A, 1989. **86**(1): p. 327-31.

Ting, H.J., Y.C. Hu, and C. Chang, *Actin monomer enhances supervillin-modulated androgen receptor transactivation.* Biochem Biophys Res Commun, 2004. **319**(2): p. 393-6.

Ting, H.J., et al., *Supervillin associates with androgen receptor and modulates its transcriptional activity.* Proc Natl Acad Sci U S A, 2002. **99**(2): p. 661-6.

Toppari, J., M. Kaleva, and H.E. Virtanen, *Trends in the incidence of cryptorchidism and hypospadias, and methodological limitations of registry-based data.* Hum Reprod Update, 2001. **7**(3): p. 282-6.

Toppari, J., et al., *Cryptorchidism and hypospadias as a sign of testicular dysgenesis syndrome (TDS): environmental connection.* Birth Defects Res A Clin Mol Teratol. 2010 **88**(10): p. 910-9.

Toth, M. and T. Zakar, *Relative binding affinities of testosterone, 19-nortestosterone and their 5 alpha-reduced derivatives to the androgen receptor and to other androgen-binding proteins: a suggested role of 5 alpha-reductive steroid metabolism in the dissociation of "myotropic" and "androgenic" activities of 19-nortestosterone.* J Steroid Biochem, 1982. **17**(6): p. 653-60.

Traeger, A., H. Noschel, and J. Zaumseil, *[Pharmacokinetics of indomethacin in pregnant and parturient women and in their newborn infants]*. Zentralbl Gynakol, 1973. **95**(1): p. 635-41.

Trasler, J.M., *Epigenetics in spermatogenesis.* Mol Cell Endocrinol, 2009. **306**(1-2): p. 33-6.

Trotter, K.W. and T.K. Archer, *The BRG1 transcriptional coregulator*. Nucl Recept Signal, 2008. **6**: p. e004.

Tsai, M.Y., et al., *Differential effects of spermatogenesis and fertility in mice lacking androgen receptor in individual testis cells.* Proc Natl Acad Sci U S A, 2006. **103**(50): p. 18975-80.

Tzortzatou, G.G., A.S. Goldman, and W.C. Boutwell, *Evidence for a role of arachidonic acid in glucocorticoid-induced cleft palate in rats.* Proc Soc Exp Biol Med, 1981. **166**(3): p. 321-4.

Urbanucci, A., et al., *Androgen regulation of the androgen receptor coregulators.* BMC Cancer, 2008. **8**: p. 219.

Vadakkadath Meethal, S. and C.S. Atwood, *The role of hypothalamic-pituitary-gonadal hormones in the normal structure and functioning of the brain.* Cell Mol Life Sci, 2005. **62**(3): p. 257-70.

Vainio, S., et al., *Female development in mammals is regulated by Wnt-4 signalling.* Nature, 1999. **397**(6718): p. 405-9.

Val, P. and A. Swain, *Mechanisms of Disease: normal and abnormal gonadal development and sex determination in mammals.* Nat Clin Pract Urol, 2005. **2**(12): p. 616-27.

Van Bokhoven, H., et al., *Mutation of the gene encoding the ROR2 tyrosine kinase causes autosomal recessive Robinow syndrome.* Nat Genet, 2000. **25**(4): p. 423-6.

Van den Driesche, S., et al., *Relative importance of prenatal and postnatal androgen action in determining growth of the penis and anogenital distance in the rat before, during and after puberty.* Int J Androl. 2011, **34,** p578–586

Vane, J.R., *Introduction: mechanism of action of NSAIDs.* British Journal of Rheumatology 1996; 35 (snppL l) : l -3

Vermes, I., J. Dohanics, and G. Telegdy, *Effect of prostaglandins on hypothalamic-pituitary-testicular function in vitro.* Acta Physiol Acad Sci Hung, 1979. **54**(4): p. 401-4.

Vidal, V.P., et al., *Sox9 induces testis development in XX transgenic mice.* Nat Genet, 2001. **28**(3): p. 216-7.

Vinggaard, A.M., et al., *Perinatal exposure to the fungicide prochloraz feminizes the male rat offspring.* Toxicol Sci, 2005. **85**(2): p. 886-97.

Virtanen, H.E., et al., *Cryptorchidism: classification, prevalence and long-term consequences.* Acta Paediatr, 2007. **96**(5): p. 611-6.

Virtanen, H.E., et al., *Testicular dysgenesis syndrome and the development and occurrence of male reproductive disorders.* Toxicol Appl Pharmacol, 2005. **207**(2 Suppl): p. 501-5.

Vladic-Stjernholm, Y., et al., *Prostaglandin treatment is associated with a withdrawal of progesterone and androgen at the receptor level in the uterine cervix.* Reprod Biol Endocrinol, 2009. **7**: p. 116.

Vo, N. and R.H. Goodman, *CREB-binding protein and p300 in transcriptional regulation.* J Biol Chem, 2001. **276**(17): p. 13505-8.

Von Euler, U.S., *On the specific vaso-dilating and plain muscle stimulating substances from accessory genital glands in man and certain animals (prostaglandin and vesiglandin).* J Physiol, 1936. **88**(2): p. 213-34.

Vottero, A., et al., *Evidence for Epigenetic Abnormalities of the Androgen Receptor Gene in Foreskin from Children with Hypospadias.* J Clin Endocrinol Metab. 2011 Dec;**96**(12):E1953-62

Walker, W.H., *Molecular mechanisms of testosterone action in spermatogenesis.* Steroids, 2009. **74**(7): p. 602-7.

Walker, W.H. and J. Cheng, *FSH and testosterone signaling in Sertoli cells.* Reproduction, 2005. **130**(1): p. 15-28.

Walsh, C., *Protein methylation. Posttranslational modification of proteins: expanding nature's inventory*, Chapter 5, Roberts and Company Publishers 2006.

Walters, K.A., et al., *Subfertile female androgen receptor knockout mice exhibit defects in neuroendocrine signaling, intraovarian function, and uterine development but not uterine function.* Endocrinology, 2009. **150**(7): p. 3274-82.

Wang, M.H. and L.S. Baskin, *Endocrine disruptors, genital development, and hypospadias.* J Androl, 2008. **29**(5): p. 499-505.

Wang, Q., et al., *Identification of an activin-follistatin growth modulatory system in the human prostate: secretion and biological activity in primary cultures of prostatic epithelial cells.* J Urol, 1999. **161**(4): p. 1378-84.

Wang, W., et al., *Purification and biochemical heterogeneity of the mammalian SWI-SNF complex.* EMBO J, 1996. **15**(19): p. 5370-82.

Wang, X., et al., *Inhibition of cyclooxygenase-2 activity enhances steroidogenesis and steroidogenic acute regulatory gene expression in MA-10 mouse Leydig cells.* Endocrinology, 2003. **144**(8): p. 3368-75.

Wang, Y., et al., *Mutation analysis of five candidate genes in Chinese patients with hypospadias.* Eur J Hum Genet, 2004. **12**(9): p. 706-12.

Warot, X., et al., *Gene dosage-dependent effects of the Hoxa-13 and Hoxd-13 mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts.* Development, 1997. **124**(23): p. 4781-91.

Webster, K.E., et al., *Meiotic and epigenetic defects in Dnmt3L-knockout mouse spermatogenesis.* Proc Natl Acad Sci U S A, 2005. **102**(11): p. 4068-73.

Weidner, I.S., et al., *Risk factors for cryptorchidism and hypospadias.* J Urol, 1999. **161**(5): p. 1606-9.

Welsh, M., et al., *Critical androgen-sensitive periods of rat penis and clitoris development.* Int J Androl. 2010 **33**(1): p. e144-52.

Welsh, M., et al., *Deletion of androgen receptor in the smooth muscle of the seminal vesicles impairs secretory function and alters its responsiveness to exogenous testosterone and estradiol.* Endocrinology. 2010 **151**(7): p. 3374-85.

Welsh, M., et al., *Androgen action via testicular peritubular myoid cells is essential for male fertility.* FASEB J, 2009. **23**(12): p. 4218-30.

Welsh, M., et al., *Identification in rats of a programming window for reproductive tract masculinization, disruption of which leads to hypospadias and cryptorchidism.* J Clin Invest, 2008. **118**(4): p. 1479-90.

Welsh, M., et al., *Androgen-dependent mechanisms of Wolffian duct development and their perturbation by flutamide.* Endocrinology, 2006. **147**(10): p. 4820-30.

Welsh, M., P.T. Saunders, and R.M. Sharpe, *The critical time window for androgen-dependent development of the Wolffian duct in the rat.* Endocrinology, 2007. **148**(7): p. 3185-95.

Welsh, M., et al., *New insights into the role of androgens in wolffian duct stabilization in male and female rodents.* Endocrinology, 2009. **150**(5): p. 2472-80.

Welshons, W.J. and L.B. Russell, *The Y-Chromosome as the Bearer of Male Determining Factors in the Mouse.* Proc Natl Acad Sci U S A, 1959. **45**(4): p. 560-6.

Werner, R., H. Grotsch, and O. Hiort, *46,XY disorders of sex development--the undermasculinised male with disorders of androgen action.* Best Pract Res Clin Endocrinol Metab.2010 **24**(2): p. 263-77.

Whalen, R.E. and J.F. DeBold, *Comparative effectiveness of testosterone, androstenedione and dihydrotestosterone in maintaining mating behavior in the castrated male hamster.* Endocrinology, 1974. **95**(6): p. 1674-9.

White, P.C. and P.W. Speiser, *Congenital adrenal hyperplasia due to 21-hydroxylase deficiency.* Endocr Rev, 2000. **21**(3): p. 245-91.

Whitfield, G.K., et al., *Steroid hormone receptors: evolution, ligands, and molecular basis of biologic function.* J Cell Biochem, 1999. **Suppl 32-33**: p. 110-22.

Wilcox, A.J., et al., *Fertility in men exposed prenatally to diethylstilbestrol.* N Engl J Med, 1995. **332**(21): p. 1411-6.

Wilhelm, D. and P. Koopman, *The makings of maleness: towards an integrated view of male sexual development.* Nat Rev Genet, 2006. **7**(8): p. 620-31.

Wilhelm, D., S. Palmer, and P. Koopman, *Sex determination and gonadal development in mammals.* Physiol Rev, 2007. **87**(1): p. 1-28.

Wilhelm, D., et al., *Antagonism of the testis- and ovary-determining pathways during ovotestis development in mice.* Mech Dev, 2009. **126**(5-6): p. 324-36.

Williams, K., et al., *Neonatal exposure to potent and environmental oestrogens and abnormalities of the male reproductive system in the rat: evidence for importance of the androgen-oestrogen balance and assessment of the relevance to man.* Hum Reprod Update, 2001. **7**(3): p. 236-47.

Williams, T.J. and M.J. Peck, *Role of prostaglandin-mediated vasodilatation in inflammation.* Nature, 1977. **270**(5637): p. 530-2.

Wilson, C.M. and M.J. McPhaul, *A and B forms of the androgen receptor are expressed in a variety of human tissues.* Mol Cell Endocrinol, 1996. **120**(1): p. 51-7.

Wilson, E.M. and F.S. French, *Binding properties of androgen receptors. Evidence for identical receptors in rat testis, epididymis, and prostate.* J Biol Chem, 1976. **251**(18): p. 5620-9.

Wilson, J.D., *Sexual differentiation.* Annu Rev Physiol, 1978. **40**: p. 279-306.

Wilson, V.S., et al., *Phthalate ester-induced gubernacular lesions are associated with reduced insl3 gene expression in the fetal rat testis.* Toxicol Lett, 2004. **146**(3): p. 207-15.

Wolf, C.J., et al., *Effects of prenatal testosterone propionate on the sexual development of male and female rats: a dose-response study.* Toxicol Sci, 2002. **65**(1): p. 71-86.

Wolffe, A.P., *Transcriptional regulation in the context of chromatin structure.* Essays Biochem, 2001. **37**: p. 45-57.

Woloszynska-Read, A., et al., *DNA methylation-dependent regulation of BORIS/CTCFL expression in ovarian cancer.* Cancer Immun, 2007. **7**: p. 21.

Wood, J.R., et al., *The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis.* J Biol Chem, 2003. **278**(29): p. 26380-90.

Woroniecki, R., A.B. Gaikwad, and K. Susztak, *Fetal environment, epigenetics, and pediatric renal disease.* Pediatr Nephrol. 2010 **26**(5): p. 705-11.

Wright, A.S., et al., *Androgen-induced regrowth in the castrated rat ventral prostate: role of 5alpha-reductase.* Endocrinology, 1999. **140**(10): p. 4509-15.

Wu, J.Y., et al., *The neuronal repellent Slit inhibits leukocyte chemotaxis induced by chemotactic factors.* Nature, 2001. **410**(6831): p. 948-52.

Xavier, F. and S. Allard, *Anti-Mullerian hormone, beta-catenin and Mullerian duct regression.* Mol Cell Endocrinol, 2003. **211**(1-2): p. 115-21.

Xita, N. and A. Tsatsoulis, *Review: fetal programming of polycystic ovary syndrome by androgen excess: evidence from experimental, clinical, and genetic association studies.* J Clin Endocrinol Metab, 2006. **91**(5): p. 1660-6.

Xu, C.J. and D. Li, *Pharmacokinetics of flutamide and its metabolite 2-hydroxyflutamide in normal and hepatic injury rats.* Zhongguo Yao Li Xue Bao, 1998. **19**(1): p. 39-43.

Xu, Q., et al., *Infertility with defective spermatogenesis and steroidogenesis in male mice lacking androgen receptor in Leydig cells.* Endocrine, 2007. **32**(1): p. 96-106.

Yamada, G., et al., *Cellular and molecular mechanisms of development of the external genitalia.* Differentiation, 2003. **71**(8): p. 445-60.

Yao, H.H., W. Whoriskey, and B. Capel, *Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis.* Genes Dev, 2002. **16**(11): p. 1433-40.

Yeap, B.B., J.A. Wilce, and P.J. Leedman, *The androgen receptor mRNA.* Bioessays, 2004. **26**(6): p. 672-82.

Yeh, S., et al., *From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells.* Proc Natl Acad Sci U S A, 1999. **96**(10): p. 5458-63.

Yeh, S., et al., *Retinoblastoma, a tumor suppressor, is a coactivator for the androgen receptor in human prostate cancer DU145 cells.* Biochem Biophys Res Commun, 1998. **248**(2): p. 361-7.

Yeh, S., et al., *From estrogen to androgen receptor: a new pathway for sex hormones in prostate.* Proc Natl Acad Sci U S A, 1998. **95**(10): p. 5527-32.

Yeh, S., et al., *Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13498-503.

You, L., et al., *Impaired male sexual development in perinatal Sprague-Dawley and Long-Evans hooded rats exposed in utero and lactationally to p,p'-DDE.* Toxicol Sci, 1998. **45**(2): p. 162-73.

You, L. and M. Sar, *Androgen receptor expression in the testes and epididymides of prenatal and postnatal Sprague-Dawley rats.* Endocrine, 1998. **9**(3): p. 253-61.

Yu, R.N., et al., *Role of Ahch in gonadal development and gametogenesis.* Nat Genet, 1998. **20**(4): p. 353-7.

Yucel, S., et al., *The effect of oestrogen and testosterone on the urethral seam of the developing male mouse genital tubercle.* BJU Int, 2003. **92**(9): p. 1016-21.

Zaviacic, M. and R.J. Ablin, *The female prostate and prostate-specific antigen. Immunohistochemical localization, implications of this prostate marker in women and reasons for using the term "prostate" in the human female.* Histol Histopathol, 2000. **15**(1): p. 131-42.

Zhang, C., et al., *Oligozoospermia with normal fertility in male mice lacking the androgen receptor in testis peritubular myoid cells.* Proc Natl Acad Sci U S A, 2006. **103**(47): p. 17718-23.

Zhang, H., et al., *The prognostic value of the orphan nuclear receptor DAX-1 (NROB1) in node-negative breast cancer.* Anticancer Res. 2011 **31**(2): p. 443-9.

Zhang, X., et al., *Malignant transformation and antineoplastic actions of nonsteroidal antiinflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts.* J Exp Med, 1999. **190**(4): p. 451-59.

Zheng, Z., et al., *SUMO-3 enhances androgen receptor transcriptional activity through a sumoylation-independent mechanism in prostate cancer cells.* J Biol Chem, 2006. **281**(7): p. 4002-12.

Zielenski, J., et al., *CFTR gene variant for patients with congenital absence of vas deferens.* Am J Hum Genet, 1995. **57**(4): p. 958-60.

Zimmerman, S.A., et al., *Diethylstilbestrol-induced perinatal lethality in the rat. II. Perturbation of parturition.* Biol Reprod, 1991. **44**(4): p. 583-9.

Zondek, T., M.D. Mansfield, and L.H. Zondek, *Amniotic fluid testosterone and fetal sex determination in the first half of pregnancy.* Br J Obstet Gynaecol, 1977. **84**(9): p. 714-6

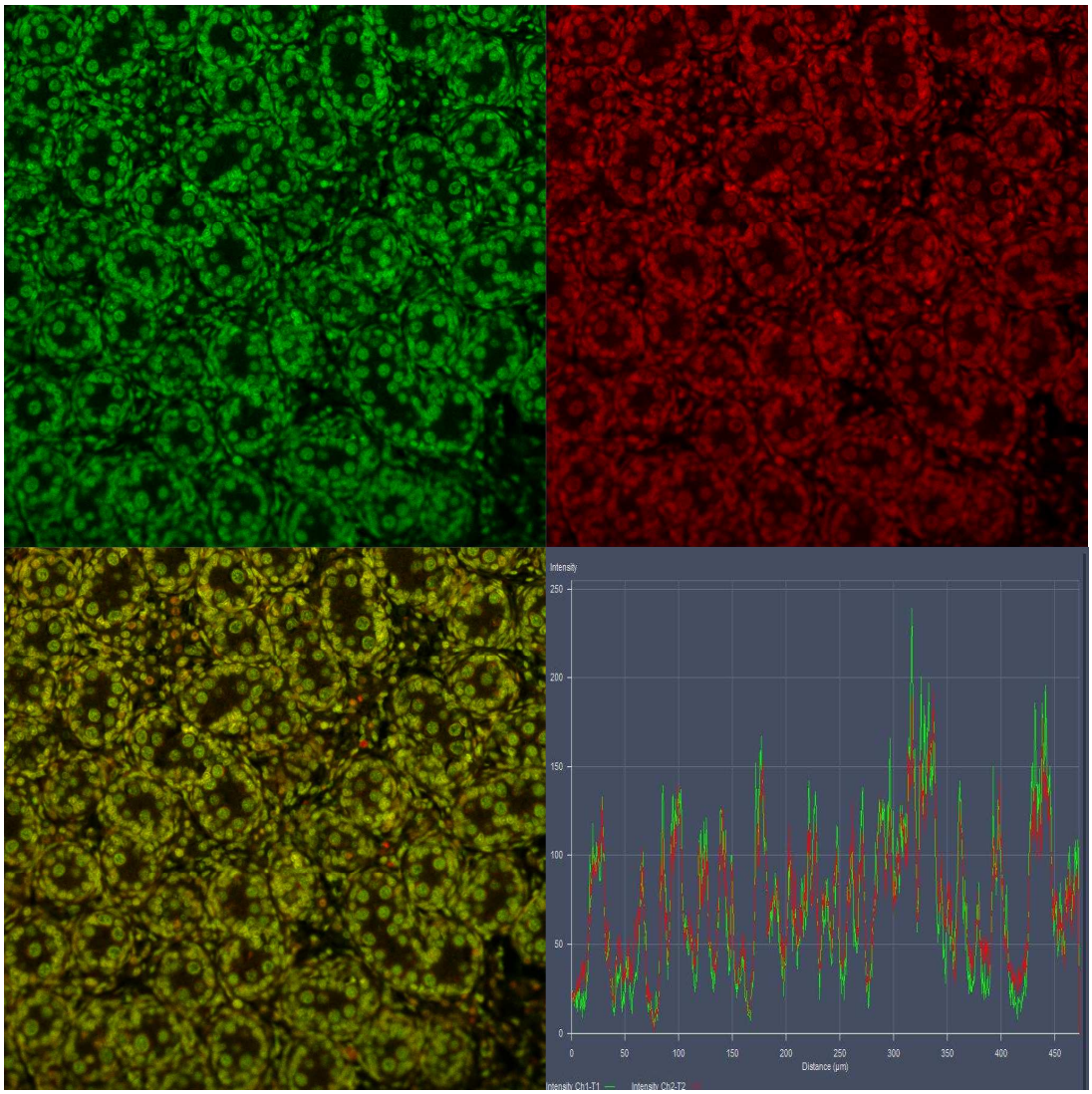
Appendix 1 – Co-regulators investigated in chapter 4

Gene identified in both microarray & GeneGo studies *	Candidate Gene -reasoning of chioce
Brg1	*Rwdd1* - novel AR regulator in Thymus
Cbp	*DNMT3L* - knock out studies cause azoospermia and involved in regulation of methylation
Fkbp5	*BORIS* -knock out studies cause oligospermia and thought to be involved in regulation of methylation
Smurf	
Trangselin	
VAV3	

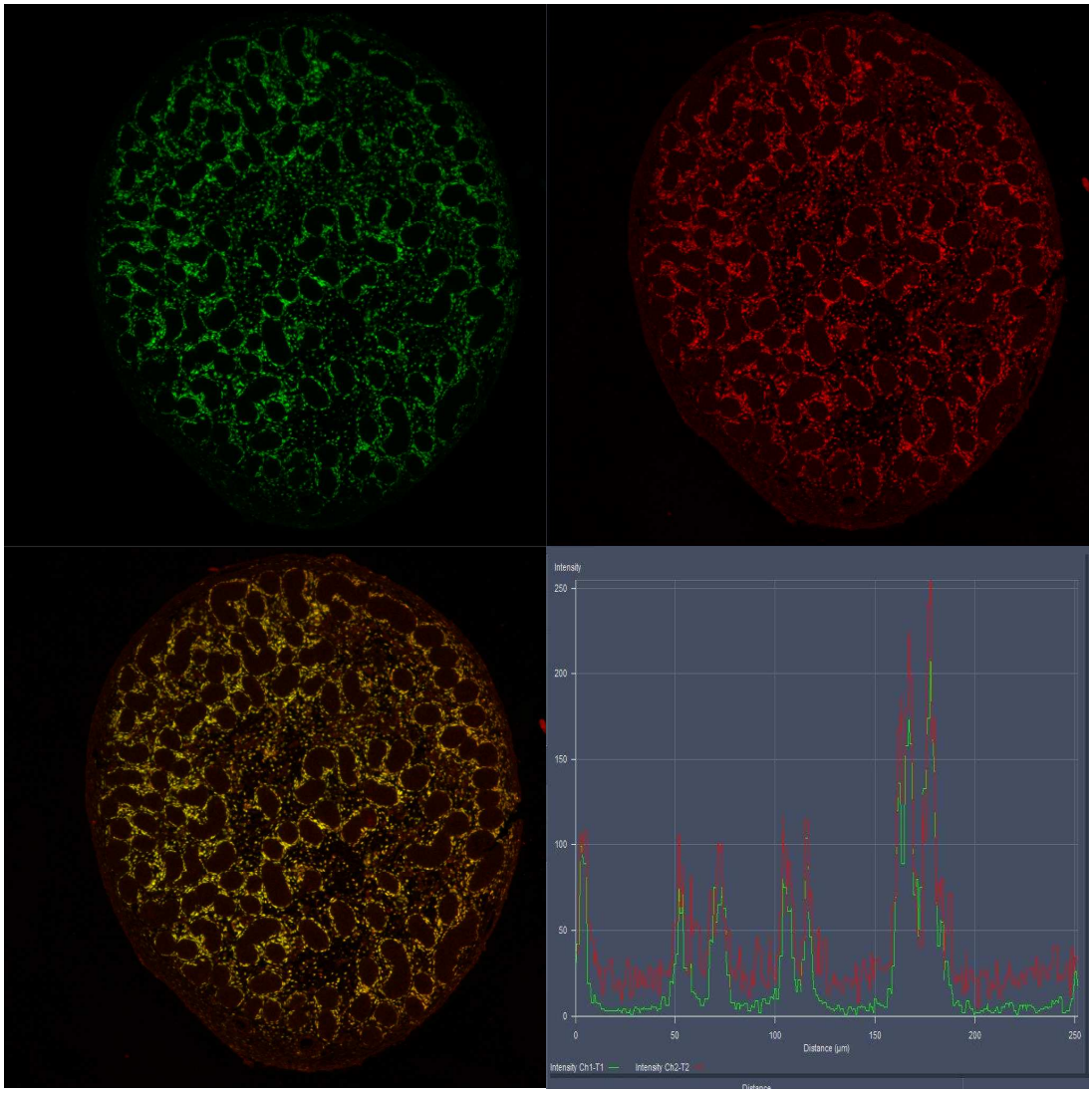
*Microarray study designed by Dr David McLeod and carried out by Affymetrix. GeneGo pathways established by bioinformatics student Kirstine Belling.

All genes were initially investigated by Taqman, those in red showed no interesting patterns of expression around the MPW in the lower genital region. The genes highlighted in green showed interesting patterns of expression around the MPW and were investigated further in chapter 4.

Appendix 2 Profile of co-localisation of BRG1 and CBP



Appendix 3 Profile of co-localisation of AR and Rwdd1



Appendix 4:

OPT software for e21.5 male control GT

Note: This is Microsoft office software and will only work on a Windows operating system.

Instructions:

Double click on dataviewer zip file
Double click dataviewer application
Click Run

In the application, go to Actions and open

Click RP6541
Double click C1_WH_1R
Double click ReconRP (separate files on CD)
Double click RP_REC0013

To speed up the movie, click on red line on tissue image (grey on left) and move up through the tissue.

To change colour click on drop down menu under colour change and change from grey to colour 1.

For X, Y Z analysis, go to action, click 'load for 3d viewing' and click on RP_REC0013 .