UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Medicine

Modelling androgen synthesis and action during human sexual differentiation

by

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ABSTRACT

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The sex of an individual is established by a complex interplay of genetic and hormonal factors during early development. Differentiation of the external genitalia concordant with genetic sex is essential, as abnormalities fundamentally compromise reproductive capacity. Much current understanding of this process comes from the study of pathological phenotypes, which provides some insight into the underlying genetics. Beyond this, because of a lack of relevant material, there is a general reliance on animal models and cancer cell lines to study the underlying pathways. Neither of these approaches is ideal. Using a very comprehensive collection of human first trimester material, this project aimed to study sexual differentiation of human external genitalia directly in the tissue of interest, and at the precise time when it occurs at 7-12 weeks post-conception.

Chapter 3 investigated the biosynthesis of testicular testosterone during the critical period of sexual differentiation, revealing that testosterone biosynthesis is unlikely to be regulated by the anterior pituitary or placental hCG during sexual differentiation; suggesting constitutive regulation occurs. In Chapter 4, characterisation of the expression of key genes in the developing human external genitalia illustrated escalating expression of nuclear androgen receptor (AR) during the first trimester in males. AR was also detected in female external genitalia; its nuclear location implied it was ligand-bound by androgen. To pursue this, in Chapter 5 an *in vitro* culture model of fetal external genitalia stromal cells was established. Male and female cells retained AR expression, which, in the absence of androgen, was diffusely cytoplasmic. Nuclear translocation occurred indistinguishably in male and female cells with thresholds of 100 pM DHT, 1 nM testosterone and 10 nM androstenedione. Androgen secretion in this range has been previously documented from the first trimester female adrenal gland. Conditioned media from the adrenal gland also elicited nuclear AR translocation in external genitalia cells. In Chapter 6, through the use of expression microarray analysis, the widespread genetic consequences of AR function during human sexual differentiation were determined.

This project provides significant insight into a critical period of human development and has identified that early human androgen secretion in males is likely to be predominantly constitutive. Strikingly, AR bioactivity appears part of normal female sexual differentiation, reinforcing a theory that in human females, sexual differentiation unfolds within a significant androgenic milieu, underlining its vulnerability to excessive androgen in conditions such as congenital adrenal hyperplasia. Furthermore, several putative candidate genes for inactivating mutations in syndromes of male under-virilisation, such as hypospadias, were identified.

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Declaration of Authorship

I, **Daniel James Asby**

declare that the thesis entitled:

Modelling androgen synthesis and action during human sexual differentiation

and the work presented in it are my own, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
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- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- none of this work has been published before submission

Signed: **Daniel James Asby**

Date: **20/02/10**

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List of Abbreviations

ACTH, adrenocorticotropic hormone
ADCYAP1, adenylate cyclase-activating polypeptide 1
AMH, anti-Müllerian hormone [also known as Müllerian inhibiting substance (MIS)]
AR, androgen receptor
cAMP, cyclic adenosine monophosphate
CAH, congenital adrenal hyperplasia
CC, corpus cavernosum
CS, Carnegie Stage
CYP11A1, cholesterol side chain cleavage enzyme
CYP11B1, 11β-hydroxylase
CYP11B2, aldosterone synthase
CYP17A1, 17α-hydroxylase / 17-20-lyase
CYP19A1, aromatase
CYP21A2, 21-hydroxylase
DEPC, diethylpyrocarbonate
DHT, dihydrotestosterone
DMEM, Dulbecco's Modified Eagle Medium
DMSO, dimethyl sulphoxide
DNA, deoxyribonucleic acid
DNase I, Deoxyribonuclease I
DSD, disorders of sex development
DTT, dithiothreitol
EG, external genitalia
ER, oestrogen receptor
FBS, fetal bovine serum
FITC, fluorescein isothiocyanate
FSH, follicle stimulating hormone
FSHR, follicle stimulating hormone receptor
GnRH, gonadotrophin releasing hormone
HBSS, Hank's Balanced Salt Solution
hCG, human chorionic gonadotrophin
HPG axis, hypothalamic-pituitary-gonadal axis
HRP, horse-radish peroxidase
HSD3B2, type 2 3β-hydroxysteroid dehydrogenase
HSD17B3, type 3 17 β-hydroxysteroid dehydrogenase
IHC, immunohistochemistry
ICC, immunocytochemistry
LH, luteinising hormone
LHR, luteinising hormone receptor
AMH, anti-Müllerian hormone (also known as Müllerian inhibiting substance; MIS)
mRNA, messenger RNA
NP, natriuretic peptide
PBS, phosphate buffered saline
PCR, polymerase chain reaction
PFA, paraformaldehyde
RNA, ribonucleic acid
RNase, ribonuclease
RT-PCR, reverse transcription-polymerase chain reaction
SA, streptavidin
SD, standard deviation
SRY, Sex determining Region of the Y chromosome
SRD5A2, 5 alpha-reductase enzyme type 2
StAR, Steroid Acute Regulatory protein
SOX2, SRY-like homeobox gene 2
TISH, tissue in situ hybridisation
UP, urethral plate
VIP, vasoactive intestinal peptide
Wpc, weeks post-conception

Dedicated to my Grandad, William Bennett.
We all miss you greatly.

And, to my Nan, Louisa Bennett.
Thank you for the cream sodas, zoids, warm ham sandwiches
and wonderful times in the Plaza and Oxford Street.

Chapter 1: Introduction

1.1 Sexual dimorphism in humans

Sexual dimorphism is found among many different types of organisms within the animal and plant kingdoms, and represents a fundamentally important part of continued species survival, acting as the conduit for procreation (Grammer *et al*., 2003; Basrur, 2006; Ashman and Majetic, 2006; Meagher, 2007). In the animal kingdom, there are myriad examples of different types of sexual asymmetry within species, which we commonly attribute to maleness or femaleness. Prominent sexual dimorphism is present in as diverse animal groups as insects, arachnids, birds, reptiles, amphibians, fish and mammals (Swain, 2002; Sarre *et al*., 2004; Ezaz *et al*., 2006; Pask *et al*., 2006; Matsubara *et al*., 2006; Stiglec *et al*., 2007; Martinez *et al*., 2008; Sanchez, 2008; Hosken and Price, 2009). The root of sexual dimorphism in humans has been subject to much speculation over the course of history (Cadden, 1995). Aristotle postulated that sexual dimorphism occurs due to differences in the temperature of semen at the time of copulation. While in the middle ages, it was believed that the contribution of female 'semen' or menstrual blood was most crucial in steering a fetus towards female development. More recently, it has been ascertained that the sex of an individual is established by a complex interplay of genetic and hormonal factors during early fetal development (Warne and Kanumakala, 2002; Wilhelm and Koopman, 2006). Broadly speaking, the processes that lead to human sex determination comprise of three sequential phases (Wilson *et al*., 1980). The first is the establishment of chromosomal sex by the pattern of *XY* or *XX* sex chromosomes instituted at conception. The second phase entails the initiation of gender-specific divergence of the gonad, which relies on the translation of chromosomal sex into genetic sex, and entails the expression of the *SRY* (sex determining region of the Y chromosome) gene in males, or absence of *SRY* in females. This differing gene expression in the bipotential gonad causes differentiation of either testes or ovaries thereby establishing gonadal sex. The third and final step is sexual differentiation, which is the development of the internal and external genitalia, and subsequently the wider sexual phenotype (e.g. breasts) in accordance with genetic sex. The phenotypic sex and gender of an individual reaches its final form at puberty (Hiort and Holterhus, 2000).

Differentiation of the internal and external genitalia concordant with gonadal sex is an essential component of human fetal development, as abnormal genitalia fundamentally compromises reproductive capacity (Warne and Raza, 2008; Katorza *et al*., 2009). Each event that contributes to establishing the final sexual dimorphism of human genitalia depends on the preceding event, and normally, chromosomal, gonadal and morphological sex are all concordant. However, there are occasions where these are inconsistent, and/or sexual differentiation is ambiguous, leading to the combination of male and female traits in a single individual; for example due to mutations in important developmental genes (Wilson *et al*., 1993; Kremer *et al*., 1995; White and Speiser, 2000; Arlt *et al*., 2004). In cases where chromosomal, gonadal, and somatic sex are incongruent in infants, rapid establishment of the diagnosis and implementation of medical and potentially later surgical management is of vital importance, because gender identity forms a major cornerstone of psychological well-being throughout life (Brinkmann *et al*., 2007). However, surprisingly, despite the significance of differentiation of genitalia and the major detrimental effects of abnormal genitalia, details of the processes underlying sexual differentiation in humans still remain poorly understood (Hughes, 2001).

1.2 Human chromosomal sex

In principle the future gender of a human being is established by the sex chromosomes that combine in the embryo at conception. Chromosomal sex acts as the first critical landmark in sex determination, predetermining genetic sex and the subsequent course of sexual differentiation (Achermann and Hughes, 2008). When an XY or XX karyotype is bestowed at conception, an individual is predestined to being either male or female, respectively. Chromosomal determination of sex was originally discovered by work in *Drosophila melanogaster* at the turn of the century (reviewed in Haqq and Donahoe, 1998). At that time it was demonstrated that XY flies develop as males, while XX fruit flies develop as females because of the double 'dose' of the *X* chromosome - accurately predicting that it is the ratio of *X* chromosomes to autosomes in fruit flies that is the critical factor for determining sex. In the 1920's this model was adopted to explain mammalian sexual dimorphism, following the discovery that female mammals have an XX karyotype, whereas males have *X* and *Y*

chromosomes. However, soon it became apparent that this model was unsuitable, when in the 1950's it was shown that the human *Y* chromosome specifies development of the testis, and appeared most important for determining sex in humans (McLaren, 1990). Later, Ford and colleagues (1959) and Jacobs and Strong (1959) were able to demonstrate that the human *Y* chromosome was associated with testicular differentiation by showing that unlike in *Drosophila*, *XO* humans are female, and, irrespective of the number of *X* chromosomes, the presence of a single *Y* chromosome was enough to prompt testis differentiation.

1.3 Genetic sex and the induction of gonadal sex

1.3.1 Assembly of the primitive bipotential gonad

Following conception, early first trimester development in humans is identical for both males and females (Schoenwolf *et al*., 2008). Development of the reproductive system begins during the fourth week of development in both sexes when the presumptive gonad begins to emerge. The human gonad arises as a bipotential primordium starting from the fifth week of gestation (Brennan and Capel, 2004). The undifferentiated presumptive gonads originate from intermediate mesoderm of the genital ridges (Fig. 1.1, A & B). These are two thickened regions covering a layer of coelomic epithelial cells anteromedial to the mesonephros (fetal renal organ) and associated with the underlying mesenchymal cells. Initial development of the bipotential gonad begins when the genital ridges are invaded by primordial germ cells (Wilhelm *et al*., 2007). Subsequently, during the sixth week of gestation, the outer epithelial layer of the genital ridge sends out finger-like projections made up of somatic support cells, which proliferate and invade the loose connective tissues of the inner medulla region, which is made up of mesenchymal cells. The extent to which these projections extend into the medullary region is dependent on the sex of the individual, growing much deeper in males and regressing in females during later sex dimorphic development of the gonad (Gassei and Schlatt, 2007). At the time when the gonad remains undifferentiated, they extend approximately midway into the medulla in both sexes and are known as the primary sex cords. The resulting bilateral structures consisting of primordial germ cells and somatic support cells of the primary sex cords are the undifferentiated bipotential gonads.

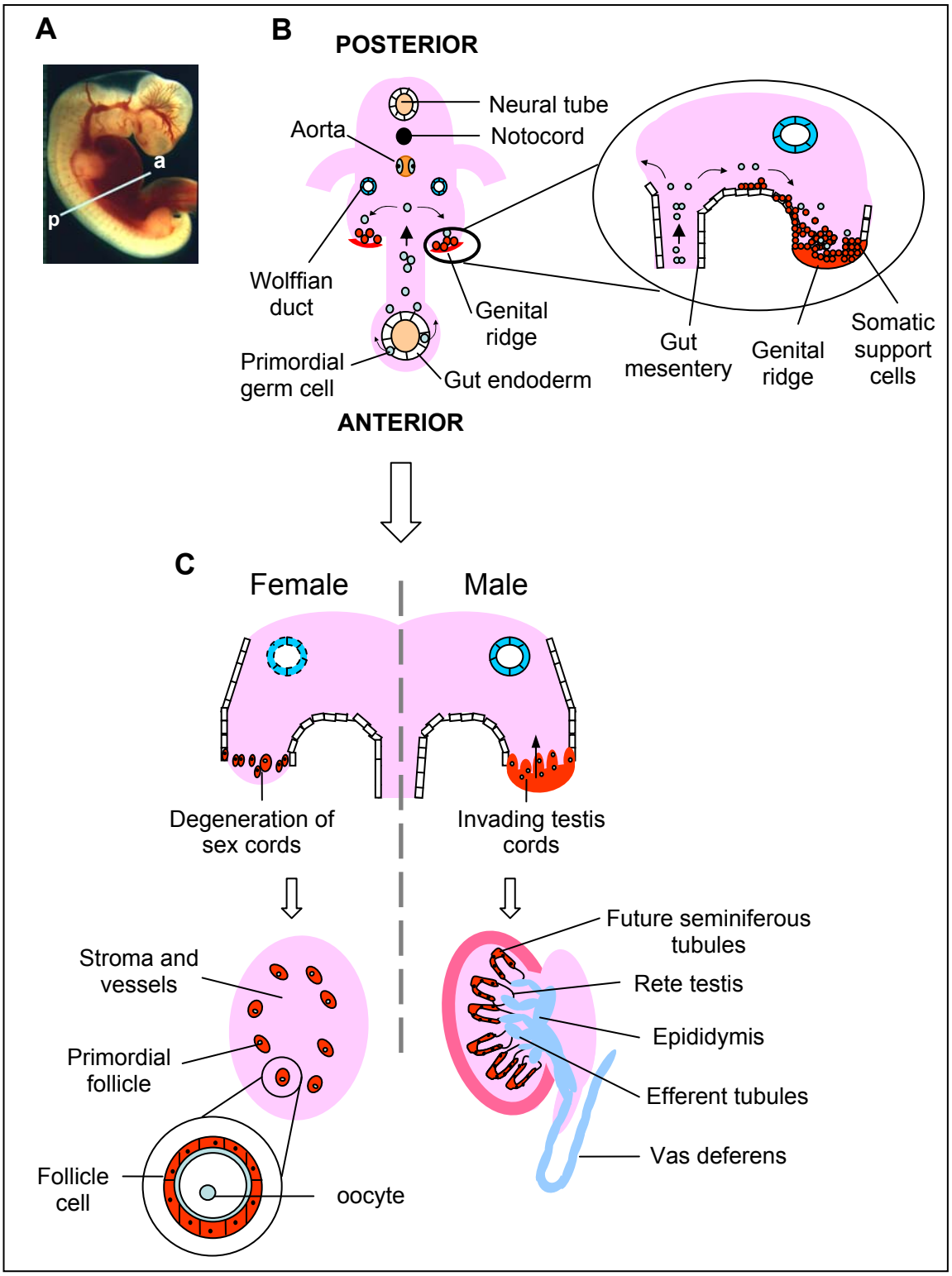

Figure 1.1: Formation of the bipotential gonad and differentiation of ovary or testis. *A, a transverse plane through the left gonadal ridge of a human embryo at 6 wpc. a, anterior; p, posterior. B, schematic cross-section of a 5-6 wpc human embryo, showing the primordial germ cells migrating from the gut endoderm to the thickening genital ridges, to form the indifferent gonads. C, During the 6th week of gestation, in males somatic support cells differentiate into Sertoli cells, which with germ cells, organize into testis cords that become the seminiferous tubules. Leydig cells appear between the testis cords that produce testosterone, maintaining the Wolffian ducts, which during the 7th week gestation differentiate into the male internal genitalia. In females, primordial follicles consisting of an oocyte and flattened surrounding support cells are formed, and the Wolffian ducts regress.*

At approximately the start of the seventh week of development, the sex chromosomal constitution directs gonadal development toward either testes or ovaries, which in turn direct reproductive organ development concordant with chromosomal sex (Achermann and Hughes, 2008). Although the pattern of sex chromosomes determines the choice between male and female developmental paths, the subsequent phases of sexual development are controlled not only by the genes on the sex chromosomes, but also by hormones and numerous other factors encoded on the autosomes (Wilson and Davies, 2007).

1.3.2 Differentiation of the testis

The classical view of the initial divergence of the sexes at this crucial stage of development in humans is that expression of a single sex-determining factor initiates a cascade of events that shifts development towards male differentiation (Goldberg, 1988). Whereas, if this key factor is absent or defective in some way, development is directed towards female differentiation; the so-called 'default' pathway (Palmer *et al*., 1989). However, it is now emerging that claiming ovarian gonadogenesis and the route towards female differentiation is a passive, rather than active process, is an oversimplification of the true situation. The sex-determining factor responsible for establishing maleness is a transcription factor encoded by the *SRY* gene (Sinclair *et al*., 1990). When this gene is expressed in somatic cells of the indifferent primitive gonad, male development ensues. This phenomenon is referred to as sex determination (Goodfellow and Lovell-Badge, 1993).

SRY is present on the *Y* chromosome and thus only expressed in males (Ross *et al*., 2006). Its expression from the seventh week of gestation triggers a cascade of gene expression that eventually leads to formation of testes (Sinclair *et al*., 1990; Koopman *et al*., 1990; Jager *et al*., 1990). Though chromosomal sex fundamentally underlies which sex an individual is destined to become, it is *SRY* expression and its effects on the expression of other downstream genes that defines the differentiation of the gonad. Under most circumstances, once this gene is expressed, the scene is irrevocably set for male development. For instance, XX humans and mice develop testes, and not ovaries, where

translocation or transgenic expression of *SRY* has occurred (Berta *et al*., 1990; Koopman *et al*., 1991). Likewise, the absence or mutation of *SRY* in males has been shown to result in complete sex reversal of males into females (Domenice *et al*., 2001).

SRY is expressed transiently and is active for only a short period in fetal human testis. Its expression is first detectable centrally within the gonad at 41 days gestation, and following peak expression at approximately 44 days, when it is also evident in cells at the cranial and caudal poles of the genital ridge, remains detectable at only a low level until day 52 (Hanley *et al*., 2000). However some studies have reported prolonged low level expression of *SRY* in humans that persist until puberty (Achermann and Hughes, 2008). During the short period of *SRY* expression, it is thought that the SRY protein must reach a certain threshold in order to sufficiently upregulate testis-specific genes and/or repress ovarian genes to bring about testis development (Bullejos and Koopman, 2005). Above this threshold, the strong influence of *SRY* causes testis-specific patterning, proliferation and epithelialisation of the bipotential gonad.

During early testis differentiation, the primary sex cords begin to extend deeper into the connective tissue of the developing gonad (Fig. 1.1, C). At this time layers of fetal Sertoli cells begin to differentiate from the mesothelial somatic support cells that make up the proliferating primary sex cords (Barrionuevo *et al*., 2009), which envelop the primordial germ cells and arrest any further development of these until adolescence (Turnpenny *et al*., 2006). Full commitment to male sex determination is signified by the maturation of the sex cords into testis cords consisting of the arrested prospermatogonia, surrounded by fetal Sertoli cells that produce factors critical to male sexual differentiation of the internal and external genitalia (Cool and Capel, 2009). Maturation of the testis cords leads to their separation from the germinal epithelium and the formation of looping structures supported by the extracellular matrix (Fig. 1.1, C), which undergo continued cell differentiation and epithelialisation during early fetal development. At puberty, these structures become canalized and develop into a system of seminiferous tubules necessary for sperm differentiation (Rhind *et al*., 2001).

Cells excluded from the cord structures are known as interstitial cells. These develop into several different cell types, including steroidogenic Leydig cells, endothelial cells, and specialised smooth muscle cells that directly surround the cords (Schoenwolf *et al*., 2008). In the region adjacent to the mesonephros that contains no germ cells, Sertoli cells organize into the rete testis; thin-walled tubules that connect the testis cords to the mesonephric ducts. Canalization of the rete testis at puberty enables formation of a contiguous conduit that originates at the seminiferous tubules and passes through the rete testis, epididymis, vas deferens and seminal vesicle (see section 1.5 for an overview of the adult male reproductive system). During testis differentiation, the testis grows much larger and becomes rounded, leading to a steady decrease in the contact area with the mesonephros and its eventual separation (Byskov, 1986). During the latter stages of development, the coelomic epithelium becomes completely dissociated from the testis cords by an intervening layer of connective tissue, the tunica albuginea, which encloses and partitions the testis. By the seventh month of gestation, the testes will have descended into the scrotal sac (Nation *et al*., 2009).

Although *SRY* expression plays a vital role in triggering gonad differentiation and male sex determination, many other factors are required in order for normal development of the testis to occur. Attempts to determine binding partners and functional interactions for *SRY* have proven difficult (Canning and Lovell-Badge, 2002). Some male-specific signalling pathways have been found that influence testis cord morphogenesis and specification of distinct cell types; although detailed understanding and the progression of events is yet to be fully elucidated (Ross and Capel, 2005). A model has been suggested in which sex determination is balanced on a molecular fulcrum and can be readily influenced one way or the other by a handful of important factors responsible for promoting one fate, while also antagonizing the opposite (Cool and Capel, 2009). Some of the genes poised to tip the balance toward testis development include: Wilms Tumor 1 (*WT1*), Steroidogenic factor 1 (*SF1*; officially known as nuclear receptor subfamily 5, group A, member 1; *NR5A1*) and genes from the fibroblast growth factor (*FGF*), platelet-derived growth factor (*PDGF*), hedgehog, neurotrophin and SRY-related homeobox gene *(SOX)* families. Of all the genes studied in this context, the gene

considered most key for directing gonadogenesis toward testes organogenesis following *SRY* expression is *SOX9* (Harley *et al*., 2003).

Experiments in mice have shown that *SOX9* is likely to be a primary target of *SRY*, with genes such as *FGF9* and *SF1* representing two important secondary targets (Lovell-Badge *et al*., 2002). In mice, reimplementing *SOX9* expression can rescue testis development when *SRY* is mutated, and *SOX9* is sufficient for complete female-to-male sex reversal when expressed ectopically in XX gonads (Bishop *et al*., 2000; Vidal *et al*., 2001; Qin and Bishop, 2005). Furthermore, XY human males with inactivating mutations in *SOX9* develop campomelic dysplasia (CMPD; OMIM #114290) due to haploinsufficiency, which in approximately 75% of cases causes sex reversal; in XX females gonad development is unaffected. *SOX9* is thought to mediate its effects via upregulation of male-specific genes, such as *WT1* and *SF1*, while also repressing expression of ovarian factors, such as *Wingless-type MMTV integration site family member 4* (*WNT4*; Clarkson and Harley, 2002).

SOX9 expression remains high throughout testis formation (Hanley *et al*., 2000). However, in the absence of FGF9 its expression gradually declines in fetal Sertoli cells. *FGF9* expression has been shown to promote proliferation of Sertoli cell precursors and maintain *SOX9* expression in these cells (Colvin *et al*., 2001). Consistent with this, in XX mice, cells or intact organs treated with exogenous *FGF9* fail to maintain female gene expression and upregulate of *SOX9*, which is normally suppressed in XX mice at this time (Kim *et al*., 2006). Downstream signalling from *SOX9*/*FGF9*-expressing pre-Sertoli cells in mice also upregulates expression of other genes such as *SF1*. Through the influence of *SOX9* and *FGF9*, coelomic epithelial cells which initially express *SF1* at only low levels, are driven to upregulate *SF1* expression, and divide and differentiate into a subset of interstitial cells (Parker *et al*., 2002). In light of this, and XY gonadal dysgenesis in humans carrying mutations in *SF1* (Achermann et al., 1999), *SF1* is clearly another key gene in testes differentiation, which acts downstream of *SOX9* and *FGF9* in the crucial *SRY*-initiated cascade. The mechanistic relationship between *SOX9*, *FGF9*, *SF1* and other key factors is still

incompletely established, but their genetic relationship is clearly critical to testis formation.

1.3.3 Differentiation of the ovary

Ovarian differentiation in the developing human female is considered the basic default pathway for the human gonad (i.e. what happens in the absence of *SRY;* Simpson and Rajkovic, 1999). The lack of *SRY* expression during the seventh week of gestation means gonadogenesis is not diverted toward testis formation, and ovarian development unfolds. Importantly, the XX somatic cells, which lack the *Y* chromosome and therefore *SRY* gene, will differentiate into follicle cells rather than Sertoli cells (Bukovsky *et al*., 2005). Because Sertoli cells govern the subsequent differentiation of all other cell types in the developing testis, in XX females or individuals lacking *SRY*, the testis cords do not differentiate, germ cell development is not inhibited, rete testis and steroidogenic Leydig cells are absent, and enlargement and rounding of the gonad is less pronounced (Schoenwolf *et al*., 2008).

In females, the primary sex cords degenerate and secondary, or cortical, sex cords emerge from the overlying coelomic mesothelium, and begin to re-invade the medullary region of the presumptive ovary (Pelliniemi and Lauteala, 1981). In the absence of Sertoli cell inhibition, female primordial germ cells differentiate into oogonia, proliferate and enter the first meiotic division, forming primary oocytes. These cells are positioned in the cortical cords, and divide and proliferate, while fragmentation of the cortical cords occurs following slight extension beyond the outer surface of the ovary. The groups of somatic support cells released from the cords are stimulated to differentiate into granulosa cells by oocytes, and form clusters surrounding a single oocyte, giving rise to the primordial follicles (Ostrer, 2000; see Fig. 1.1, C). Oocyte development is arrested in the primordial follicles, which will go on to form ova during puberty. Follicles are localised to the cortical region, while the medullary region of the developing ovary contains vasculature, nerves and connective tissue stroma. The latter supports the follicles and gives rise to theca cells which form an overlying layer of cells around the outward facing basal lamina of the granulosa cells

(Byskov, 1986). Towards the end of the seventh week of gestation, the ovaries separate from the mesonephroi and become suspended in the pelvis by their mesentery (Schoenwolf *et al*., 2008).

The genetic basis of ovarian differentiation is less well understood than for testis differentiation. In principle our knowledge of the genetic basis of ovarian development stems from those genes that are repressed by *SRY*, *SOX9* and other key testis determining factors. Apart from the need for two *X* chromosomes - illustrated by the abnormal development of ovaries in Turner's Syndrome *XO* patients (Sybert and McCauley, 2004) - and the need for exclusion of the *Y* chromosome - as shown by testis development in Klinefelter's syndrome *XXY* patients (Lanfranco *et al*., 2004) - detailed knowledge of 'pro-ovarian' pathways involved in female development is yet to be determined. Some genes that have been shown to have significant roles in mammalian ovarian differentiation include *forkhead box L2* gene (*FOXL2;* Harris *et al*., 2002), *follistatin* gene (*FST;* Yao *et al*., 2004), *WNT4* (Jeays-Ward *et al*., 2003) and *Folliculogenesis specific basic helix-loop-helix* gene (*FIGLA*; also known as Factor in Germline Alpha, *FIGα;* Huntriss *et al*., 2002). In mouse, *FIGLA* is expressed by oocytes and the released factor is required for recruitment and differentiation of granulosa cells to form the primordial follicles (Soyal *et al*., 2000). WNT4 is also required for normal oocyte development, represented in XX mice null for *WNT4*, where only 10% of the normal number of oocytes are found (Vainio *et al*., 1999). It has been postulated that WNT4 acts to repress factors necessary for testis determination, rather than play an active role in ovary development. Hence the aforementioned downregulation of *WNT4* by SRY and SOX9 in males is thought to be necessary to prevent the repression of 'pro-testis' factors during human testis development (Jordan *et al*., 2003). WNT4 expression has been shown to be maintained by another important pro-ovarian factor R-SPONDIN1 – a gene found mutated in some cases of human XX sex reversal (Tomizuka *et al*., 2008).

One further gene known to be highly important to normal development of the ovary is *DAX1* (dosage-sensitive sex reversal, adrenal hypoplasia congenita (AHC)-critical region of the *X* chromosome, gene 1; officially known as *NR0B1*). *DAX1* is considered vitally important for follicular organisation and the early

stages of female differentiation (Kucinskas and Just, 2005). However, its mode of action remains incompletely understood in humans and in mouse models (Beverdam and Koopman, 2006). *DAX1* is thought to antagonise *SRY* during sex cord development and, like WNT4, is described as an ovarian-promoting factor due to 'anti-testis' activity (Sharpe, 2006). Despite this, it has also been shown to be essential for normal testis development, with *DAX1* knock-out XY mice exhibiting testis dysgenesis (Meeks *et al*., 2003). Thus it has been theorised that DAX1 has dosage dependent effects, where specific levels of DAX1 are essential within a narrow window of time for normal gonadogenesis to occur in males and females (Achermann and Hughes, 2008).

1.4 Steroidogenesis

Steroid biosynthesis is an essential part of human physiology. It is involved in a number of different important processes, such as inflammation (Critchley *et al*., 1999; Maia and Casoy, 2008), control of circulating volume (Stewart, 2008), psychological well-being (Weber, 1998), as well as aspects of human sexual differentiation (Siiteri and Wilson, 1974; Geissler *et al*., 1994; Can *et al*., 1998; Hanley and Arlt, 2006). There are a number of steroid hormones that play a significant role in reproductive functioning in the human body. Particularly important are the steroidogenic sex hormones, including the androgens testosterone and, its 5α–reduced form, dihydrotestosterone (DHT), which are essential for virilisation in males, and oestrogens and progesterone necessary for the female menstrual cycle. All the major steroid sex hormones are made through a common set of interconnected pathways referred to collectively as steroidogenesis (Kronenberg *et al*., 2008; see Fig 1.2).

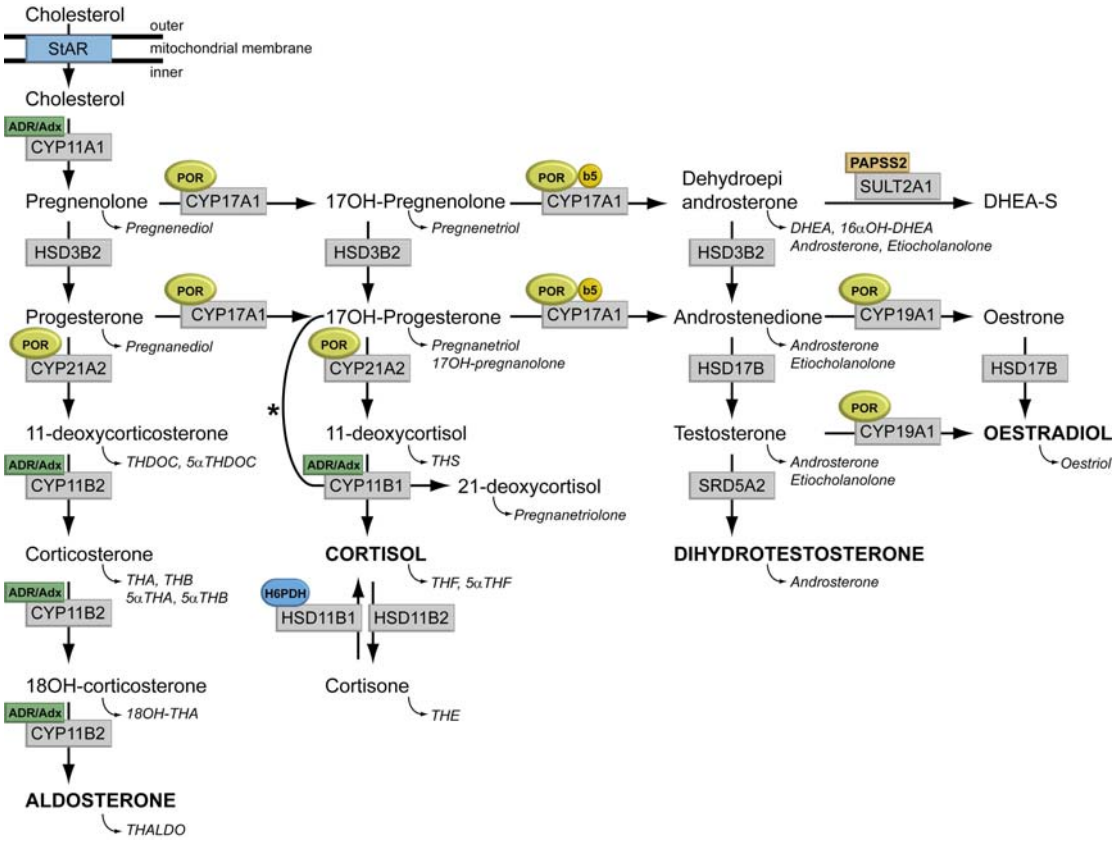

Figure 1.2: Steroidogenic biosynthetic pathways responsible for the production of gonadal and adrenal steroid hormones from cholesterol. *Mitochondrial CYP type I enzymes (CYP11A1, CYP11B1 and CYP11B2) requiring electron transfer via adrenodoxin reductase (ADR) and adrenodoxin (Adx) are marked with a labelled box "ADR/Adx". Microsomal CYP II enzymes (CYP17A1, CYP21A2 and CYP19A1) that receive electrons from P450 oxidoreductase are marked by a circled "POR". The 17,20-lyase reaction catalysed by CYP17A1 requires cytochrome b5 in addition to POR, as indicated by a circled "b5". Hexose-6-phosphate dehydrogenase (H6PDH) is the cofactor to HSD11B1. Urinary steroid hormone metabolites are given in italics below the plasma hormones. The conversion of androstenedione to testosterone is catalysed by HSD17B3 in the gonad and HSD17B5 in the adrenal cortex. StAR, Steroidogenic Acute Regulatory protein; CYP11A1, P450 side-chain cleavage enzyme; HSD3B2, 3β-hydroxysteroid dehydrogenase type 2; CYP17A1, 17α-hydroxylase; CYP21A2, 21-hydroxylase; CYP11B1, 11β-hydroxylase; CYP11B2, aldosterone synthase; HSD17B, 17β-hydroxysteroid dehydrogenase; CYP19A1, aromatase; SRD5A2, 5α-reductase type 2; SULT2A1, sulfotransferase 2A1; PAPSS2, 3'-phosphoadenosine 5'-phosphosulfate synthase 2.*

Steroidogenesis is a complex cascade of enzymatic reactions. All of the steroid hormones of the adrenal cortex, placenta, ovaries and testes originate from steroidogenesis, including mineralocorticoids, glucocorticoids and gonadal steroids (Hanley and Arlt, 2006). The products of steroidogenesis all originate from the common steroid precursor cholesterol, which is obtained mostly from

circulating low-density lipoprotein, and also from local conversion of acetate. The final steroid product generated is determined by the precise complement of enzymes involved (Christenson and Devoto, 2003). The coordinated expression of steroidogenic enzymes delineates the steroidogenic phenotype of a particular organ or tissue within the endocrine system, with each steroidogenic organ expressing its own characteristic complement of enzymes.

In steroidogenic cells, cholesterol is transported from the outer mitochondrial membrane to the inner mitochondrial membrane, where steroidogenesis occurs. This is mediated by the steroidogenic acute regulatory protein (StAR). StAR plays a key role in the acute regulation of steroid hormone synthesis. Within the inner membrane of mitochondria, the initial conversion step of steroidogenesis is the side-chain cleavage of cholesterol, which occurs universally in all steroidogenic cells, performed by CYP11A1. Following this, there is an intrinsic tissue-specific and/or cell-specific restriction on what steroids can be synthesised that is predetermined by the expression of the appropriate sequence of enzymes in the tissue or cells in question (Miller, 2005). For example in the adult adrenal cortex, following conversion of cholesterol to pregnenolone, expression of CYP21A2 in both the zona glomerulosa and zona fasciculata directs steroidogenesis towards mineralocorticoid and glucocorticoid synthesis, respectively. However, it is the expression of CYP11B2 exclusively in the zona glomerulosa that leads to the production of aldosterone. Whereas the lack of CYP11B2 in the zona fasciculata prevents aldosterone production, and the expression of CYP11B1 permits cortisol production (Stewart, 2002). Likewise, the presence of HSD17B3 activity in the gonads, directs steroid biosynthesis towards the production of testosterone in the testes, which is further converted to estradiol in the ovary by CYP19A1 (aromatase) expression (Bashin *et al*., 2002). All conversion steps following pregnenolone synthesis are performed either on the inner surface of the mitochondria (e.g. CYP11B1 and CYP11B2) or in the endoplasmic reticulum (e.g. CYP21A2, CYP17A1, CYP19A1 and HSD3B2) of steroidogenic cells.

1.4.1 Androgen biosynthesis in the human testis

One of the key factors produced by the developing testis that promotes male differentiation and virilisation of tissues is testosterone. Following testes determination and the initial establishment of male morphological sex, the biosynthesis of testosterone by fetal testicular Leydig cells at approximately eight weeks gestation, is crucial to continuing the cascade of events that lead to the male phenotype (Siiteri and Wilson, 1974). Like all steroid hormones, it is produced from cholesterol. In the so-called 'classical pathway', the sequence of steroidogenic reactions that leads to testosterone biosynthesis utilizes the steroidogenic enzymes CYP11A1, HSD3B2, CYP17A1 and HSD17B3, to ultimately produce testosterone from cholesterol (Fig. 1.3). Testosterone has a major role initiating male differentiation of the reproductive system in the fetus, and therefore has critical importance as an androgen in humans.

Testosterone can be converted into the more potent androgen DHT (Kim *et al*., 2002). Following secretion of testosterone from the testis, DHT is produced locally in tissues by the action of the enzyme type 2 5α-reductase, which is a membrane-bound enzyme encoded by the gene *SRD5A2* (Ntais *et al*., 2003). Tissues where DHT is produced typically express *SRD5A2,* commonly mesenchymal cells, allowing for conversion of testosterone in target cells (Berman *et al*., 1995). Interestingly, it has been demonstrated in humans that *SRD5A2* is active from a very early stage in development and is able to convert testosterone to DHT even before the fetal testis acquires the ability to produce testosterone (Fisher, 2008). DHT plays a vital role in normal male sexual differentiation during embryogenesis, and is also important for many androgen-mediated events during male puberty. Studies in mice indicate that DHT acts during embryonic life to amplify hormonal signals that can only be mediated by testosterone at higher concentrations (Wilson *et al*., 2002). Therefore indicating that DHT is a more potent androgen than testosterone. This is consistent with reports of inactivating mutations in SRD5A2 that have caused micropenis, hypospadias and partial sex reversal in males (Can *et al*., 1998; Nordenskjold and Ivarsson, 1998; Chavez *et al*., 2000; Sasaki *et al*., 2003).

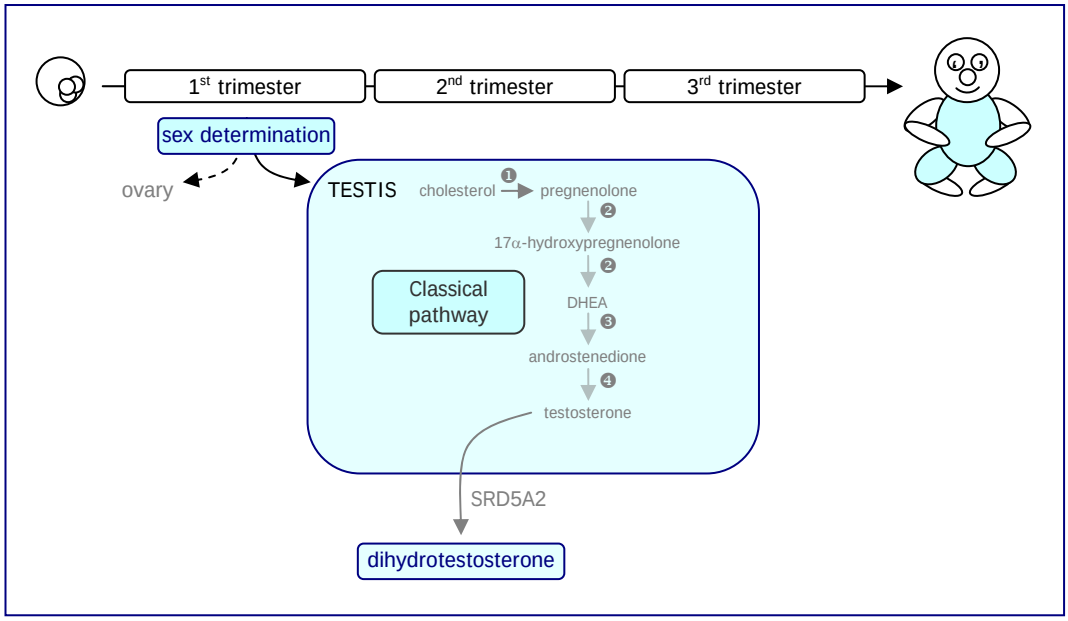

Figure 1.3: Classical pathway of testosterone biosynthesis in the developing human testis.
Sex determination at 7 wpc generates either a testis or an ovary. In the testis, steroidogenesis commences in the fetal Leydig cells to generate testosterone. Testosterone is converted to dihydrotestosterone in target tissues to elicit male sexual differentiation. ❶, CYP11A1, P450 side-chain cleavage enzyme; ❷, CYP17A1, 17α-hydroxylase / 17-20-lyase; ❸, HSD3B2, type 2 3β-hydroxysteroid dehydrogenase; ❹, HSD17B3, type 3 17β-hydroxysteroid dehydrogenase. Type 2 5α-reductase (SRD5A2) is shown on the diagram.

1.4.2 The alternative pathway to dihydrotestosterone

Alongside the classical pathway is the more recently discovered alternative pathway to potent androgen (Auchus, 2004; Arlt *et al*., 2004; Leihy *et al*., 2004; Shaw *et al*., 2006). In the classical pathway of androgen production, the end product is testosterone, which can serve as a precursor for 5α–reduction to the more potent DHT at the sites of androgen action. However, it has been demonstrated in animal models that DHT can be formed by another pathway. In the testis of the tammar wallaby (*Macropus eugenii*) pouch young and in the immature mouse testis, it is thought that the accumulation of 17-hydroxyprogesterone (17-OHP) prompts to the conversion of 17-OHP into DHT via a testosterone-independent route of steroidogenesis (Wilson *et al*., 2003; Mahendroo *et al*., 2004). This is thought to occur through the conversion of 17-OHP firstly to 5α-pregnane-17α-ol-3,20-dione by 5α reductase activity (presumed to be the type 1 enzyme, *SRD5A1*), which is followed by a sequential

series of enzyme reactions converting it to 5α-pregnane-3α,17α-diol-20-one (5-pdiol) by aldo-keto reductase (HSD3A) activity, then androsterone by CYP17A1 action, androstanediol (5-adiol) by 17 β-hydroxysteroid dehydrogenase (HSD17B) activity, and finally to DHT by HSD3A activity (Fig. 1.4).

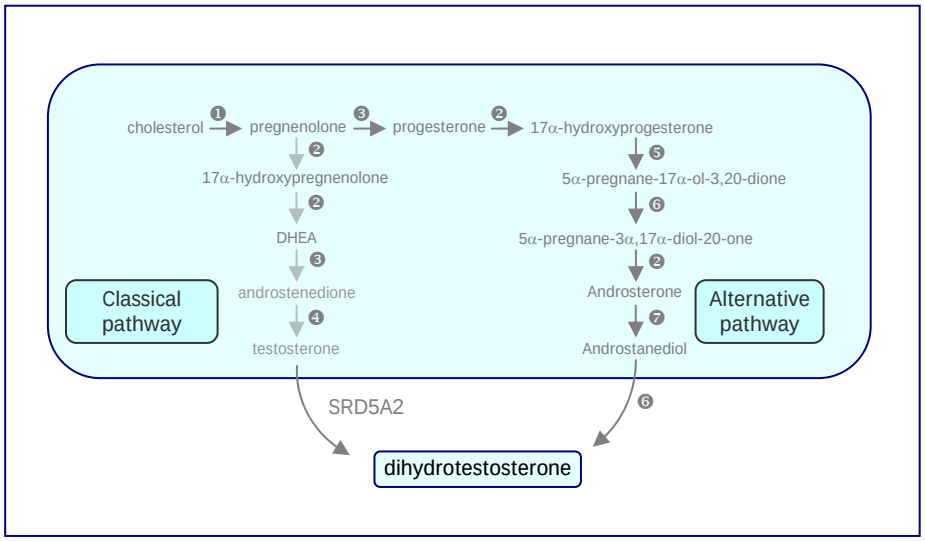

Figure 1.4: Alternative or 'backdoor' pathway of potent androgen production, compared to classical pathway.
❶, CYP11A1, P450 side-chain cleavage enzyme; ❷, CYP17A1, 17α-hydroxylase / 17-20-lyase; ❸, HSD3B2, type 2 3β-hydroxysteroid dehydrogenase; ❹, HSD17B3, type 3 17β-hydroxysteroid dehydrogenase; ❺, SRD5A, 5α-reductase activity; ❻, HSD3A, 3α-hydroxysteroid dehydrogenase / aldo-keto reductase activity; ❼, HSD17, 17β-hydroxysteroid dehydrogenase activity; ❽, CYP11B1, 11β-hydroxylase. Type 2 5α-reductase (SRD5A2) is shown on the diagram.

In the tammar wallaby, 5-adiol plays a key role in the formation of the male urogenital system and copious amounts have been detected in the testes of pouch young (Shaw *et al*., 2000). If female pouch young are treated with 5-adiol the urogenital tract and the external genitalia become virilised (Leihy *et al*., 2001). Furthermore, in several rodent species 5-adiol is the major androgen formed in immature male testes (Chase and Payne, 1983; Ge *et al*., 1999; Frungieri *et al*., 1999), and is readily secreted in regenerating Leydig cells of adult rats following treatment with cytotoxic drugs (Risbridger and Davies, 1994). In rodents it has also been demonstrated that the capacity to form 5-adiol declines during late fetal development, when there is a shift towards testosterone production (Chase and

Payne, 1983; Ge and Hardy, 1998), which can be accelerated by chorionic gonadotrophin (Moger, 1977).

The role of the alternative pathway in male sexual differentiation in humans remains unclear and it is unknown to what extent the alternative pathway operates within the human fetal testis (Auchus, 2004). However, there is the possibility it could represent an important pathway to DHT during early development, especially when we consider that the steps of the alternative pathway leading from 5-pdiol to 5-adiol avoid two energy inefficient steps used to synthesise testosterone. Because 17-OHP is superior to testosterone as a substrate for 5-reductase (Pratis *et al*., 2000), and 5-pdiol appears to be the best substrate for the 17,20-lyase activity of CYP17A1 (Wilson *et al*., 2003), the use of 5-pdiol in the alternative pathway suggests a more efficient route to production of 19-carbon steroids. Hence the formation of 5-adiol in fetal testes could represent the most efficient route to DHT due to inherent properties of the steroidogenic enzymes involved. However, the fact that the fetal testis secretes high amounts of testosterone (Siiteri and Wilson, 1974) and that mutations in *HSD17B3* and *SRD5A2* (both clear parts of the classical pathway) cause profound under-virilisation (Imperato-McGinley and Zhu, 2002), suggests the classical pathway predominates in humans during normal male differentiation, although a role for the alternative pathway can not be fully excluded.

1.4.3 Regulation of androgen steroidogenesis in adults

The pathways and associated enzymes involved in androgen biosynthesis are typically subject to regulation consisting of trophic hormone stimuli and negative feedback mechanisms. In terms of testosterone biosynthesis, in the normal human adult testis regulation is primarily controlled by the hypothalamic-pituitary-gonadal (HPG) axis, though production of luteinizing hormone (LH). The HPG axis comprises of the hypothalamus, anterior pituitary gland and testes in males or ovaries in females (Fig. 1.5), and represents a ‘closed-loop’ endocrine system subject to negative feedback control (Low, 2008). The hypothalamus is located at the base of the forebrain and is directly connected to the pituitary stalk, which is in turn continuous with the posterior lobe of the

pituitary gland. The hypothalamus is also very closely associated with the anterior pituitary through a dense capillary network, the median eminence capillary plexus, which lies at the interface between the hypothalamus and anterior pituitary. The central function of the hypothalamus is to organize the secretion of different hormones in response to multiple inputs from the circulatory and nervous systems regarding the homeostatic state of the body (Mastorakos *et al*., 2006). In terms of the HPG axis, hypothalamic releasing hormones are secreted in response to relevant inputs, and carried locally in the bloodstream where they are detected by specialised secretory cells (gonadotrophs) in the anterior pituitary gland (Melmed and Kleinberg, 2008). In response to the releasing hormones, the anterior pituitary is prompted to release its own hormones, but in much larger quantities, which go on to regulate hormone biosynthesis and secretion from the gonads.

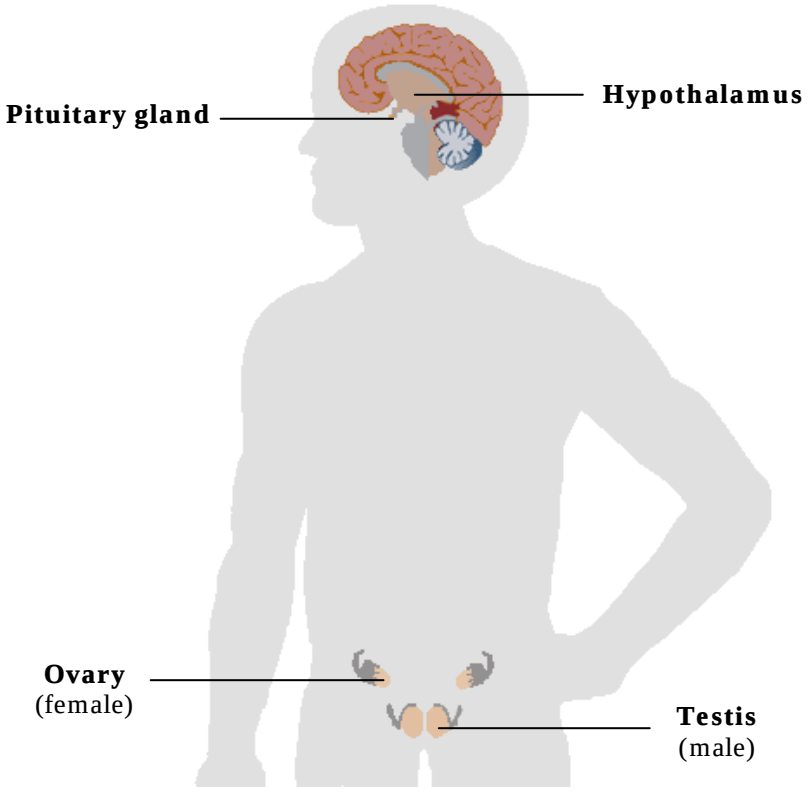

Figure 1.5: Locations of the organs of the hypothalamic-pituitary-gonadal (HPG) axis.
Figure indicates the locations for the hypothalamus, pituitary gland and testes/ovaries (gonads), which together make up the HPG axis. Adapted from niaaa.nih.gov.

The HPG axis is central to both the development and maintenance of the reproductive system in males and females. In humans the hypothalamic secretion of gonadotrophin-releasing hormone (GnRH; also known as Luteinizing hormone-releasing hormone, LHRH) stimulates gonadotroph cells in the anterior pituitary to release large quantities of glycoprotein gonadotrophins (Fig. 1.6), specifically luteinizing hormone (LH) and follicle stimulating hormone (FSH; Huirne and Lambalk, 2001). These are secreted into the body's general circulation and soon reach the target gonad tissues.

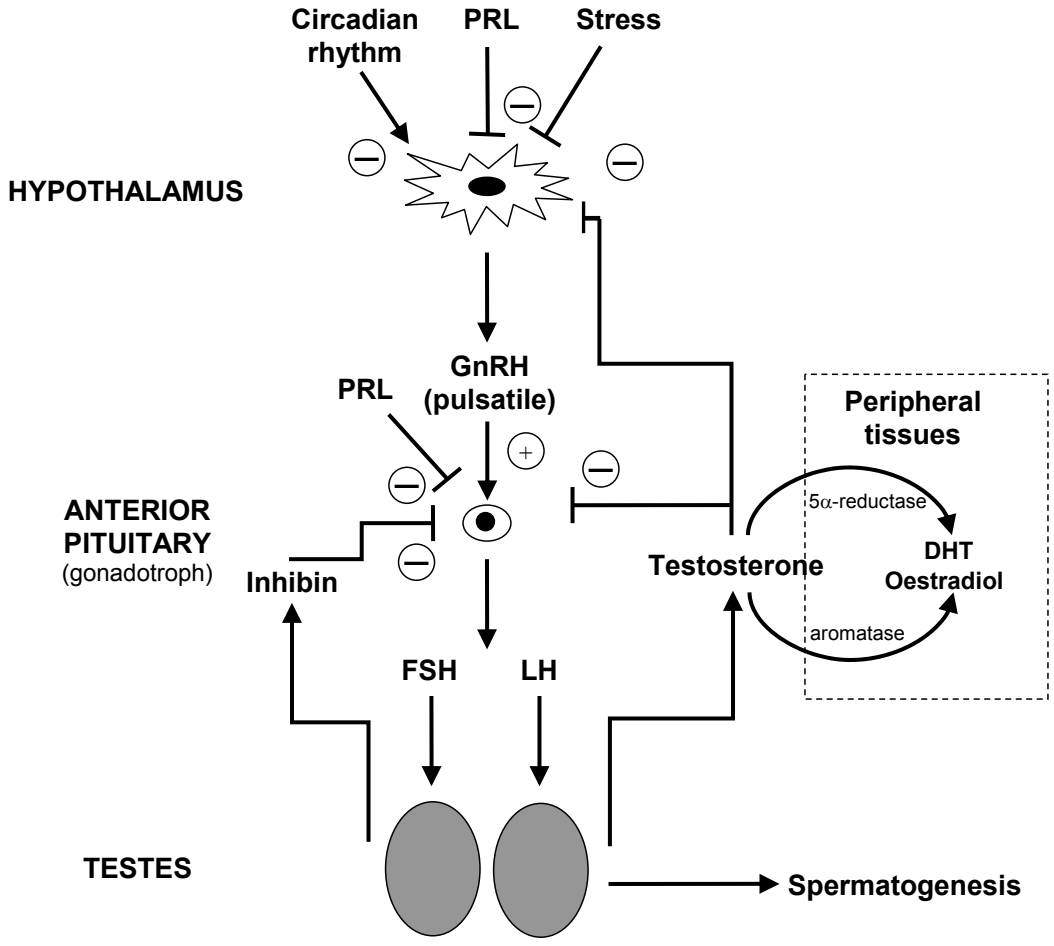

Figure 1.6: Hormonal action of the adult HPG axis in males.
Figure indicates hormones produced by each organ of the HPG axis in males and their relative effects on target tissues. Various inputs at the top of the axis dictate the production of gonadotrophin-releasing hormone (GnRH) from the hypothalamus, which stimulates the gonadotroph cells of the anterior pituitary to produce the gonadotrophins FSH and LH. LH-mediated testosterone biosynthesis in the testis is crucial to the development of secondary sex characteristics in males and providing substrate for dihydrotestosterone (DHT) and oestradiol production. Note the negative feedback loop that arises from testosterone and inhibin secretion. PRL, prolactin; FSH, follicle stimulating hormone; LH, luteinizing hormone. Adapted from Holt and Hanley, 2006.

1.4.4 Regulation of androgen biosynthesis during fetal development

The regulatory mechanisms underlying androgen steroidogenesis in adult males is well documented. However, despite its critical importance during early development of the reproductive tract, the regulation of testicular androgen production during the first trimester has remained unclear (Sobel *et al*., 2004). Generally three candidates for regulation have been considered: (i) LH from the HPG axis - similar to adult regulation; (ii) hCG from the placenta; or (iii) constitutive secretion of androgen from the fetal testis.

Current understanding of androgen biosynthesis during early development has been achieved primarily through biochemical studies where steroid intermediaries are traced from initial substrate to final products, and by the phenotypes observed in individuals with mutations in key biosynthetic enzymes. The importance of LHR, inferred from similar mutational analysis, suggests its ligands LH and/or hCG could be important for regulation of fetal testicular androgen biosynthesis (Ascoli *et al*., 2002). Severe inactivating LHR mutations causes a disorder of sex development (DSD) in 46,XY males, which can include complete failure to virilise and the appearance of female-type external genitalia at birth (Themmen and Huhtaniemi, 2000). LHR belongs to a family of G protein coupled receptors with seven transmembrane helices, and structurally resembles the thyrotrophin receptor (Ji and Ji, 1991). It is encoded by a single gene on chromosome two, which has 11 exons and 10 introns. A number of tissue specific splice variants of LHR have been identified, although only two well characterised splice products exist: the full length protein that is composed of 699 amino acid residues and the smaller splice variant composed of 685 amino acid residues (Ryu *et al*., 1998; Minegishi *et al*., 2007). However, despite the apparent importance of LHR, it appears that the gonadotrophs that produce LH are functionally immature during the first trimester, making LH production unlikely at this stage of sexual differentiation.

An obvious alternative candidate to LH regulation is hCG. Although hCG is synthesized abundantly during the first trimester, it would seem unlikely that it is responsible for androgen regulation, as it would be required to cross the fetal-placental barrier. Furthermore, testosterone secretion has been illustrated prior to

LHR expression in the developing fetus, which could suggest constitutive secretion of androgens and the potential importance of Leydig cell nuclear transcription factors in the regulation of testosterone biosynthesis (e.g. SF1). It has been documented that inactivating mutations in SF1 result in a lack of gonadogenesis, and sex reversal in 46,XY males, including ambiguous internal genitalia (Achermann *et al*., 1999; Lin *et al*., 2007). It has also been shown that mutations causing constitutive activation of LHR lead to a normal male phenotype, although precocious puberty occurs do to androgen excess (Min *et al*., 1998a).

1.5 Development of the human genitalia

The development of the internal and external genitalia in males and females begins at approximately the fourth week of gestation and continues through puberty, reaching its final form in adulthood (Achermann and Hughes, 2008). In adults, the male reproductive system (Fig. 1.7) is composed of: the testes; internal genitalia, consisting of the epididymides, vasa deferentia, prostate gland and seminal vesicles; and external genitalia, consisting of the penis and scrotum (Johnson and Everitt, 2000). Together these have two major functions: the production of male gametes (spermatozoa) through the process of spermatogenesis, and copulation and ejaculation of spermatozoa. In contrast the female reproductive system (Fig. 1.8) is made up of: the ovaries; the internal genitalia, consisting of the Fallopian tubes, uterus, cervix and vagina; the external genitalia consisting of the vaginal opening, clitoris, labia minora and labia majora; and the mammary glands of the breasts (Johnson and Everitt, 2000). The female reproductive system essentially performs four main functions: the formation of female gametes (oocytes) through oogenesis, to host fertilization and pregnancy; parturition; and lactation. Although adult reproductive functions are not investigated in the present study, their anatomical integrity is crucial, and is ensured by appropriate development that is initiated in the latter half of the first trimester.

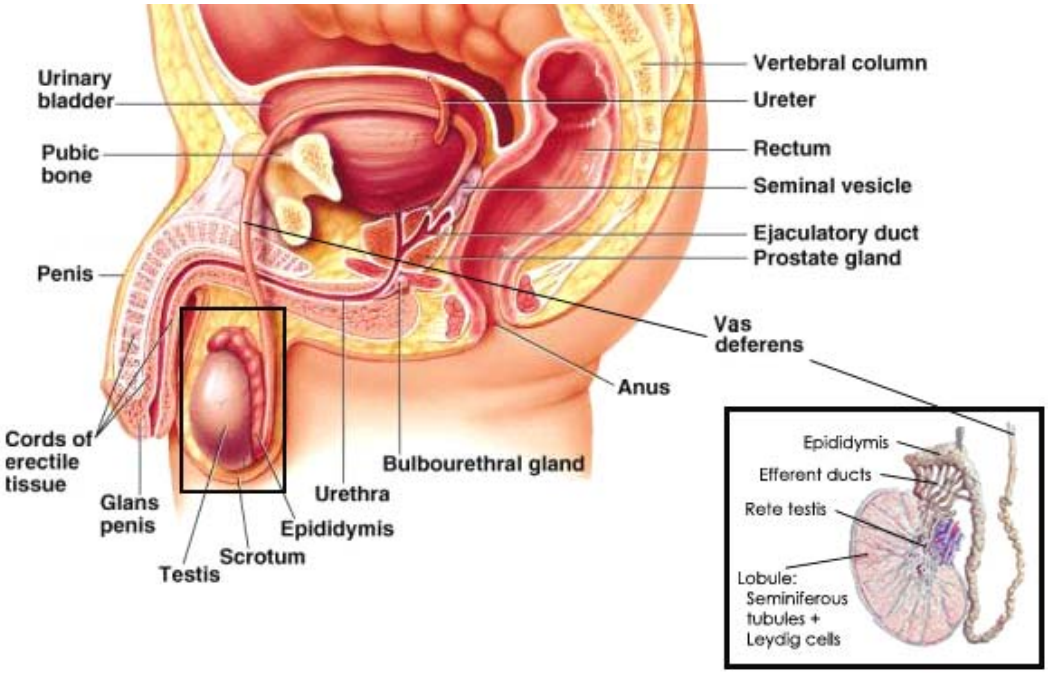

Figure 1.7: The human adult male internal and external genitalia. *Schematic diagram of the fully developed male reproductive system, depicting the major elements of the internal and external genitalia, and testis microstructure (boxed area). Adapted from uh.edu(a).*

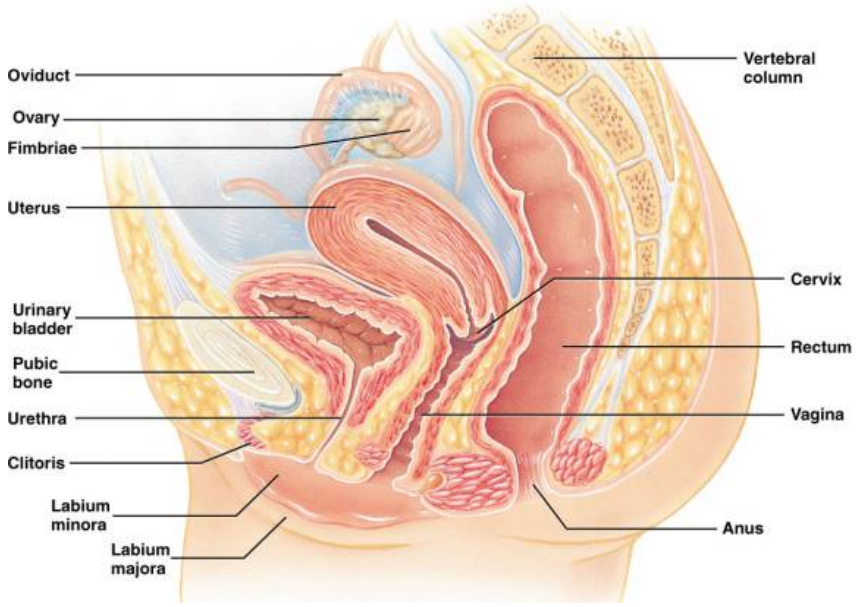

Figure 1.8: The human adult female internal and external genitalia. *Schematic representation of the fully developed female reproductive system, depicting the elements of the internal and external genitalia. Adapted from uh.edu(b).*

Initially, during the fourth week of gestation, two sets of genital ducts are formed from the embryonic mesoderm, which mature into the majority of structures of the internal genitalia for both sexes (Johnson and Everitt, 2000; Schoenwolf *et al*., 2008). Five mesenchymal swellings covered with ectoderm also emerge proximal to the cloacal membrane, which develop into the external genitalia (Cummings and Kavlock, 2004). Between weeks four and seven of gestation, these bipotential structures are indistinguishable in males and females. In males, activation of *SRY* during week seven, and the subsequent differentiation of the testes, leads to production of male sex hormones and virilisation of the internal and external genitalia between weeks 8 and 12 – making this period a 'critical window' of sexual differentiation (Hughes, 2001). In the absence of testes, virilisation does not occur and sexual differentiation of the bipotential genitalia leads to female internal and external genitalia. Therefore in principle it is the testis and ensuing synthesis of key male hormones that determines the route of major sexual differentiation during this crucial time in fetal development; as was first shown in the classic experiments by Alfred Jost more than 50 years ago (Jost *et al.*, 1953). There are two hormones responsible for male sexual differentiation: testosterone and anti-Müllerian hormone (AMH; also known as Müllerian inhibiting substance, MIS); produced by fetal Leydig cells and fetal Sertoli cells, respectively (Sobel *et al*., 2004). The synthesis of these important hormones alone in males is enough to elicit virilisation of the internal and external genitalia; the first marked feature of extragonadal sexual dimorphism in human development. In females, differentiation of the internal and external genitalia is thought to occur in an 'androgen-free' environment, and as with ovarian organogenesis, is considered a default developmental pathway.

Because the route of sexual differentiation of the internal and external genitalia is determined early on in the developing fetus, the clear differences between fetal and adult reproductive endocrinology means our relatively detailed understanding of the latter does not provide for a good understanding of the former. Fetal reproductive endocrinology is characterized by distinctive functioning of fetal endocrine organs, several hormones and metabolites with prominent fetal roles, adaptation of fetal endocrine tissues to special intrauterine functions, and specialised mechanisms to neutralize the biological actions of

potent hormones in appropriate scenarios (Fisher, 2008). In terms of sexual differentiation, there are three key features of the fetal reproductive/endocrine system: (i) mesonephroi; a pair of fetal organs akin to primitive kidneys which develop into elements of the internal genitalia (Holt and Hanley, 2006); (ii) the fetal adrenal glands; bilateral organs with a unique metabolic profile and *in utero* a key source of cortisol (Goto *et al*., 2006) and androgen precursors (Asby *et al*., 2009); and, (iii) the fetal testes, which are principally responsible for virilisation of male fetuses during the critical period of sexual differentiation (Ostrer, 2000). Determining how these unique aspects of fetal reproductive endocrinology may influence sexual differentiation of the human external genitalia is central to the current study. Hence what follows is a brief description of current understanding of the differentiation of the internal genitalia, followed by a more detailed account of the differentiation of the external genitalia.

1.5.1 Sexual differentiation of the internal genitalia

The internal genitalia initially develops as two bilateral pairs of genital ducts: the Wolffian (mesonephric) ducts, which drains urine from the mesonephros and forms most of the adult male internal genitalia; and the Müllerian (paramesonephric) ducts, which lies lateral to the mesonephros and develops into a significant portion of the adult female internal genitalia (Achermann and Hughes, 2008). During the eighth week of gestation, the fetal Sertoli cells begin to differentiate in response to *SRY* and start secreting AMH (Wilson and Davies, 2007). AMH is a catabolic glycoprotein hormone expressed exclusively in Sertoli cell, which causes the Müllerian ducts to regress rapidly between the eighth and tenth week of gestation (see Fig. 1.9). AMH acts on the AMH type II receptor (AMHR-II), expressed by the mesenchymal cells surrounding the Müllerian ducts. Evidence for this comes from the study of inactivating mutations in the *AMH* or *AMHR-II* genes in human XY males, which cause persistent Müllerian duct syndrome (Josso *et al*., 2005). Affected individuals develop internal structures normally associated with female development (e.g. the uterus), alongside androgen-dependent elements of the male internal and external genitalia.

At the same time as initial AMH secretion, fetal Leydig cells begin to produce testosterone (Sobel *et al*., 2004). Testosterone prevents regression of the Wolffian ducts and induces them to develop further, differentiating into a contiguous system of accessory organs (ducts) - the epididymis, vas deferens and seminal vesicle - that by the third month of development form a fully integrated connection from the fetal rete testis to the urethra (Kucinskas and Just, 2005). The prostate gland arises in approximately the tenth week from a cluster of endodermal outgrowths from the pelvic urethra, induced by the adjacent mesenchyme, via the action of DHT (Timms, 2008). Further condensation of the surrounding mesenchyme, urethral tissue and parts of the Wolffian ducts give rise to the mature prostate gland, a composite organ made up of several glandular and non-glandular components tightly fused within a common capsule.

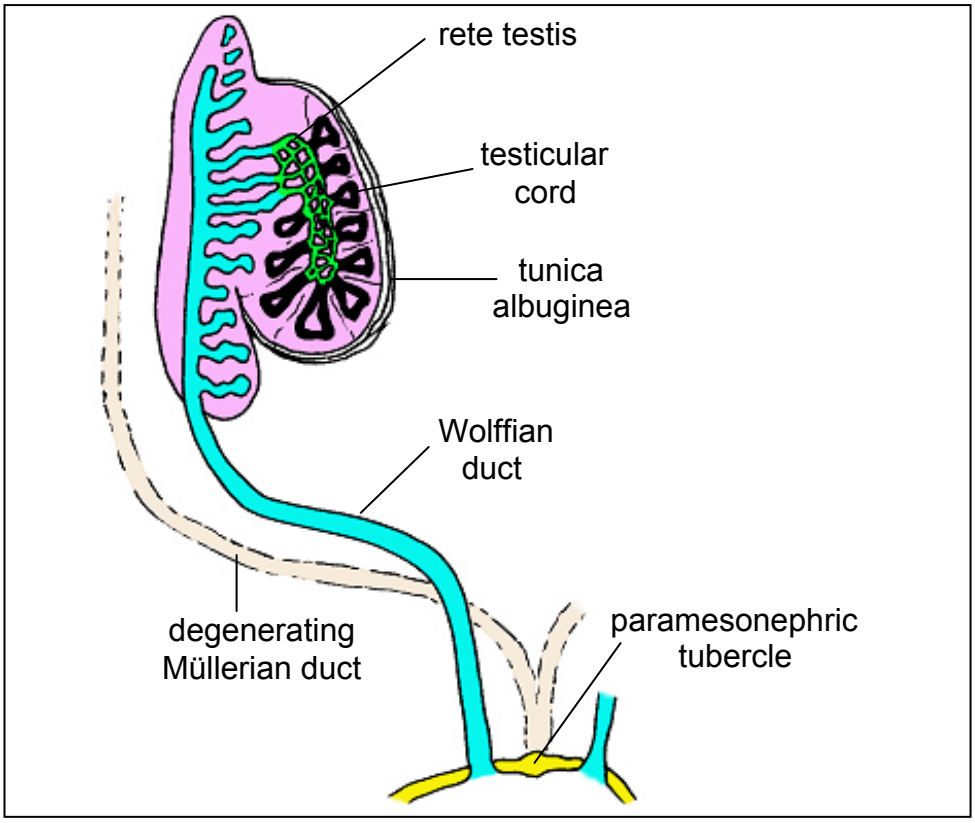

Figure 1.9: Differentiation of the male internal genitalia.
The Müllerian duct (pale brown) regresses due to the action of testicular AMH, secreted from the fetal Sertoli cells, while the Wolffian ductal system (blue) is maintained and differentiates, owing to the secretion of testicular testosterone from fetal Leydig cells. Rete testis, green; testicular cords, black.

In the absence of *SRY* and subsequent expression of genes associated with male sex determination, fetal Sertoli and Leydig cells do not differentiate in the XX female gonad. This results in a lack of hormone action from AMH and androgens at the critical stage of differentiation of the genital duct system, and reveals the inherent tendency of the genital ducts to feminize (Achermann and Hughes, 2008). Hence, in XX females and XY males with gonadal dysgenesis, the Wolffian ducts and associated mesonephric tubules rapidly regress due a lack of testosterone needed to maintain them. Although steroidogenic theca cells (the homologue of the Leydig cells in the developing ovary) are capable of some androgen secretion, the low level of expression of steroidogenic enzymes required for testosterone biosynthesis is well below the threshold required to promote Wolffian duct differentiation (Bardin and Lipsett, 1967). Hence, the Müllerian ducts develop uninhibited, due to the lack of AMH secretion from Sertoli cells. The Müllerian ducts will eventually differentiate into the Fallopian tubes proximally, and the distal portion forms the uterus, cervix and is likely to also contribute to the distal portion of the vagina (see Fig. 1.10), though the origin of the vagina remains contentious (Shapiro *et al*., 2000). The remainder of the female internal genitalia consists of the inferior portion of the vagina, thought to form from the endodermal tissue of the sinusal tubercle in the posterior urethra and surrounding mesenchyme, and the hymen, which is formed from the cloacal membrane covering the urogenital orifice that the vagina develops from (Schoenwolf *et al*., 2008). It should be noted that the development of the female internal genitalia remains poorly understood and much is still to be elucidated about the specific processes involved.

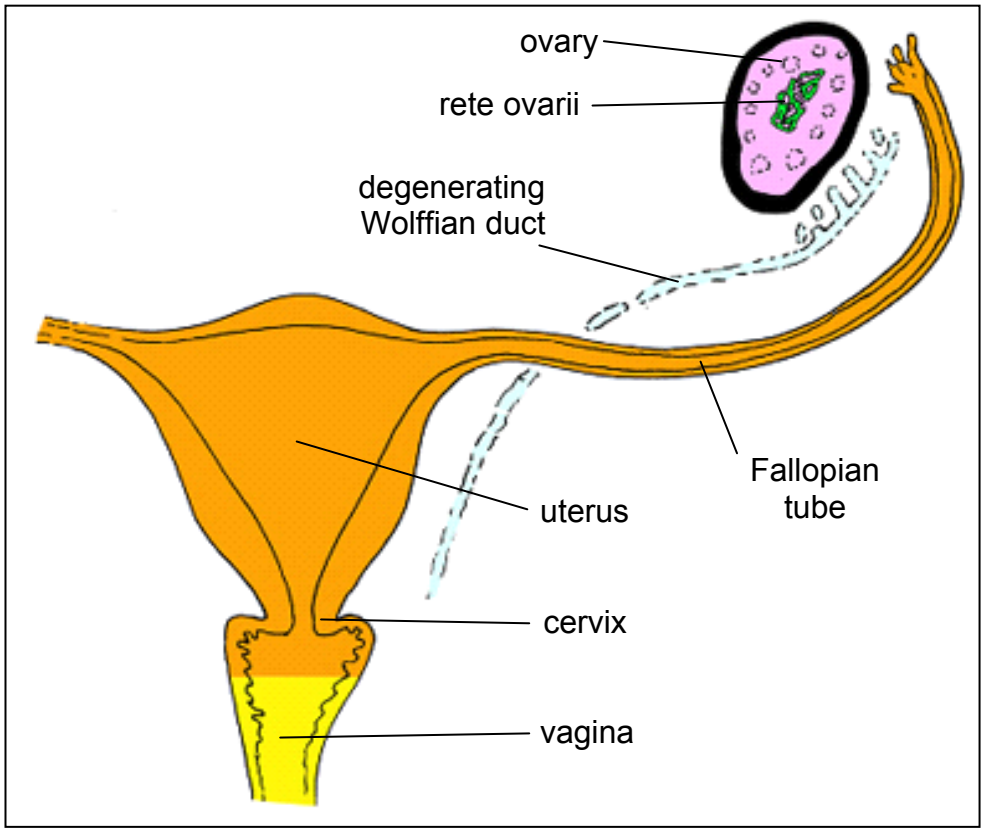

Figure 1.10: Differentiation of the female internal genitalia.
The Müllerian ducts differentiates in the absence of testicular AMH, to form the Fallopian tubes, uterus, cervix, and upper third of the vagina (orange), whereas the Wolffian ducts regress (pale blue) in the absence of potent androgen. The rete ovarii (green) within the ovary (purple) corresponds to the male rete testis.

1.5.2 Sexual differentiation of the external genitalia

1.5.2.1 Assembly of the presumptive bipotential external genitalia

The presumptive structures of the external genitalia form during the fourth week of gestation, developing identically in males and females, but with the potential to develop dimorphically from the eighth week of gestation onwards (Ostrer, 2000). The cloacal membrane demarcates the position where hindgut endoderm meets external ectoderm. In the third week of human development mesenchymal cells migrate to either side of the cloacal membrane forming cloacal swellings (Asby *et al*., 2009). Over the following weeks, a septum develops internally that eventually reaches the cloacal membrane, thus forming the perineum. This septum divides the hindgut posteriorly from the anterior urogenital structures.

Similarly, by the seventh week of development, at the surface cloacal membrane, the posterior anal membrane is now physically partitioned from the anterior urogenital membrane. With this segregation of the cloacal membrane into anus and urogenital structures, the anterior cloacal swellings are now termed the urethral folds, which at their superoanterior limit are fused into the genital tubercle. Lateral to the urethral folds are the labioscrotal swellings. The genital tubercle is comprised of the glans and the corpora cavernosa anlages. Accordingly, by seven weeks gestation, the external genitalia are clearly apparent as the bipotential anlagen, in preparation for major sexual differentiation over the remaining weeks of the first trimester (Goto *et al*., 2006).

1.5.2.2 Differentiation of the male external genitalia

Sexual differentiation of the male external genitalia begins during the eighth week of gestation, in parallel with sexual differentiation of the internal genitalia (Hughes, 2001). Morphologically, the external genitalia undergo marked remodelling and enlargement of the bipotential structures in male fetuses, while regression and relatively subtle remodelling occurs in females between 8 to 12 weeks gestation. As alluded to previously, the male external genitalia originate from three main presumptive structures: the genital tubercle; two urethral folds, which surround the urogenital groove (also known as the urogenital slit); and two labioscrotal swellings, which flank the urethral folds (Achermann and Hughes, 2008; see Fig. 1.11). During sexual differentiation the genital tubercle enlarges and lengthens to form the distal portion of the penis (glans penis). Primordial corpora cavernosa are also formed from the differentiating genital tubercle – two trabeculated sinus spaces surrounded by a tough fibrous capsule continued in the body of the phallus, which in the fully developed adult provide the main erectile tissue and mediate the haemodynamic changes needed for penile erection (Johnson and Everitt, 2000).

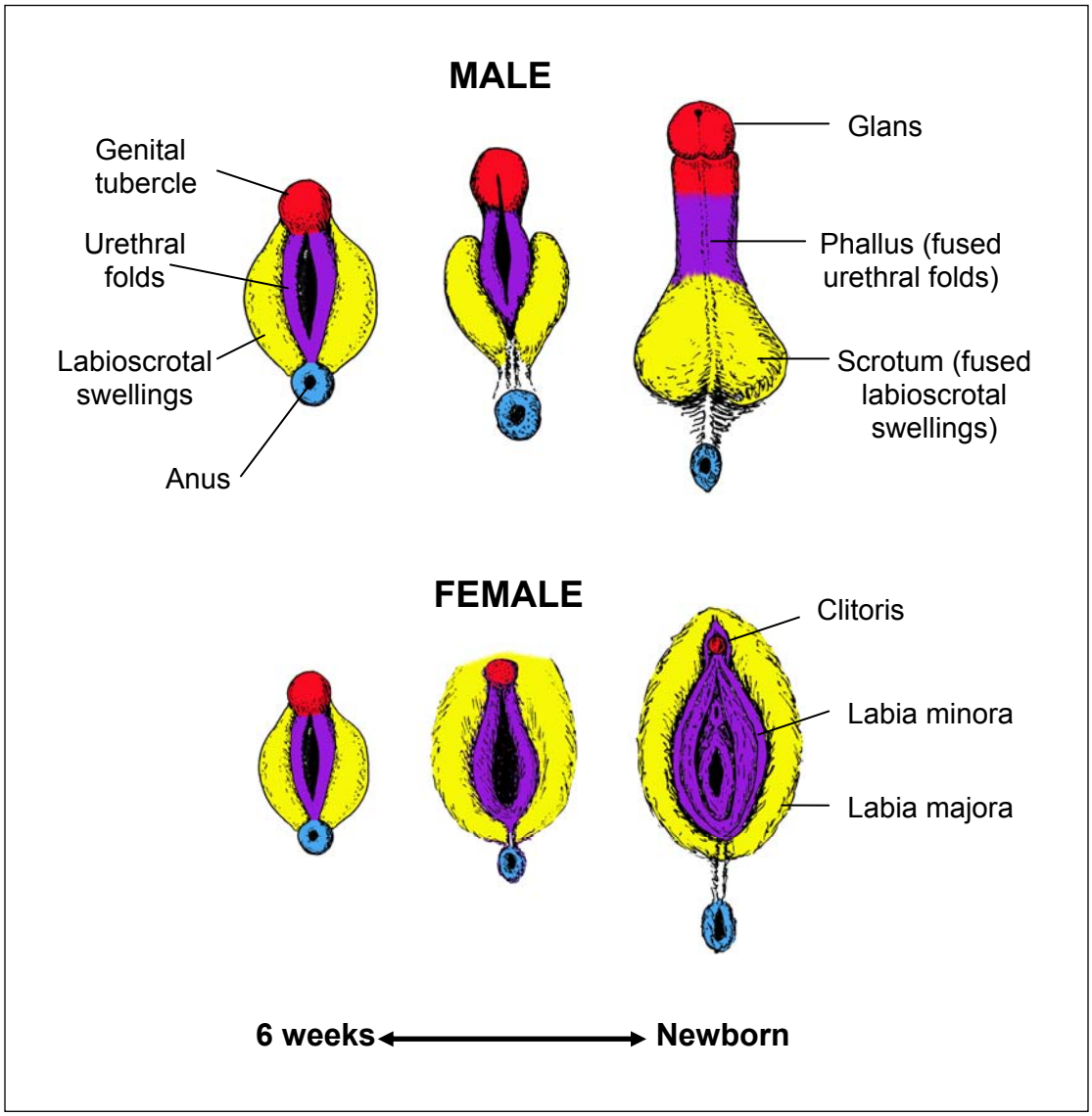

Figure 1.11: Sexual differentiation of the male and female external genitalia. *The external genitalia are shown from 6 wpc to the newborn period. Derivatives of the genital tubercle are coloured red; derivatives of the urethral folds in purple; and derivatives of the labioscrotal swellings in yellow. At 6 wpc, the developing external genitalia are identical in males and females. Between 8 and 12 wpc the external genitalia differentiates concordant with gonadal sex. In males the urethral folds fuse to completely enclose the urethra and create the phallus, while the genital swellings enlarge and fuse in the midline to create the scrotum, which hold the descended testes. In females, the urethral folds remain separate forming the labia minora and the genital swellings enlarge but remain separate to create the labia majora.*

As the genital tubercle differentiates, the urethral folds move towards each other and eventually fuse in the midline, beginning at the proximal end in the perineal region and extending distally toward the glans penis (Schoenwolf *et al*., 2008). The fusion of the urethral folds forms the shaft of the penis and corpus spongiosum tissue that encloses the phallic urethra. Fusion of the urethral folds to enclose the penile urethra is complete by 12 to 14 wpc. The urethra is formed from tubularisation of the urogenital groove, which is gradually closed off at the ventral surface of the penis as the urethral folds fuse (Kim *et al*., 2002). The urethral plate region (a solid multilayered cord extension of the urogenital groove that extends distally into the glans penis), is also thought to contribute to the urethra by canalisation of tissues, to form the distal end of the urethra within the glans penis (glans urethra) and external penile meatus. The third main component of the indifferent external genitalia, the fleshy labioscrotal swellings, slip posteriorly and fuse in the midline to form the scrotum (Achermann and Hughes, 2008). The genital tubercle and urethral folds remain covered in ectoderm, which subsequently forms the skin covering the penis. The ectoderm covering the glans penis will break down to form the foreskin (prepuce) later in development.

As stated previously, differentiation of the male external genitalia is dependent on the functional integrity of the fetal testis and the ability of cells to respond appropriately to androgen signalling. However, whereas testosterone is considered the main protagonist of virilisation of the internal genitalia, *SRD5A2* expression profiling data and observations from human *SRD5A2* mutation sex reversal syndromes, have indicated that the induction of male differentiation of external genitalia is promoted principally by DHT (Wilson *et al*., 1993; Kim *et al*., 2002). DHT is regarded as a more potent form of testosterone for sexual differentiation of the external genitalia. The use of *in vitro* reporter gene assays in mouse has shown that when both steroid SRD5A isozymes are disrupted by homologous recombination, only partial virilisation of the external genitalia occurs in the absence of DHT (Thigpen *et al*., 1992). Testosterone alone appeared unable to mediate complete sexual differentiation at physiological levels. Thus, DHT seems to act during embryonic life to amplify hormonal signals that can only be achieved by significantly higher concentrations (approximately 10-fold more) of testosterone (Wilson *et al*., 2002). Accordingly,

during human sexual differentiation, testosterone acts as a pro-hormone, which is delivered to the presumptive tissues of the external genitalia via the bloodstream and then converted to DHT by SRD5A2 at the site of sexual differentiation. DHT has a number of potent effects during development of the male external genitalia. It is required for stimulating elongation of the genital tubercle, fusion of the urethral folds over the urogenital groove, and posterior movement, approximation and fusion of the labioscrotal swellings (Nef and Parada, 2000). For correct mediation of these processes by DHT the functional integrity of SRD5A2 and the receptor for DHT, the androgen receptor (AR), are required (Meyer, III *et al*., 1975; Ohno, 1978; Gottlieb *et al*., 1999).

The expression profile of SRD5A2 and AR has been characterised in the male external genitalia after 12 weeks gestation, but not before this period (Kim *et al*., 2002). AR is expressed in the surface epithelium of the external genitalia, the epithelium of the developing urethra, as well as in the underlying stromal cells, especially those areas condensed as the corpora cavernosum. SRD5A2 expression appears similarly located during development. Following first trimester development and differentiation, further virilisation of the external genitalia involves much less tissue remodelling in favour of growth and enlargement of pre-existing structures, including preparation of the scrotum to receive the descending testes during late gestation under the influence of androgen and insulin-like 3 (INSL3; Tomboc *et al*., 2000). Therefore, if male sexual differentiation has not occurred appropriately during the early first trimester, there is little potential to remedy this later. Furthermore, the early androgenic milieu is at least partially modified later in development by fetal expression of the CYP19A1 enzyme, aromatase, which converts testosterone to estradiol (Grumbach and Auchus, 1999). This limits the substrate for conversion to DHT, thus moderating the supply of ligand for AR, while only adding minimally to the total pool of oestrogens that are already synthesized by the placenta.

Although there is relatively good understanding of human androgen biosynthesis, 5α-reduction in target tissues and the binding of potent androgen in tissues,

knowledge of the molecular mechanisms responsible for male sexual differentiation largely ends at this point, and there is a general problem that little is known about what signalling and developmental pathways are utilized after activation of AR. After this point, current understanding stems from the phenotypes observed at birth in infants with known genetic syndromes and studies performed in cancer cell lines. For example, in individuals with anopthalmia-esophageal-genital syndrome (AEG; OMIM 206900), under-development of the external genitalia in males is observed at birth. The recent discovery that AEG results from mutations in *SOX2* suggest this gene may be downstream of AR in androgen signalling in the fetal external genitalia and important for sexual differentiation (Williamson *et al*., 2006). Similar genital abnormalities are seen in coloboma-heart anomaly-choanal atresia-retardation-genital and ear anomalies syndrome (CHARGE; OMIM 214800) due to mutations in the chromodomain helicase DNA-binding protein 7 (*CHD7*) (Vissers *et al*., 2004). Both *SOX2* and *CHD7* are also associated with tracheo-esophageal fistula, although, at present, interactions between the two genes or their encoded proteins are unknown and it is unclear how AR signalling impacts on *SOX2* and/or *CHD7*, or vice-versa, during sexual differentiation. In addition, studies in LNCaP have revealed genes, such as that encoding prostate-specific antigen (*PSA*; officially known as kallikrein-related peptidase 3, *KLK3*), as androgen-regulated. However, it is unclear whether the importance of these genes in malignancy translates into a key role in normal human development. LNCaP cells and the role of *KLK3* as an androgen-regulated gene are discussed further in Chapter 5 and 6.

1.5.2.3 Differentiation of the female external genitalia

The processes that mediate female sexual differentiation are less obvious than in males, and do not entail such considerable remodelling of the early bipotential external genitalia. In XX fetuses, the seeming absence of potent androgen secretions prevents the possibility of virilisation of the external genitalia taking place. Generally, the differentiation of the external genitalia in females is characterised by processes representing the opposite to those witnessed in males. Hence, the urethral folds remain separated and the labioscrotal swellings do not

move posteriorly during the critical window of sexual differentiation. In females these structures mature into the labia minora and labia majora, respectively (Schoenwolf *et al*., 2008; see Fig. 1.11). Similarly, the genital tubercle does not undergo extensive elongation, but is believed to bend inferiorly, and remains contained at the distal end of the urethral folds. In this position it develops into a small mass of highly sensitised erectile tissue - the glans clitoris (clitoris) - that is analogous to the glans penis.

1.6 The androgen receptor

All androgens bind a common intracellular receptor – the androgen receptor (AR; Beato and Klug, 2000; McPhaul, 2002). Male sexual differentiation, the development of secondary sexual characteristics in adolescence, and the processes surrounding spermatogenesis all require androgen binding to the AR (Matsumoto *et al*., 2005). AR is a ligand-activated transcription factor and a member of a super-family of steroid nuclear receptors known as the activated class I steroid receptors. It is one of four closely related nuclear receptors, the others being the glucocorticoid, mineralocorticoid and progesterone receptors, which all bind to DNA via a distinct consensus sequence found at specific regulatory sites known as hormone response elements (HRE). Several groups have cloned and sequenced the gene for the human AR (Chang *et al*., 1988; Lubahn *et al*., 1988; Trapman *et al*., 1988; Tilley *et al*., 1989; Brown *et al*., 1989). The gene encoding AR has been mapped to the X chromosome (Xq11-q12) and is composed of eight exons, encoding a protein of 919 amino acid residues. It has been found that in some tissue types a smaller AR isoform (AR-A) is expressed, which is truncated at the N-terminus and accounts for between 4% and 26% of the total AR protein level (Li and Al-Azzawi, 2009). The significance of this isoform in humans is unknown, but it has been reported that 46,XY patients that only express functional AR-A, due to mutations in *AR*, are significantly under-virilised. It has been suggested that AR-A may act as a dominant inhibitor of transactivation induced by the full length AR (Zoppi *et al*., 1993; Holterhus *et al*., 1997).

Both the AR-A and full length AR-B proteins contain four major functional domains (Li and Al-Azzawi, 2009), which resemble those found in other members of the steroid receptor superfamily (Liao *et al*., 1989). These are: an N-terminal transactivation domain (NTD) encoded by exon 1; a central DNA binding domain (DBD) encoded by exons 2 and 3; a C-terminal ligand binding domain (LBD) encoded by exons 4 to 8; and a hinge region connecting the DBD to the LBD (Fig. 1.12). Within the NTD, homopolymorphic stretches of amino acids have been found. A repeated CAG sequence in exon 1 encodes a stretch of glutamine residues, typically ranging from 11 to 31 repeats. The length of this CAG repeat has been found to affect AR function, with the number of repeats being inversely proportional to the activity of liganded AR as a transcription factor (Rocha *et al*., 2008).

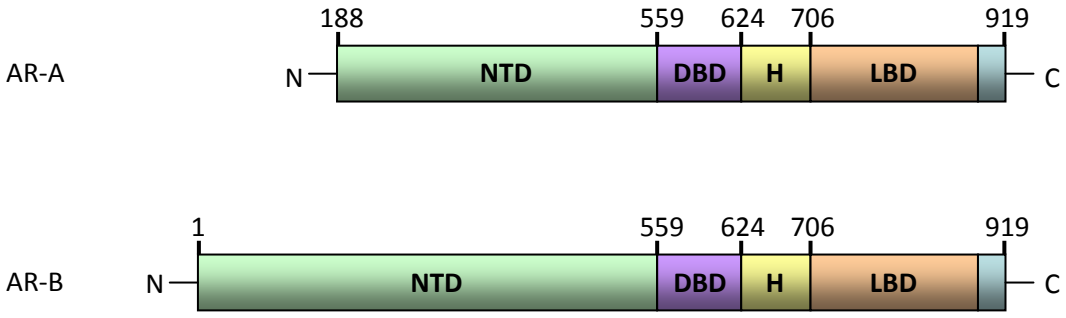

Figure 1.12: Structural domains of the two isoforms (AR-A and AR-B) of the human androgen receptor.
Numbers above the the domains indicate the number of amino acid residues where each domain is found in the overall protein structure, starting from N-terminus (N; left) to the C-terminus (C; right). NTD, N-terminal domain; DBD, DNA-binding domain; H; hinge region; LBD, ligand-binding domain.

Androgens have been shown to exert some limited non-genomic effects on human physiology, such as attenuation of intracellular calcium concentration and modification of cell membrane permeability (Foradori *et al*., 2008). Similarly, AR also has the potential to perform functions in the absence of genomic effects, for example activation of second messenger pathways (Kousteni *et al*., 2001).

However, most androgen-mediated functions arise from the classical genomic effects of androgens following binding to AR. Once liganded, AR is made 'active' and begins to exert effects on gene transcription. Transcriptional regulation of target genes by AR entails a complex sequence of events that are not yet fully understood. This involves binding of androgen, phosphorylation steps, dissociation of chaperone proteins, dimerisation, intracellular trafficking, and DNA binding leading to regulation of gene expression and a plethora of biological effects (Fig. 1.13). Briefly, in its unliganded state, the AR is located in the cytoplasm complexed to HSPs, which in turn are bound to chaperone proteins. Liganding of AR leads to dissociation of HSPs, followed by translocation into the nucleus and phosphorylation of AR. AR forms homodimers in the nucleus and these are able to bind specific DNA sequences known as androgen response elements (AREs), typically found upstream of androgen-regulated genes (Schuur *et al*., 1996; Cleutjens *et al*., 1996; Verrijdt *et al*., 1999). Binding to AREs leads to transcriptional regulation of downstream target genes, either increasing or decreasing expression. AR-induced transcriptional activity can be modulated by co-regulatory proteins that interact with the ligand-bound dimerised AR (Marcelli *et al*., 2006). AR-ligand interactions, intracellular trafficking of bound AR and binding of AR to DNA are discussed in more depth in Chapters 5 and 6.

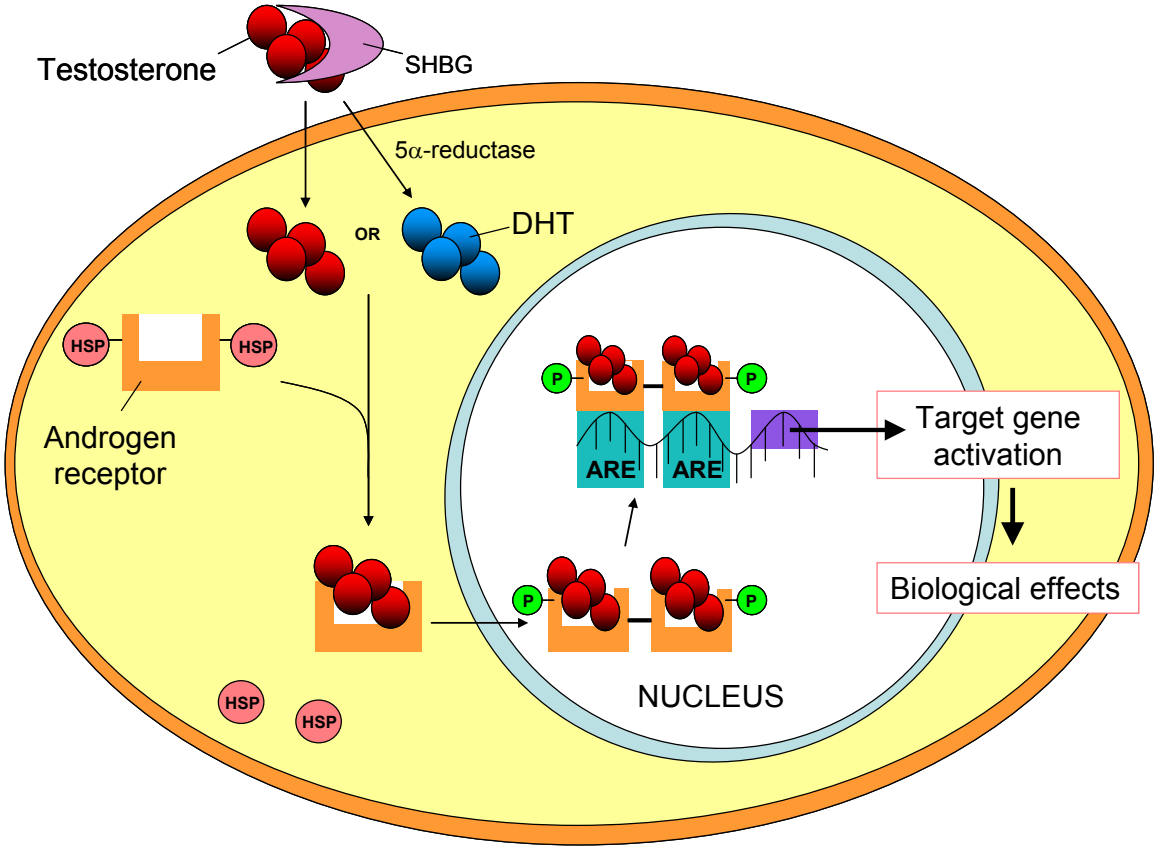

Figure 1.13: Schematic diagram of androgen action in a target cell. *Circulating testosterone bound to sex hormone binding globulin (SHBG) enters the cell in its free form where it can be converted to dihydrotestosterone (DHT) prior to binding AR, or bind directly to cytoplasmic androgen receptors (AR). Bound AR undergoes conformational changes and disassociation from heat shock proteins (HSP) and co-chaperones, followed by phosphorylation, dimerisation and translocation into the cell nucleus. In the nucleus, AR dimers (along with other cofactors) bind androgen response elements typically found upstream of transcription start sites and mediate myriad biological effects via modulation of transcription of target genes. P, phosphorylation.*

Currently we know nothing of AR target genes in the primary cells of the differentiating external genitalia at the time of sexual differentiation. Identifying AR targets is of critical importance if we are to understand the specific regulatory roles played by androgens during normal human sexual differentiation, as well as in disorders of sex differentiation. Although knowledge of AR target genes is very limited in primary cells, advances have been made in recent years regarding the proteins that interact with AR to modulate AR signalling (Janne *et al.*, 2000; Reddy *et al.*, 2006). This research has been

conducted primarily in cancer cell lines, particularly in the androgen-sensitive human prostate adenocarcinoma cell line LNCaP (Wang *et al*., 2005). Experiments have shown that AR interacts with several other transcription factors, and AREs have been shown to occur frequently in regulatory regions of genes, in tandem with those of OCT1 and GATA2 and FOXA2 (Wang *et al*., 2007b). Cooperative action of AR with OCT1 and GATA2 has been demonstrated, with additional potential interactions between AR and FOXA2. These newly discovered partner transcription factors, and their wider family members, play important roles in development, making it quite likely that they or closely related family members will impact on AR action during human sexual differentiation. Interestingly, studies comparing normal genital fibroblasts with those from individuals who lack a functional AR, revealed several differences in gene expression. However those genes that were not expressed when AR was mutated, were not direct AR target genes within normal adult genital fibroblasts (Holterhus *et al*., 2003). Some evidence also exists to suggest that AR has the capacity for auto-regulation; the implications of which are unknown. Further details regarding AR target genes and their study in LNCaP cells, as well as AR auto-regulation can be found in Chapters 5 and 6.

1.7 Disorders of sex development

There exists a definitive division between the sexes in humans – sexual dimorphism. At birth, this distinction is most readily apparent in the external genitalia, which, under normal circumstances, permits instantaneous sex assignment and the establishment of gender. However, in approximately 1 in 4000 births (Sax, 2002) significant ambiguity of the external genitalia prevents straightforward sex assignment and special intervention is required to distinguish whether the individual should be raised as male or female (Brinkmann *et al*., 2007; Mieszczak *et al*., 2009). Such circumstances undoubtedly cause major emotional stress for both parent and child. In light of the relatively high incidence of genital birth defects and the distress it can cause, any additional data regarding the mechanisms underlying sexual differentiation will not only increase our general understanding of the basic science, but have clinical value. Equally, many of the advances in our understanding of normal sexual

development stems from detailed clinicopathologic assessment of cases of disordered sex differentiation (DSD; Bouvattier *et al*., 2002; Nikolova and Vilain, 2006; Hersmus *et al*., 2008)

Congenital defects of sexual development are caused by mutations and/or chromosomal anomalies which can affect both the autosomes and the sex chromosomes (Hughes, 2008). As might be expected, dramatic alterations result from mutations of *SRY* and deletions or duplications of the sex chromosomes (Jager *et al*., 1990; Lanfranco *et al*., 2004; Sybert and McCauley, 2004). However, most do not arise in this way and many genital abnormalities occur as a result of mutations of the autosomes and autosomal genes (Cameron *et al*., 1996a; Bilbao *et al*., 1998; Canto *et al*., 2004; Lin *et al*., 2007). A huge range of DSD phenotypes exist, which may present at parturition or during the development of secondary sexual characteristics in adolescence. Each individual DSD is determined by both the underlying mutation and its phenotypic penetrance. Although much work has gone into the understanding and treatment of DSD, and a plethora of candidate genes have been identified in mice, only a small number of DSD can be directly attributed to a specific gene or genetic defect. For example, the percentage of patients with disorders of gonad development that can be diagnosed at the molecular level is as low as 15-20 % (Achermann and Hughes, 2008).

The recently introduced DSD categorisation covers a wide range of congenital conditions where chromosomal, gonadal or anatomical sex is atypical, many of which entail disruption of sexual differentiation of the external genitalia (Table 1.1). The present study is focused in large on development and differentiation of the external genitalia, knowledge of which stems from the study of pathological phenotypes of these structures. In principle two opposing pathological scenarios underpin the majority of cases of DSD involving the external genitalia: male under-development (or ‘under-virilisation’) syndromes, and female virilisation due to excessive androgen action. Disorders related to abnormal sex determination and gonadogenesis, result in major disruption to sexual differentiation for the reasons that have been alluded to previously, but shed little light on the specific processes involved in the morphological development of the

external genitalia. In contrast, DSD resulting from disorders relating to androgen synthesis or its effects (see 46,XY DSD section B and 46,XX DSD section B in Table 1.1) tell us much about the morphological development of the external genitalia and are observed relatively frequently in patients. Common amongst the pathological phenotypes of the external genitalia witnessed in DSD are hypospadias in males (Caione, 2009) and congenital adrenal hyperplasia (CAH) in females (White, 2009). These are discussed below.

Sex Chromosome DSD	46,XY DSD	46,XX DSD
A: 47,*XXY* (Klinefelter's syndrome) **B: 45,*X* (Turner's syndrome)** **C: 45,*X*/46,*XY* (mixed gonadal dysgenesis)** **D: 46,*XX*/46,*XY* (chimerism)**	**A: Disorders of gonadal (testis) development** 1. Complete or partial gonadal dysgenesis (e.g. *SRY, SOX9, SF1, WT1, DHH*, etc) 2. Ovotesticular DSD 3. Testis regression	**A: Disorders of gonadal (ovary) development** 1. Gonadal dysgenesis 2. Ovotesticular DSD 3. Testicular DSD (e.g. *SRY translocation,* duplicated *SOX9*)
	B: Disorders in androgen synthesis or action 1. Disorders of androgen synthesis *Smith-Lemli-Opitz syndrome* *StAR mutations* *LHR mutations* *CYP11A1 mutations* *HSD3B2 mutations* *CYP17A1 mutations* *HSD17B3 mutations* *SRD5A2 mutations* 2. Disorders of androgen action *AIS* *Environmental modulation*	**B: Androgen excess** 1. Fetal *HSD3B2 mutations* *CYP21A2 mutations* *CYP11B1 mutations* *Glucocorticoid mutations* 2. Fetoplacental *Aromatase deficiency* *Oxidoreductase deficiency* 3. Maternal *Maternal virilising tumours* *Androgenic drugs*
	C. Other conditions 1. Syndromic associations of male genital development 2. Persistent Müllerian duct syndrome 3. Vanishing testis syndrome 4. Isolated hypospadias (*Cxorf6*) 5. Congenital hypogonado-trophic hypogonadism 6. Cryptorchidism (*INSL3; Leucine-rich repeat-containing G protein-coupled receptor 8; LGR8*) 7. Environmental influences	**C. Other conditions** 1. Syndromic associations 2. Müllerian duct agenesis / hypoplasia 3. Uterine abnormalities 4. Vaginal atresias 5. Labial adhesions

Table 1.1: Classification system for disorders of sex development (DSD).
Shown here is an abridged version of the basic framework of the recently introduced classification system for the more common DSD. Adapted from Achermann and Hughes, 2008.

1.7.1 Under-development of the male external genitalia and hypospadias

Defects anywhere along the pathway of androgen synthesis and target organ action can result in impaired virilisation leading to 46,XY DSD. Such conditions cause a spectrum of genital defects; however most common among these is male hypospadias (Belman, 1997; Baskin, 2004). This pathological phenotype is the most frequently observed congenital abnormality of the penis and is characterised by anomalous formation of the external genitalia, in which a part of the urethral canal is open on the ventral surface of the penis or perineum (Stokowski, 2004; Leung and Robson, 2007). It is also accompanied by deficient foreskin on the ventral penile surface and abundant foreskin on the dorsal surface, and is typically associated with other abnormalities; most commonly cryptorchidism and inguinal hernia (Khuri *et al*., 1981; Leung and Robson, 2004). Although hypospadias is well-characterised in terms of the abnormal phenotypes observed as a result of different male under-development DSD, the genetic and molecular basis of hypospadias is not understood and no obvious underlying cause has been assigned (Boehmer *et al*., 2001). Because of this the only treatment for hypospadias is post-partum surgical repair of the affected tissues, between 6 and 12 months of age (Leung and Robson, 2007).

As cholesterol is the starting substrate for all steroidogenesis, defects in its synthesis lead to a broad range of abnormal phenotypes, including microcephaly, cardiac deficiencies, polydactyly, micropenis and also severe hypospadias; as represented in Smith-Lemli-Opitz syndrome (Porter, 2008). In these conditions, the testes develop normally, but 65% of 46,XY patients have abnormal external genitalia, varying from micropenis and hypospadias, to a complete failure to virilise (complete sex reversal; Kelley and Hennekam, 2000). Similarly, inactivating mutations in StAR protein in 46,XY fetuses restrict movement of cholesterol into mitochondria, leading to a marked deficiency in testosterone biosynthesis, which results in the development of phenotypically female external genitalia (46,XY DSD; Bardakjian and Schneider, 2005). StAR mutations can also cause lipoid congenital adrenal hyperplasia, a severe form of primary adrenal failure, caused by severe glucocorticoid and mineralocorticoid deficiencies owing to the lack of substrate for steroidogenesis (Kim *et al*., 2010).

As was mentioned above, loss of function mutations in LHR can also lead to abnormal differentiation of the male external genitalia. Though the role of LHR in sexual differentiation of the external genitalia is yet to be confirmed, it is presumed that LHR defects cause lowered responsiveness to LH and hCG in fetal Leydig cells, leading to impaired testosterone secretion. Typically, 46,XY males possessing LHR mutations have significantly feminised external genitalia (Kremer *et al*., 1995). However, milder disease phenotypes have also been described, including micropenis, due to subnormal androgen synthesis (Misrahi *et al*., 1998). Likewise, mutations in the genes that encode the key steroidogenic enzymes required for testosterone synthesis (i.e. *HSD17B3, CYP11A1, HSD3B2* and *CYP17A1*) have all been shown to result in under-developed external and internal genitalia in 46,XY patients, which can range from under-virilised male to ambiguous or normal female external genitalia. *HSD17B3* mutations typically are associated with male to female complete sex reversal owing to the vital function of the HSD17B3 enzyme in testicular testosterone biosynthesis (Andersson *et al*., 1996). For *CYP11A1*, *HSD3B2* and *CYP17A1* mutations, hypospadias is commonly displayed accompanied by adrenal insufficiency; all due to the impaired steroidogenesis in both the testis and adrenal cortex (Rheaume *et al*., 1992; Rosler *et al*., 1996; Suzuki *et al*., 1998; Kim *et al*., 2008). Unlike the aforementioned forms of DSD, SRD5A2 deficiency does not tend to lead to widespread lack of virilisation. Instead, normal internal genitalia is usually observed, while only the external genitalia shows under-virilisation, which can vary greatly, but typically this is less well developed, at least partially ambiguous, and with a hypospadiac phallus (Andersson *et al*., 1991; Wilson *et al*., 1993). This is because mutation of *SRD5A2* does not impede testosterone biosynthesis, but does prevent its conversion to DHT, which is necessary for normal differentiation of the external genitalia, but can be partially compensated for by less potent testosterone.

Inhibition of androgen action due to end-organ resistance to androgens results in a subset of DSD that fall into a group of disorders called androgen insensitivity syndrome (AIS; OMIM #300068). AIS is caused by loss-of-function mutations in the *AR* gene and only effects 46,XY individuals due to X-linked inheritance (Hiort and Holterhus, 2000). The clinical presentation observed in AIS patients is

a result of the defective androgen action in androgen dependent tissues, despite normal androgen biosynthesis in the fetal testis. Hence, 46,XY males will display a lack of virilisation of the internal and external genitalia, the degree of which depends on the extent of the loss of function of the AR. The complete form (CAIS) results in complete 46,XY sex reversal, while the partial form (PAIS) and mild/minimal form (MAIS) can lead to ambiguous genitalia, various types of hypospadias or normal external genitalia with infertility (Kucinskas and Just, 2005). Other types of androgen insensitivity also occur, where the AR appears normally but the patient displays under-virilisation. Very few of these types of disorders have been assigned a molecular or genetic cause, due to our inadequate understanding of AR downstream signalling and its target genes. However, in patients with AEG and CHARGE syndromes, where under-development of the external genitalia commonly occurs (described above), the associated mutations in *SOX2* (Williamson *et al*., 2006) and *CHD7* (Sanlaville *et al*., 2006), respectively, implies that these genes may be important for sexual differentiation, and therefore represent putative therapeutic targets for treatment of hypospadias.

1.7.2 Virilisation of the female external genitalia and congenital adrenal hyperplasia

As has been outlined previously, in order for the normal female genitalia to develop, an absence of testes (and therefore potent androgen), must persist through the critical window of sexual differentiation. In cases where there is androgen excess during sexual differentiation, 46,XX DSD results and the external genitalia develops ambiguously or appearing male. 46,XX DSD due to androgen excess is characterised by the presence of normal ovaries and Müllerian derivatives, and lack of testes, with abnormalities limited to virilisation of the external genitalia (van Mil and Hiort, 2008). The stage of development when the female fetus is exposed to androgen dictates the degree of virilisation of the external genitalia. Crucially, by about the twelfth week of gestation, any androgen excess will only result in clitoral hypertrophy (Grumbach and Ducharme, 1960). Müllerian duct derivatives such as the Fallopian tubes and uterus develop normally in even the most severe cases of virilised external

genitalia due to androgen excess, because AMH is required for Müllerian duct regression and androgens can not mimic this function.

CAH accounts for most cases of 46,XX DSD (White and Speiser, 2000). There are many varieties of CAH, each characterised by distinctive biochemical consequences and clinical features (Miller, 1994). The common feature of all types of CAH is impairment of cortisol biosynthesis in the fetal adrenal cortex. This leads to over-expression of the *POMC* gene and hypersecretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary, and consequently hyperplasia of the adrenal cortex. Many types of CAH in females also lead to androgen excess. The most common virilising form of female CAH is caused by mutations in the gene that encodes for the steroidogenic enzyme CYP21A2. Other forms of CAH are due to mutations in the genes encoding CYP11B1, P450 oxidoreductase (POR), and to a lesser extent HSD3B2.

The 'classical' forms of CAH due to CYP21A2 deficiency, and CAH due to CYP11B1 deficiency, are broadly similar in terms of their pathological phenotypes and underlying cause, owing to the sequential position of the two enzymes in the steroidogenic pathway to cortisol and aldosterone synthesis. In both these disorders the developing fetus is exposed to an overproduction of adrenal androgens and androgen precursors (Stewart, 2008). Affected 46,XY males present with no abnormalities of the external genitalia, whereas developing 46,XX females have ambiguous or male external genitalia at birth. Mutations in *POR* produce genital abnormalities in female newborns similar to those observed in CAH due to CYP21A2 (Arlt, 2007). This is because POR is a key mediator of electron transfer and plays a central role in regulating the activity of all microsomal steroidogenic enzymes, including CYP21A2. In contrast to CYP21A2 and CYP11B1, mutations in HSD3B2 result only in modest clitoromegaly, accompanied by glucocorticoid deficiency (Mermejo *et al*., 2005). The mild virilisation that occurs in CAH due to mutations in HSD3B2 is thought due to conversion of excess DHEA to testosterone by type 1 3β-hydroxysteriod dehydrogenase (HSD3B1), which is expressed in the placenta and peripheral tissues of the fetus. However, new steroidogenic pathways have been described

complicating our understanding of this, such as the alternative pathway to testosterone production (Auchus, 2004) described earlier in section 1.4.2.

CAH due to CYP21A2 deficiency is the most common cause of ambiguous genitalia in female newborns. The milder non-classical, late-onset form occurs in approximately 1 in 500 to 1 in 1,000 individuals, while the classic, severe form of the condition, has an incidence of approximately 1 in 10,000 to 1 in 15,000 (Therrell, 2001; Krone and Arlt, 2009). Classic CAH due to CYP21A2 deficiency results from complete or near-complete loss of function of CYP21A2, and can be sub-classified into the salt wasting form or simple virilising, non-salt wasting form (approximately 3:1, respectively). Salt wasting occurs due to impaired sodium/potassium exchange in the fetal kidneys as a result of aldosterone deficiency; accompanying symptoms of loss of cortisol production found in the non-salt wasting form (Spandri *et al*., 2004). Crucially in CAH due to CYP21A1 deficiency, decreased CYP21A2 activity in adrenal steroidogenesis, and therefore decreased cortisol production, results in an absence of negative feedback to the hypothalamic-pituitary unit by cortisol. Increased ACTH causes the adrenal cortex to produce excessive cortisol precursors, particularly androgen precursors such as progesterone and 17-hydroxyprogesterone which lie proximal to the obstructed stage of steroidogenesis. This excess of androgen precursors supplies androgen biosynthetic pathways in the adrenal cortex, bringing about a significant elevation of circulating levels of androgen. If apparent during the critical window of sexual differentiation, this causes varying degrees of female virilisation, including labioscrotal fusion and enlargement of the developing clitoris (Hughes *et al*., 1987).

Aside from CYP21A1 CAH, 46,XX DSD due to androgen excess in females can also be caused by mutations in *CYP19A1*. *CYP19A1* encodes for the steroidogenic enzyme aromatase, which plays a crucial role in synthesising circulating oestrogens from the ovary at the time of puberty and is responsible for local conversion of androgens to oestrogens in a number of different tissues, including the placenta (Zhou *et al*., 1991). Aromatase plays a critical role in the fetoplacental unit. Its expression at approximately 12 weeks gestation in the placenta serves to protect the developing fetus from continued and/or excessive

androgen exposure *in utero* by converting circulating testosterone and androstenedione to oestrogens. Hence, aromatase deficiency leads to a large amount of these androgens in the fetus, which cross into the maternal circulation. Affected fetuses are born with clitoromegaly and various degrees of fusion of the labioscrotal swellings (Shozu *et al*., 1991).

1.8 The current study

1.8.1 Use of a human fetal tissue resource

It has been alluded to throughout this chapter that a significant deficit of information regarding human sexual differentiation still exists. Furthermore, much of what is understood about the molecular mechanisms that contribute to these processes is derived from either studies performed using animal models (particularly rodents) or in cancer cells lines such as LNCaP. Some understanding does exist regarding the genes contributing to development of the external genitalia in humans, but on the whole these have been elucidated from observations of pathological mutations phenotypes, where it can be difficult to delineate normal gene function, as well as direct and/or secondary effects of the defective gene/s that contribute to abnormalities. Direct study of sexual differentiation in the human fetus is rare and such studies are sporadic in the literature (Kalloo *et al*., 1993; Bastos *et al*., 1999; Kim *et al*., 2002). This is because of a lack of human fetal tissue for use in such studies. In the present study privileged access has been gained to a supply of human fetal tissue collected from first trimester termination of pregnancy, providing the means to directly address some of the currently unanswered questions regarding differentiation of the external genitalia, through direct study of human genital tissue during the critical window of sexual differentiation.

In recent years, studies in human embryos and fetuses have highlighted how developmental processes can differ greatly between humans and other mammals, and that many human disorders are not faithfully reproduced in other species (Strachan *et al*., 1997). Hence human fetal tissue research not only provides crucial understanding of the normal biology of our own species, but also aides in identifying associations between congenital disorders and their genetic causes, as

well as general understanding of the underlying human pathophysiology. The study of sex determination and sexual differentiation has been important for emphasizing many features of human development not found in animal models. Comparative studies in mice and humans have demonstrated that although cellular context and gene repertoire are generally similar during sexual development, there can be important variations in gene expression patterns and a lack of genotype–phenotype correlation (Swain and Lovell-Badge, 1999). For example, in both mice and humans *SRY* has been shown to act as a key factor in switching sexual development towards male differentiation and promoting testis differentiation (Berta *et al*., 1990). However, in mice, *SRY* expression in the presumptive gonad peaks on a single day of development (Lovell-Badge *et al*., 2002), whereas human *SRY* demonstrates continued expression following activation in the seventh week of gestation, followed by a gradual decrease after testis differentiation (Hanley *et al*., 2000). Moreover, in terms of DSD, it has been documented that the condition of CAH due to CYP21A2 deficiency is not analogous to any pathological conditions in mice, owing to the unique nature of the structure and function of the adrenal glands in higher primates including humans (Cutler, Jr. *et al*., 1978; Abbott and Bird, 2009). Hence study of this condition can only be conducted in human tissues, and not model rodents.

In the present study we have utilized arguably the largest, most comprehensive collection of fetal first trimester human material to study sexual differentiation of the human external genitalia. With ethical approval and informed consent, the collection of fetal material from procedures of first trimester social/voluntary termination of pregnancy has been performed by our group for more than a decade to amass a resource of human tissue covering the period of between approximately three and 14 weeks gestation. The material collected can be used for a variety of different molecular biology investigations and functional analyses. Through utilization of a readily available large resource of human fetal tissue, all the work shown herein has been performed directly in the human tissues of interest during the developmental stage of interest

1.8.2 Overall aims and objectives

The current study aims to investigate the fundamental processes underlying sexual differentiation of the human external genitalia, which despite its vital importance in human development, is still very poorly understood. Previous work has highlighted the immense importance of testosterone to mediate normal sexual differentiation concordant with genetic sex in males. However, as yet the signalling pathways responsible for regulation of testicular testosterone secretion in the developing fetus during the first trimester are still unclear. One of the primary aims of the present study was to address this issue by examining the functions of LH and hCG in the developing fetus and whether they participate in regulating testosterone biosynthesis in fetal Leydig cells during the critical window of sexual differentiation. This was accomplished using a variety of methods that assessed LH and hCG expression in the developing fetus and their potential effects on testicular testosterone secretion.

Similarly, previous work has also made it clear that the AR is instrumental for mediating normal sexual differentiation in males. However, it has not been shown when AR is expressed during the critical window of sexual differentiation and its distribution in primary human tissue. Through the use of immunohistochemistry and mRNA tissue *in situ* hybridisation, we characterised expression of AR and SRD5A2, in addition to SOX2 and SOX9, in the developing human external genitalia and have illustrated their spatiotemporal expression patterns to provide a backdrop for analysing downstream androgen-regulated events. Identification of AR target genes is of paramount importance to understand how androgens bring about sexual differentiation of the male external genitalia, and also comprehend the causes of genital abnormalities observed in DSD. The study of AR target genes firstly required development of an *in vitro* culture model. This model was then used to compare the transcriptome of genital fibroblasts treated with androgens, with that from androgen deprived cells, to assess transcriptional regulation by AR in the developing external genitalia. Furthermore, there was specific interest in evaluating the expression of genes identified in LNCaP cells as regulated by AR and their importance in primary human cells, as well as an evaluation of the hypothesized autoregulation of AR

by ligand-bound AR. Utilization of microarray technology enabled investigation of putative AR target genes in the external genitalia on a genome-wide scale.

Chapter 2: Materials and Methods

2.1 Chemicals and reagents

Chemicals were purchased from Fisher as AnalaR grade (Fisher Scientific UK Ltd, Leicester, UK), or equivalent, unless otherwise stated. All solutions were prepared using deionised water produced by reverse osmosis using the Barnstead water purification system and subsequently autoclaved at 15 psi for 15 min. Solutions for nucleic acid and tissue manipulations related to non-radioactive tissue *in situ* hybridisation work (see below) were prepared using diethylpyrocarbonate (DEPC; Sigma-Aldrich Corp., MO, USA) treated water (DEPC-water [0.1%]). All disposable polypropylene tubes and tips were purchased from Camlab (Camlab Ltd., Cambridge, UK) and sterilised by autoclaving at 18 psi for 30 min. All glassware was sterilised by heating to 170^{o}C for a minimum of 1 hr. Phosphate buffered saline (PBS) solutions were made up using PBS tablets (Sigma) dissolved in deionised water [0.1%]. Unless otherwise indicated, DNA modification enzymes were purchased from Promega (Promega Corp., Southampton, UK).

2.2 Collection of human embryonic and fetal material

The collection and use of human embryonic and fetal material was performed by Prof. Hanley and Prof. Wilson (project supervisors) with ethical approval from the Southampton & South West Hampshire Joint Local Research Ethics Committee, in accordance with the guidelines outlined by the Polkinghorne Committee. Embryos were collected from medical (RU486) or surgical termination of first trimester pregnancies, following written consent from donors. Material was staged immediately after collection by stereomicroscopy according to the Carnegie classification system (O'Rahilly and Muller, 1987; Bullen and Wilson, 1997), as shown in Table 2.1 and Fig. 2.1. Beyond the latest stage of Carnegie staging, Carnegie stage 23, which covers development up to 56 days post-conception, gestational age of fetal material was determined by direct measurement of fetal foot length, which were equated to weeks post-conception (wpc). For the purpose of the present study, gestational age of material generally ranged between 7 and 12 wpc (see Table 2.2). The various tissues collected for use in the present study (principally external genitalia, gonad, adrenal gland and

placenta) were routinely used in one of three ways: (i) tissues were either placed in culture media, disaggregated by chemical digestion and passing tissue through a P1000 pipette tip, and then taken directly into culture; (ii) stored in RNA Later RNA Stabilization Reagent (Qiagen Ltd., West Sussex, UK) at -80°C prior to RNA extraction; or (iii) fixed for between 3 hrs and 18 hrs (dependent on specimen size) in methanol acetone [2:2:1, methanol:acetone:DEPC-water], stored at 4°C in 70% DEPC-ethanol.

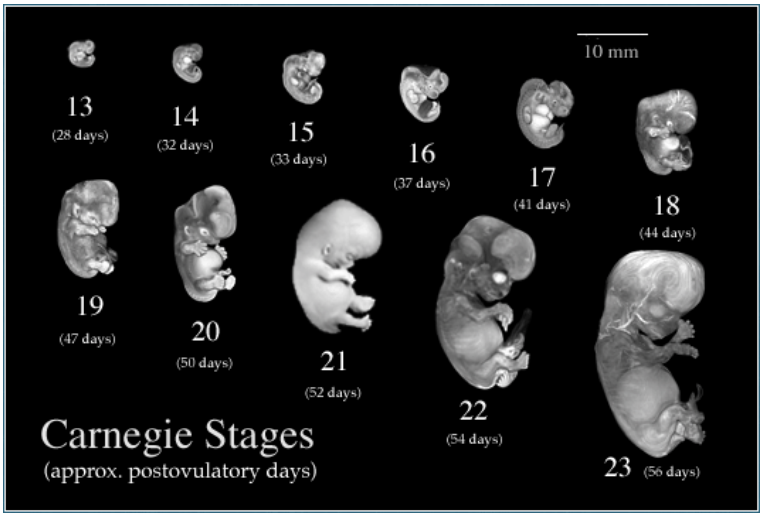

Figure 2.1: The Carnegie Stages of human embryonic development
Used for precise staging of gestational age. Adapted from umich.edu.

Carnegie Stage	Approx. age (days)	Size (mm)	Key developmental features
13	28 - 32 (week 5)	4 - 6	leg buds, lens placode, pharyngeal arches **Somite Number 30**
14	31 - 35	5 - 7	lens pit, optic cup
15	35 - 38	7 - 9	lens vesicle, nasal pit, hand plate
16	37 - 42 (week 6)	8 - 11	nasal pits moved ventrally, auricular hillocks, foot plate
17	42 - 44	11 - 14	finger rays
18	44 - 48 (week 7)	13 - 17	ossification commences
19	48 - 51	16 - 18	straightening of trunk
20	51 - 53 (week 8)	18 - 22	upper limbs longer and bent at elbow
21	53 - 54	22 - 24	hands and feet turned inward
22	54 - 56	23 - 28	eyelids, external ears
23	56 - 60	27 - 31	rounded head, body and limbs

Table 2.1: Standard staging criteria for classification of human embryos
Adapted from UNSW Embryology

Fetal Foot length (mm)	Gestational age (weeks post-conception)
4	7.5
5-6	8
7	9
8	10
10	11
14	12

Table 2.2: Conversion table for determining gestational age from fetal foot length

2.3 Determination of chromosomal sex by use of fluorescence in situ hybridisation (FISH)

The karyotyping of placental tissue was used to determine the chromosomal sex of fetuses. To achieve this, placental tissue obtained during the collection of fetal material was firstly flattened between two 'Superfrost' glass microscope slides (BDH, Dorset, UK) that had been cleaned with methanol, to leave a smear sample on each slide. Slides were then placed in cooled methanol:glacial acetic acid fixative [3:1] for 30 min, at -20^{o}C. Following the fixing of cells, 5 µl of mixed *X* (containing FITC dye) and *Y* (containing Texas Red dye) chromosome DNA probes (prepared by Dr. Spalluto and Miss Wilkinson, Human Genetics Division, Southampton University) were added to the cells and a coverslip placed over the slide, which was sealed down with rubber cement. Slides were then incubated overnight at 37^{o}C. The following day, slides were washed several times in 0.1x saline sodium citrate (SSC; [15 mM NaCl, 1.5 mM sodium citrate (Sigma)]) at 60^{o}C, with the coverslips removed, and mounted using Vectashield mounting medium with DAPI (Vector Labs, CA, USA). Slides were examined using fluorescent microscopy, utilizing a Zeiss Axiovert/Axiovision imaging system (Carl Ziess Ltd., Herts, UK) to visualise the *X* and *Y* chromosome probes and determine the karyotype of cells (see Fig. 3.1 for examples of FISH).

2.4 Preparation of paraffin wax-embedded tissue sections

2.4.1 Embedding tissue

Fixed tissues were incubated in graded DEPC-ethanol solutions (70%, 80%, 90%, and 100%; 2 hrs each) to dehydrate tissues, and then placed in chloroform overnight. Tissues were then placed in a new tube, covered in molten paraffin wax (BDH) and heated to 72^{o}C for 2 hrs. This was repeated twice, with the final treatment being conducted in a vacuum to aid penetration of wax throughout the tissue. Subsequently, the tissue was transferred to a metal histology cassette (Fisher), where it was carefully embedded in a pool of molten wax. Wax blocks were cooled and stored at 4^{o}C, ready to be cut.

2.4.2 Slide coating

Microscope slides were treated with 3-aminopropyltriethoxysilane (TESPA; Sigma) for the mounting of embryonic material tissue sections. Prior to this, slides received a 5 min treatment in DEPC-water and two 5 min DEPC-ethanol treatments, followed by a 20 min drying period in a filtered air stream. Slides were then treated for 1 min each with DEPC-ethanol:HCl [70% DEPC-ethanol containing 10% HCl solution], followed by DEPC-water and then DEPC-ethanol, before being dried once more in filtered air. Finally, slides were treated with 2% TESPA (v/v) in dry acetone for 15 sec, washed six times in DEPC-water, and then dried and heated at 37^{o}C overnight.

2.4.3 Microtome sectioning

Wax blocks were cut on a dry microtome (Leica RM 2135, Leica Instruments, Wetzlar, Germany) in RNase-free conditions. Tissue was cut into 5 μm thick sections and mounted on RNase-free TESPA-coated glass slides. Firm adhesion of sections to slides was achieved by floating sections on DEPC-water, briefly heating to 45°C to expand the paraffin wax, and aspirating the water from beneath the tissue sections until fully adhered. Slides were incubated at 37^{o}C for ~18 hrs to complete the adhesion process and dry sections. Slides were stored at 4^{o}C.

2.5 Reverse Transcriptase PCR

2.5.1 Total RNA extraction

Cells were centrifuged to form a pellet (and supernatant discarded), or tissues removed from, RNA Later RNA Stabilization Reagent (Qiagen). Total RNA was extracted using Tri Reagent (Sigma) in accordance with the manufacturer's protocol, utilizing DEPC-treated reagents throughout to ensure RNase-free conditions. Initially, a suitable amount of Tri Reagent was added to tissue/cells followed by repeated passaging through a 1 ml pipette tip to dissociate cells. The cell suspension was incubated at room temperature for 5 min to allow complete dissociation of nucleoprotein complexes. Following this, one tenth volume of chloroform was added, the solution mixed vigorously for 15 sec and then allowed to stand for 15 min at room temperature. Next, the sample was centrifuged at 10,000 rpm for 15 min at 4°C to separate the mixture into three phases: an upper colourless aqueous phase containing RNA and lower red organic phase containing protein, separated by an interphase containing DNA.

The RNA phase was carefully removed from the mixture and transferred to a new tube. RNA was then precipitated by incubating at room temperature for 10 min with an equal volume of isopropanol. Following this, the RNA precipitate was pelleted by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was removed and discarded, and the pellet washed in 75% ethanol by vortexing briefly and centrifuging at 7,500 rpm for 5 min, at 4°C. The precipitate was air dried and the pellet resuspended in DEPC-water. For those samples not being used for cDNA synthesis straight away, 1 U/µl of RNase inhibitor (Promega) was added and the RNA sample was stored at -80^{o}C.

2.5.2 First strand cDNA synthesis

cDNA synthesis primarily relied on the use of an oligo(dT) primer (Operon), which hybridises to the 3' poly(A) tail sequences found in most eukaryotic mRNAs, to convert 1-5µg of total RNA into "first strand" cDNA. RNA was quantified using a Nanodrop ultra-low-volume spectrophotometer (Nanodrop Technologies Inc., DE, USA). In order to remove any genomic DNA contaminants from the RNA preparation, RNA was treated with DNase I (Sigma)

prior to cDNA synthesis, following the manufacturer's protocol for the DNase treatment kit. Briefly, the sample was incubated with DNase I for 15 min at room temperature, followed by the addition of EDTA solution to deactivate the enzyme, heating at 70^{o}C for 10 min, and final 1 min incubation on ice. cDNA was synthesised by firstly preparing an RNA/primer mixture containing 1-5 μg of total RNA, 0.5 mM dNTPs (Roche) and 2.5 μM Oligo(dT) in 13 μl DEPC-water. The RNA mixture was denatured by heating to 65°C for 5 min, and then added to this was the RT-PCR reaction mixture [first strand buffer (50 mM Tris-HCl pH8.3, 75 mM KCl, 3 mM $MgCl_2$; Invitrogen Life Technologies, Paisley, UK), 10 mM DTT (Invitrogen) and either 1 μl of SuperScript III reverse transcriptase enzyme (RT, Invitrogen) or DEPC- water]. cDNA synthesis was carried out at 50°C for 55 min. The reaction was terminated by heating to 70°C for 15 min. cDNA samples were stored at –20°C. The reaction mixture that used DEPC-water, rather than RNA polymerase, served as a negative control in subsequent PCR reactions.

2.6 Polymerase chain reaction

Primer design was achieved using the 'EditSeq' and 'Primer Select' computer programmes (DNASTAR Inc., WI, USA). Gene base sequence information was obtained from the National Centre for Biotechnology Information website (NCBI, http://www.ncbi.nlm.nih.gov) and downloaded into the EditSeq programme, before appropriate primer pairs were generated using Primer Select. The list of primers created ranked the quality of primers according to thermodynamic properties of the annealing reactions and the presence of intrinsic flaws in the base sequences of primers (i.e. hairpin loops and/or primer dimerisation). The choice of primer pairs was based on product length, position within the DNA sequence, similar annealing temperatures between pairs, absence of hairpin loops and low probability of dimerisation. Unless the gene of interest lacked introns, primers were also selected to include pairs that spanned introns, in order to minimize amplification of any contaminating genomic DNA in the PCR reaction. The optimal primer pair chosen was ordered from Operon Biotechnologies, Inc. (Cologne, Germany).

The typical composition of each PCR reaction consisted of 100 ng template DNA mixed with DNA polymerase reaction buffer [50 mM KCl, 10 mM Tris-HCl pH 3.8, 1.5 mM MgCl2 (Promega)], 250 µM dNTPs (Roche Applied Science, East Sussex, UK), 5 µM of each primer, and 1 U Thermus aquaticus (Taq) polymerase (Promega). For GC rich template DNA, 5% dimethyl sulphoxide [DMSO (Sigma)] was also added. When necessary the concentrations of MgCl2 and DMSO were varied to optimize the reaction. The standard thermal cycler programme for a PCR reaction is shown in Table 2.3, although this was varied on occasion in order to optimize individual primers. A list of all the primer pairs used in the present study are shown in Table 2.4.

No. of cycles	**Purpose**	**Conditions**
1 cycle	Initial denaturation	95^{o}C, 4 min
35-40 cycles	i. Denaturation ii. Annealing iii. Extension	95^{o}C, 45 sec 55-65^{o}C, 45 sec 72^{o}C, 45 sec
1 cycle	Extension	72^{o}C, 10 min

Table 2.3: Standard programme used to perform PCR

Target gene (gene symbol)	Primer pair sequences (5' → 3')	Annealing temperature
ADAMTS1	GGGGCCGGAAGTGACCTC TCCCCATGACCATTATCCAGAAA	56°C
AP1G2	TCCTGACCTACGGGCTGACTGTGC CCTGGCGGATGCGGTTGTTGT	62°C
AP3S1	CAACAGCAAATCATCAGGGAGACT AGAGGGCAGGTTTGGCACTTT	56°C
AR	CGGGGACATGCGTTTGGAGA CAGATCAGGGGCGAAGTAGAGCAT	58°C
ATP1A1	CCCTCACTCCCCCTCCCACTACTC CGTCTCCAGGGGGTTTTCATTTGT	60°C
BMP7	TCGGAGGAGGGCTGGCTGGTGTT TGGCGTGGTTGGTGGCGTTCAT	65°C
CCNG2	GCTACCCCGGAGAATGATAACACT AAGGCACAAGGCTAATACAGATGG	56°C
CYBB	CCATCCGGAGGTCTTACTTTGAA CTTGGACACCTTTGGGCACTTGA	58°C
CYP24A1	CGCTCGCTTGCCGCCTTCCT CCCGCTGCCAGTCTTCCCCTTCC	65°C
CYP3A7	CTATGATACTGTGCTACAGT TCAGGCTCCACTTACGGTCT	60°C
DDIT4	GCCCTGGCGTCTGTCCTCAC CCGCACGGCTCGCTGTAG	58°C
DKK1	AGGCGTGCAAATCTGTCTCG TGCATTTGGATAGCTGGTTTAGTG	55°C
FBLN2	AGCCCAATACCTGCAAAGACAAT GACATGGCTCGGACAGTGACG	56°C
FGF9	CCAGGGAACCAGGAAAGA GACACAGCGAATCAATAAGAAC	55°C
FGFBP1	CCCCCTTGCACTCTACCTGAC CCTTCCTGGGCTTTGTGTTCC	56°C
FST	GCGGGCTGGATGGGAAAACCTAC TCGCTGGCATAAGTGGCATTGTCA	64°C
GATA2	ACCCGGGCCTGGCGCACAACTAC CCCCGCGGAAGATGAGGCTGGAGA	65°C

Table 2.4: List of primer pairs used in the current study
Primers were designed so that they were intron-spanning, and thus could be used for RT-PCR. Where a gene had no introns, -RT sample were prepared alongside +RT samples, to clarify that genomic DNA was not amplified during PCR. Full gene names shown in relevant chapters.

Target gene (gene symbol)	Primer pair sequences (5' → 3')	Annealing temperature
GATA6	TGCTGGAAAAATTGCAACAA CAACCTGCCTGTGGGTTAGT	62°C
hCGB	GCTGCTGCTGTTGCTGCTGCTGAG TTATTGTGGGAGGATCGGGGTGTC	64°C
HPRT	CCTGGCGTCGTGATTAGTGATGAT AGCTTGCGACCTTGACCA	56°C
IL6	CCCAGTACCCCCAGGAGAAGATT GTTGGGTCAGGGGTGGTTATTG	56°C
KIAA0232	TCCGGAGAATGCGTTGAGA TGTTCCTGCAGGTCGTAGTC	55°C
KIAA1727	CAGCGGGATGGTGAACTTTGTC CCGGGGCAGGCTGTGGAACT	60°C
KLF13	CCCCGCAGAGGAAGCACAAG CAAAAGGAAAGGCCAGGGACTCAC	60°C
KLK2	GCTTCCCACACCCGCTCTAC GGGCCCCATGATGTGATACCT	56°C
KLK3	GCTTCCCACACCCGCTCTACGA GGGACAAGGGCCAGGACCATCT	62°C
KRT5	GCCCGGCTGCTGCGTGAGTA CGGGAGGAGGAGGTGGTGGAGAC	62°C
KRT73	CCGCCCGGGGGTATCCAT AGCTGCGTCCACGTCCTTCTTA	60°C
LHR	CCACAGGGCCGAAAACC GAGCCCCATGCAAAAGTCTG	55°C
LHX2	AAAAGACAAAGCGCATGCGGC CAGGCACAGAAGTTAAGACTG	58°C
LIFR	GCCCGGAGAAGAGTATGTATGTGG TGCGGCTGGGTTTGGTATTTC	58°C
LOC441453	ACCGGTTTGTAGCCATCTGTC ACGCCTGTTCCATAAAATAAGC	55°C
LSM4	ACCTGGTGAGCTGCGACAACTGG GCGCCTGCCTCTTCCTTTCTGA	62°C
MMP7	AAACTCCCGCGTCATAGAAATAAT TGAGTTGCAGCATACAGGAAGTT	55°C
MUC1	CCAGCCCGGGATACCTACCAT GGAGCCCCCACCACAACACTT	60°C
MYC	CGACGCGGGGAGGCTATTCTGC CCGCCACCGCCGTCGTTGTCT	66°C
NFKB2	TGTGCGGGGTGGAGATGAAGTTTA GTAGGCCAGGGAGGAGGGGAAGAA	62°C

Table 2.4 continued (part 2)

Target gene (gene symbol)	Primer pair sequences (5' → 3')	Annealing temperature
NPY5R	TGCCACAAAGTTAGAAGAAAGGAT TGAAACACTGGAAGGGGAGAA	52°C
PAX2	GTGGTGAGGCAGCGCATCGTGGAG AGGGGAGGCCGTGCTGGGAACAAT	66°C
PCDH1	CCCCATAGGCCACTCGGTCATCC TGCCAGAGTCCACAGCCACAATC	62°C
PDGFRA	ACAACAGCGGCCTTTTTGTG AATGTGCCTGCCTTCAAGCTC	58°C
PLK2	ACGCCGCAAAAATTATTCCTCACA GGGGTACCACATATCGTTCTCCTT	58°C
PMVK	CGAGGAGGGGCGCGATTGTTC GTCTGCGTCACGGCCCCATAGG	64°C
RORC	GGGCCCTCATATTCCAACAACTT GGCCCGGCACATCCTAACC	58°C
SERPINA12	TGCAGCCACAGCGTAAGTTTTTG GTGCCCGTCATGTGGAGTCTGG	60°C
SHROOM1	CATCCGGCGCTGGCTAAGTTTGAA GAGGCATGGGGGTCTGGTGGTGAT	65°C
SLC12A7	CGCGGGGCCGAGAAGGAGTGG GGTGCGCGGCGGTGGTGTCG	66°C
SLC22A3	CCCCAACTGGCAAGGAATC CAAGGCGCTCAATGGTTAGT	55°C
SLC26A2	GATGGGCGGGGAAAGGGACAG GGCCAGCCAGCAGGGAATAAGC	63°C
SLC30A1	AGGTAATGGGGGCTCTGGTGA ACTTCTGGGGTTTTCTGGGTCTGC	58°C
SLC34A1	TGATGCAGGCGGGGGACAGGAC CCAGGTGAAGGGGGCAGGGAAGT	65°C
SLC9A2	CCGGCGACACAGTTTGCGAGAA GCGGCTTGGGCGGGATGC	64°C
SLCO2A1	CTTGGGGATGCTGTTTGGAGGAAT TGTAGAGGGGGTTGTGGGAGATGC	62°C
SOAT2	CTGGGCCGCCTCTGTGTTCCTGT GGGCCGGTGCGCTGGTCAT	65°C
SOX2	GGCACCCCTGGCATGGCTCTTG TTCTTGTCGGCATCGCGGTTTTTG	58°C
SOX5	TGCCTGGTGGATGGCAAAAAGC GTTAATGTGCTTGGCCAC	59°C
SOX6	GCAGTGATCAACATGTGGCCT CGCTGTCCCAGTCAGCATCT	58°C

Table 2.4 continued (part 3)

Target gene (gene symbol)	Primer pair sequences (5' → 3')	Annealing temperature
SOX8	TGGAGTCTGGTGCCTATGCCTGT GCCGAGCACTGCATCAGCTTTGT	58°C
TBX6	GGCTACCGCTACCCCGAACTG GGCTGCCCGAACTAGGTGTATGC	60°C
TDRD3	GACGGCTGCTATTGCTGAAGTT GCTGCTGTGGCTGTGATTTAGG	56°C
TMCO5	CGGGGCCTGGTGGAAGA CCCTCATTATTTTGGCTGGTAT	55°C
WNT4	CGTTCGCTGCGCCTCCTCGTCT CCTTCCTGCCGGCCTCATTGTTGT	65°C
WNT6	CGGGGACGCGGAGACATC TGGAAGCGGCACAGGCAGTT	60°C

Table 2.4 continued (part 4)

2.7 Agarose gel electrophoresis

PCR products were typically analysed on 1.5% agarose gels (although 1-2% gels were also used for some purposes). 1.5 g of agarose powder (Sigma) was added to 100 ml TAE buffer [40 mM Tris-Acetate, 1 mM EDTA pH 8.0 (Fisher)] and dissolved by heating at high temperature. To visualise DNA under UV, approximately 200 μg/ml of ethidium bromide (Sigma) was added to the dissolved agarose solution prior to pouring the gel. Gel electrophoresis took place in a gel tank containing 1x TAE buffer, and was run at 80-90V for 40-60 min, dependent on the analysed DNA fragment size. Samples were electrophoresed with size standards and visualised under a UV transilluminator (Ultraviolet Products (UVP) Inc., CA, USA). Digital photographs were taken on an Olympus digital camera (Model C-5060; Olympus Corp, Japan). Image processing was carried out using Doc-It imaging software version 2.4 (UVP).

2.8 Purification of PCR Products

Gel electrophoresis was used to separate PCR products, and the resultant bands were excised from the gel with a sterile scalpel, followed by extraction of DNA using a Qiaquick gel extraction kit (Qiagen). Gel extraction proceeded according to the manufacturer's instructions, which in principle employs spin-column

technology and pre-optimised buffers to achieve efficient recovery of DNA, while removing contaminants at the same time.

2.9 DNA sequencing of PCR products

PCR products were extracted and purified as outlined in section 2.7. 50 ng of template DNA was used in each sequencing reaction. Automated sequencing reactions were performed using ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin Elmer Life Sciences Inc, Boston, MA). 4 µl of DNA template (12.5 ng/µl) and primer [3.2 pM] was added to terminator ready reaction mix, in a total volume of 20 µl [contains G-dye terminator, A-dye terminator, T-dye terminator, C-dye terminator, dITP (in place of dGTP to minimize band compression), dATP, dTTP, dCTP, Tris-HCL (pH 9.0), $MgCl_2$, thermal stable pyrophosphate and AmpliTaq DNA polymerase]. The solution was placed on a thermal cycler and initial extension was carried out by denaturation at 96°C for 5 min, followed by 25 cycles consisting of: 96°C for 15 sec (denaturing); 50°C for 20 sec (annealing); and 60°C for 4 min (extension). Ethanol precipitation was then used to remove unincorporated dye terminators and each reaction was transferred to a fresh tube containing 2.0 µl of 3M sodium acetate (pH 4.6) and 50 ml of 95% ethanol. This was subsequently vortexed and incubated on ice for 10 min. Next, labelled DNA was pelleted by centrifugation at 13,000 rpm for 15-30 minutes. The supernatant containing unincorporated dye terminators was carefully removed, and the pellet washed with 70% ethanol. Samples were centrifuged once again at 13,000 rpm for 5 min and the supernatant aspirated carefully. Pellets were dried in a vacuum centrifuge for 1-2 hours and sequenced by the DNA sequencing facility within the Human Genetic Division, University of Southampton.

2.10 Non-radioactive mRNA tissue in situ hybridisation

Non-radioactive mRNA tissue *in situ* hybridisation (TISH), and associated techniques, were performed in a dedicated RNase-free lab space, under strict RNA-free conditions (including swabbing of equipment with 0.1% DEPC-ethanol containers and pre-sterilization of metal- and glass-ware by heating at 170°C for 4 hrs at atmospheric pressure.

2.10.1 Production of cDNA template for mRNA TISH probes

To generate cDNA for use as template for *in vitro* transcription of cRNA probes, we adopted a strategy whereby 3-rounds of PCR were used, which resulted in the production of a cDNA oliognucledotide conjugated to a T7 RNA polymerase promoter sequence needed for *in vitro* transcription. Table 2.5 shows the primer pairs used for each round of PCR.

The initial step involved using PCR to generate cDNA that contained a T7 promoter terminal sequence for each gene of interest (adapted from; Cone and Schlaepfer, 1997). This was performed by firstly amplifying the target region of the gene through standard PCR, using either genomic DNA as a template (e.g. *SRD5A2*) or reverse transcribed cDNA from extracted mRNA (e.g. *AR*). Following gel electrophoresis and gel extraction, the three first round products were used in a second round of PCR, where nested primer pairs were utilised to increase amplicon specificity. In addition, one of these was tailed with an extra 16 bases representing the 3' end of the T7 RNA polymerase promoter. The second round of PCR led to the generation of two PCR products for each gene of interest, each with a short T7 promoter 'tag' sequence at the 5' end of the mRNA strand, representing a lower T7 primer and upper T7 primer). Because each PCR product was transcribed individually, there was no need to make use of different promoters for the anti-sense and sense strands (i.e. T7 and T3 promoters). Gel electrophoresis and gel extraction followed the second round of PCR.

PCR Round	Previous primer pair used	Primer Pair	Primer sequences 5'➔3' (italics indicate T7 RNA polymerase recognition sequence)
1st	n/a	a) *HSD17B3* U *HSD17B3* L	CCCAGCAAGGCCAGTCCAGATT GGCCCCCACCCCTTGTTG
	n/a	b) *SRD5A2* U *SRD5A2* L	ACAATCCCCGGAAAACTGGTATGG AGCGTTCGGCCCCTTCCTTAGAGA
	n/a	c) *AR* U *AR* L	CGGGGACATGCGTTTGGAGA CAGATCAGGGGCGAAGTAGAGCAT
2nd	a	d) *HSD17B* U *HSD17B3* T7 L	CCCAGCAAGGCCAGTCCAGATT *ACTCACTATAGGGAGA*ACCCCTTGTTGTGTCTCC
	a	e) *HSD17B3* L *HSD17B3* T7 U	GGCCCCCACCCCTTGTTG *ACTCACTATAGGGAGA*AGGCCAGTCCAGATTCTTTGGG
	b	f) *SRD5A2* U *SRD5A2* T7 L	ACAATCCCCGGAAAACTGGTATGG *ACTCACTATAGGGAGA*AGCGTTCGGCCCCTTCCTTAGA
	b	g) *SRD5A2* L SRD5A2 T7 U	AGCGTTCGGCCCCTTCCTTAGAGA *ACTCACTATAGGGAGA*ATGAACCTGGGTGGCTTATGAGA
	c	h) *AR U* *AR* T7 L	CGGGGACATGCGTTTGGAGA *ACTCACTATAGGGAGA*AGTAGAGCATCCAGGA
	c	i) *AR L* *AR* T7 U	CAGATCAGGGGCGAAGTAGAGCAT *ACTCACTATAGGGAGA*CGTTTGGAGACTGCCA
3rd	d	*HSD17B3 U* T7 universal	CCCAGCAAGGCCAGTCCAGATT TAAGCTTTAATACG*ACTCACTATAGGGAGA*
	e	*HSD17B3 L* T7 universal	GGCCCCCACCCCTTGTTG TAAGCTTTAATACG*ACTCACTATAGGGAGA*
	f	*SRD5A2 U* T7 universal	ACAATCCCCGGAAAACTGGTATGG TAAGCTTTAATACG*ACTCACTATAGGGAGA*
	g	*SRD5A2 L* T7 universal	AGCGTTCGGCCCCTTCCTTAGAGA TAAGCTTTAATACG*ACTCACTATAGGGAGA*
	h	*AR U* T7 universal	CGGGGACATGCGTTTGGAGA TAAGCTTTAATACG*ACTCACTATAGGGAGA*
	i	*AR L* T7 universal	CAGATCAGGGGCGAAGTAGAGCAT TAAGCTTTAATACG*ACTCACTATAGGGAGA*

Table 2.5: PCR primers for generation of cDNA template for creating riboprobes used in TISH

During the first round of PCR standard PCR was performed using a typical primer pair combination based on the target gene sequence; subsequent rounds. Subsequent rounds of PCR used combinations of the relevant primer pair, in addition to a standard primer combined with part of the T7 RNA polymerase recognition sequence. HSD17B3, 17 β-hydroxysteroid dehydrogenase 3; AR, androgen receptor; SRD5A2, type 2 5 α-reductase; U, upper primer; L, lower primer; n/a, non-applicable.

For the third and final round of PCR, diluted amplification products from the previous round were re-amplified to extend the previously introduced 'abridged' T7 sequence and complete the 23 base pair sequence required for recognition by the T7 RNA polymerase. This was achieved through the use of a 'T7 universal primer', used in conjunction with the appropriate standard primer used in previous amplifications. This third round of PCR was introduced into the cDNA template generation process, principally to reduce the cost of primers. The cost of six different PCR primers with complete T7 promoter tails would be far greater than six primers with shortened T7 tags, plus one multipurpose universal T7 primer to generate the full T7 sequence in all six final cDNA products.

2.10.2 Precipitation of cDNA template for mRNA TISH probes

cDNA template from PCR reactions was precipitated by firstly adding DEPC-water up to approximately 10 x volume of initial cDNA solution, followed by 1/10 volume 3 M sodium acetate and 3x volume DEPC-ethanol. The solution was mixed thoroughly and incubated at –20^{o}C overnight. Samples were then centrifuged at 13,000 rpm for 30 min at 4^{o}C, the supernatant removed, and the pellet washed in 70% DEPC-ethanol, before being left to air-dry. Finally, the dry pellets were resuspended in DEPC-water and a sample gel electrophoresed to check for quality. Precipitated cDNA was stored at –20^{o}C.

2.10.3 Synthesis of digoxigenin-labelled riboprobes

Precipitated cDNA template was used for *in vitro* transcription to generate cRNA probes labelled with digoxigenin (DIG). Preparation of riboprobes was performed using 1 µg of cDNA, in a reaction mix containing 1 x RNA polymerase transcription buffer (Promega), 10 U RNase Inhibitor (Roche), 10 mM NTPs/DIG-UTP (DIG-mix, Roche), and 40 U T7 RNA polymerase (Roche), made up to 50 µl with DEPC-water. The reaction mixture was then incubated at 37^{o}C for 2 hrs to catalyse *in vitro* transcription. A portion of the reaction product was analysed following incubation on a thin 1.5% TBE agarose gel to confirm cRNA synthesis. To the main reaction mixture, 20 U of deoxyribonuclease I (DNase I; Promega) was added, and the mixture incubated at 37^{o}C for 15 min for

the removal of template DNA, followed by addition of 0.4 mM EDTA. The cRNA transcripts were precipitated at -20^{o}C overnight in DEPC-ethanol containing 20 mM lithium chloride and 0.1 mM filter sterilised yeast tRNA (Invitrogen). Precipitated cRNA products were resuspended in DEPC-water to give a final concentration of approximately 0.1 μg/μl, and stored at -80^{o}C with 1 U RNase inhibitor. The insulin riboprobes used in the present study were provided by Miss Louise Williams, Human Genetics Division, University of Southampton.

2.10.4 Pre-hybridisation treatments and hybridisation of riboprobes

Pre-treatment of tissue sections was performed to enable labelled riboprobes to access tissue more efficiently. Initially, serial tissue sections were de-waxed and rehydrated, through a series of treatments: xylene (two 5 min treatments); xylene:ethanol [1:1] (30 sec treatment) and diminishing graded ethanol treatments of 100%, 90%, 70%, 50% and 30% (3 min per treatment). Sections were then washed in DEPC-PBS prior to a series of pre-hybridisation treatments that are shown in Table 2.6. These treatments included the use of pepsin, glycine, PFA and acetic anhydride/triethaloamine (TEA), which were all performed in 450 ml RNase-free stainless steel canisters and slide racks, pre-baked at 180^{o}C. Following the pre-treatments outlined in Table 2.6, slides were dehydrated through a series of two minute ethanol treatments in the following order: 30%, 50%, 70%, 90% and two 100% treatments. Lastly, slides were dried in filtered air for 30-60 min.

Treatment	Temperature	Duration	Effect
0.1% pepsin, 0.5% 2 M HCl, in DEPC-water	37°C	4 min	Improve riboprobe accessibility to target mRNA through protease digestion of tissue in acidic conditions.
0.07% glycine in DEPC-PBS	Room temp	30 sec	Raise pH to alkaline conditions to inactivate pepsin
DEPC-PBS	Room temp	30 sec (x2)	Wash
4% PFA in DEPC-PBS	Room temp	20 min	Fix tissue to maintain integrity
DEPC-PBS	Room temp	5 min (x2)	Wash
25 mM acetic anhydride in 0.1 M triethanol-amine (pH 8.0)	Room temp	10 min, with agitation.	Reduces non-specific probe binding by blocking cationic free amino acid residues produced during slide coating
DEPC-PBS	Room temp	2 min (x2)	Wash

Table 2.6: Pre-hybridisation treatments for slides used in TISH
All solutions were prepared using 0.1% DEPC-water or 0.1% DEPC-PBS (pH7.4).

For probe hybridisation, sense and anti-sense cRNA probes were mixed in hybridisation buffer [50% formamide (Sigma), 10% dextran sulphate, 5x SSC (0.75 M NaCl, 75 mM sodium citrate), 1x Denhardt's solution [0.02% Ficoll 400 (Sigma), 0.02% polyvinylpyrrolidone (Sigma), 0.02% bovine serum albumin(Sigma)], 10 mM dithiothreital (DTT; Sigma), 500 µg/ml yeast tRNA, 1 µg/ml RNase inhibitor] to a final concentration of 1-2 µg/ml. All sections on a single slide were covered in hybridisation mix containing sense riboprobes, and the consecutive slide containing neighbouring tissue sections covered with anti-sense riboprobes. Slides were incubated overnight at 52°C in a humid chamber.

2.10.5 Post-hybridisation treatments

Following overnight incubation with riboprobes, slides were removed from the humidity chamber and sections washed in a series of sequential SSC solutions to remove residual hybridisation mix and riboprobes (see Table 2.7 for all post-hybridisation treatments). This was followed by a Tris-HCl wash and blocking

solution treatment, to prepare for immunological detection of the DIG-labelled riboprobes. An RNase treatment was not used, as it was deemed likely to decrease specific signal. This is because, due to cross linking of tissue components and the conformation of mRNA, full length hybridization of riboprobes would not be achieved, therefore leaving single-stranded areas available for RNase degradation (Wilkinson, 1998).

Treatment	Temperature	Duration	Effect
5x SSC*	52°C	15min	Remove non-specifically bound riboprobes
2x SSC/50% formamide	52°C	20min (x2)	As above (formamide used to increase stringency)
2x SSC (with gentle agitation)	37°C	30min (x2)	As above
0.2x SSC (with gentle agitation)	37°C	30min	Final high stringency washes to increase removal of non-specifically bound riboprobes
0.2x SSC	37°C (cooling on bench)	30min	As above
Tris-HCl Buffer [100 mM Tris-HCl (pH 7.6), 150 mM NaCl]	Room temp.	5 min (x2)	Wash slides in preparation for immunological detection
Blocking Solution [10% fetal calf serum in Tris-HCl Buffer]	Room temp.	1 hour	Reduce non-specific binding during antibody detection of DIG-labelled probes

*1x SSC is 0.15M NaCl, 15 mM sodium citrate

Table 2.7: Post-hybridisation washes for TISH slides

2.10.6 Immunological detection of digoxygenin

For immunodetection of DIG-labelled probes, tissue sections were covered with alkaline phosphatase-conjugated anti-DIG antibody (Roche) diluted in blocking solution [Tris-HCl buffer, 2% fetal calf serum] to a final concentration of 1:150. Slides were then incubated overnight at 4^{o}C, in horizontal slide holders placed within a plastic box containing PBS-soaked tissue (humidity chamber). Following incubation, slides were washed three times (3 min per wash) in Tris-HCl buffer and incubated for 2 min in detection solution (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$). Slides were then placed in the dark for 5-24 hrs while the chromogenic reaction took place, brought about by the addition

of Nitro Blue Tetrazolium Chloride (NBT; Roche) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP; Roche), diluted in detection buffer (4.5 µl/ml NBT [100 mg ml^{-1}], 4.5 µl/ml BCIP [50 mg ml^{-1}]). Sections were washed in water to stop the colour reaction and mounted in Aquamount (BDH). Staining was visualised using bright field microscopy.

2.11 Immunohistochemistry

Optimal conditions immunohistochemistry (IHC) were determined for each antibody by testing the use of different concentrations of antibodies and varying treatment time for antigen-unmasking. In principle the methodology involved the use of indirect immunological detection of antigens, through the use of a primary and either: (i) a secondary antibody directly conjugated to a fluorochrome (e.g. FITC, Texas Red); (ii) a biotinylated secondary antibody, followed by streptavidin detection conjugated to a fluorochrome; or (iii) a biotinylated secondary antibody, followed by streptavidin detection conjugated to horse radish peroxidase (SA-HRP; Vector), which is detected enzymatically. Dual IHC combined the use of '(i)' and '(ii)'.

Tissue sections mounted on slides were first de-waxed in xylene (3 min wash, x2) and then rehydrated in 100%, 90% and water for 2 min each. Slides were then incubated for 20 min in 3 % (v/v) hydrogen peroxide in PBS to inactivate endogenous peroxidases, followed by washes in PBS. Sections were permeablised to facilitate antigen unmasking by standing slides in boiling 10 mM sodium citrate buffer (pH 6.0) for 10 min. Following a further wash in PBS, slides were incubated overnight at 4°C with primary antibody. Each primary antibody was diluted in buffer solution [0.1% Triton X-100 (Sigma), PBS] containing 3% serum (species of origin dependent on species in which secondary antibody was raised) to reduce background binding by secondary antibody. Details of primary antibodies used, and their dilution are shown in Table 2.8.

Primary antibody	Raised in	Dilution	Source/Reference
Monoclonal anti-androgen receptor (AR)	Mouse	1:100	Lab Vision Corp., Cheshire, UK
Monoclonal anti-luteinizing hormone (LH), beta subunit	Mouse	1:200	Lab Vision Corp., Cheshire, UK
Monoclonal anti-human chorionic gonadotrophin (hCG), beta subunit	Mouse	1:100	Lab Vision Corp., Cheshire, UK
Polyclonal anti-LH/hCG receptor	Rabbit	1:500	MBL International Corp., MA, USA
Anti-SOX2	Rabbit	1:500	Chemicon Int., Hampshire, UK
Anti-SOX9	Rabbit	1:800	Chemicon Int., Hampshire, UK
Polyclonal anti-CYP11A1	Rabbit	1:1500	Gift from Prof. A. Payne (Stanford University, CA, USA)
Polyclonal anti-HSD3B2	Rabbit	1:500	(Doody *et al*., 1990)
Polyclonal anti-CYP17A1	Rabbit	1:1000	(Hanley *et al*., 2001)

Table 2.8: Primary antibodies used for immunohistochemistry

The following day, slides were washed in PBS (5 min x3) and incubated with either biotinylated, or fluorescently-labelled secondary antibody for two hours at 4^{o}C. The different secondary antibodies used were diluted in buffer [0.1% Triton X-100, in PBS] as follows: biotinylated anti-rabbit 1:800 (Vector); biotinylated anti-mouse 1:100 (Vector); fluorescently-labelled Texas Red anti-rabbit 1:150 (Vector); fluorescently-labelled Fluorescein Isothiocyanate (FITC) anti-mouse 1:64 (Sigma). Following secondary antibody incubation, slides were again washed in PBS (5 min x3).

For sections treated with biotinylated antibodies, slides were then incubated with SA-HRP, diluted to 1:200 in buffer [0.1% Triton X-100, PBS], and left at room temperature in a humidity chamber for one hour. To produce colour staining one diaminobenzidine tablet (DAB; Merck Laboratories, Darmstadt, Germany) was used dissolved according to the manufactures instruction and containing 0.1% hydrogen peroxide. Exposure was for 3 min, followed by washing briefly in PBS. Slides were then counter-stained using 0.01% toludine blue, rinsed in water

and sections dehydrated using 70%, 90% and 100% ethanol. Finally, slides were immersed in xylene and sections mounted using Entellan (Fisher). In the case of fluorescently-labelled secondary antibodies, slides only required rehydration and mounting following secondary antibody incubation. These slides were mounted using VectaShield Mounting Medium with DAPI (Vector).

2.12 Immunocytochemistry

Cells were removed from cultures during sub-culturing and plated onto 8-chamber glass slides (LAB-TEK, Nalge Nunc Int., Rochester, NY) coated with fibronectin (Chemicon) and containing the appropriate growth media. Slides were incubated at 5% CO_2 at 37°C to enable sufficient growth of cells where required. Once at ~50% confluency, cells were fixed with either PFA [4% in PBS] for 4 min or with methanol:acetone:water [2:2:1] for 3 min, followed by dehydration in 50%, 70% and 100% ethanol for 2 min each. When not used immediately for immunocytochemistry (ICC), dehydrated slides were stored at 4^{o}C. The ICC procedure followed the same steps outlined in section 2.10, beginning with rehydration of cells.

2.13 Microscopy and image processing for IHC, ICC and TISH

All IHC, ICC and TISH slides were examined using a Zeiss Axioplan 2 light microscope (Carl Zeiss Ltd), used in combination with a Zeiss FluoArc lamp (Carl Zeiss Ltd) for fluorescence microscopy; a Zeiss AxioCam Hrc camera (Carl Zeiss Ltd) for capturing digital images; and Zeiss AxioVision 4.5 image processing software (Carl Zeiss Ltd) to capture and format images; and Adobe Photoshop CS2 (Adobe Systems Inc., CA, USA) used for further image formatting and adjusting file compatibility.

2.14 Hormone chemiluminescent immunoassays

Assaying of hormones was performed by Dr Peter Wood at Southampton University Hospitals NHS Trust. Hormone assays were performed directly on 0.5 ml media aliquots from explant cultures of fetal testes to measure concentrations of testosterone and hCG, or equivalent cultures of anterior pituitary glands to

measure LH concentration. Hormones were assayed according to the manufacturer's instructions using automated chemiluminescent immunoassays. All hormones tested were undetected in control media samples. The testosterone immunoassay has previously been validated by gas chromatography/mass spectrometry (Goto *et al*., 2006). All other assays undergo regular quality-control analysis as part of clinical diagnostic service provision.

2.15 Immunoblotting

2.15.1 Preparation of cell lysates

Growth media was removed and cells were washed briefly in warmed PBS. Next, a small amount of ice cold lysis buffer [150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 0.5% Triton X-100, protease inhibitors (Roche)] was added to cover cells, which were then placed on ice for 30 min, with gentle rocking. The resulting cell lysate was taken into a tube and stored at -80^{o}C.

2.15.2 SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli (Laemmli, 1970). This entailed using single dimension SDS-PAGE, performed vertically in a Mini-Protean 3 Cell (Bio-Rad, Hertfordshire, UK), assembled according to the manufacturer's instructions. 10% acrylamide resolving gels [10% bisacrylamide (Bio-Rad), 375 mM Tris-HCl (pH 8.8), 0.1% SDS (Sigma), 0.1% ammonium persulphate (APS; Sigma) and 0.05% N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma)], were overlaid with 5% acrylamide stacking gels [5% bisacrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% APS and 0.1% TEMED]. Prior to electrophoresis, protein samples were prepared by adding an equal volume of 2x SDS gel-loading buffer (100 mM Tris-HCl [pH 6.8], 200 mM DTT, 4% SDS, 20% glycerol and 0.2% bromophenol blue) and boiling at 95^{o}C for 5 min. A prestained protein marker (New England BioLabs (NEB) Ltd., Hitchin, UK) was boiled for 5 min along with the protein samples and then loaded on to the gel. Electrophoresis was conducted in 1x SDS-PAGE buffer (25 mM Tris-HCl [pH 8.3], 250 mM glycine, 0.1% SDS) at 55 mA until proteins were separated, as indicated by the prestained marker.

2.15.3 Western Blotting

Electrophoretic transfer of proteins from SDS-PAGE to a nitrocellulose membrane (Hybond-ECC membrane; GE Healthcare/Amersham Biosciences, Buckinghamshire, UK) was performed in transfer buffer [48 mM Tris-HCl pH 8.3, 39 mM glycine, 20% methanol using a semi-dry horizontal blotter (Mini Trans-Blot Electrophoresis Transfer Cell; Bio-Rad). Blotting was conducted at a constant current of 250 mA, for 2 hrs. After transfer, the membrane was blocked overnight using blocking buffer [5% non-fat milk powder, 0.1% Tween-20 (Sigma) in PBS] at 4^{o}C. Next, membranes were washed three times in PBS containing 0.1% Tween-20 (PBS-Tween) for 5 min, with mild agitation. Anti-AR primary antibody (Lab Vision) was diluted to 1:500 in blocking buffer, and incubated with the membrane for 2 hrs at room temperature. The membrane was subsequently washed three times in PBS-Tween for 10 min, and then incubated with peroxidase-labelled anti-mouse secondary antibody (Amersham) diluted 1:100,000 in blocking buffer for 1 hr. β-actin was detected by incubating the membrane with mouse anti-β-actin peroxidase antibody (Sigma) and acted as a positive control, giving an indication of relative total protein content between individual samples. Membranes were then washed three times in PBS-Tween and once with PBS. Membranes were developed on Kodak G.R.I. Autoradiography film by application of Advanced Enhanced Chemiluminescence (ECL) reagents (Amersham).

2.16 Cell culture

2.16.1 Culture Conditions and Cell Maintenance

All cells were cultured in 5% CO_2 at 37°C. All primary human cells/tissues from the collection of fetal material were maintained in Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12 serum-free medium (PAA laboratories, Somerset, UK), supplemented with 1% premixed Penicillin/Streptomycin solution (Invitrogen), 2% UltraSer SF [steroid-free (Pall Life Sciences/Biosepra, Cergy-Saint-Christophe, France,)] and 1% insulin-transferrin-selenium-X (ITS; Invitrogen). For the purpose of culturing external genitalia explants, freshly harvested tissue was mechanically disaggregated using a scalpel and passing

through a 1 ml pipette tip, prior to culture. LNCaP cells were obtained from the American Tissue Culture Collection (ATCC) and initially maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 1% premixed Penicillin/Streptomycin solution and 10% fetal bovine serum (FBS; PAA); this was optimal conditions for growth and therefore increasing cell numbers. However sub-culturing led to extended growth in DMEM/Ham's F12 (2% UltraSer, 1% ITS), prior to use in experiments comparing androgen treated and androgen deprived cells.

For the passaging of cells, 1 x Trypsin/EDTA (Invitrogen) was added in sufficient quantity to cover the surface of the culture vessel and then cells were incubated at 37°C, until adherent cells detached (2-5 minutes). Trypsin was inactivated by adding horse serum (PAA) and DMEM/Ham's F12 medium [1:1:6, trypsin:horse serum:medium], and the cell suspension transferred to a fresh tube. Further media was then added, and the cells subjected to two rounds of centrifugation at 1000 rpm for 4 min. The final cell pellet was resuspended in fresh culture media and diluted to an appropriate density (~1:5 dilution for routine passaging), before being reseeded on to fresh 25 or 75cm^2 culture flasks.

2.16.2 Freezing and thawing cells

Cells were trypsinised and pelleted as described in section 2.16.2. The final cell pellet was resuspended at a density of ~6 x 10^6 cells/ml in media containing 50% FCS (PAA) and 10% DMSO (Sigma), and aliquotted into cryotubes. Cells were frozen at diminishing temperatures at a rate of 1^{o}C/min to -80^{o}C, prior to storage in liquid nitrogen. Frozen cells were thawed rapidly at 37^{o}C, diluted with 10 ml of pre-warmed culture media and centrifuged at 1,000 rpm for 4 min at 4˚C. The cell pellet was resuspended in fresh culture media and transferred to a culture flask.

2.17 Preparation of steroid hormones

Steroid hormones (dihydrotestosterone [DHT], testosterone, androstenedione and DHEA) were purchased in powdered form from Sigma. Each was made up in glass to a stock solution of 10 mM in ethanol, and stored at -20^{o}C wrapped in foil

to prevent light degradation of steroids. Stock solutions were diluted as required in ethanol, using a serial dilution method. Ethanol was used as a vehicle control for all steroid hormone treatments (negative control; e.g. "- DHT").

2.18 Microarray analysis

2.18.1 RNA extraction

Total RNA was extracted from fetal genital fibroblast cells either following androgen deprivation or the addition of 10 nM DHT. Further analysis was performed by the Microarray Core Facility in the Faculty of Life Sciences, University of Manchester. RNA quality was assessed using the RNA 6000 Nano Assay, and analyzed on an Agilent 2100 Bioanalyser (Agilent Technologies, CA, USA). RNA was quantified using a Nanodrop ultra-low-volume spectrophotometer (Nanodrop Technologies).

2.18.2 Synthesis of fragmented biotinylated cRNA

Approximately 15 µg total RNA was utilized to synthesize cDNA for use as template for cRNA. Synthesis was carried out using an SuperScript double-stranded cDNA synthesis kit (Invitrogen), with the use of 100 pM T7-(dT)24 primer (Operon). A GeneChip Sample Clean Up Module kit (Qiagen/Affymetrix Plc, High Wycombe, UK) was used for cDNA cleanup and the quality of the cDNA was assessed using the RNA 6000 Nano Assay, combined with analysis on an Agilent 2100 Bioanalyser. The Genechip IVT labelling kit (Affymetrix) was then used to produce biotinylated cRNA from the cDNA template. Biotinylated cRNA was purified using the GeneChip Sample Clean Up Module and quantified using the Nanodrop spectrophotometer. Fragmentation of 15 µg cRNA was carried out by incubating at 94°C for 35 min in 5x fragmentation buffer.

2.18.3 Hybridization, scanning and analysis

A hybridisation cocktail containing the fragmented cRNA, made as described in the GeneChip Expression Analysis Technical Manual, was incubated for 16 hrs at 45°C with human genome U113 plus 2.0 array slides in a rotisserie box rotating at 60 rpm. Following hybridization, the arrays were loaded onto a

Fluidics station for washing and staining with R-phycoerythrin conjugated to streptavidin (Molecular Probes, Inc., OR, USA) using the EukGe W S2v5 program controlled using Affymetrix GCOS (V1.4) software. The arrays were then scanned by loading them onto an Agilent GeneArray 3000 high resolution scanner, using Affymetrix GCOS (V1.4) software and the signal intensity converted to a signal intensity value, represented in the microarray raw data produced. The U113 plus 2.0 array slides used comprised over 47,000 probe sets, and allowed for analysis of gene expression across the entire human genome, including multiple independent measurements for each transcript to increase accuracy and reproducibility.

Quality control checks and control hybridizations were performed to assess the likelihood of technical problems with the microarray analysis and examine reproducibility of the results. These procedures revealed no technical problems, and the presence of a statistically significant biological effect as a result of the androgen treatment used to treat cells. Microarray analysis, along with technical quality control checks and statistical analyses of data, were performed by Dr. Leo Zeef in the Faculty of Life Sciences Core Microarray Facility (University of Manchester). Technical quality control was performed with dChip (Version: 2008) using the default settings.

2.19 Chromatin immunoprecipitation

Chromatin was cross-linked for 10 min at room temperature by addition of 1/10 volume of fresh 11% formaldehyde solution directly into culture flasks containing confluent monolayer cultures in appropriate culture medium. Next, 1/20 volume of 2.5 M glycine was added to cells to quench the formaldehyde, followed by two 5 min washes in PBS. Cells were then harvested using a silicon scraper (Greiner Bio One Ltd., Gloucestershire, UK) and centrifuged at 4000 rpm for 10 min, at 4^{o}C. The resulting pellets were snap frozen in liquid nitrogen and stored at -80^{o}C. Sonication steps and incubation with antibody pre-bound magnetic beads were not performed in the present study; this will be carried out prior to future ChIP-Seq analyses.

2.20 Quantitative real time PCR

Probe-based quantitative real-time PCR (qPCR), also known as TaqMan PCR, was used to quantify the relative gene expression of genes in cultured human fetal genital fibroblasts, either treated with 10 nM DHT or grown in the absence of androgens. This method required the use of a pair of PCR primers, combined with an oligonucleotide fluorogenic probe, which contains a reporter fluorescent dye and quencher dye to facilitate accurate monitoring of the synthesis of PCR product in real time, via recordings of changes in fluorescence (purchased as TaqMan gene expression assays; Applied Biosystems (ABI), CA, USA). qPCR reactions were performed in accordance with the manufacturer's instructions outlined in the ABI TaqMan gene expression assay guide book (http://www.appliedbiosystems.com). For this, each qPCR reaction contained 1-100 ng of cDNA prepared from extracted RNA (see section 2.8), added to a 20 µl reaction mixture containing: 1x TaqMan gene expression assay [900 nM of each primer, 250 nM 6-FAM dye-labelled MGB TaqMan probe] and 1x ABI TaqMan Universal PCR master mix (no AmpErase UNG). qPCR reaction mixtures were loaded on to 96-well plates (ABgene Ltd, Epsom, UK), sealed with adhesive PCR film (ABgene), and qPCR carried out in triplicate for each sample, using an ABI 7900HT sequence detection system. Data were analysed using the delta-delta-Ct method (ABI) and normalised to the expression of the *TBP* and *HPRT* genes (both of which are not regulated by androgen) for initial validation of the technique and sets of primers and probes, before routinely using *TBP* as a negative control for data collection

Chapter 3: Initiation and regulation of androgen biosynthesis in the human fetal testis

3.1 Introduction

The aim of this chapter was to assess the signalling pathway(s) responsible for initiating and maintaining testosterone production during early fetal sexual differentiation. Hence, a great deal of the emphasis in this chapter is on the HPG-axis, which is not only responsible for directing androgen production during adulthood, but has also been considered as an endocrine signalling axis likely to mediate initial testosterone biosynthesis in the fetus (discussed in Chapter 1). The chapter also examines the role the placenta may play in regulating fetal androgen production, namely through human chorionic hormone (hCG) production; another theory for regulation of testosterone production during sexual differentiation (discussed in Chapter 1).

The two principle hormonal candidates for stimulating testosterone production, Luteinizing hormone (LH) from pituitary gonadotrophs and placental hCG (see Chapter 1), were investigated. Primarily this was achieved through the use of IHC and chemiluminescent immunoassay of culture media and *in vitro* hormone secretion, to characterise spatiotemporal expression of key components of the two putative pathways during the critical window of sexual differentiation in fetal development.

In order to achieve these aims, firstly the expression of a number of gonadal steroidogenic enzymes critical to classical testosterone biosynthesis were investigated (CYP11A1, HSD3B2, CYP17A1 and HSD17B3), along with expression of gonadal LH receptor (LHR); expression of both components would be necessary for either LH- or hCG-driven testosterone production through the classical pathway. Subsequently, production of LH and hCG themselves were assessed, as was testosterone secretion with and without addition of the chemical reagent forskolin, which mimics the LH/hCG-mediated cAMP signalling pathway.

As a preface to these data, and in fact all the data in subsequent chapters, the chapter begins with the inclusion of an additional results subsection regarding how the chromosomal sex of fetal specimens used in subsequent experiments was determined. It should be emphasised that the technique described, and accompanying results data in this subsection are pertinent to all data in the current study, and illustrate a reliable, robust technique used to determine the chromosomal sex of all fetal specimens collected for the current study and a plethora of other studies within the Human Genetics Division of the University of Southampton.

3.2 Aims

- To establish a method to determine chromosomal sex of human fetal tissue.
- To characterise the expression of *CYP11A1*, *HSD3B2*, *CYP17A1* and *HSD17B3* in the human fetal testis during late first trimester development; to ascertain presence of steroidogenically active Leydig cells, and assess testicular androgen biosynthesis pathways.
- To investigate what factors are responsible for regulation of testosterone biosynthesis during human sexual differentiation, through an analysis of *hCGβ* and *LHβ* expression, secretion of associated hormone and expression of their mutual receptor, the LHR.

3.3 Results

3.3.1 Determination of genetic sex of fetal specimens using fluorescence in situ hybridisation

Following the collection of fetal material for use in investigative experiments, it was a necessity to know the chromosomal sex of specimens. This was accomplished using fluorescence *in situ* hybridisation (FISH) analysis of placental tissue (see Chapter 2). Through the use of fluorescent probes, fragments from either the *Y* and/or the *X* chromosomes were highlighted within

the nuclei of each single cell; indicating XY male, or XX female chromosomal sex, respectively. As shown in the representative examples in Fig. 3.1, this technique produced clear, unequivocal data directly showing the sex chromosome composition of fetal placental cells and therefore establishing the chromosomal gender of each fetus used in the study.

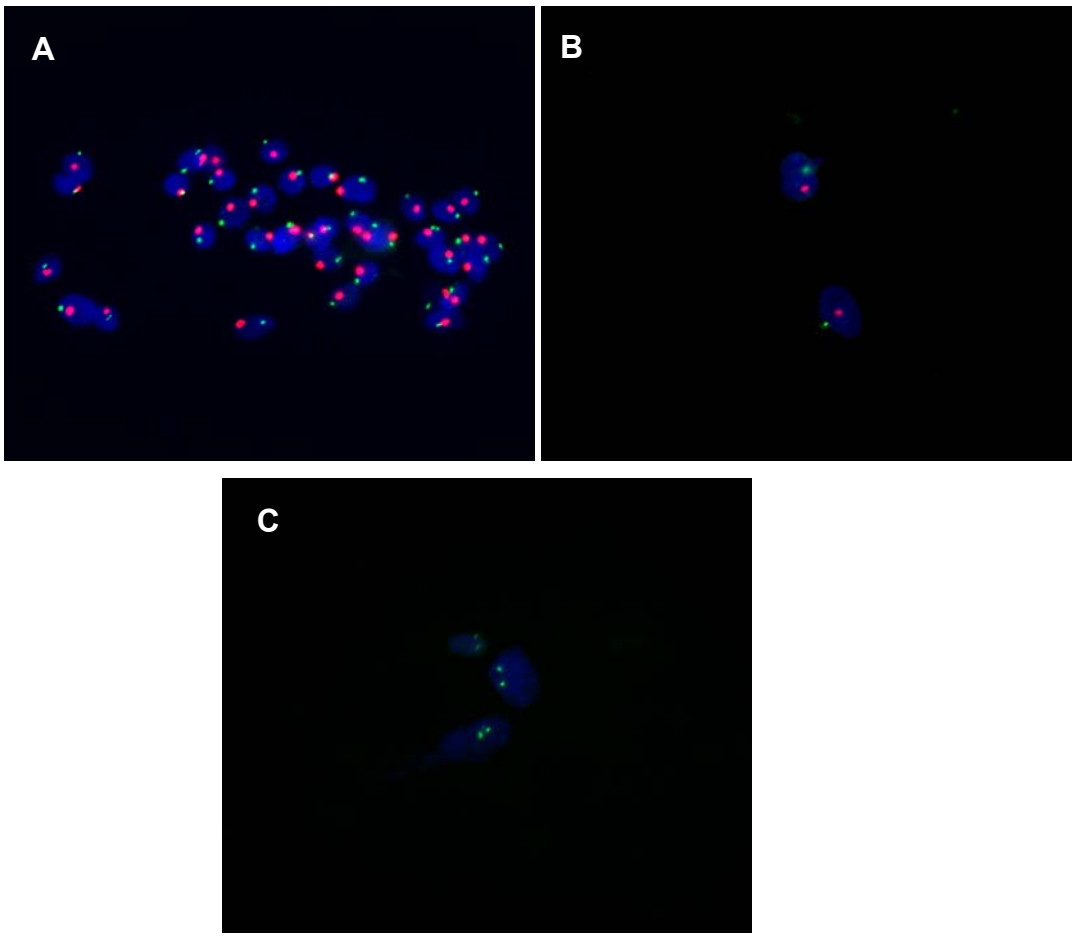

Figure 3.1: Examples of FISH performed on placental tissue to determine sex
Visible in panels A and B is a single X (green) and Y chromosome (red) within the nuclei of each cell (blue), thereby denoting chromosomal sex as male in these two examples. In contrast, panel C shows two X (green) chromosomes present within each cell nucleus, indicating the chromosomal sex is female.

3.3.2 Expression of CYP11A1 in the developing 46,XY human testis

CYP11A1 was first detected at low levels in the fetal testis at 8 wpc using IHC (Fig. 3.2, A). Expression was localised to interstitial cells within the testis and

was absent within the testis cords, consistent with CYP11A1 being expressed in developing Leydig cells and not in the Sertoli or germ cell lineages. Detection of CYP11A1 was much increased by 9.5 wpc (Fig. 3.2, B) and even more so at 11 wpc (Fig. 3.2, C). Similar to detection in 8 wpc specimens, expression appears localised to the interstitium at these older time points.

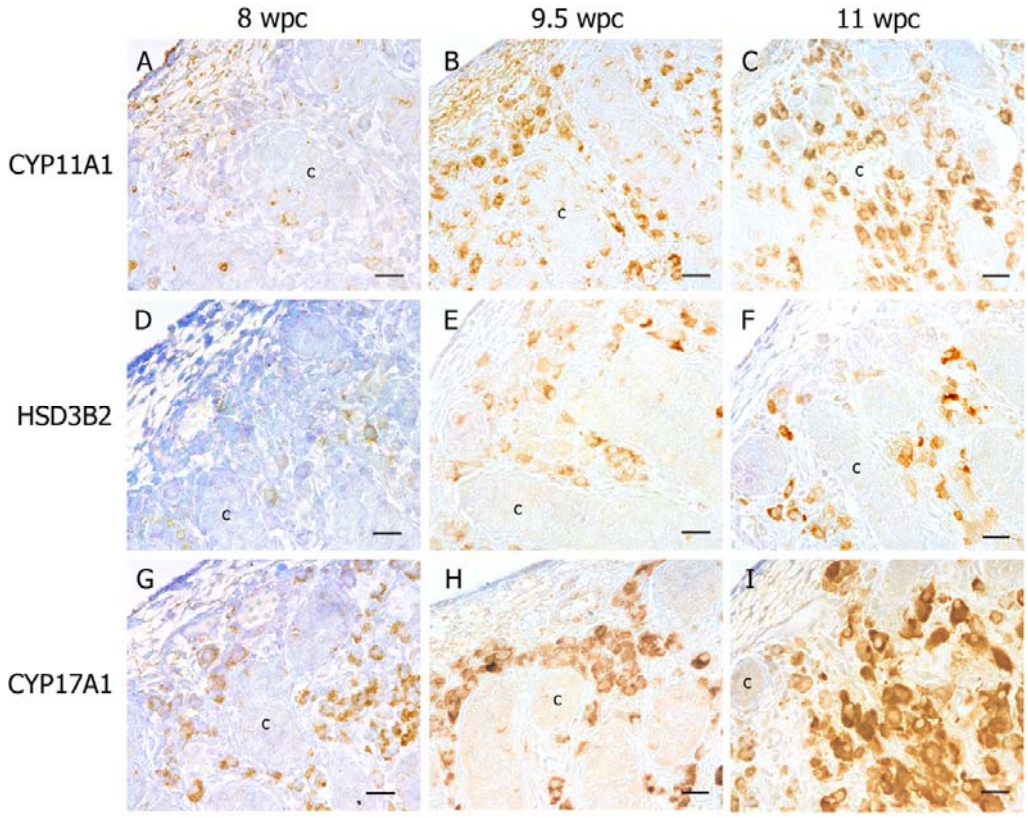

Figure 3.2: Immunohistochemistry analysis of the expression of key steroidogenic enzymes in transverse sections of 46,XY human testis at 8 to 11 wpc
Fetal age is displayed above in weeks post-conception (wpc) and enzyme names shown down the left-hand side: CYP11A1 (A-C), HSD3B2 (D-F), and CYP17A1 (G-I). c, testis cord. Scale bar represents 20 μM.

3.3.3 Expression of HSD3B2 in the developing 46,XY human testis

HSD3B2 was expressed at very low levels in fetal testis at 8 wpc (Fig. 3.2, D), with expression appearing to be concentrated outside of the testis cords. Expression of HSD3B2 was increased considerably by 9.5 wpc and clearly concentrated in the interstitial cells, implying only early Leydig cells express HSD3B2 (Fig. 3.2, E). At 11 wpc, a comparable level of expression persisted that remained concentrated in the interstitium (Fig. 3.2, F).

3.3.4 Expression of CYP17A1 in the developing 46,XY human testis

CYP17A1 was expressed at relatively high levels during early development of the testis at 8 wpc, in comparison to CYP11A1 and HSD3B2 expression at this time (Fig. 3.2, G), but still showed the same pattern of expression, remaining localised to the interstitium. At 9.5 wpc, expression of CYP17A1 was increased, and appeared to be expressed at high levels in all but a few interstitial Leydig cells (Fig. 3.2, H). This intense level expression was maintained at 11 wpc, where once again the majority of interstitial cells show very pronounced staining for CYP17A1 (Fig. 3.2, I).

3.3.5 Expression of HSD17B3 in the developing 46,XY human testis

Detection of HSD17B3 transcript by RT-PCR in fetal testis was apparent as early as 7.5 wpc, and also evident at 8.5 wpc (Fig. 3.3). Excision and sequencing of the RT-PCR bands confirmed this. By use of non-radioactive mRNA tissue *in situ* hybridisation, at 11 wpc HSD17B3 was detected in interstitial cells of the testis, but not the testicular cords, when compared to control sense staining (Fig. 3.4).

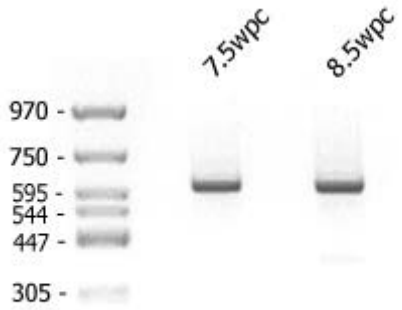

Figure 3.3: RT-PCR analysis of expression of HSD17B3 in 46,XY human testis
mRNA extracted from fetal testes at 7.5 wpc and 8.5 wpc. Ladder fragments, measured in base pairs, shown on left.

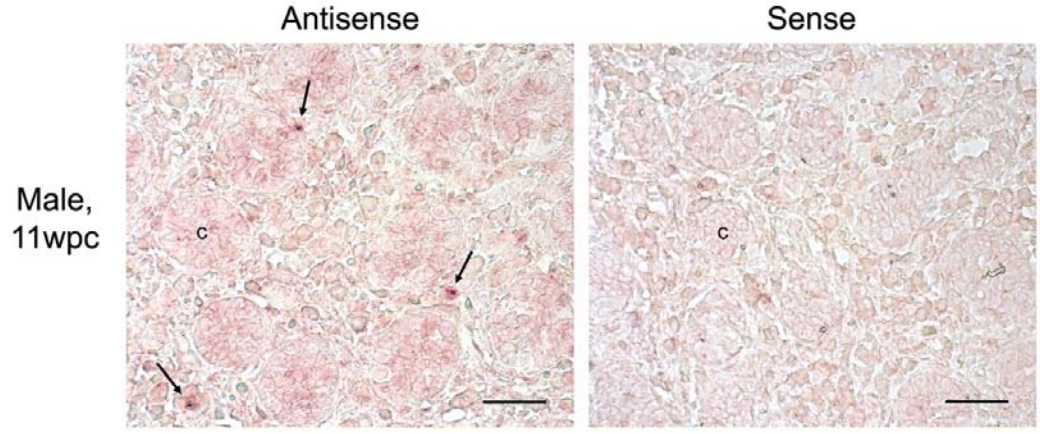

Figure 3.4: TISH analysis of HSD17B3 expression in sections of 46,XY human testis at 11 wpc
HSD17B3 anti-sense probe (left) and control sense probe (right) are shown. c, testis cord. Scale bar represents 50 uM

3.3.6 Expression of LHR in the developing 46,XY human testis and 46,XX human ovary

LHR expression in the fetal testis follows a similar cellular pattern to that of the steroidogenic enzymes discussed above. LHR expression was first detected convincingly at 8 wpc (Fig. 3.5, B). IHC staining around the cell perimeters at this stage and in older specimens (see arrows in Fig. 3.5), is consistent with the expression of a cell surface receptor such as LHR. The robustness of this detection profile was intensified in the developing testis as a function of age, increasing moderately at 8.5-9 wpc, and more strikingly at 10-10.5 wpc (Fig. 3.5, C and D, respectively). In 46,XY specimens at 7.5 wpc, expression of LHR was not detectable in gonadal tissue by RT-PCR analysis using up to 40 cycles of amplification and with varying annealing temperatures covering a range of +/- 5^{o}C around the optimal annealing temperature found for older 46,XY specimens (Fig. 3.6). This implied that the weak IHC staining visible at 7.5 wpc is most likely non-specific background staining (Fig. 3.6, A). In the RT-PCR analysis, LHR was strongly detected at 8.5 wpc, which concurs with the IHC data. Sequencing of the two components of the double band detected by RT-PCR at 8.5 wpc revealed both PCR products had strong homology with LH-R transcripts, with the smaller product appearing to be a truncated form of LHR mRNA. This is consistent with reports of alternative splice variants of LHR

(Nakamura *et al*., 2004; Apaja *et al*., 2006). Some staining was apparent in the developing human ovary during the same time period (Fig. 3.6, E-H), but the peripheral cellular distribution profile was not apparent, and staining did not intensify with age as observed in male specimens. These findings suggest LHR is at best only weakly expressed in the fetal ovary or that the staining is non-specific.

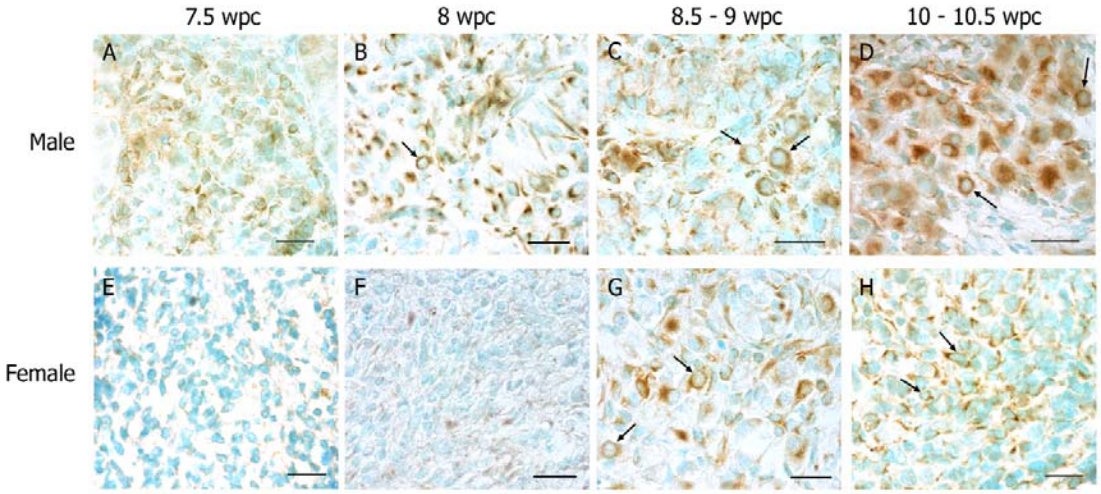

Figure 3.5: Immunohistochemical analysis of LHR expression in transverse sections of 46,XY and 46,XX human gonad, at 7.5 to 10.5 wpc
IHC performed at several fetal ages, shown above in weeks post-conception (wpc). Arrows highlight areas of prominent cell surface staining. Scale bar represents 50 μM.

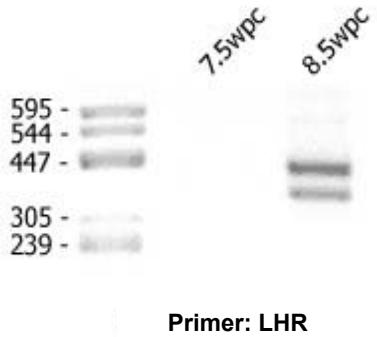

Figure 3.6: RT-PCR analysis of LHR expression in 46,XY human testis at 7.5 to 8.5 wpc
mRNA extracted from fetal testis at 7.5 wpc and 8.5 wpc. Ladder fragments, measured in base pairs, shown on left.

3.3.7 hCG secretion in the 46,XY developing human testis

In 46,XY male fetuses, low levels of hCG expression were detected within the interstitium of the developing testis from the onset of sexual differentiation at 7.5 wpc (Fig. 3.7, A) and were still detectable at 9 wpc (Fig. 3.7, B), Staining was more robust at 10.5 wpc (Fig. 3.7, C). However, this overall level of hCG detection within the testis was minimal compared to staining of the placenta from the same fetus (Fig. 3.7, D).

Consistent with this IHC profile, as shown in Fig. 3.8 *in vitro* hCG secretion from the testis at 7.5-8 wpc was also relatively low (mean +/- standard deviation (SD): 31.6 +/- 12.3 IU/L), and only marginally greater than background levels detected in control media (3.0 +/- 11.2 IU/L). In contrast, hCG was enormously abundant in the media following overnight culture with a fragment of placenta at the same developmental age (42195.0 +/- 23946.9 IU/L). Testicular hCG was comparable to levels secreted by the adrenal gland (89.94 +/- 31.9 IU/L). The low level of hCG in the media following overnight culture of human fetal testes, correlated to the detection of transcripts by RT-PCR of the hCG β-chain (Fig 3.9). hCG transcript was most readily detected at 7.5 wpc, but was waning at 8 wpc, and not detected at 8.5 wpc (Fig. 3.9).

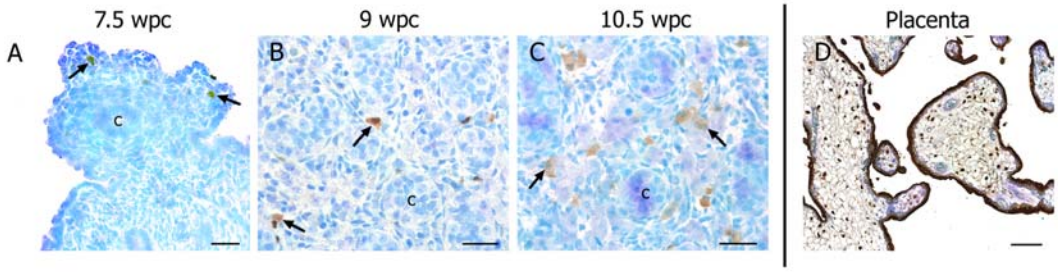

Figure 3.7: Immunohistochemistry analysis of hCG biosynthesis in transverse sections of 46,XY human testis at 7.5 to 10.5 wpc
Fetal age is displayed above in weeks post-conception (wpc) and placental positive control in panel D. c, testis cord; arrows highlight prominent positive staining Scale bar represents 20 μM.

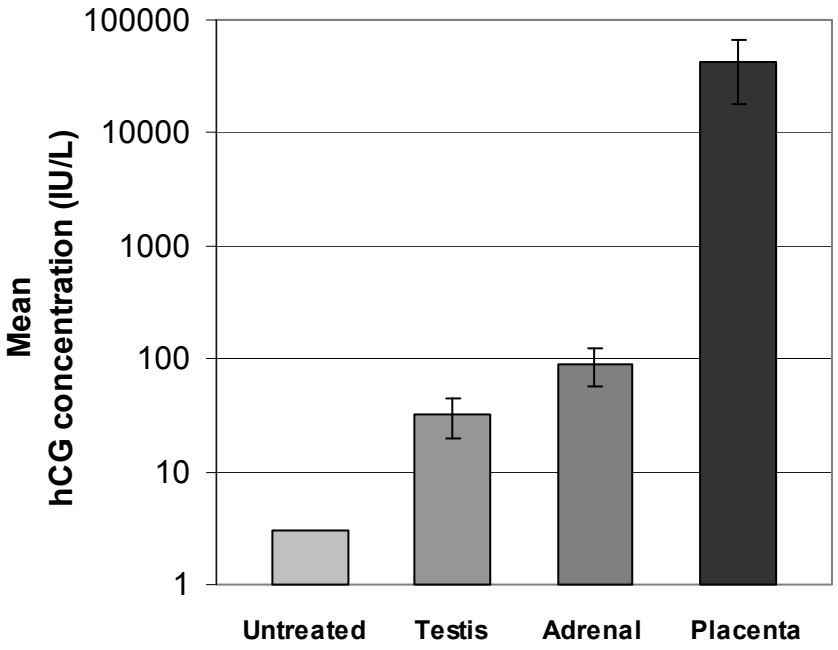

Figure 3.8: Chemiluminescent immunoassay analysis of hCG secretion in 46,XY human testis at 7.5 to 8 wpc
A single testis, adrenal gland and piece of placental tissue were collected from two 46,XY fetuses (7.5 and 8 wpc). Organ culture was performed for 24 hours and the conditioned media collected and analysed for hCG. Error bars indicate +/- standard deviation.

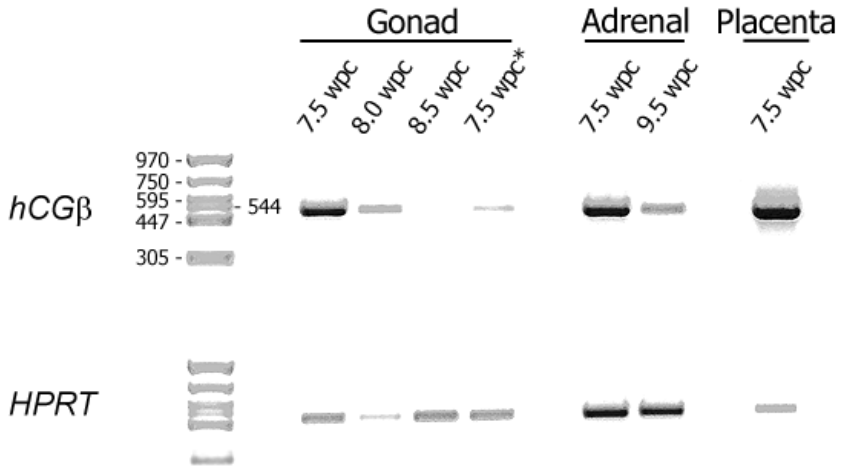

Figure 3.9: RT-PCR analysis of hCG expression in 7.5 to 8.5 wpc 46,XY and 46,XX human gonad, adrenal gland and placenta
mRNA was extracted from fetal testis and ovary (*), adrenal gland and placenta from different fetuses. Fetal age shown at the top of each band. Bands for expression of hCG and the house-keeping gene HPRT are shown (upper and lower bands, respectively. Ladder fragments shown on left (measured in base pairs). wpc, weeks post-conception

3.3.8 Morphological development of the pituitary gland

At 8 wpc the developing pituitary gland had begun to exhibit a bilobed appearance and also appeared highly vascularised (Fig 3.10). Relative to the adjacent CNS tissue that exhibited little vascularisation, the prevalent blood supply to the fetal pituitary gland at this time implies the possibility of functioning gonadotrophs.

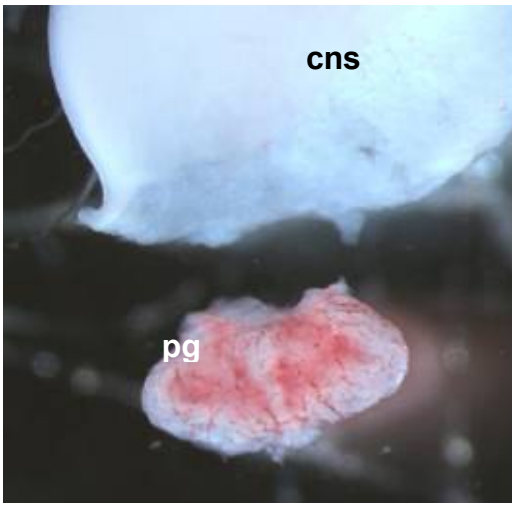

Figure 3.10: Dissected 46,XY developing human pituitary gland at 8 wpc
cns, developing central nervous system; pg, pituitary gland. Note the highly vascular pituitary tissue compared to the CNS.

3.3.9 LH secretion in 46,XY developing human anterior pituitary gland

LH was detected within 46,XY anterior pituitary gland tissue sections at 8 wpc (Fig. 3.11, A and B). LH showed a scattered staining pattern within a few cells at 8 wpc and 8.5 wpc (Fig. 3.11, C). This limited detection of immunostaining correlated to the minimal LH secreted into media during overnight organ culture of 46,XY 8 wpc fetal anterior pituitary glands (mean: 0.04 IU/L; n=3). Similarly low secretion was apparent in female specimens (mean: 0.45 IU/L; n=3), when IHC analyses detected negligible LH *in situ* (Fig. 3.11, D).

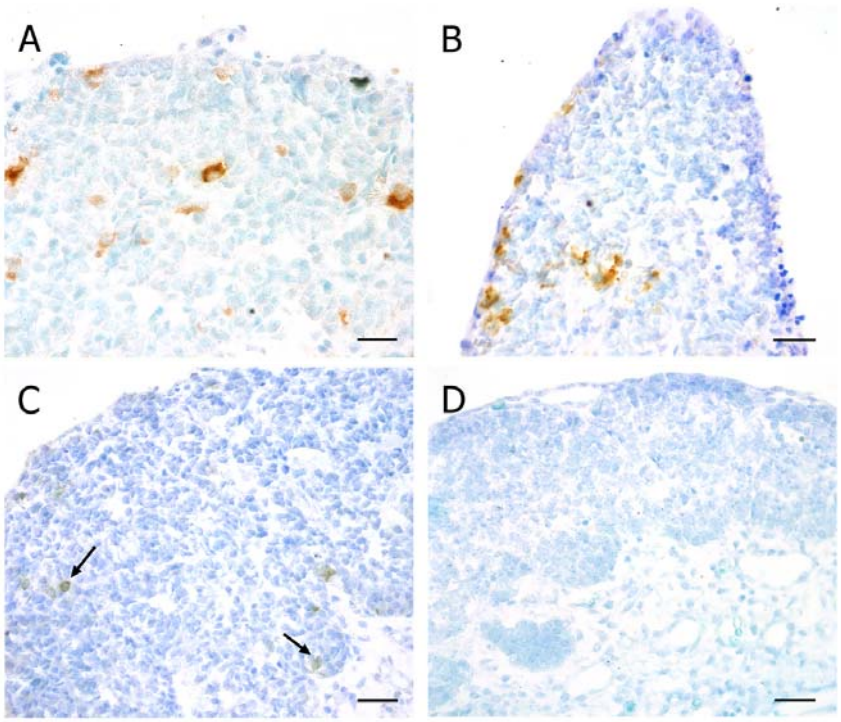

Figure 3.11: Immunohistochemical analysis of LH biosynthesis in transverse sections of 8 to 8.5 wpc 46,XY and 46,XX human anterior pituitary gland
Panels depict most heavily stained areas of tissue. A and B, 8 wpc 46,XY fetus; C, 8.5 wpc 46,XY fetus; D, 8 wpc 46,XX fetus. Arrows point to positive IHC stain in cells. Scale bar represents 20 μM.

3.3.10 Testosterone secretion in 46,XY human testis

Analysis of testosterone secretion showed that the fetal testis at 8 wpc robustly secreted testosterone into the media following overnight culture (mean: 296.99 nmol/L; n = 4), when compared to untreated control media samples (<0.3 nmol/L). Furthermore, testosterone secretion was largely unaffected by the addition of forskolin (10 μM), which produced only a marginal stimulatory effect (mean fold induction +/- SD: 1.01 +/- 0.33; n = 4 [done in parallel with testosterone secretion analysis]).

3.4 Discussion

Identifying what regulates testosterone secretion in the first trimester and how this is achieved, is critical to our understanding of normal sexual differentiation, with potential implications for the treatment of cases of aberrant under- and over-virilised development. This chapter describes efforts to identify the key factors which contribute to the regulation of testosterone biosynthesis during human fetal sexual differentiation. Comparisons have been made between the expression of hCG and LH, along with characterisation of the expression of their shared receptor LHR and steroidogenic enzymes critical to producing testosterone. The data provide novel insight into the expression of these key genes during early human development, producing findings that resolve some issues on the regulation of early fetal testosterone secretion in humans; specifically, indicating that perhaps neither LH or hCG has a substantial influence on early fetal testosterone biosynthesis. Hence these data imply the existence of other possibly unknown factors that could regulate these processes in the absence of a key role for either hCG or LH.

3.4.1 Steroidogenesis during human fetal sexual differentiation

Taken together the IHC expression data for CYP11A1, HSD3B2 and CYP17A1, and RT-PCR and *in situ* results for HSD17B3, strongly imply that differentiation of functional Leydig cells has occurred by 8 wpc in humans. While this is not a novel assertion, illustration of the expression of the key steroidogenic enzymes directly in first trimester human gonadal tissue is not well documented, and clarification of this supposition also reflects the integrity of the tissue-processing and detection methods used in the present study. Because these genes are important mediators of classical testosterone biosynthesis, their expression during the critical window of human sexual differentiation between 8 and 12 wpc, would also imply that fetal testosterone necessary for sexual differentiation is produced predominantly via the classical pathway of androgen biosynthesis, and does not readily utilise the "alternative pathway". This is consistent with the association of male under-virilisation in individuals possessing mutant forms of *HSD17B3* (Geissler *et al*., 1994; Rosler *et al*., 1996; Bilbao *et al*., 1998) and *SRD5A2* (Andersson *et al*., 1991; Can *et al*., 1998; Sasaki *et al*., 2003), the gene

products of which do not form part of the alternative pathway. In collaboration with Prof. Wiebke Arlt (University of Birmingham), we are currently working to clarify our assumption that the classical pathway is most important for normal androgen production during sexual differentiation through analyses of the conversion of common steroidal substrates in fetal testis organ cultures. By determining what steroid products are synthesised *in vitro* by late first trimester fetal testes, we will be able to validate which pathway is principally utilised for sexual differentiation.

3.4.2 Interpreting the role of the LH signalling pathway during fetal sexual differentiation

Steroidogenesis in the adult human testis is a hormonally regulated, multi-step process in which LH has a critical function as a trophic regulator (Takahashi *et al*., 1982; Huhtaniemi and Toppari, 1995; Zhang *et al*., 2004). However, the current study provides convincing evidence that LH/LHR signalling is not essential for the onset of testosterone production during human fetal development. Specifically, we showed that LH secretion from the anterior pituitary gland at 8 wpc was negligible and only minimal LH was expressed within the anterior pituitary at 8-8.5 wpc. Moreover, forskolin, which stimulates adenylate cyclase - a major component of the signalling pathway that propagates LH signalling (Ahluwalia *et al*., 1974; Moger and Anakwe, 1983; Browne *et al*., 1990) - had only a minimal effect on testicular testosterone production at 8 wpc, strongly implying that LH signalling via cAMP is not part of normal androgen-dependent early male sexual differentiation.

3.4.3 Interpreting the role of the hCG signalling pathway during fetal sexual differentiation

The problem of analyzing the importance of LHR is complicated further by its second function as the receptor for hCG. Indeed, a number of previous studies have concluded that it is hCG, and not LH, that is the vital mediator of testosterone production during the first trimester (Reyes *et al*., 1974; Huhtaniemi *et al*., 1977; Misrahi *et al*., 1998; Hiort and Holterhus, 2000; Murray *et al*., 2000). Predominantly based on clinical findings and biochemical analysis of

plasma concentrations of hCG, and reinforced by descriptions of positive expression of LHR only from approximately 10-12 wpc, it has been concluded in such studies that hCG is likely to regulate early testosterone production; at least from 10 wpc onwards. However, placental hCG, although abundant, would have to cross the fetal-placental barrier. Therefore, we analysed whether paracrine or autocrine hCG may regulate early fetal testosterone production. Using different methods we showed that hCG regulation of early testosterone production seems unlikely. Staining by IHC was sparse and hCG secretion from the fetal testis barely detectable at a stage when copious testosterone was secreted. Therefore hCG is unlikely to be a regulator of initial testosterone production during sexual differentiation. Hence if LHR does have a vital function during early development of the genital tract, it is not an implication of its role as the receptor for hCG.

3.4.4 Assessing the function of LHR during sexual differentiation, in the absence of a significant role for LH and hCG

Contrary to the apparent lack of a role for anterior pituitary LH signalling or hCG signalling in early testosterone biosynthesis, the current data do indicate that LHR is expressed in 46,XY fetuses from 8 wpc, and therefore during the critical period of male sexual differentiation. The presence of Leydig cell LHR in humans at this early stage has never been described before. Previously, LHR expression was found only after 10-12 wpc at the earliest (Huhtaniemi *et al*., 1977; Molsberry *et al*., 1982). In the literature it is apparent that LHR does have an important role later in fetal development, which is most probably related to LH secretion from the anterior pituitary. It is well documented that LH expression increases during the second trimester, reaching significant plasma concentrations of 8-10 ng/ml by around 15-20 wpc (Kaplan *et al*., 1976; Kaplan and Grumbach, 1978). Also at this point, a marked increase in testosterone production is observed that correlates well with the initiation of LH secretion (Siiteri and Wilson, 1974; Tapanainen *et al*., 1981; Misrahi *et al*., 1998). Hence it is thought that during this period, LH is important to testosterone biosynthesis, signalling via the LHR. Consistent with this, individuals born with pituitary hypoplasia demonstrate under-virilisation characterised by micropenis. The most

startling indication that LHR is important in sexual differentiation comes from patients with LHR mutations. Severe inactivating mutations in the LHR gene result in near complete failure to virilise in individuals and 46,XY testicular DSD. Less severe mutations have been shown to cause micropenis, ambiguous genitalia, and/or hypospadias (Kremer *et al*., 1995; Themmen and Brunner, 1996; Themmen *et al*., 1998; Min *et al*., 1998; Ascoli *et al*., 2002). Hence, indicating an important role for LHR during the critical window of male sexual differentiation between 8 to 12 wpc.

In contrast to the human data collected in the present study, research on LHR function in mouse has shown that testosterone secretion during early sexual differentiation can be enhanced upon exposure to forskolin (Zhang *et al*., 2004). This could imply LHR is more important to early sexual differentiation in rodents, however, Zhang and colleagues also showed that forskolin had a positive effect on testosterone secretion even in LHR knock-out (LuRKO) mice; emphasising that perhaps cAMP signalling is being initiated via a different G protein coupled receptor, not LHR itself. Moreover, when circulating LH is absent in mice (hypogonadal mice) or mice lack the anterior pituitary gland (T/ebp mice), initial sexual differentiation still progresses normally and animals show no physical abnormalities during the early stages of fetal development (O'Shaughnessy *et al*., 1998; Pakarinen *et al*., 2002). These data suggest LHR is not needed in mice to regulate testosterone during the earlier stages of sexual differentiation and that perhaps a signalling pathway similar to LHR regulation is employed during sexual differentiation that must utilise cAMP; thereby explaining why forskolin artificially induces secretion.

3.4.5 Other potential endocrine sources that could initiate and regulate testosterone biosynthesis during sexual differentiation

In light of the above arguments against the importance of the most commonly regarded hormone regulators of early testosterone production (e.g. LH and hCG), logic dictates that there must be an alternative regulatory system responsible for performing this task. The following hormones are potential key regulators of early testosterone biosynthesis.

3.4.5.1 ACTH

One possible candidate for regulation of testosterone biosynthesis is adrenocorticotropic hormone (ACTH). Expression of the principle receptor for ACTH, the melanocortin type-2 (Mc2) receptor, along with the Mc5 receptor, has been reported in the fetal mouse testis (Baker *et al*., 1999; O'Shaughnessy *et al*., 2003; Nimura *et al*., 2006; Nimura *et al*., 2008), as has the expression of the Mc1 receptor in human testis (Thornwall *et al*., 1997), which can also bind ACTH. Furthermore, it has been shown in mice that ACTH at normal circulating concentrations is able to stimulate expression of testicular StAR protein and increase cAMP production (Johnston *et al*., 2007), as well as induce a 10-fold increase in androgen output from fetal testis (O'Shaughnessy *et al*., 2003). However, these studies also demonstrated that fetal testicular androgen secretions are normal in POMC-null mice, which lack the ability to produce ACTH due to a deletion in the gene for *proopiomelanocortin* (*POMC*) polypeptide precursor, indicating that ACTH is not essential for normal virilisation during early development, even if it does have some capacity for testicular androgen stimulation.

In humans there is only very limited evidence to suggest ACTH is capable of stimulating testicular androgen secretions. Our group has shown that McR2 is not expressed by human fetal testis at 8 wpc (Goto *et al*., 2006). Domenice and colleagues (2001) showed that chronic ACTH secretion stimulated androgen production in a patient with X-linked adrenal hypoplasia congenita (AHC). Due to the nature of the patient's disorder and deficit of adrenocortical steroidogenic cells, in theory the androgens detected might only stem from the steroidogenic Leydig cells, indicating a direct link between ACTH and Leydig cell function in humans. Similar conclusions based on this paradigm were drawn in, or could be extrapolated from, a number of other clinical cases involving male patients with X-linked AHC who exhibited signs of precocious puberty (e.g. (Bertrand *et al*., 1965; Petersen *et al*., 1977; Marilus *et al*., 1981; Wittenberg, 1981; Yeste *et al*., 2009).

However, this 'direct link' is in fact quite tenuous when we consider the many other possible explanations for the increased androgen output. This includes the

possibility of the androgen being derived from fetal adrenocortical cells that can persist into early childhood in patients with adrenal hypoplasia congenita (Petersen *et al*., 1977); increased LH levels resulting from the adrenal insufficiency or the congenital mutation itself, which have some association with such disorders (Wittenberg, 1981) and would stimulate androgen output independently of the increased circulating ACTH; and there is also evidence to suggest that mutations in the *NR0B1* (DAX1) gene that cause X-linked AHC, can bring about Leydig cell hyperplasia and confer autonomous growth and function to Leydig cells (Yu *et al*., 1998; Seminara *et al*., 1999; Domenice *et al*., 2001) .

3.4.5.2 Vasoactive intestinal peptide

Vasoactive intestinal peptide (VIP) was originally isolated from the porcine gastrointestinal tract (Said and Mutt, 1970) and then various animal neural tissues (Said and Rosenberg, 1976; Loren *et al*., 1979; Emson *et al*., 1979), with later identification in animal and human neuroendocrine tissues, functioning as an autocrine regulator of anterior pituitary hormone secretion (Arnaout *et al*., 1986; Linder *et al*., 1987; Nagy *et al*., 1988; Koves *et al*., 1990). Homologous to ADCYAP1 (discussed below), VIP belongs to the secretin/glucagon/vasoactive intestinal peptide family of proteins and utilises cAMP as its primary second messenger (Sreedharan *et al*., 1991; Ishihara *et al*., 1992). VIP has been shown to be capable of promoting testicular steroidogenesis and could play a vital role in regulating testosterone production during the gonadotrophin-independent period of androgen biosynthesis that coincides with human early fetal sexual differentiation (El-Gehani *et al*., 1998b; El-Gehani *et al*., 1998c).

The first evidence advocating a role for VIP in testicular steroidogenesis stemmed from the spatiotemporal expression patterns of vasoactive intestinal peptide receptor 2 (VIPR2). A member of the G protein-coupled receptor family, VIPR2 has strong affinity for VIP, as well as ADCYAP1 and peptide histidine isoleucine (PHI) (Usdin *et al*., 1994; Svoboda *et al*., 1994). Amongst other locations, VIPR2 has been identified in rat testis, where it is strongly expressed in fetal Leydig cells and can mediate stimulation of steroidogenesis when liganded by VIP (El-Gehani *et al*., 1998a). VIPR2 is absent in adult-type Leydig

cells, and its marked effect on steroidogenesis occurs exclusively during the fetal and early neonatal period (Usdin *et al*., 1994).

VIP has been shown to increase both cAMP production and steroidogenesis in a dose-dependent manner, with noticeably high sensitivity to VIP in the testis, compared to other tissues responsive to VIP (e.g. adrenocortical, anterior pituitary and granulosa cells) (Kasson *et al*., 1986; El-Gehani *et al*., 1998b). The effect of VIP on Leydig cells is thought to be augmentation of HSD3B2 and CYP11A1 activity, hence ultimately it could up-regulate fetal androgen biosynthesis (Kasson *et al*., 1986). Sufficient circulating levels of VIP are present in fetal rat, which shows a steady decline as early development progresses (Tatsuno *et al*., 1994; Hill *et al*., 1996), with VIP maximal potency coinciding with the early critical periods of fetal organogenesis and peak fetal Leydig cell activity (El-Gehani *et al*., 1998c). However, it has been noted that the stimulatory effects attributed to VIP, could also be brought about by ADCYAP1 liganding VIPR2, as both are present locally in fetal testis at sufficient levels to mediate increased steroidogenesis (El-Gehani *et al*., 1998b).

3.4.5.3 Thyroid hormone

There exists a limited amount of data that suggests thyroid hormones contribute to the differentiation of adult-type Leydig cells during postnatal development. It has been shown in rat that artificially induced hypothyroidism and hyperthyroidism can restrict or promote Leydig cell generation, respectively, suggesting thyroid hormone may be an important factor in initiating Leydig cell differentiation (Mendis-Handagama *et al*., 1998; Ariyaratne *et al*., 2000). These data also indicated thyroid hormone levels could also effect the production of testosterone and 5α-reduced androgens. Although this appears to be an indirect effect, it does raise the possibility that thyroid hormones could have some importance in initiating or regulating early testosterone production.

3.4.6 A paracrine or constitutive basis for the initiation and regulation of testosterone biosynthesis during sexual differentiation

The data from the present study have shown that LH and hCG are unlikely regulators of early testosterone biosynthesis. If we also accept that the placental unit is unlikely to directly stimulate fetal testicular hormone secretion function, then along this line of reasoning, the one remaining likely source of hormonal influence on testicular function is from the testes themselves, i.e. paracrine/autocrine regulation or alternatively, constitutive testosterone secretion. A short list of regulatory factors that could be involved in local or constitutive fetal androgen production during early sexual differentiation follows.

3.4.6.1 Adenylate cyclase-activating polypeptide 1

A possible paracrine factor that could play a prominent role in testosterone secretion during fetal sexual differentiation is the hormone adenylate cyclase-activating polypeptide 1 (ADCYAP1) [also known as pituitary adenylate cyclase-activating polypeptide (PACAP)]. Originally isolated from ovine hypothalamic tissues, and sharing some sequence homology with growth hormone-releasing hormone, ADCYAP1 is a neuropeptide capable of stimulating cAMP production through activation of adenylate cyclase (Miyata *et al*., 1989; Hosoya *et al*., 1992). ADCYAP1 has been shown to be highly abundant in rat testis at various stages of life, where it represents one of the most highly concentrated peptides therein (Arimura *et al*., 1991; Shioda *et al*., 1994; Hannibal and Fahrenkrug, 1995). Previous studies have also demonstrated the presence of ADCYAP1 receptors (ADCYAP1R1; also known as PAC1) on immature mouse TM3 Leydig cells (Matsumoto *et al*., 2008), recorded high levels of circulating ADCYAP1 that would allow liganding and activation of these receptors (Banks *et al*., 1993), and produced some evidence to suggest ADCYAP1 has the capacity to directly up-regulate steroidogenesis in both fetal rat (Romanelli *et al*., 1997; Rossato *et al*., 1997; El-Gehani *et al*., 2000) and mouse testis (Lacombe *et al*., 2006), with a proposed specific stimulatory effect on testosterone secretion (Matsumoto *et al*., 2008). Taken together these studies suggest ADCYAP1 contributes to normal testicular steroidogenesis in a paracrine and/or endocrine fashion, could regulate early testosterone biosynthesis during human sexual differentiation.

3.4.6.2 Natriuretic peptide hormones

Found in humans and in animal models, the natriuretic peptides comprise a family of three highly homologous proteins known as atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP) (Oikawa *et al*., 1984; Steinhelper, 1993; Levin *et al*., 1998). Thus far, three distinct receptors for the natriuretic peptides have been identified: natriuretic peptide receptor A (NPR-A), NPR-B, and the putatively biologically silent NPR-C [also known as ANP-clearance receptor] (Fuller *et al*., 1988; Chinkers *et al*., 1989; Schulz *et al*., 1989). The natriuretic peptides have been shown to be capable of regulating the function of numerous types of steroidogenic cells in human, rat, mouse and bovine species (Higuchi *et al*., 1986a; Higuchi *et al*., 1986b; Nawata *et al*., 1991; Kawai *et al*., 1996), with comprehensive evidence in rat and mouse for modulation of Leydig cell function and the ability to stimulate testosterone production (Foresta and Mioni, 1988; Khurana and Pandey, 1993; Pandey *et al*., 1999). Furthermore, local expression of all the natriuretic proteins has been demonstrated in testis, and BNP and CNP have been localized in the Leydig cells (Vollmar *et al*., 1990; Collin *et al*., 1997; Middendorff *et al*., 1997).

ANP and BNP may be important during the gonadotrophin-independent phase of human fetal testicular steroidogenesis. It has been found that both ANP and BNP can stimulate testosterone production in a dose-dependent manner in fetal rat Leydig cells, resulting in more than a two-fold induction at physiologically relevant levels (Khurana and Pandey, 1993; El-Gehani *et al*., 2001). Of the two peptides, BNP has the greater potency in eliciting increased testosterone production (approximately 10-fold greater than ANP). These findings, coupled with data confirming expression of BNP and its principle receptor NPR-A in rodent fetal Leydig cells (Cameron *et al*., 1996b; El-Gehani *et al*., 2001), indicates BNP could perform a crucial role in regulating testosterone production via an autocrine mechanism. A role for natriuretic peptides in early androgen production is made even more likely in light of the results from the present study regarding forskolin's effect on testosterone biosynthesis during the period of sexual differentiation (section 3.3.10). It was found that forskolin, and therefore stimulus of the cAMP pathway in fetal testis, did not increase testosterone

output. This could be explained if the main signalling pathway was via a second messenger system other than cAMP, for example cGMP; the principle second messenger utilised by the natriuretic peptides (Chinkers *et al*., 1989; Drewett and Garbers, 1994).

3.4.6.3 Constitutive testosterone production

The lack of clear evidence supporting a role for either the archetypal regulators of androgen secretion (gonadotrophins), atypical putative endocrine mediation or a paracrine/autocrine mechanism controlling early fetal testosterone could indicate that testosterone is simply produced constitutively at this time. Constitutive expression is exhibited by many different cell types (e.g. chondrocytes (Recklies *et al*., 2005) and osteoblasts (Rifas *et al*., 1989)) and can be driven by constitutively active G protein-coupled receptors, similar to LHR (Olesnicky *et al*., 1999). Given that regulation of early testosterone production can still not yet be attributed to a major hormone, despite much research in this field, it is possible that this unknown factor does not exist and that testosterone production is an intrinsic property of early fetal Leydig cells in humans, regulated by transcription factors such as SF-1 (official designation NR5A1) or DAX1 (official designation NR0B1).

3.4.7 Conclusion

In summary this chapter identifies robust testosterone secretion by the human fetal testis at 8 wpc – the start of major male sexual differentiation. This is likely to occur via the classical pathway of androgen steroidogenesis. It has been demonstrated that during this period the recognised ligands of the LHR, LH and hCG, appear highly unlikely to regulate fetal testosterone biosynthesis. These findings imply that intra-testosterone mechanisms, still involving the LHR, and/or constitutive secretion are likely to be of major importance.

Chapter 4: Immunolocalisation of the androgen receptor, type 2 5 alpha-reductase, SOX2 and SOX9 in the developing human fetal external genitalia

4.1 Introduction

The previous chapter dealt with the production and regulation of testicular androgens during fetal sexual differentiation. The present chapter focuses on the downstream effects of these androgens and the means by which their virilising actions are propagated during this important phase of fetal development. Following sex determination, the primitive testis produces several steroid and peptide hormones that contribute to sexual differentiation and result in the phenotypic sex observed at birth (Siiteri and Wilson, 1974; Teixeira *et al*., 2001; Ghayee and Auchus, 2007). The androgenic steroid hormones are fundamental to the induction, growth and differentiation of the male external genitalia from the genital tubercle and urogenital folds (Rey and Picard, 1998). The most important of these in this regard are testosterone and its 5α-reduced form dihydrotestosterone (DHT), both of which mediate their effects via the androgen receptor (AR; Brown, 1995).

A full understanding of the mechanisms that contribute to the embryology of the external genitalia is yet to be realised. Weeks eight to twelve post-conception are considered most key to the development and growth of the fetal male external genitalia, because this is when androgens first appear in the normal male fetus when their activity results in formation of the penis and fusion of the urethral folds and labioscrotal folds to form the phallic urethra and scrotum, respectively (Achermann and Hughes, 2008). In recognizing the clear importance of androgens in these processes a number of studies in animals have examined the distribution of AR in the developing external genitalia. Early on it was shown that AR is expressed in the genital tubercle of fetal rabbits (Veyssiere *et al*., 1985). Later work revealed that in mouse the AR persisted through early development in the urogenital sinus and Wolffian ducts, and in other areas such as the epididymis, ductus deferens, seminal vesicles and prostate (Cooke *et al*.,

1991). Type 2 5 alpha-reductase (SRD5A2) expression was also demonstrated in portions of the Wolffian ducts and appeared vital to the development of the external genitalia in fetal mice (Tsuji *et al*., 1994). Furthermore clear expression was noted in the genital tubercle during early rat sexual differentiation (Tian and Russell, 1997). Increasingly, these studies also offered some initial evidence for the presence of AR and SRD5A2 in female as well as male external genitalia.

Early research into AR expression in humans indicated significant expression in male prepubertal corpus spongiosum fibroblasts (Gearhart *et al*., 1988), prepubertal and adult preputial skin (Fichman *et al*., 1981; Razel *et al*., 1985; Roehrborn *et al*., 1987), and the adult corpus cavernosum (Nonomura *et al*., 1990). There were also some reports indicating adult female genital skin was AR positive (Mowszowicz *et al*., 1981; Ruizeveld de Winter *et al*., 1991; Choudhry *et al*., 1992; Hodgins *et al*., 1998). However, until recently studies examining AR expression in humans and animals utilized only biochemical assay techniques and analyses of steroid-binding activity, and did not directly illustrate AR distribution in genital tissues. Immunohistochemistry has been employed in more recent research, but there are only a limited number of studies that have been performed in this fashion, most of which do not look at AR expression during normal fetal development. Most tend to fall into one of three broad categories: (1) studies on adult and infant genitalia (Ruizeveld de Winter *et al*., 1991; Blauer *et al*., 1991; Choudhry *et al*., 1992; Hodgins *et al*., 1998; Celayir *et al*., 2007); (2) characterisation of cancerous tissues, usually of the prostate gland (Sadi *et al*., 1991; Tilley *et al*., 1994; Yoshikawa *et al*., 1996); or (3) analysis of AR expression in genital cell cultures (Sultan *et al*., 1982; Quarmby *et al*., 1990a).

A few studies have looked directly at AR expression in the fetal external genitalia. However, these have predominantly focussed on the second and third trimester, by which time the key events of sexual differentiation have already taken place (Kalloo *et al*., 1993; Kim *et al*., 2002). Nevertheless the results from these studies are pertinent to our understanding of sexual differentiation and provide a backdrop to the results from the present study. Most crucially they have provided novel insight into AR distribution in the developing external genitalia, indicating that AR expression tends to be most notable in epithelial

cells, the urethral plate and corpus cavernosum with only weak expression observed in stromal cells. These studies also raised questions regarding the significance of AR in female external genitalia, having observed similar AR expression in the analogous structures of the external genitalia of males and females (Kalloo *et al*., 1993; Sajjad *et al*., 2004).

In this chapter we strived to gain further insight into the normal development of the male external genitalia and the essential developmental processes that contribute to this organogenesis of the penis during the period of sexual differentiation. A secondary objective was to also attempt to establish important bio-markers for normal sexual development in males and to examine these in the analogous female structures of the external genitalia. In order to achieve this we observed how morphological development of the normal external genitalia progresses in 46,XY males and 46,XX females, and also characterised the expression of key genes vital to sexual differentiation in these tissues. To enable a better understanding of the role of androgens in the development of the fetal external genitalia the distribution of SRD5A2 and AR was examined, to ascertain where DHT could be produced in the tissues of the external genitalia, and predict its target cells and putative effects. Similarly, AR expression was analysed to examine the specific target areas of testosterone and DHT, hence indicating where specifically virilisation occurs during sexual differentiation and possible downstream effects.

The expression of SOX2 (SRY-related HMG-box gene 2) and SOX9 was also studied as previous research has implied both are critical to normal sexual differentiation. SOX2 mutations have been associated with disorders of sex differentiation (DSD), with particularly good evidence of this association coming from the study of Anopthalmia-esophageal-genital syndrome (AEG; OMIM 206900). SOX2 mutations are commonly found in patients with AEG, where a common feature is under-virilisation resulting in ambiguous male genitalia and pseudohaemaphroditism. SOX9 is also associated with DSD, where mutations in males cause campomelic dysplasia (OMIM 114290), a feature of which is abnormal external genitalia. This phenotypic manifestation of SOX9 mutations is most likely due to a functional role in sex determination, where it has been

identified as a strong candidate for SRY protein interactions (Sekido and Lovell-Badge, 2008), discussed in detail in Chapter 1.

4.2 Aims

- To observe how the normal morphological development of the developing fetal external genitalia progresses during the period of sexual differentiation, and compare and contrast the changes that occur in male and female fetuses.
- To use tissue *in situ* hybridisation and immunohistochemistry techniques to characterise the expression patterns of SRD5A2 and AR in the phallic tissues of the developing external genitalia of males and female fetuses during sexual differentiation, and determine the molecular mechanisms underlying morphological changes during early development.
- To use immunohistochemistry to characterise the expression of SOX2 and SOX9 as important bio-markers of normal differentiation of the male external genitalia, and consider the functions of these proteins in normal male sexual development, particularly in reference to their expression in fetal female external genitalia.

4.3 Results

4.3.1 Morphological development of the external genitalia

The gross morphology of the human 46,XY and 46,XX fetal external genitalia at three different stages during major sexual differentiation is shown in Fig. 4.1. Evident in the 46,XY specimens (Fig. 4.1, A-C), is a natural progression of the union of the two labioscrotal folds, and a second fusion event involving the urethral folds. The former were spread wide apart at 7.5 wpc (Fig. 4.1, A), but were in closer proximity at 9 wpc (Fig. 4.1, B). The clear beginnings of the scrotum are taking form by 10 wpc, where significant fusion and enlargement of the folds is evident, although there is still some separation along the ventral axis of the penis at this time (Fig. 4.1, C). This separation was absent later in

development and both aspects were fused at 14 wpc (Fig. 4.2). Similarly, the urethral folds also demonstrate progressive fusion as a function of time (Fig. 4.1). The urethral folds appear fully patent at 7.5 wpc, but then display increasing fusion along the ventral axis of the penile shaft from the proximal end between 9 and 10 wpc, and complete closure of the urethra in later development (Fig 4.2). The glans has also developed from the genital tubercle anlagen by 10 wpc, widening and extending up from the distal region of the phallus, with complete fusion along its ventral axis.

During this period the general morphology of the external genitalia in 46,XX fetuses was initially very similar (Fig. 4.1), with dimorphism apparent as development progressed. At 7.5 wpc there was no obvious difference in the appearance of the female external genitalia compared to that of males (Fig. 4.1, D). In later female specimens (Fig. 4.1, E and F), the labioscrotal folds remained separated on either side of the phallus between 7.5 and 10 wpc (eventually developing into the labia majora in a normal female). Furthermore, the urethral folds showed no fusion as development progressed, other than at the extreme distal and proximal ends (permitting formation of the labia minora and the vulval vestibule).

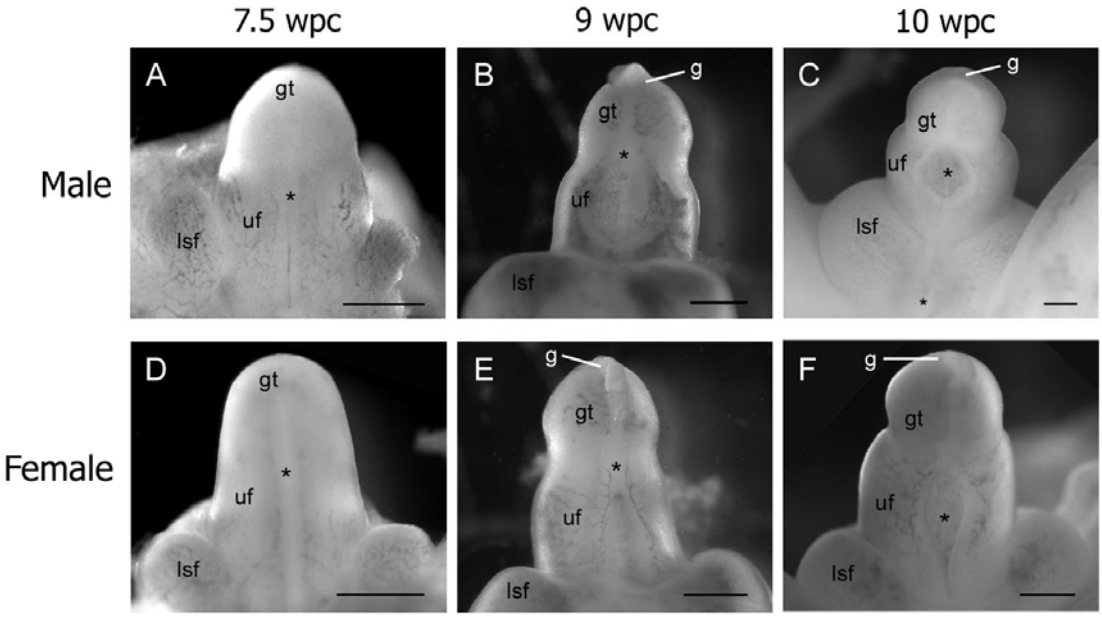

Figure 4.1: Transillumination micrographs of the human fetal external genitalia during the period of sexual differentiation.
A-C, 46,XY male external genitalia: A, Undifferentiated, indifferent external genitalia with open urethra. B and C, Differentiation of labioscrotal folds and fusion of urethral folds at 9-10 wpc. D-F, 46,XX female external genitalia lacking major differentiation of the labioscrotal folds and fusion of the urethral folds between 7.5 and 10 wpc. gt, genital tubercle; uf, urethral folds; lsf, labioscrotal folds; g, developing glans. Asterisks mark patent regions. Scale bars: 500 μm.

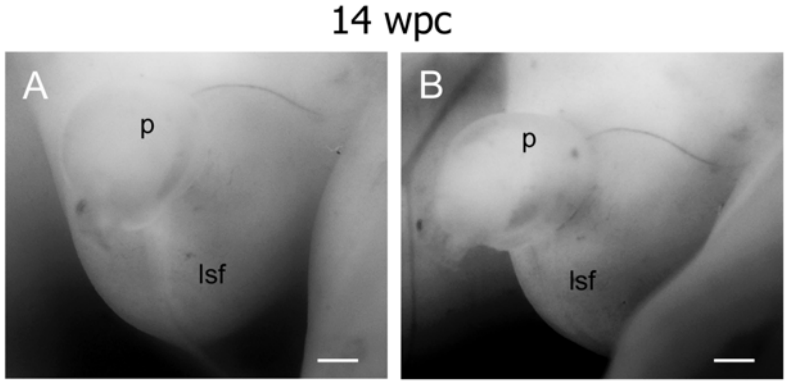

Figure 4.2: Transillumination micrographs of the human fetal male external genitalia at 14 wpc
A and B, the 46,XY male external genitalia viewed from different angles just following major sexual differentiation. At this stage fusion of the labioscrotal folds and urethral folds has taken place along the midline, to form the scrotum and shaft of the penis, respectively. p, early phallus/penis formed from the genital tubercle and fusion of the urethral folds; lsf, labioscrotal folds. Scale bars: 500 μm.

4.3.2 Expression of 5 alpha-reductase type 2 in the developing 46,XY and 46,XX human external genitalia

4.3.2.1 TISH optimization

Prior to analysis of SRD5A2 expression, the detection of *insulin* transcript in developing pancreas was used to optimize the non-radioactive mRNA tissue *in situ* hybridisation (TISH) technique utilised in this part of the study and as a positive control for experiments on SRD5A2 (Fig. 4.3). Digoxyigenin labelled antisense probes for insulin readily produced staining following optimization of the technique (Fig. 4.3, A), revealing the locations of islet formation (Fig. 4.3, B). As would be expected, the use of sense probes showed no staining in the same area of pancreas tissue (negative control).

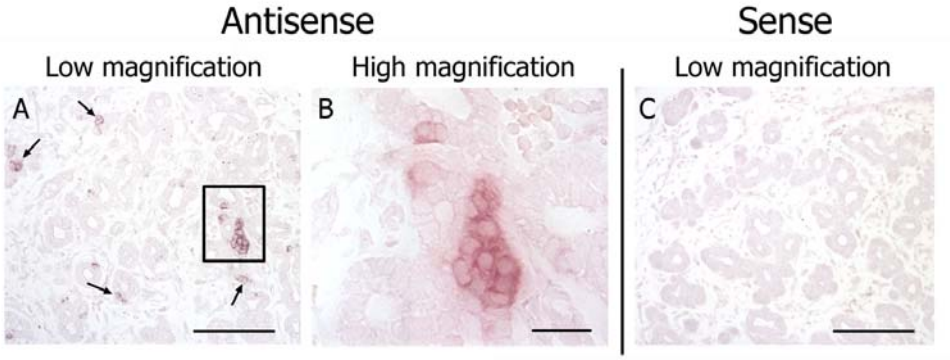

Figure 4.3: TISH positive control - Insulin expression in section of fetal pancreas
A boxed area indicates magnified region in B. Arrows indicate areas of most intense insulin expression. Sense probe staining shown in C. 100 µM scale bar on low magnification micrographs and 10 µM on B. Probe dilution 1:20,000.

4.3.2.2 TISH analysis

Representative TISH staining of SRD5A2 during sexual differentiation is shown in Fig. 4.4. SRD5A2 transcript was detected in the external genitalia of 46,XY fetuses at 8.5 wpc (Fig. 4.4, A & B), where it is strongest in the epithelial cells of the genital skin and urethral plate, and also in cells of the corpora cavernosa anlagen, with weaker expression in the stromal cells. Prior to 8.5 wpc, SRD5A2 expression was not detected (data not shown). Detection of staining indicative of

SRD5A2 expression was increased at 11 wpc (Fig. 4.4 D), particularly in the urethral plate around the newly forming urethra and in the cells of each developing corpus cavernosum. The intensity of staining in the stromal cells relative to the sense probe also appeared increased in later specimens. Detection in the 46,XX external genitalia was generally weaker, but surprisingly still noticeable in some areas. SRD5A2 transcript was not as prominent in the urethral plate relative to the 46,XY tissue, although it is still quite distinct in the genital skin and, to a lesser extent, in the cells of the developing corpora cavernosa (Fig. 4.4, G and H). Expression of SRD5A2 was only apparent in 46,XX fetuses at 10 wpc or older. Table 4.1 summarizes the expression patterns of SRD5A2.

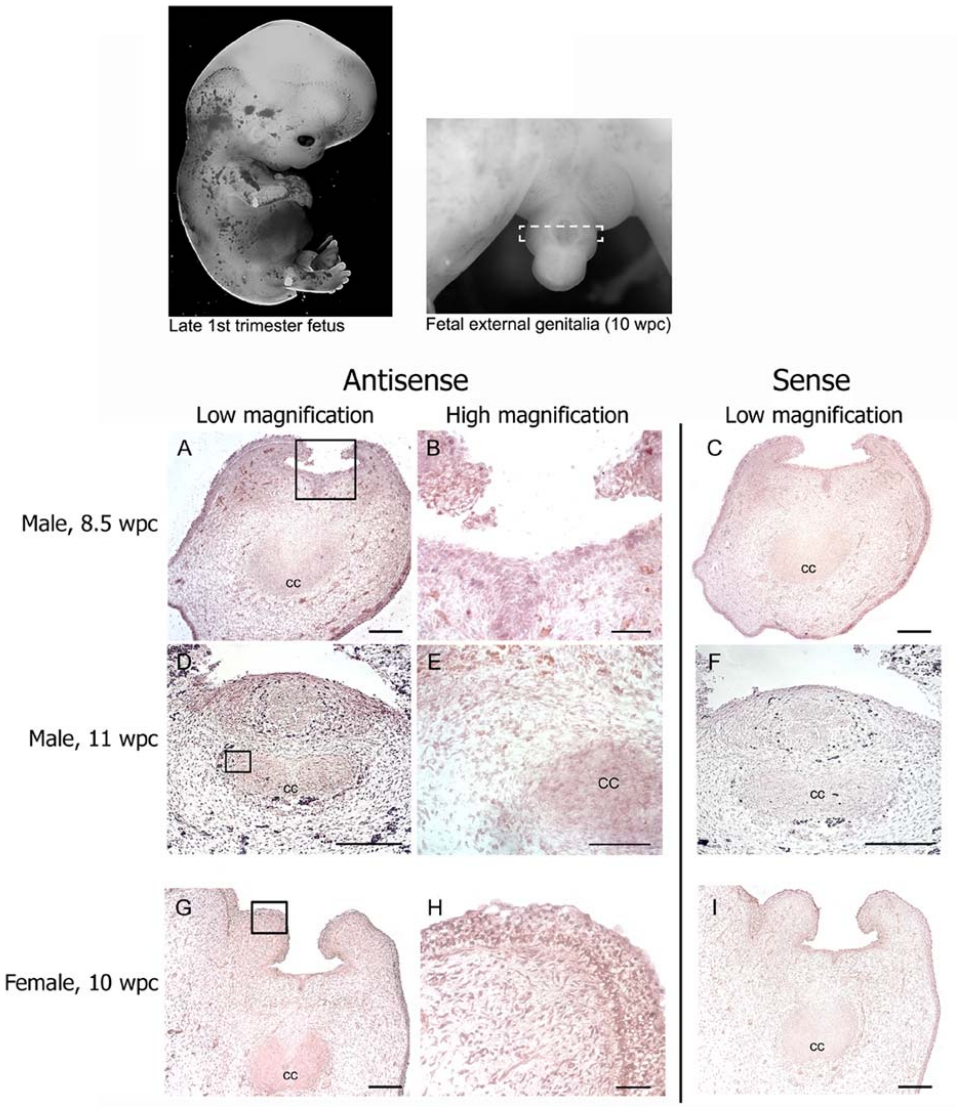

Figure 4.4: Expression of SRD5A2 in 46,XY male and 46,XX female human external genitalia

Transverse sections through the phallus. Probe dilution: 1:20,000 throughout. CC, developing corpora cavernosa. Boxed areas indicate region magnified in adjacent picture (right). 100 μM scale bar at low magnification and 20 μM for high magnification micrographs.

4.3.3 Expression of AR in the developing 46,XY and 46,XX human external genitalia

Representative immunohistochemical staining patterns of the AR in the developing external genitalia between 7.5-11 wpc are shown in Fig. 4.5. AR expression was nuclear and was more robustly detected as a function of time, with the first signs of expression from 7.5 wpc and very pronounced expression by the end of the period of sexual differentiation. At 8.5 wpc AR expression in 46,XY fetuses during sexual differentiation is strongest in the corpora cavernosal tissue and stromal cells around the developing urethra (compared to the broader stroma), and generally weakest in the surface genital epithelial layer. At 11.5 wpc in 46,XY specimens (Fig 4.5, I and J) there is relatively strong expression in the epithelial cells of the skin and developing urethra, approaching the same intensity as the strongest stromal stain.

In the 46,XX external genitalia, AR immunoreactivity was strongest in the stromal cells surrounding the developing urethra, and AR was weakly detected in all other areas of the tissue. In contrast to the 46,XY specimens, AR expression remained almost completely absent in the cells of the developing corpora cavernosa, although this area was still morphologically similar in older specimens of 46,XY and 46,XX external genitalia (Fig. 4.5, K). In and around the region of the urethral plate, the 46,XX specimens displayed moderately stronger AR detection than in the general stroma. However, this relative difference was not of the magnitude observed in the 46,XY specimens. Generally detection of AR expression in the 46,XX external genitalia was more prevalent in younger relative to older specimens (Fig. 4.5, C and D), such that peak AR expression in female fetuses was prior to 10.5 wpc. However, all detections in 46,XX specimens was nuclear, implying transcriptionally active, ligand-bound AR and the presence of androgen in developing female external genitalia. Table 4.1 summarizes these expression patterns of AR.

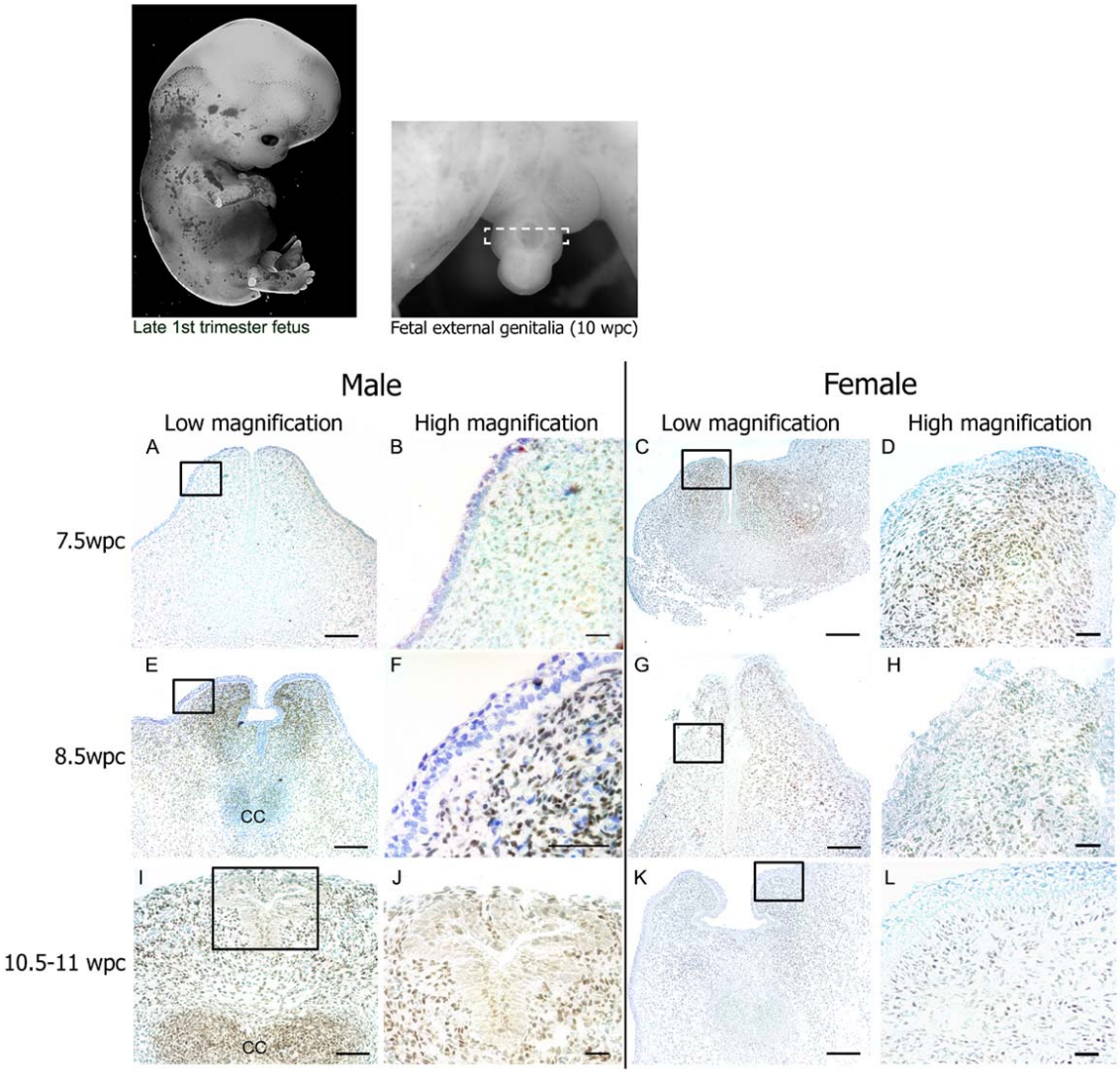

Figure 4.5: Expression of AR in 46,XY male and 46,XX female human external genitalia

Transverse sections through phallic tissue. CC, developing corpora cavernosa. Boxed areas indicate magnified region in adjacent high magnification micrograph (right). Inset (top) shows representative fetus and approximate location of tissue sections taken from external genitalia. 100 μM scale bar at low magnification and 20 μM for high magnification micrographs, apart from figure 'F' where scale is set at 50 μM

4.3.4 Expression of SOX2 in the developing 46,XY and 46,XX human external genitalia

Representative immunohistochemical staining of SOX2 at 7.5-11 wpc in the developing fetal external genitalia is shown in Fig. 4.6. SOX2 expression was clearly detected most intensely in the surface epithelial cells overlying the external genitalia, and in the developing urethra. This pattern was similar at 7.5 wpc and 8.5 wpc. However, the skin epithelium at 10.5-11 wpc in 46,XY tissue

was only weakly SOX2-positive, with conspicuous strong immunoreactivity limited to the urethral epithelial cells (Fig. 4.6, I and J). Moreover, older male fetuses do appear to express limited amounts of SOX2 in each developing corpus cavernosum. There appears to be no difference in the expression pattern in males and females, although during the latter stages of sexual differentiation SOX2 reaction in 46,XX fetuses showed greater intensity in basal cells of the differentiating epithelium compared to the superficial epithelial cells. Table 4.1 summarizes the expression patterns of SOX2.

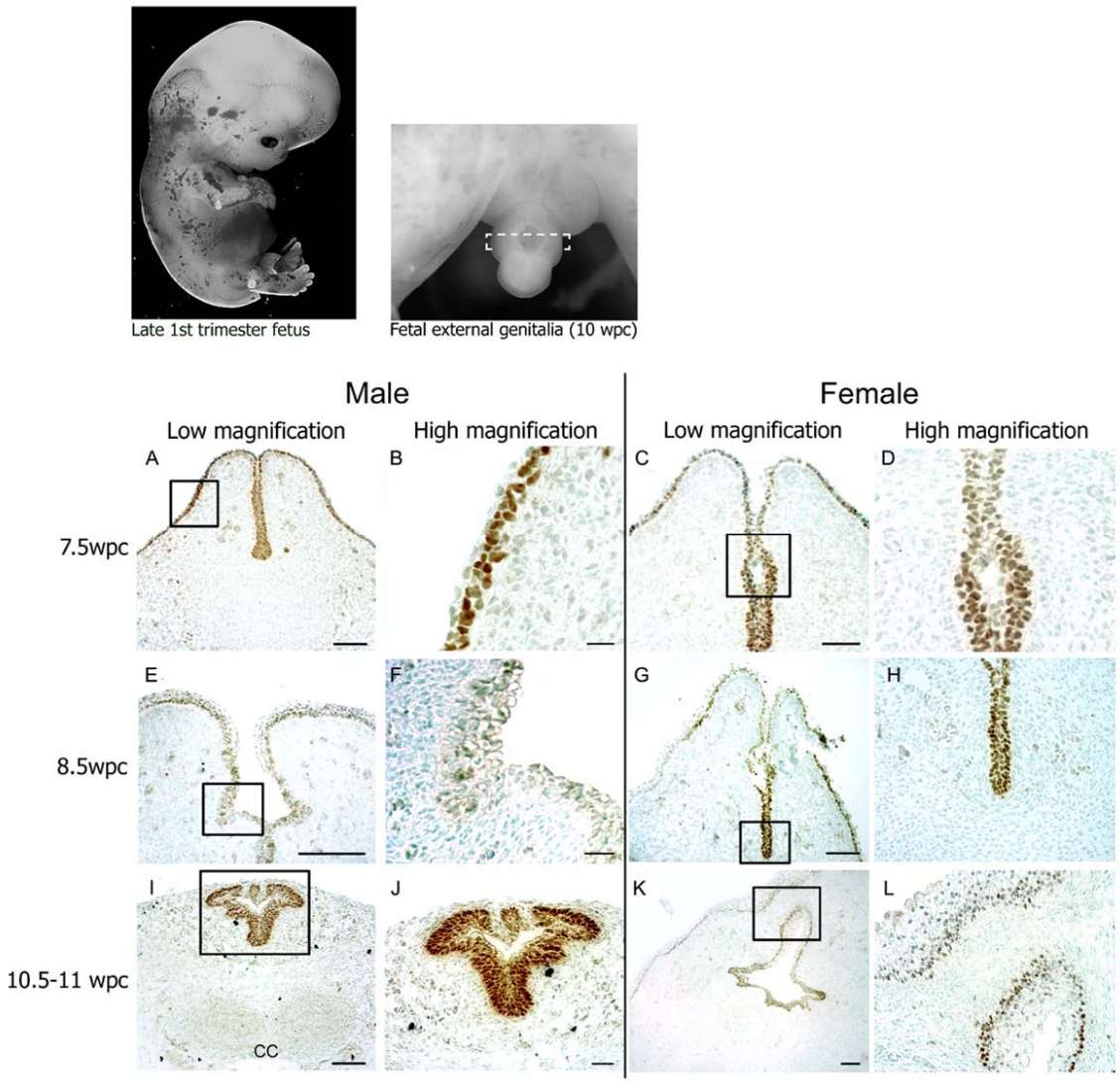

Figure 4.6: Expression of SOX2 in 46,XY male and 46,XX female human external genitalia

Transverse sections through phallus. CC, developing corpora cavernosa. Boxed areas indicate magnified region in adjacent high magnification micrograph (right). Inset (top) shows representative fetus and approximate location of tissue sections taken from external genitalia. 100 μM scale bar at low magnification and 20 μM for high magnification micrographs.

4.3.5 Expression of SOX9 in the developing 46,XY and 46,XX human external genitalia

Representative immunological staining patterns of SOX9 at 7.5-11 wpc in the human fetal external genitalia are shown in Fig. 4.7. Within both 46,XY and 46,XX external genitalia SOX9 reactivity was strongest in the skin, developing urethral epithelium and corpus cavernosal tissues, and only weakly positive in stromal cells, particularly in the urethral plate region. Furthermore, both males and females SOX9 expression becomes restricted to the basal cells of the epithelium than in the superficial epithelial cells; emphasised at later stages as the skin differentiates into a thicker structure. Levels of SOX9 expression in male and female external genitalia remained relatively constant throughout sexual differentiation. Table 4.1 summarizes the expression patterns of SOX9.

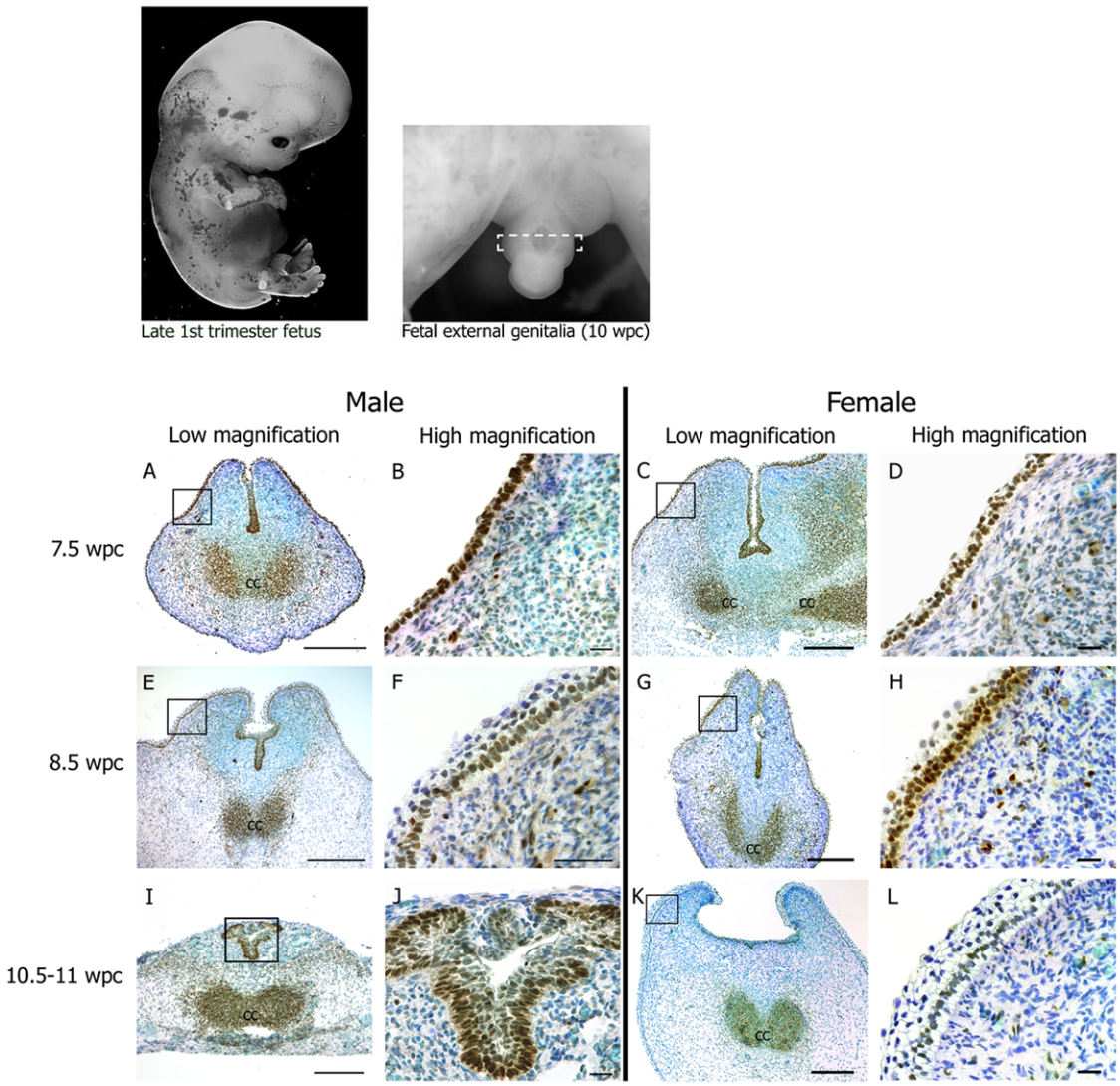

Figure 4.7: Expression of SOX9 in 46,XY male and 46,XX female human external genitalia

Transverse sections through phallus. CC, developing corpora cavernosa. Boxed areas indicate magnified region in adjacent high magnification micrograph (right). Inset (top) shows representative fetus and approximate location of tissue sections taken from external genitalia. 200 μM scale bar at low magnification and 20 μM for high magnification micrographs, except F where scale bar 50 μM.

Tissue	SRD5A2		AR		SOX2		SOX9	
	male	female	male	female	male	female	male	female
Skin epithelium	++	++	+ ↑	+	+++ ↓	+++	+++	+++
Urethral epithelium	++ ↑	+	+ ↑	+	+++ ↑	+++	+++	++
Urethral plate	+++ ↑	+	+++ ↑	+++ ↓	–	–	+	+
Corpora cavernosa	+++ ↑	++	++ ↑	+	+ ↑	–	+++	+++
General stroma cells	+	+	++ ↑	++ ↓	–	–	++	++

Table 4.1: Overview of immunohistochemical expression of SRD5A2, AR, SOX2 and SOX9 in 46,XY and 46,XX human external genitalia during sexual differentiation
The number of + symbols is an approximate indication of the average amount of each protein detected over the course of the gestation period examined in each region of the external genitalia. A - symbol indicates that no expression was detected and arrows denote the overall increase/decrease in expression between 7.5 and 11 wpc.

4.4 Discussion

This chapter describes the histological localization of AR, SRD5A2, SOX2 and SOX9 within the developing 46,XY and 46,XX external genitalia during the critical period of sexual differentiation. The results revealed a tissue-specific expression pattern for each of these important proteins, all known to be associated with sexual development and/or the organogenesis of the external genitalia. AR expression was maintained and more widespread in 46,XY fetuses compared to female specimens, consistent with its importance for morphogenesis of the penis. Immunoreactivity was most prominent in male stromal cells, particularly around the urethra and corpora cavernosa, with intensity increasing in later development. Furthermore, because AR expression in cells tends to coincide with that of SRD5A2, detection of which was also greater in 46,XY tissue, this suggests that these cells could represent key sites of DHT synthesis in males. In contrast, both SOX2 and SOX9 generally demonstrate a similar equivalent expression pattern in males and females, localizing strongly in the epithelial cells of the skin and urethra. This suggests a potential role for these transcription factors in the general formation and remodelling of genital tissue in these areas, regardless of chromosomal or genetic sex, more so in the case of SOX9, which also displayed high immunoreactivity in the primitive corpora cavernosa of both sexes. On the whole, the expression of each of the four

proteins increased as a function of gestational age in 46,XY fetuses, but tended to remain constant or decrease in 46,XX external genitalia. These results raise interesting questions about the function of these key proteins in sexual development, and their relationship to the different hormone milieu in which they reside during various stages of development in each sex.

4.4.1 The function of AR and SRD5A2 in the developing external genitalia during sexual differentiation

The AR is a signal-transducing steroid hormone receptor required for normal sexual differentiation and development of the male phenotype. Normally resident in the cell cytoplasm, it is a ligand-activated transcription factor, which once bound by androgen, translocates to the nuclei of cells and binds to regulatory elements of target genes (Rundlett *et al*., 1990). Specifically, the binding of its ligand results in a conformational change in the AR protein and disaggregation of heat shock proteins, followed by dimerisation with a second AR protein, transfer into the nucleus and finally binding to specific DNA response elements via a conserved DNA-binding domain (Khorasanizadeh and Rastinejad, 2001). In the present study, AR immunoreactivity was almost exclusively in the nuclei of 46,XY cells in fixed tissue sections implying a receptor-ligand interaction had occurred. Interestingly, this was also true in cells of 46,XX fetuses during early development, suggesting that AR is not only present but that bioactive androgens are also secreted during the normal formation of the female external genitalia. However, the visible decrease in AR expression observed in later development of 46,XX tissue, suggests that whatever factor drives an increase in AR expression in males, is absent in females.

The conspicuous presence, and broad distribution, of AR in the developing 46,XX external genitalia was somewhat surprising in the general context of sexual differentiation. The traditional view of female genital development is that of a passive or ‘default’ process, which takes place in the absence of fetal gonads, amid an ‘androgen-free’ environment (Jost, 1972; Achermann and Hughes, 2008). In light of this, it seems counterintuitive to find nuclear AR during female development. Generation of a normal female phenotype necessitates strictly regulated androgen exposure to prevent malformation (Asby

et al., 2009). It could be argued that expression of AR can only risk potential pathological development by facilitating a means of virilisation. Unfortunately most of our current understanding of normal female sexual differentiation is derived from clinical disorders of excess androgen. Surprisingly little is known about physiologically normal development. What is undeniable is that appropriate growth, vascularisation and innervation of the developing female external genitalia has to be initiated during the first trimester, in order to attain normal development (Sajjad *et al*., 2004; Achermann and Hughes, 2008).

There is some limited data available on AR expression in early female development. AR immunoreactivity has been observed at the end of the first trimester in female external genitalia, which then diminishes, and is generally only evident in the clitoris beyond the start of the second trimester (Kalloo *et al*., 1993; Shapiro *et al*., 2000). The relevance of AR expression in the female genital tract following 12 wpc is somewhat academic in terms of the effects of androgens, as by approximately 12 wpc aromatase (CYP19A1) expression begins in both males and females, limiting the supply of AR ligand and reducing the effects of potent androgens by converting them into oestrogenic hormones (Grumbach and Auchus, 1999). Hence it is the earlier expression of AR that is most significant physiologically and pathologically. From clinical data, we are aware that female fetuses that undergo abnormal exposure to androgens during the first trimester, can exhibit severe virilisation of the external genitalia, such as in cases of CAH due to CYP21A2 deficiency (Walsh *et al*., 1978; Speiser and White, 2003; Berenbaum and Bailey, 2003). Abnormal androgen exposure after this time affects clitoris development only, which is consistent with previous characterisation of AR expression. Abnormal virilisation in females due to androgen excess necessitates the presence of functional AR, therefore perhaps it is not wholly surprising to discover its expression in 46,XX tissue in the present study. However, the occurrence of nuclear AR implies an additional propensity for androgen production by normal female fetuses and that these androgens are at concentrations sufficient to cause translocation of AR to the nucleus.

Some androgen production has been shown previously in 46,XX fetuses during the first trimester. Our group demonstrated this originated from adrenal gland

steroidogenesis (Goto *et al*., 2006). The present findings seem to substantiate the production of adrenal androgens during early female development, but moreover appear to represent a new paradigm of female development, whereby physiological androgen secretion is potent enough to drive AR nuclear translocation in the external genitalia and thus presumably facilitate expression of AR target genes. Whether the AR is absolutely necessary for normal female development or not is unknown. The marked similarities apparent in the gross morphology of the external genitalia of both males and females during sexual differentiation, suggest both genders are subjected to similar influences early in development and implies there could be a normal role for AR and androgens in female development. Thus far there exists anecdotal evidence to suggest that defective AR can lead to under-development of the female external genitalia (data not shown), but this is not a common observation (McPhaul, 2002; Shiina *et al*., 2006). In contrast, the fact that complete androgen insensitivity in males with AR mutations develop 'normal' female genitalia, would indicate that AR is not vital to progression towards a female phenotype (Brown *et al*., 1990; Wilson, 1992). Thus there is an obvious need to try to understand the significance of androgens in normal female development. Furthermore, it will be important to investigate the relative amounts of androgen found during normal development of males and females, along with a measure of responsiveness in target cells. Ultimately this would give clues as to where the separation exists between normal sex differentiation and pathological virilisation. This topic is explored in Chapter 5.

The development of the male external genitalia is dependent on androgens. It was therefore not surprising to detect AR expression in the 46,XY tissue and its presence in the nuclei of positively-stained cells. Initial detection of AR coincided with the surge of testicular testosterone biosynthesis that begins at approximately 8 wpc and peaks at around 14 wpc (Siiteri and Wilson, 1974; Drews *et al*., 2001). A rise in AR, and also SRD5A2 detection was then observed after 8 wpc and this increased as a function of gestational age in most cell types, mirroring the normal androgen surge found in males. A lack of data from previous studies specifically recording changes in AR and SRD5A2 temporal expression during human first trimester development means this correlation has

not been documented previously. However, a similar temporal increase in AR was suggested in human penile urethral epithelium during second trimester development (Kim *et al*., 2002) and also in the mouse genital tubercle, where analysis of AR mRNA expression demonstrated a gradual increase between gestational days 12-18 (Agras *et al*., 2006).

The association between AR and SRD5A2 expression in 46,XY external genitalia, and known patterns of androgen production in male fetuses, suggests these two phenomena could be directly related. One possibility could be that a downstream effect of activation of AR by androgens is the biosynthesis of more testicular androgen via an endocrine signal; thus producing more substrate for AR binding and continually driving forward androgen production during early development. Alternatively, liganding of AR could promote transcriptional activity whereby expression of AR itself is induced through a self-regulatory, positive feedback mechanism. Some evidence for AR self-regulation has been found previously (Grad *et al*., 2001). This theory would be consistent with events in the developing 46,XX external genitalia, which demonstrates AR translocation, if a threshold level of androgens was not ordinarily reached for positive feedback. Thus self-regulation and virilisation would be dose-dependent and rely on a tipping-point that is not achieved in normal female development. With so little data available on the downstream targets of AR in normal tissue it is difficult to fully explore the correlation between androgen production, AR expression and its consequences. AR auto-regulation is investigated further, and discussed in greater depth, in Chapter 5.

Because differentiation of the male external genitalia is dependent on androgens, particularly DHT (Wilhelm and Koopman, 2006), detection of cells expressing AR and SRD5A2 in 46,XY external genitalia point to key sites of virilisation, and provide clues to which tissues and cell types undergo androgen-mediated growth and differentiation that lead to the male phenotype. For example, the presence of strong AR and SRD5A2 immunoreactivity in periurethral cells indicates that DHT may directly influence the folding and fusion of the urethral folds to form the urethra and ventral aspect of the penis; especially in light of their absence in the same region of 46,XX tissue. Similarly, conspicuous AR and

SRD5A2 expression in the stromal cell aggregations of the primordial corpora cavernosa suggest a role for DHT in mediating differentiation and proliferation of cells, which in this instance results in the formation of a relatively large area of densely packed cells characteristic of the early corpora cavernosa (Bastos *et al*., 1999). Furthermore, the appearance of AR-positive urethral epithelium cells in older 46,XY fetuses, where the urethral epithelium has become contiguous, could indicate that androgens play a role in canalization of the urethra after fusion of the urethral folds.

The patterns of AR localization observed in 46,XY fetuses in the present study differ from some previous findings. For example, Kim and colleagues (Kim *et al*., 2002) reported that fetal genital skin epithelial cells are consistently positive for AR and that stromal cells are largely negative. However, their study examined expression at a slightly later stage of development (12-14 wpc and 16-20 wpc), which may account for this difference. Furthermore, Blauer and colleagues (Blauer *et al*., 1991) also showed AR-positive cells in the genital skin, in this instance in adults, suggesting that AR has a function only in fully differentiated skin and is possibly limited to expression in dermal structures, such as hair follicles, where androgen has influences on hair growth (Trueb, 2002; Alonso and Rosenfield, 2003).

Other studies have observed an AR expression pattern closer to the current data. One such study, which again examined AR expression during second trimester development (18-20 wpc), demonstrated skin epithelial cells did not express AR and the periurethral stromal cells were strongly positive for AR (Kalloo *et al*., 1993). Similarly, an examination of first trimester urethral fold tissue yielded data indicating strong AR immunoreactivity in stromal cells from 8 to 12 wpc, but little AR expression in the urethral epithelium prior to 11 wpc (Sajjad *et al*., 2004). Animal studies have also revealed that AR expression is mostly mesenchymal early on in sexual differentiation, and only later appears in the epithelium (Cooke *et al*., 1991; Bentvelsen *et al*., 1995). These studies, along with the present findings, emphasise the notion that AR expression is probably more important in highly differentiated genital skin once it is established, and not vital for bringing about the initial differentiation. However, this does not

preclude some influence by androgens in remodelling genital skin, as an effect could still be exerted indirectly through altered gene expression in adjacent stromal cells. It has been shown that mesenchymal cells are capable of influencing epithelium development via interactions with the extracellular matrix and expression of tissue-specific secretary proteins (Sharpe and Ferguson, 1988; Hay and Zuk, 1995). This type of interaction can regulate epithelial differentiation and proliferation, and has been demonstrated in humans and animals during the development of numerous organ systems, including the genitourinary tract (Chung and Cunha, 1983; Kedinger *et al*., 1986; Kurzrock *et al*., 1999; Nakaya and Sheng, 2008).

The pattern of SRD5A2 detection observed was largely consistent with what has been detected previously in older fetuses. For example, between 16 and 22 wpc, SRD5A2 was shown to be strongly localized to the stromal cells of the developing corpora cavernosa and urethral plate, particularly within the region that corresponds to urethral fold fusion (Levine *et al*., 1996; Kim *et al*., 2002). This is consistent with the strong staining in the corpora cavernosa and urethral plate found in the present study. The SRD5A2 detection in the periurethral cells was especially strong towards the latter end of sexual differentiation (i.e. 8.5-11 wpc), where it co-localized with conspicuous AR staining. Taken together this indicates that DHT may be actively concentrated in this region, prompting robust AR effects that facilitate urethral fold fusion and urethral seam remodelling. A vital importance for SRD5A2 in this region during sexual differentiation would explain why deficiencies or mutations in the *SRD5A2* gene result in varying degrees of hypospadias (Andersson *et al*., 1991; Can *et al*., 1998; Silver and Russell, 1999; Sasaki *et al*., 2003; Achermann and Hughes, 2008).

4.4.2 The function of SOX2 and SOX9 in the developing 46,XY and 46,XX external genitalia during sexual differentiation

The SOX family of transcription factors comprises 20 different genes that have been widely studied during fetal development and are known to impinge upon cell fate and differentiation in a number of developmental processes, such as neurogenesis and skeletogenesis (Lefebvre *et al*., 2007). The current data

represents the first time that SOX2 and SOX9 expression has been studied in the developing external genitalia of human 46,XY or 46,XX fetuses. As was apparent for the AR and SRD5A2 data, a characteristic cell-type-specific expression pattern was evident from the immunohistochemical staining of SOX2 and SOX9, which is largely maintained throughout the period of sexual differentiation, albeit with some small alterations in intensity. Specifically, both transcription factors localized to the nuclei of epithelial cells within the developing external genitalia, including the epithelial cells of the urethral plate and luminal cells of the male urethra following urethral fold fusion. This nuclear expression is consistent with an active SOX transcription factor, and with marked localization in and around the urethra, could suggest an importance for SOX2 and SOX9 in urethra formation and urethral seam remodelling. It is interesting to find a pattern that persists throughout sexual differentiation, and in both genders, as this would suggest that the expression of these genes are unaffected by the changing hormone milieu associated with early development, and in particular the presence or absence or androgens. However, differing functions for these proteins could still occur during different phases of development or differ between sexes, due to for example the influence of co-factors or distinct down stream targets, which are regulated temporally or through virilising signalling pathways.

Closer analysis of the localisation of SOX2 and SOX9 in both sexes reveals that expression within the epithelium is limited to the basal epithelial cells. In younger specimens both proteins appeared to be expressed throughout the entire skin and urethral epithelia. However in older specimens, where maturation and cell differentiation of tissues has begun in earnest, expression was limited to only the basal epithelial cells, and no expression is found in the more superficial cells that emerge during later development. This is consistent with the association of SOX2 protein with ‘stemness’ and pluripotency during early development (Takahashi and Yamanaka, 2006; Wernig *et al*., 2007; Tay *et al*., 2008) and the role of SOX9 in maintaining stem cell compartments in hair follicles and acting to facilitate cell differentiation, including that of fetal Sertoli cells (Morais da *et al*., 1996; Blache *et al*., 2004; Wang *et al*., 2007a). Therefore this could imply a function for both proteins maintaining a population of undifferentiated epithelial

cells, which enables the generation of the upper layers of the genital skin, and possibly also facilitating tissue remodelling events such as fusion of the urethral folds.

In addition to epithelial expression, SOX9 also strongly localizes to the developing corpora cavernosa. In light of a well documented associated between SOX9 and bone formation, due to SOX9's effects on chondrocyte differentiation (Bell *et al*., 1997), this expression could be an evolutionary relic of os penis (baculum) formation. However, preliminary study of collagen type II expression revealed no localisation in the corpora cavernosa (data not shown), making this unlikely. Alternatively, expression in the corpora cavernosa could indicate SOX9's function to do with cell proliferation. A high level of proliferation is predicted in the corpora cavernosa based on the large, cell-dense character of this region observed in the male tissue. This could be facilitated by SOX9, which has known mitogenic activity and has been shown to stimulate expression of growth factors (Schaeffer *et al*., 2008). This putative role becomes more likely when we consider the reported induction of SOX9 expression by androgens (Schaeffer *et al*., 2008) and the high concentration of AR detected in the developing corpora cavernosa. If SOX9 was providing a proliferation stimulus, driven by signalling from AR, this would provide an explanation for the rapid proliferation in this region in males and the relatively weaker expression of SOX9 in 46,XX corpora cavernosa, which was particular evident in older specimens when androgen levels would be most dimorphic between males and females.

The lack of any previous data on *SOX2* or *SOX9* expression in the external genitalia means there is no direct reference point for interpreting the current findings; making it difficult to substantiate any theories regarding their putative function. However, prior studies have made it clear that both SOX2 and SOX9 have some role in sexual development and could contribute to the formation of the external genitalia. The involvement of SOX2 in the development of the genitals is apparent primarily from studies of anopthalmia-esophageal-genital syndrome (AEG). Affected infants (and animals) commonly display under-virilisation of the male external genitalia, alongside a range of other congenital abnormalities, due to mutations in the gene encoding SOX2 (Kelberman *et al*.,

2006; Williamson *et al*., 2006). Mutations in *SOX2* were first found to be responsible for AEG as recently as 2003 (Fantes *et al*., 2003), but known cases can be traced back over the past 20 years (Arroyo *et al*., 1992). There are approximately 23 novel *SOX2* mutations now recognised as being associated with AEG (Wang *et al*., 2008a), all of which result in at least some form of genital tract abnormality. This ranges from cryptorchidism (Arroyo *et al*., 1992) to micropenis and varying degrees of hypospadias (Ulman *et al*., 1996; Bardakjian and Schneider, 2005).

The current data suggests that the role of SOX2 in sexual development could relate to tissue remodelling and maintenance of the character of the skin's basal epithelium. The clinical data has implied a link between SOX2 and hypospadias, and with clear expression of SOX2 in the genital skin epithelium, it is easy to speculate how SOX2 could directly or indirectly help facilitate urethral fold fusion either through influences on differentiation of superficial epithelial cells, proliferation of epithelial cells, or even apoptosis of epithelial cells at the urethral fold junction. This could occur directly under the control of SOX2 or under the influence of a secondary signal, such as AR. The general lack of knowledge on AR targets means it can not be ruled out that *SOX2* could be an AR target gene, and hence when mutated, genital abnormalities would arise even in the presence of normal androgen levels and AR. Microarray analysis has provided some very limited evidence to suggest that SOX2 expression in normal human genital fibroblasts is up-regulated by a short exposure to DHT, but that in cases of AIS this is not the case, therefore indicating that SOX2 could be a target of normal AR signalling (Holterhus *et al*., 2003). Prolonged exposure to DHT over a number of days did not have the same relative effect on SOX2 expression. Furthermore, the study also showed that baseline levels of SOX2 were increased in AIS individuals, suggesting that perhaps in the unliganded state, wild-type AR might actually down-regulate SOX2.

The similarities in SOX2 expression observed in 46,XY and 46,XX external genitalia somewhat detracts from the possibility of SOX2 as a target of AR; although it does not rule it out. Another possibility would be that SOX2 acts upstream of AR, consistent with instances of female AEG where genital

abnormalities have been recorded. While much rarer in the literature, reports of AEG in females have been documented, and although abnormal external genitalia appears to be less common in these patients, some reports have identified defects in the genital tracts of females (Chitayat *et al*., 1996). Such reports signify that the role of SOX2 in genital development is not limited to male virilisation and imply it possibly possesses a more fundamental importance, independent of interaction with high levels of androgens and AR. This fits well with limited evidence, chiefly from animal studies, that directly demonstrate that SOX2 is involved in a variety of other processes related to sexual differentiation, indicating its importance stretches beyond a key role in the development of the external genitalia. Specifically this includes associations with sex determination (Beverdam and Koopman, 2006; Chen *et al*., 2008b), genital ridge formation (Payen *et al*., 1997) and prostate development (Sattler *et al*., 2000; Kasper, 2008); and in adults SOX2 has also been associated with normal ovary function (Avilion *et al*., 2003) and, when down-regulated, erectile dysfunction (Sullivan *et al*., 2005). These examples propose SOX2 is a basic requirement of organogenesis and normal functioning in the genital tract.

Like SOX2, an implied importance for SOX9 in sexual development stems from analysis of mutant forms of the gene and the associated clinical data. Mutations in SOX9 are most commonly associated with the condition campomelic dysplasia (CMD; Foster *et al*., 1994; Cameron *et al*., 1996a; Pop *et al*., 2005). Thus far upward of 24 novel mutations of the SOX9 gene have been described that result in CMD (Meyer *et al*., 1997). A congenital disease, CMD is a short-limb skeletal dysplasia primarily characterized by bowing and angulation of long bones, along with other skeletal defects (Lee *et al*., 1972). The condition is also usually associated with anomalies of the tracheobronchial tree, and in two thirds of affected XY individuals, defects of the genital tract or complete sex reversal (Velagaleti *et al*., 2005). First described by Hovmoller and colleagues (Hovmoller *et al*., 1977), the manifestation of abnormalities of the external genitalia in 46,XY patients can present in varying degrees, including abnormal male external genitalia with bifid scrotum and hypospadias (Mansour *et al*., 1995), ambiguous genitalia (Cameron *et al*., 1996a), abnormal female external

genitalia with enlarged clitoris (Mansour *et al*., 1995), and phenotypically normal female external and internal genitalia (Hall and Spranger, 1980).

As well as the established connection between SOX9 and CMD, there is also mounting evidence to suggest that SOX9 is a downstream target of SRY (Morais da *et al*., 1996; Sekido and Lovell-Badge, 2008). Mutations in SOX9 therefore would have a fundamental impact on sex determination and in turn sexual differentiation, and this is probably what accounts for the DSD associated with CMD. Hence, unlike in the case of SOX2, it seems most likely that the connection between SOX9 and the development of the external genitalia is secondary to its regulation of testicular differentiation. However, direct study of SOX9, not focused on mutational analysis, has shown that SOX9 is expressed in the basal epithelial cells of adult prostate, where it interacts directly with AR protein and may also regulate AR gene expression (Wang *et al*., 2007a). It was suggested in this study that the role of SOX9 was to maintain the proliferative capability of basal cells or possibly regulate basal-cell specific proteins to aid in supporting the luminal epithelium. SOX9 could then be performing a similar role in the developing external genitalia, where it is expressed in a pattern reminiscent of that observed in the adult prostate. Previous data provides a substantial link between SOX9 and sexual development, and the current findings demonstrate the expression and presumed function of SOX9 in the developing external genitalia. However, its specific function in this regard still remains to be elucidated.

4.4.3 Conclusion

In this chapter the spatiotemporal expression patterns of SRD5A2, AR, SOX2 and SOX9 have been illustrated in the developing human external genitalia during the critical period of sexual differentiation, implying possible functions of these important proteins. It has been shown that AR expression increases in males between 8 and 12 wpc, and is nuclear in both male and female external genitalia during this period. The function of bioactive AR in females during sexual differentiation is not yet known. There is a clear need to elucidate the genetic targets of AR, in order to comprehend its function in normal differentiation of the male external genitalia, and investigate its functionality in the developing female fetus.

Chapter 5: Identification of androgen-regulated genes through the use of a genital fibroblast culture model

5.1 Introduction

As has been discussed in previous chapters, the development of the male genitalia during fetal life is induced by potent androgenic steroids (i.e. testosterone and DHT). In order for these androgens to correctly mediate sexual differentiation their common receptor, the androgen receptor (AR), is of paramount importance. There is understanding on AR ligand-receptor interactions (Farla *et al*., 2005; Marcelli *et al*., 2006; Kung and Evans, 2009), as well as an appreciation of the mechanisms by which liganded AR moves from the cell cytosol to the nucleus (Poukka *et al*., 2000; Kanno *et al*., 2007; Kaku *et al*., 2008; Cutress *et al*., 2008), where it is able to bind elements of the genome and regulate gene transcription. However, very little is known about which genes are regulated by AR in primary cells and hence what specific effects androgens have on their target cells that lead to the broad changes observed in tissue morphology and function mediated by androgens. This chapter begins to fill this gap, continuing where the two previous chapters ended, by investigating features of androgen-mediated signalling; the consequences that follow the production of androgen (studied in Chapter 3) and the translocation of AR to the nucleus of primary cells (characterised in Chapter 4).

For approximately two decades since the initial discovery of the oestrogen receptor (ER) in the early 1960s, it was generally thought that all unliganded steroid hormone receptors resided in the cytoplasm of cells until ligand activation prompted movement into the nucleus (Gorski *et al*., 1968; Jensen *et al*., 1968). However, during the 1980s and 90s, conflicting data were produced regarding the localisation of unliganded steroid hormone receptors (Welshons *et al*., 1984; Perrot-Applanat *et al*., 1985; Ennis *et al*., 1986; Liao *et al*., 1989; McGimsey *et al*., 1991), leading to dispute over the established model and whether unliganded steroid receptors were in fact located partly or entirely in the nuclear compartment. Further work finally confirmed that localisation of unbound AR is

cytosolic, proving the original theory was correct, at least for AR (Simental *et al*., 1991; Jenster *et al*., 1991); exceptions are present among other steroid hormone receptors, i.e. the ER and progesterone receptor (Tyagi *et al*., 2000). Through the re-examination of AR localisation in the cell, and the dynamic nature of the protein, further insight was gained into receptor/ligand interactions for AR.

Unliganded AR is located in the cytoplasmic compartment of cells bound to heat-shock proteins (HSPs) such as HSP70 and HSP90, which are in turn complexed to co-chaperone proteins such as FKBP52 (O'Malley, 1990; Nemoto *et al*., 1992; Fang *et al*., 1996; Heinlein and Chang, 2002; see Fig, 1.13). Upon androgen exposure, the liganding of AR induces an allosteric change in the AR protein structure that induces its dissociation from the accompanying proteins (Kuiper and Brinkmann, 1995; Hiort and Holterhus, 2000). The 'remodelled' AR in the AR-androgen complex is then able to dimerise with other similar AR-androgen complexes via the dimerisation motif which, in turn, presents a protein dimer suitable for translocation from the cytosol to the nucleus. It is thought this occurs because of the unmasking of the nuclear localization sequence within the AR protein structure that allows importin proteins to bind and transport AR homodimers through the nuclear pore complex (Jenster *et al*., 1993; Zhou *et al*., 1994; Kaku *et al*., 2008).

Once in the nucleus, the AR homodimer can interact with specific sequences within the genome via its DNA-binding domain (DBD). These high affinity binding sites are known as androgen response elements (AREs; Grad *et al*., 1999; Khorasanizadeh and Rastinejad, 2001). All steroid hormone receptors bind to hormone response elements (HREs) that consist of two palindromic half-site sequences organized as inverted six base pair repeats separated by three nucleotides; classically: 5'-AGAACAnnnTGTTCT-3' where the first and last bases in the sequence are most variable, and 'n' represents any base (Roche *et al*., 1992; Beato and Klug, 2000). Subtle differences in the HRE sequence and the position in the genome determine receptor specificity. AREs may comprise of classical inverted repeats but, more commonly, specificity for androgens has been associated with direct repeats or several contiguous binding sites, each

composed of sequences based on the consensus HRE (Claessens *et al*., 1996; Kasper *et al*., 1999; Verrijdt *et al*., 1999; Schoenmakers *et al*., 2000; Verrijdt *et al*., 2003). AREs are found in regions proximal to the promoter of target genes, typically several kilobases (kb) upstream of the transcriptional start site, within introns, but also occasionally in exonic DNA (Rundlett *et al*., 1990; Celis *et al*., 1993; Ho *et al*., 1993; Cleutjens *et al*., 1997; Devos *et al*., 1997; Thornton *et al*., 1998). This binding to 'target' DNA influences the transcription of these genes.

In order for AR to correctly regulate the transcription of target genes, a number of coregulatory proteins are required. These are thought to facilitate modification of the chromatin structure and/or help attach the AR homodimer to the basal transcriptional apparatus, either to augment or diminish gene expression (Agoulnik and Weigel, 2009). A multitude of putative AR coactivators (which increase transcription) and corepressors (which inhibit transcription) are known, including the relatively AR-specific coactivators SRA, ARA24, ARA54, ARA55 and ARA70 proteins (Yeh *et al*., 1998; Lanz *et al*., 1999; Kang *et al*., 1999; Miyoshi *et al*., 2003; Kurisu *et al*., 2006; Harada *et al*., 2008; Agoulnik and Weigel, 2009), and the p160 family of generic steroid receptor coactivators, which includes SRC-1 (Agoulnik *et al*., 2005), TIF2/SRC-2 (Agoulnik *et al*., 2006), and RAC3/AIB1/SRC-3 (Zhou *et al*., 2005). To date little is known regarding mechanisms of AR corepression and only a handful of AR-specific corepressors have been identified, including: cyclin D1 (Knudsen *et al*., 1999), calreticulin (Dedhar *et al*., 1994), and HBO1 (Sharma *et al*., 2000). Phosphorylation of AR that occurs during translocation also influences its ability to interact with cofactors and hence can further modulate the transcriptional activity of AR.

The biological consequences of androgen action result from the effects of the proteins encoded by AR-regulated genes (Hiort and Holterhus, 2000). Therefore identification of AR target genes would fundamentally expand our knowledge of the specific roles of androgens in normal and abnormal physiology. In recent years, potential target genes have been identified using genome-wide, as well as subtractive hybridization or enrichment approaches, typically using LNCaP cells (Bolton *et al*., 2007; Jariwala *et al*., 2007; Bao *et al*., 2008; Leveille *et al*., 2009).

Hitherto these studies emphasise that the wide-ranging effects of androgens on cellular functions are mostly dependent on increased transcription of target genes rather than AR-mediated downregulation. This could be an intrinsic feature of AR action, although it is arguably more likely a result of experimental bias.

The most well-studied AR target gene is the androgen-regulated prostate-specific antigen (*PSA*; officially known as kallikrein-related peptidase 3, *KLK3*) gene, which has been extensively investigated as a model of AR-mediated gene transcription (Balk *et al*., 2003). *KLK3* has been shown to contain multiple upstream AREs (Riegman *et al*., 1991; Schuur *et al*., 1996; Cleutjens *et al*., 1996; Cleutjens *et al*., 1997) and demonstrated some variance in expression correlated to androgen treatment of LNCaP cells (Montgomery *et al*., 1992; Xing *et al*., 2001; Wang *et al*., 2005; Massie *et al*., 2007). There has been much interest in the *KLK3* gene and its regulation by AR as a biomarker of prostate cancer. The prostate gland serves an essential role in male reproduction and is the most frequent site of non-skin cancer in men (Ntais *et al*., 2003). Androgens play an important role in the development and physiology of the prostate gland, as well as tumour cell proliferation (Hsing *et al*., 2008). The apparent dependence of prostate cancer on androgen production means androgen deprivation therapy is the primary treatment for metastatic prostate cancer. However, the relatively short response to androgen deprivation therapy prior to recurrence (typically <2 years), along with evidence for AR reactivation and increased AR expression in recurrent tumors (Bantis and Vasiliou, 2009), have encouraged efforts to increase our knowledge of the factors that regulate AR action and prostate AR target genes, to enable the development of improved prostate cancer therapies.

Another gene that has been debated as an AR target gene is the *AR* gene itself. It has long been proposed that the AR is capable of positive and/or negative autoregulation. A number of studies have shown attenuation of *AR* gene expression in various cell lines as a result of androgen exposure (Quarmby *et al*., 1990b; Shan, Rodriguez & Janne, 1990; Wiren *et al*., 1997; Yeap, Krueger & Leedman., 1999), while others have demonstrated the likely presence of 2-4 AREs in the coding region of the *AR* gene in a 6.5 kb region between exons 4 and 5 (Burnstein *et al*., 1995; Grad *et al*., 1999; Grad *et al*., 2001). However,

there has been much conflict of opinion as to whether autoregulation fundamentally promotes increased (Wiren *et al*., 1997) or decreased *AR* expression (Quarmby *et al*., 1990b), if the effects on AR gene expression are via modulation of gene transcription and/or translation (Shan *et al*., 1990; Wolf *et al*., 1993), and what mechanism underlies the effect. Furthermore, studies on autoregulation have tended to be performed in either abnormal cells (i.e. cancer cell lines, such as LNCaP) or animal cells, with very little effort made to examine this phenomenon in normal human cells, particularly in the developing external genital where its influence would be most pronounced and most relevant.

The central purpose of this chapter was to develop a model to identify putative AR-regulated genes in the external genitalia, in order to gain further insight into the specific functions of androgens in regulating human sexual differentiation. There is a lack of information regarding AR target genes in physiologically normal human cells, especially those from a fetal origin at the time of major sexual differentiation. To study AR target genes requires a robust and reliable model system. The initial phase of work aimed to establish an external genitalia primary cell culture model.

The most readily available facile assay for androgen responsiveness was to analyse AR translocation to the nucleus by immunocytochemistry. It was proposed that cultures would be assessed for homogeneity and ability to demonstrate AR translocation following each passage. RT-PCR and/or immunoblotting would demonstrate whether cells had retained their phenotype. The goal was to culture primary human fetal tissue from the external genitalia to a point where sufficient material would be available for use in microarray experiments at the lowest passage number. Hence the first task was to establish a protocol for the growth of monolayer cultures that led to consistent growth of cells from the fetal external genitalia. Genital fibroblasts taken from the fetal phallus were chosen for this to allow: direct study of an AR expressing cell type; analysis of cells from a structure that undergoes remarkable changes during sexual differentiation; and relatively straightforward culture *en masse*.

Once a culture system was established, further experiments would compare effects from different androgens at varying concentration and assess androgen-dependent changes in gene expression. The culture model would be investigated both in male fetuses, where androgen under-activity would result in under-virilisation, and female fetuses, where androgen over-activity would mimic the events of congenital adrenal hyperplasia due to CYP21A2 deficiency (CAH). For the initial analysis of potential androgen-regulated genes within the culture model a broad literature search was proposed to establish a list of genes suspected to be AR target genes. These genes would then be subject to analysis initially by RT-PCR.

5.2 Aims

- To establish a robust culture model using human fetal genital fibroblasts from the period of major sexual differentiation that permits the identical growth of cells with or without androgen, in a repeatable manner
- To validate the culture model
- To assess the effects of different androgens on AR intracellular trafficking and signalling
- To identify and analyse an initial cohort of putative AR target genes in the cell type where they were first identified (LNCaPs) and in primary human cells (culture model)
- To investigate the effect of androgen on AR expression and elucidate if AR autoregulation occurs in the developing external genitalia

5.3 Results

5.3.1 Developing a model system to culture genital fibroblasts in monolayer

Initial attempts to culture genital fibroblasts were not successful and a variety of culture methods had to be tested before finding a satisfactory means of culturing cells, which was robust and could be easily replicated. In essence this involved taking freshly dissected fetal external genitalia (mostly phallus tissue) and

mechanically disaggregating this with a scalpel to gradually release cells from the tissue macrostructure. Chemical digestion of this initial suspension was found to be dispensable so this step was omitted. The final optimised approach saw mechanically processed tissue resuspended in cell culture media and cultured directly in tissue culture flasks. Small lumps of tissue adhered and cell outgrowths eventually formed colonies of monolayer fibroblasts (Fig. 5.1). These cells were then passaged after 7-10 days using trypsin so that cells could be re-seeded onto new culture flasks. The dispersed cells showed increased growth of colonies, forming uniform confluent cell monolayers. Two or three passages maximised cell numbers over 5 – 10 T75 flasks. At each passage a small number of cells were plated on fibronectin coated glass slides to allow ICC analysis of AR expression and translocation in response to 10 nM DHT ('Check slides').

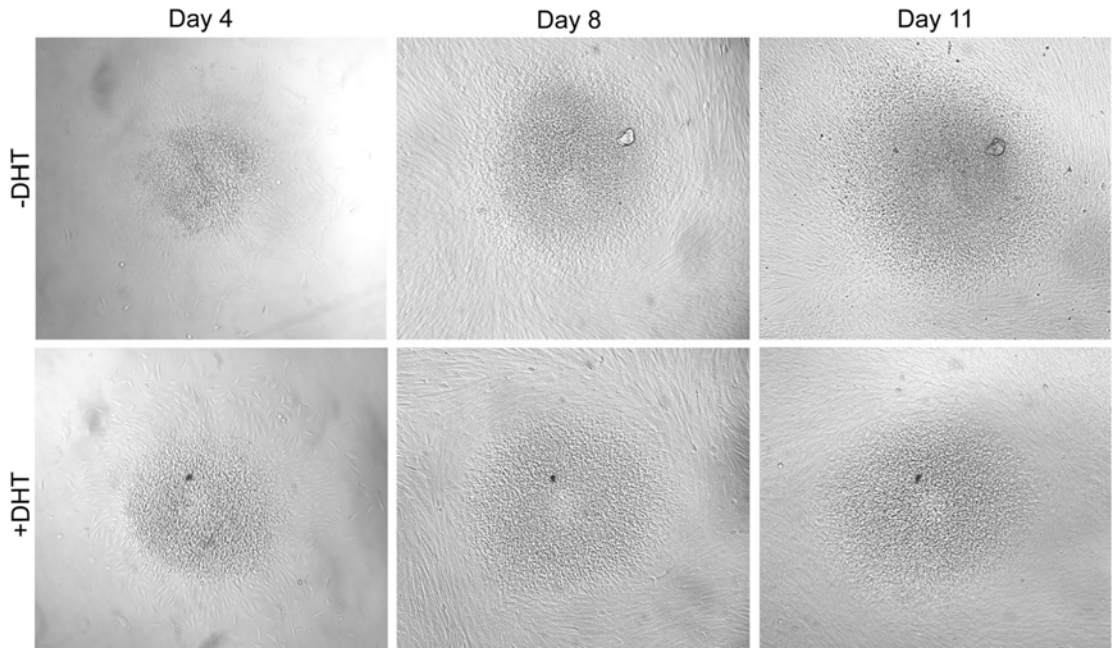

Figure 5.1: Examples of human fetal genital fibroblast cultures
Top row, untreated cells; bottom row, cells treated with 10 nM DHT. Cells are seen to be forming a colony around a tissue fragment at day 4, and expanding in a uniform monolayer at day 8 and 11.

These initial experiments to optimise conditions generated a robust protocol that enabled samples to be stored for further cell culture and analysis of RNA and protein, opening the way for a number of important experiments investigating AR functionality and the identification of androgen-regulated genes in both male and female fetuses. No differences were identified nor morphological differences seen in cultures from male or female fetuses. In total, 11 male and 13 female genital fibroblast cultures were established from fetuses at 7.5-10.5 wpc (Table 5.1)

Fetus	Age of fetus (wpc)	Chemical digestion?	Stored cells?	Stored mRNA?	Stored protein?
Male 1	8	✓	✓	✓	✓
Male 2	10.5	✓	✓	✓	✓
Male 3	8.5	✓		✓	
Male 4	8.5		✓	✓	✓
Male 5	7.5		✓	✓	
Male 6	8			✓	
Male 7	8			✓	
Male 8	8			✓	
Male 9	9		✓	✓	✓
Male 10	8		✓	✓	✓
Male 11	8		✓	✓	✓
Female 1	9.5	✓		✓	
Female 2	8	✓		✓	
Female 3	8.5	✓	✓	✓	✓
Female 4	8	✓	✓	✓	✓
Female 5	8.5		✓	✓	✓
Female 6	9		✓	✓	
Female 7	7.5		✓	✓	
Female 8	9.5		✓	✓	
Female 9	7.5			✓	
Female 10	7.5			✓	
Female 11	9			✓	
Female 12	8		✓	✓	✓
Female 13	9.5		✓	✓	✓

Table 5.1: List of specimens of fetal external genitalia collected and cultured for use in the culture model, and details of material amassed for future work.

All cell cultures were maintained for prolonged periods (minimum of three passages) and +/- DHT cultures grown in parallel. All cultures were periodically checked for normal AR translocation activity and showed comprehensive response to 10 nM DHT (see section 5.3.2 below). Stored cells, indicates cells were frozen in freeze down media and taken into liquid nitrogen for long-term storage. Stored mRNA indicates cell pellet was taken into RNA Later mRNA preservation medium and frozen at -80^{o}C. Stored protein indicates cell lysates were taken and frozen at -20^{o}C. wpc, weeks post-conception.

5.3.2 Nuclear translocation of AR following androgen treatment

Initial experiments using the culture model studied the effect of androgen treatment (10 nM DHT) on cell growth and morphology, and AR translocation. In terms of the former, there appeared to be no difference between those cells grown in culture media containing no DHT and androgen-treated cells (Fig. 5.1). In the absence of DHT, AR was consistently localised in the cytoplasmic compartment of genital fibroblasts (Fig. 5.2 A), and was strongly detected within the peri-nuclear region. However, in cells treated with 10 nM DHT the AR became heavily concentrated in the nucleus (Fig. 5.2 B). These observations imply the *in vitro* cultured fibroblast cells possess a similar phenotype to that of the phallic cells with nuclear AR characterised *in situ* in the previous chapter, which possessed nuclear AR. The expression of AR, and its translocation in response to androgen treatment, was present in all male (46,XY) fetuses tested (n=11) and all female (46,XX) fetal tissue samples (n=13), and was also comparable across a range of fetal ages from 8-11 wpc (Fig. 5.2 C-H).

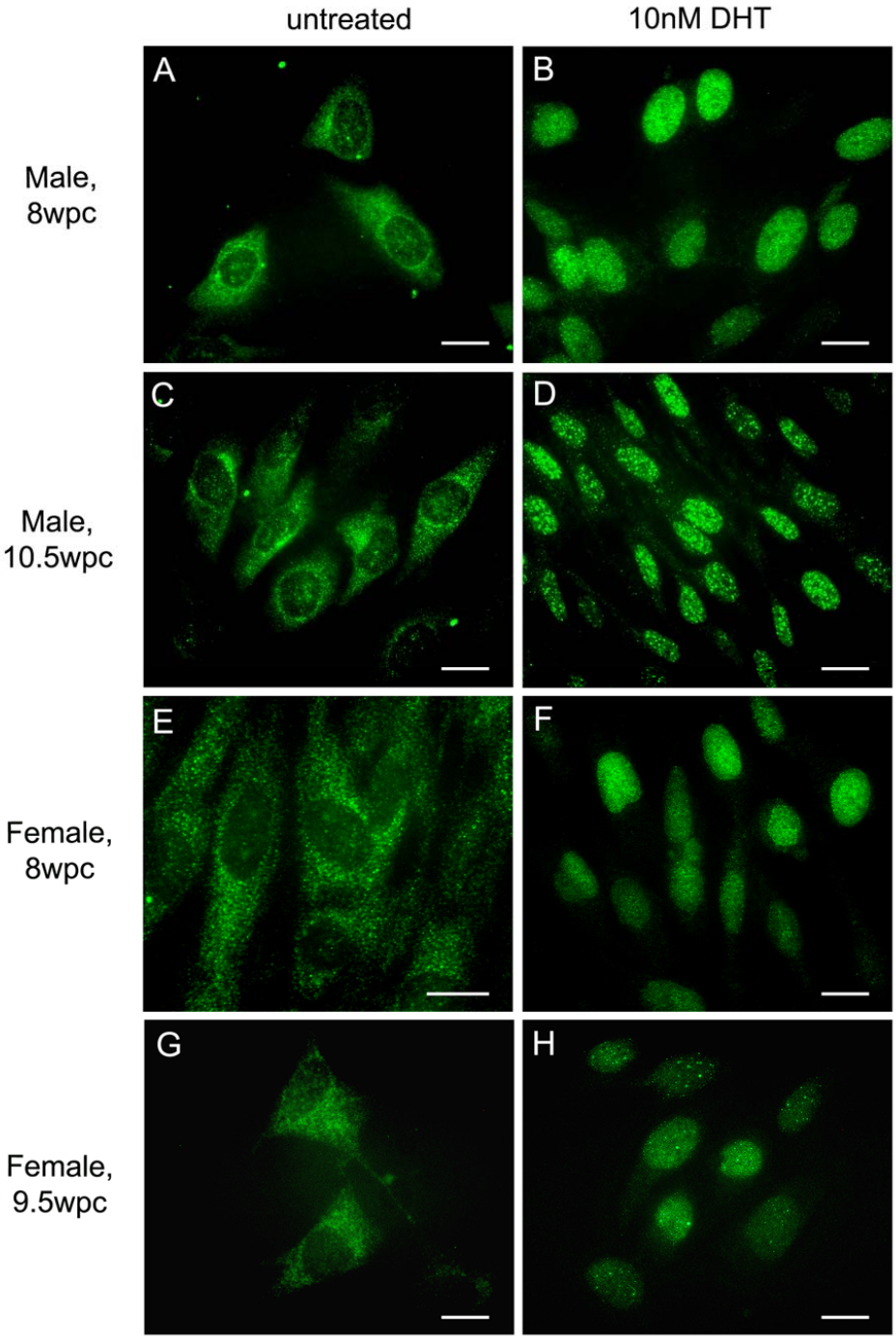

Figure 5.2: Immunocytochemical analysis of AR expression in untreated fetal genital fibroblasts and cells treated for 18 hr with 10 nM DHT
Scale bar 50 μM

5.3.3 Potency of androgens in inducing nuclear translocation of AR

The dynamic nature of AR localisation and its regulation by androgens was investigated further by determining the specific concentrations at which translocation of AR takes place for three physiologically relevant androgens. Using parallel cultures from the same fetus and cell passage, dose-response analyse were performed by the addition of a range of androgens overnight (~18 hr) to the culture media (100 nM, 10 nM, 1 nM, 100 pM, 10 pM, and untreated cells). The androgens used were three common steroids known or likely to be present in the fetal circulation during early development. The androgens used were: DHT, testosterone and androstenedione along with the androgen precursor DHEA; the first two having an obvious relevance to sexual differentiation and AR signalling.

DHT demonstrated AR immunofluorescence almost exclusively in the nuclear compartment at 10 nM and 100 nM (Fig. 5.3 E and F; K and L; Q and R; and W and X). Although AR translocation is still apparent at concentrations of 100 pM and 1 nM (Fig. 5.3 C and D; I and J; O and P; and U and V), immunofluorescence was less strongly detected in the nucleus, and AR began to appear in the cytoplasm; illustrating an indistinct, mixed pattern of AR localisation. Hence for this key range of DHT concentrations, staining for AR was never predominantly nuclear or cytoplasmic, and mixed patterns of nuclear/cytoplasmic stain was observed in all cultures, and across individual cells from the same culture well. In those cultures treated with 10 pM DHT (Fig. 5.3 B; H; N; and T), AR was almost exclusively cytoplasmic, emulating untreated cells and reflecting a deficiency of androgen to cause AR translocation. In summary, these data indicate that a minimum of approximately 100 pM DHT is required to trigger induction of AR nuclear translocation and approximately 1 nM to generate more complete levels of nuclear detection. The observed patterns of immunofluorescence were the same in both male and female fetal tissue cultures, and over the range of fetal ages.

Testosterone dose-response treatments also resulted in similar clear patterns of AR localisation. In addition to the range of concentrations used for DHT treatments (100 nM, 10 nM, 1 nM, 100 pM and 10 pM), another concentration

was added at the upper end of the range for the less potent androgens tested. The resulting data showed that nuclear AR immunofluorescence was predominant from 10 nM to 1 μM (Fig. 5.4 D-F; J-L; and P-R), but became more mixed between the nuclear and cytoplasmic compartments at 1 nM (Fig. 5.4 C, I and O), and distinctly cytoplasmic below 1 nM testosterone (Fig. 5.4 A and B; G and H; and M and N). Hence, these data indicate that the minimum concentration of testosterone required to induce AR nuclear translocation is approximately 1 nM; a 10-fold increase relative to DHT treatment. Once more there was some variation across specimens, but generally there was good correlation between concentration and AR localisation, which was unaffected by fetal age and sex of the starting specimen.

The study of the least potent of the common androgenic steroids revealed similar trends between AR translocation and androgen treatment. Specifically, in the case of androstenedione treatment it was found that at least 10 nM of the androgen was required to induce translocation, with increasing levels prompting greater nuclear localisation (Fig. 5.5). With DHEA considered an androgen precursor rather than demonstrating androgenic aetirity *per se*, a highly concentrated treatment was necessary to cause translocation, with a 100 nM treatment producing some small signs of AR localisation. 1 μM DHEA resulted in clear nuclear rather than cytoplasmic localisation (Fig. 5.6). Hence, androstenedione appeared to be approximately 10-fold less potent than testosterone, and 100-fold less potent than DHT in this context. DHEA possessed an even poorer capacity to result in AR translocation. However, as with the more potent androgens, both sets of results for androstenedione and DHEA were similar in males and females, and remained unaffected by the age of specimens.

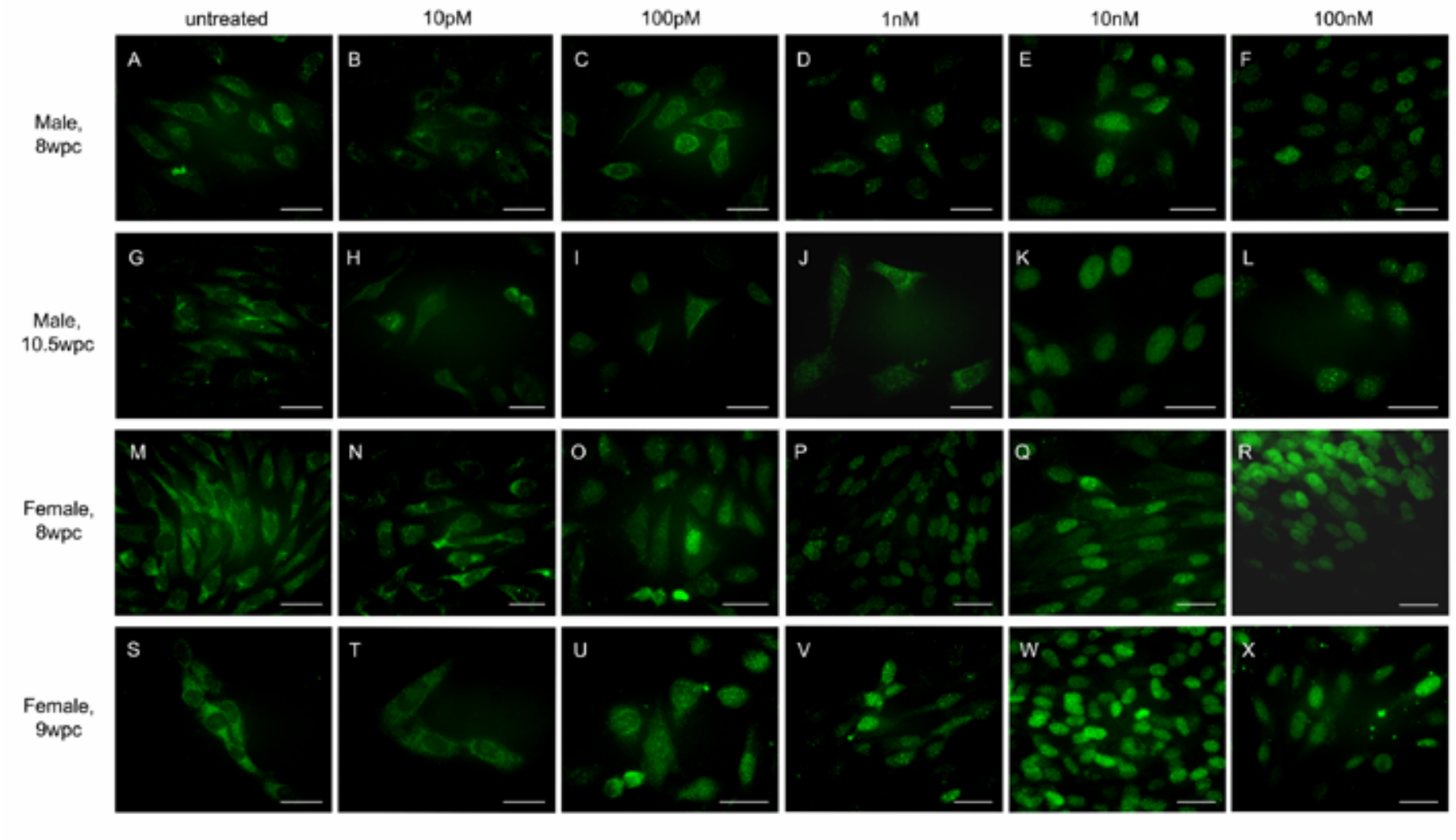

Figure 5.3: Immunocytochemical analysis of AR expression in untreated genital fibroblasts and cells treated for 18 hr with 100 nM, 10 nM, 1 nM, 100 pM and 10 pM DHT
Scale bar 50 μM

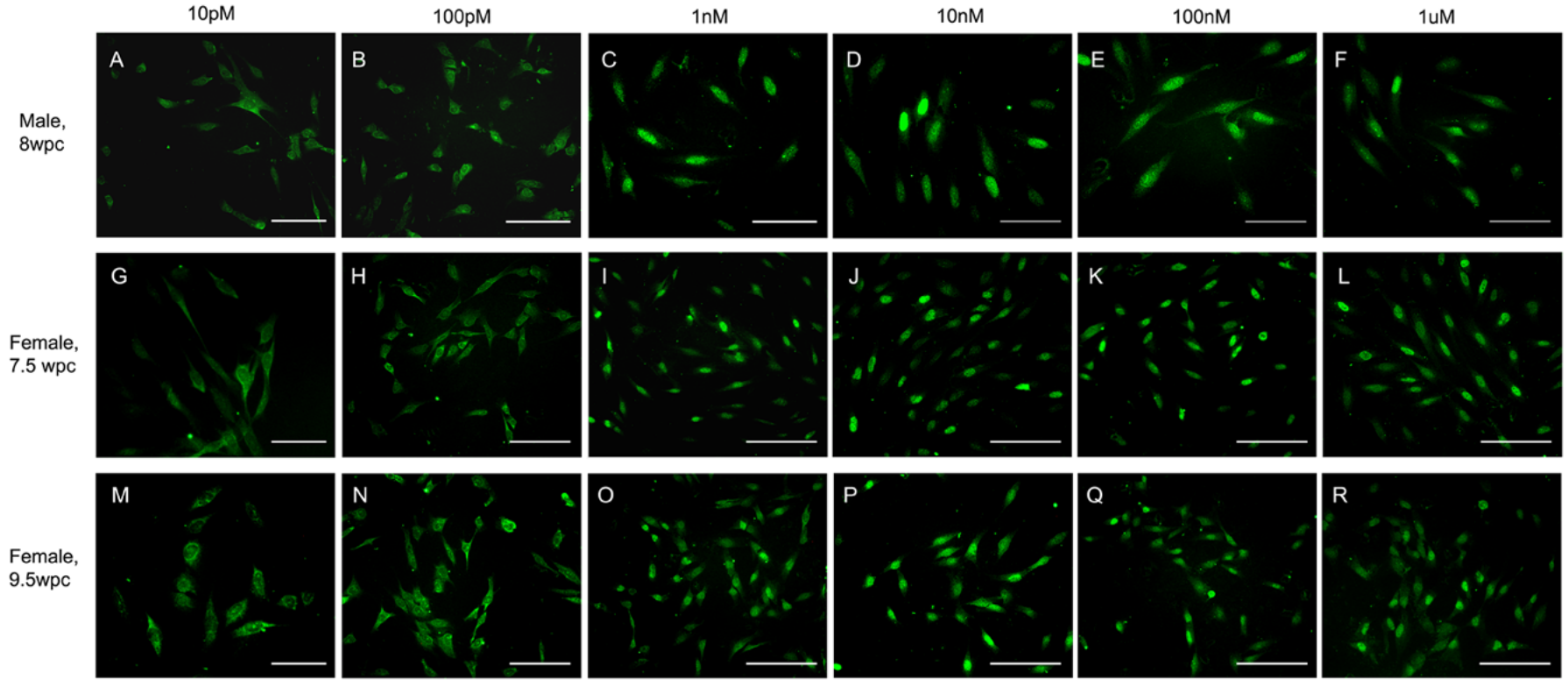

Figure 5.4: Immunocytochemical analysis of AR expression in genital fibroblasts treated for 18 hr with 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM testosterone
Scale bar 50 μM

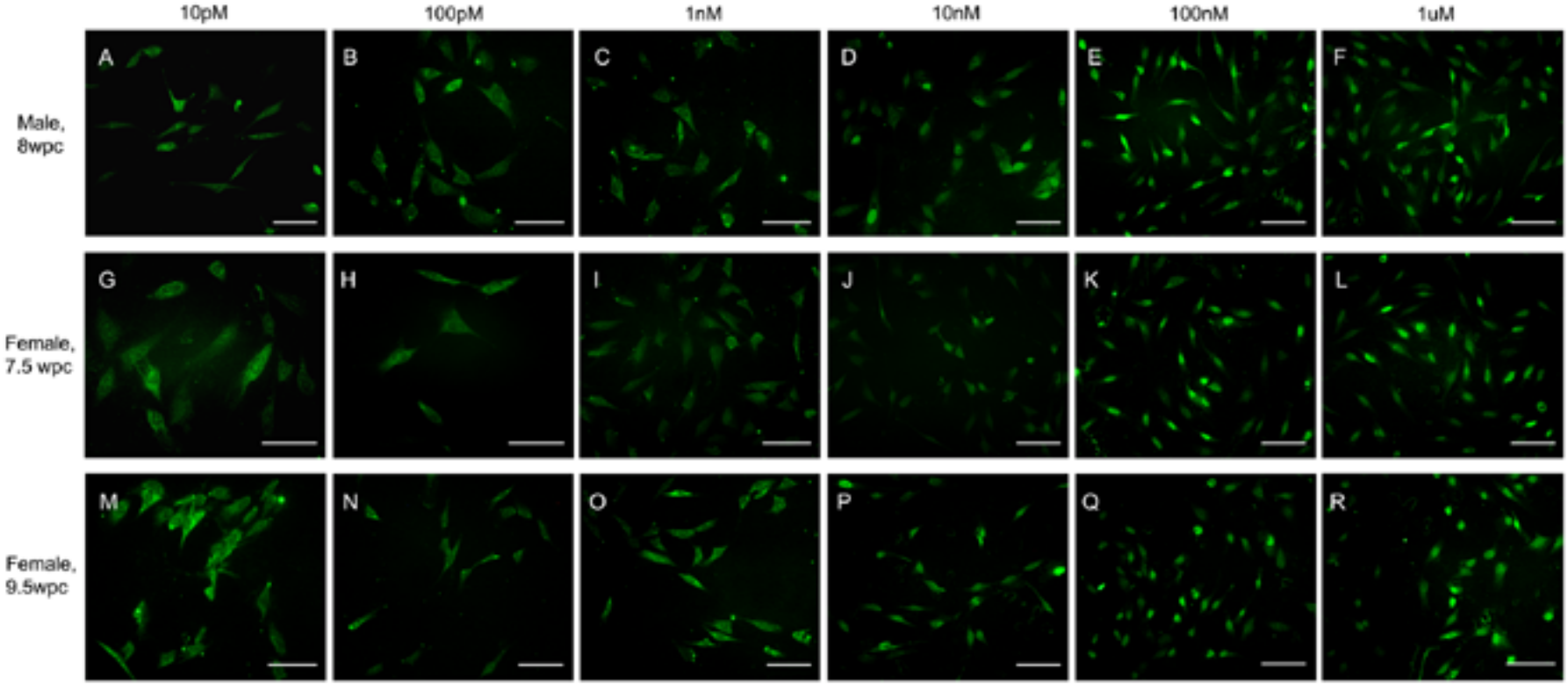

Figure 5.5: Immunocytochemical analysis of AR expression in genital fibroblasts treated for 18 hr with 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM androstenedione
Scale bar 50 μM

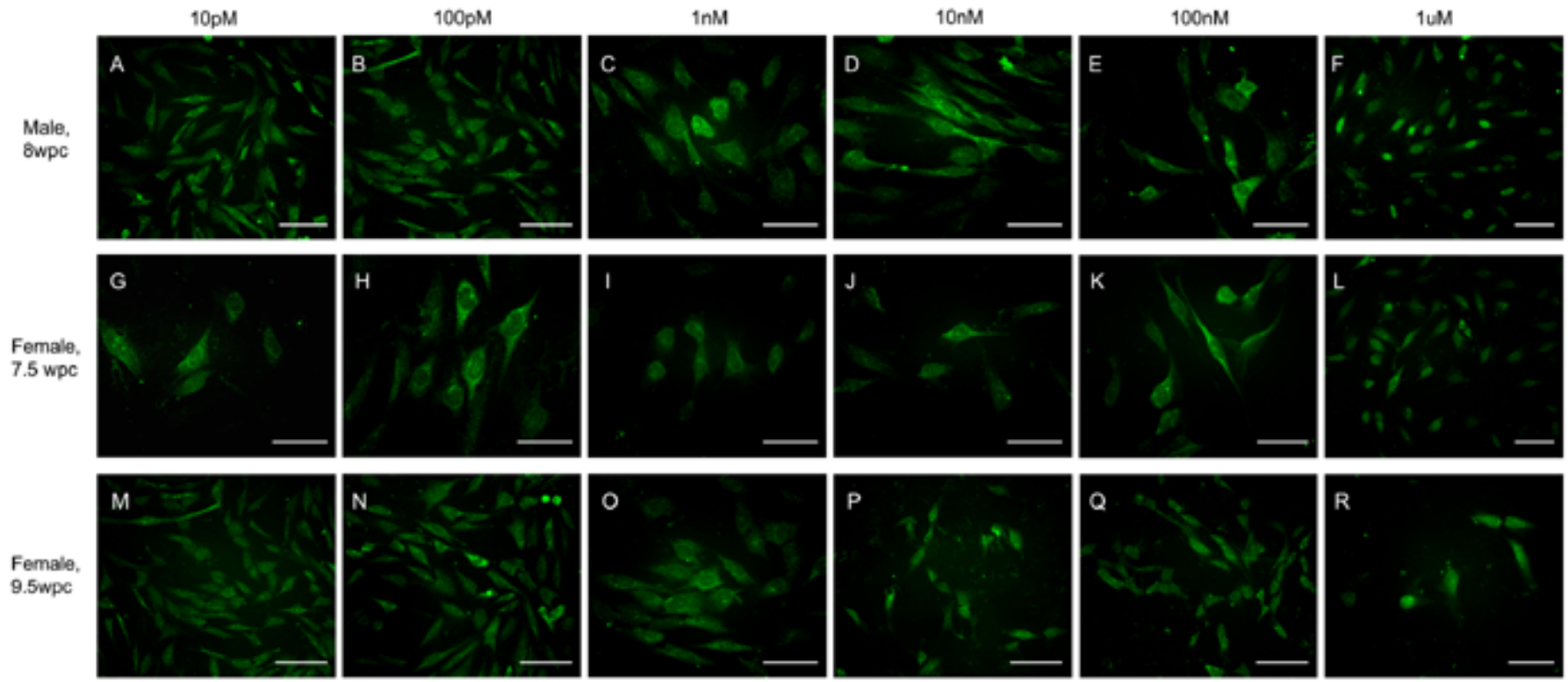

Figure 5.6: Immunocytochemical analysis of AR expression in genital fibroblasts treated for 18 hr with 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM DHEA
Scale bar 50 μM

5.3.4 Induction of AR translocation by organ co-culture

Due to previous work from our group demonstrating the importance of the fetal adrenal gland's influence on sexual differentiation (Goto *et al*., 2006), and its relevance to female virilisation seen in CAH due to CYP21A2 deficiency, the effect of the adrenal gland on AR translocation was investigated using the cell culture model. Using culture media that had been conditioned overnight (~18 hr) by a freshly retrieved fetal adrenal gland at 8 wpc, it was found that 46,XY genital fibroblasts previously deprived of any androgen, demonstrated clear nuclear AR translocation following a 12 hr treatment with the conditioned media (Fig.5.7 D). Identically cultured cells from the same cell culture, and originating from the same fetal external genitalia, when treated with 10 nM DHT for the same time period also displayed comprehensive nuclear localisation of AR (Fig.5.7 C); as did cells continuously cultured with 10 nM DHT from their initial seeding (Fig.5.7 A). These data imply that the normal fetal adrenal cortex steroid secretion is capable of mediating the same effects as potent androgens. However, this finding does not rule out that androgens or their precursors may also arise from other organs.

In further experiments, media conditioned by other organs was used alongside that conditioned using an adrenal gland. This included the conditioning of media for ~18 hr with the fetal kidney (a non-steroidogenic organ/negative control), followed by 12 hr treatment of genital fibroblast cultures previously deprived of androgens. In addition, fetal ovary was also utilised. Both ovary and kidney treatments resulted in a complete lack of AR translocation to the nucleus compared to that achieved by the adrenal gland (Fig. 5.8, C and D). These follow-up experiments were conducted using 46,XX fetal material and suggested that the effect of the adrenal gland in respect to androgen biosynthesis was equivalent in both male and female fetuses sourced from the period of sexual differentiation.

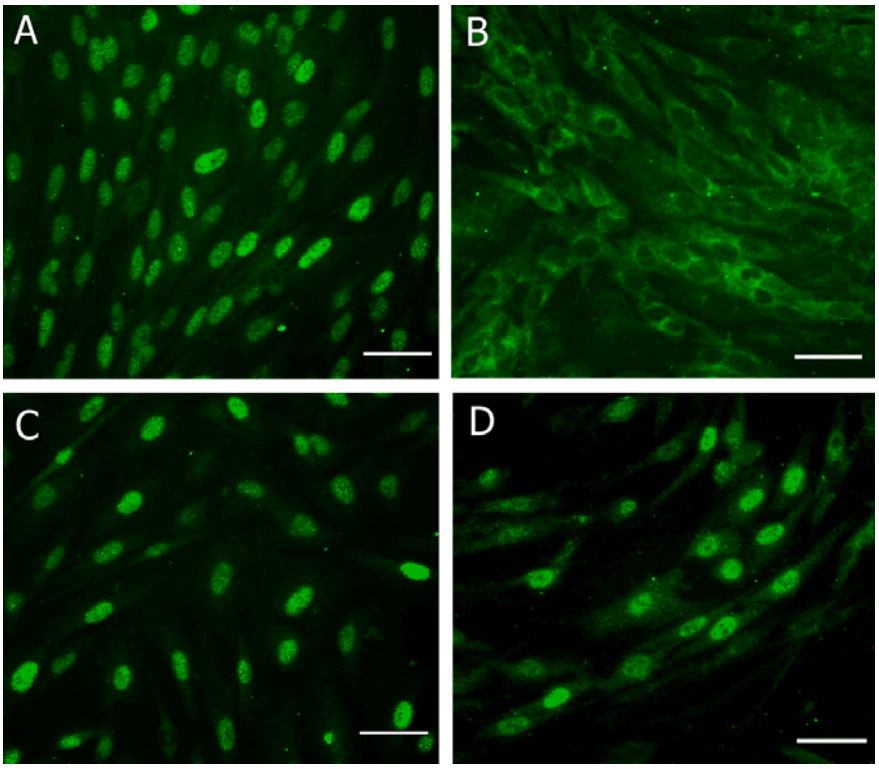

Figure 5.7: Immunocytochemical analysis of AR expression in 8 wpc 46,XY genital fibroblasts following different androgen treatments or organ co-culture conditioned media treatment

A, Cells treated continuously with 10 nM DHT; B, untreated cells; C, untreated cells treated with 10 nM DHT for 12 hrs; and D, untreated cells treated for 12 hrs with adrenal cortex conditioned media. Scale bar 50 μM

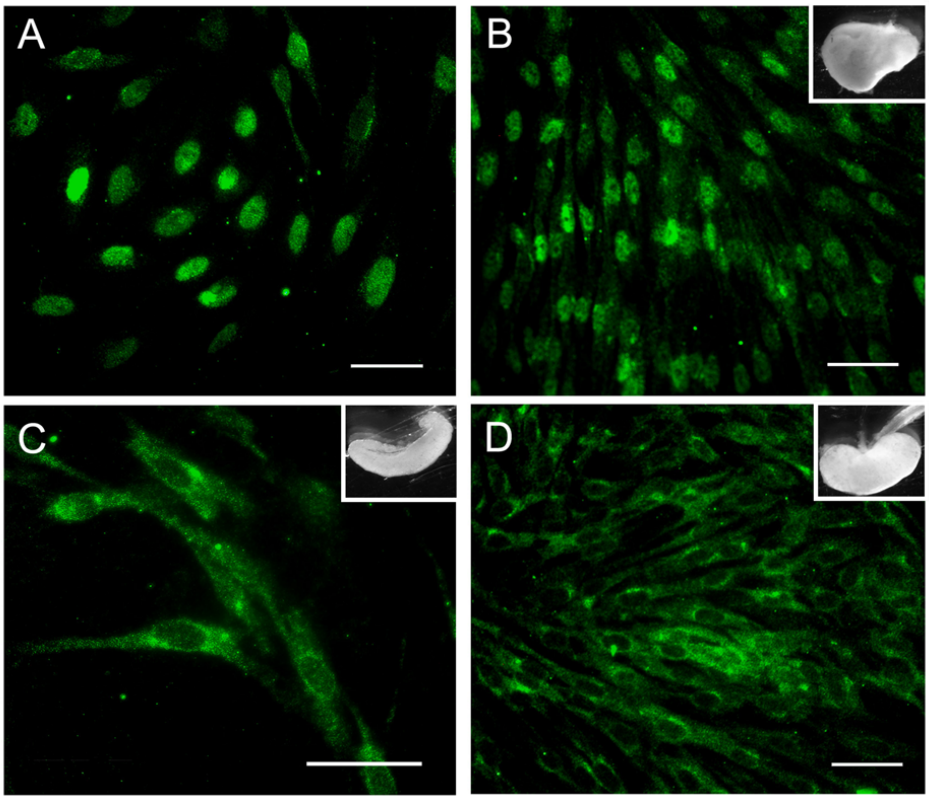

Figure 5.8: Immunocytochemistry analysis of AR expression in 8.5 wpc 46,XX co-cultured genital fibroblasts
A, cells treated with 10 nM DHT for 12 hr; B, untreated cells treated for 12 hr with adrenal cortex conditioned media; C, untreated cells treated for 12 hr with ovary conditioned media; D, untreated cells treated for 12 hr with kidney conditioned media. N.B. All organs and cultured genital fibroblasts originated from the same fetus. Inset shows the fetal organs used for co-culture overnight conditioning of media. Scale bar 50 μM

5.3.5 Analysis of previously identified AR target genes in human fetal genital fibroblasts following androgen treatment

The AR translocation witnessed in cells from the culture model system implies activation of AR as a transcription factor, and therefore implies effects on androgen-regulated genes. To assess this hypothesis, we assessed the relative levels of expression of putative AR target genes with or without androgen treatment. Initially this involved identifying genes likely to be regulated by androgens by means of literature searches, which resulted in the compiling of the list shown in Table 5.2 (primarily from Bolton *et al*., 2007; Massie *et al*., 2007; Prescott *et al*., 2007; Jariwala *et al*., 2007). Expression of these genes was then assessed by use of RT-PCR analysis of mRNA isolated taken from LNCaP cells and a range of genital fibroblast cultures aged 7.5-9.5 wpc, including samples originating from cells used in previous experiments in this chapter. The use of

LNCaP cells provided an expected positive control. All fetal mRNA samples originated from cells that had been rigorously checked at each stage for appropriate AR translocation in response to androgens, hence predicting functional androgen signalling. A minimum of four separate RT-PCR reactions were performed for each gene, on RNA extracted from different cell cultures, including LNCaP and at least one reaction using genital fibroblast mRNA.

Of the genes selected for analysis, many were not expressed in detectable quantities or results were not reproducible. Genes that were readily expressed in LNCaP cells and the human fetal external genitalia, and illustrated promise as an androgen-regulated gene are shown in the RT-PCR analyses in Fig. 5.9. From this, it was surprising that androgen treatment lacked a conclusive impact on gene transcription, although some effects were apparent, typically among the LNCaP cells. Specifically, *SLC22A3*, *ATP1A1* and *SLC26A2* demonstrated potential as AR targets upregulated by androgens. Likewise, *AP1G2* demonstrated some potential as a downregulated androgen target in the genital fibroblasts; despite it being a putatively upregulated gene in the literature (Bolton *et al*., 2007). *SLCO2A1*, *SLC12A7*, *ADAMTS1* and *MYC* all demonstrated limited evidence for androgen regulation. *KLK3*, widely regarded as a gene directly regulated by AR, based primarily on data from LNCaP cells, demonstrated consistent androgen-mediated upregulation in LNCaP cells. However, this was not apparent in genital fibroblasts. Interestingly, the expression of the *AR* gene itself seemed unaffected by androgen treatment, despite some evidence in the literature showing the contrary is true in a number of different cancer cell lines (Shan, Rodriguez & Janne, 1990; Wiren *et al*., 1997; Yeap, Krueger & Leedman, 1999; Burnstein, 2005).

Official name	Official symbol	Putative effect of androgen on expression
Adaptor-related protein complex 1, gamma 2 subunit	*AP1G2*	Upregulation
Adaptor-related protein complex 3, sigma 1 subunit	*AP3S1*	Upregulation
ATPase, Na+/K+ transporting, alpha 1 polypeptide	*ATP1A1*	Upregulation
Androgen receptor	*AR*	Upregulation?
cyclin G2	*CCNG2*	Upregulation
GATA binding protein 2 (stem cell transcription factor)	*GATA2*	Upregulation
KIAA0232	*KIAA0232*	Upregulation
KIAA1727	*KIAA1727*	Upregulation
kallikrein-related peptidase 2	*KLK2*	Upregulation
kallikrein-related peptidase 3	*KLK3 (PSA)*	Upregulation
Keratin 5	*KRT5*	Upregulation
Keratin 73	*KRT73*	Upregulation
Matrix metallopeptidase 7	*MMP7*	Upregulation
Mucin 1, cell surface associated	*MUC1*	Upregulation
Phosphomevalonate kinase	*PMVK*	Upregulation
Solute carrier family 12 (potassium/chloride transporters), member 7	*SLC12A7*	Upregulation
Solute carrier family 12 (sodium/chloride transporters), member 3	*SLC22A3*	Upregulation
Solute carrier family 26 (sulfate transporter), member 2	*SLC26A2*	Upregulation
Solute carrier family 30 (zinc transporter), member 1	*SLC30A1*	Upregulation
Solute carrier family 9 (sodium/hydrogen exchanger), member 2	*SLC9A2*	Upregulation
solute carrier organic anion transporter family, member 2A1	*SLCO2A1*	Upregulation
sterol O-acyltransferase 2 (cholesterol syn)	*SOAT2*	Upregulation
leukemia inhibitory factor receptor alpha	*LIFR*	Upregulation
A disintegrin-like and metallopeptidase with thrombospondin type 1 motif, 1	*ADAMTS1*	Downregulation
Cytochrome P450, family 24, subfamily A, polypeptide 1	*CYP24A1*	Downregulation
Dickkopf homolog 1 (Xenopus laevis)	*DKK1*	Downregulation
DNA-damage-inducible transcript 4	*DDIT4*	Downregulation
Fibroblast growth factor binding protein 1	*FGFBP1*	Downregulation
Follistatin	*FST*	Downregulation
Interleukin 6	*IL6*	Downregulation
v-myc myelocytomatosis viral oncogene homolog (avian)	*MYC*	Downregulation
Polo-like kinase 2 (Drosophila)	*PLK2*	Downregulation

Table 5.2: List of putative AR-regulated genes tested for response to androgen using RT-PCR

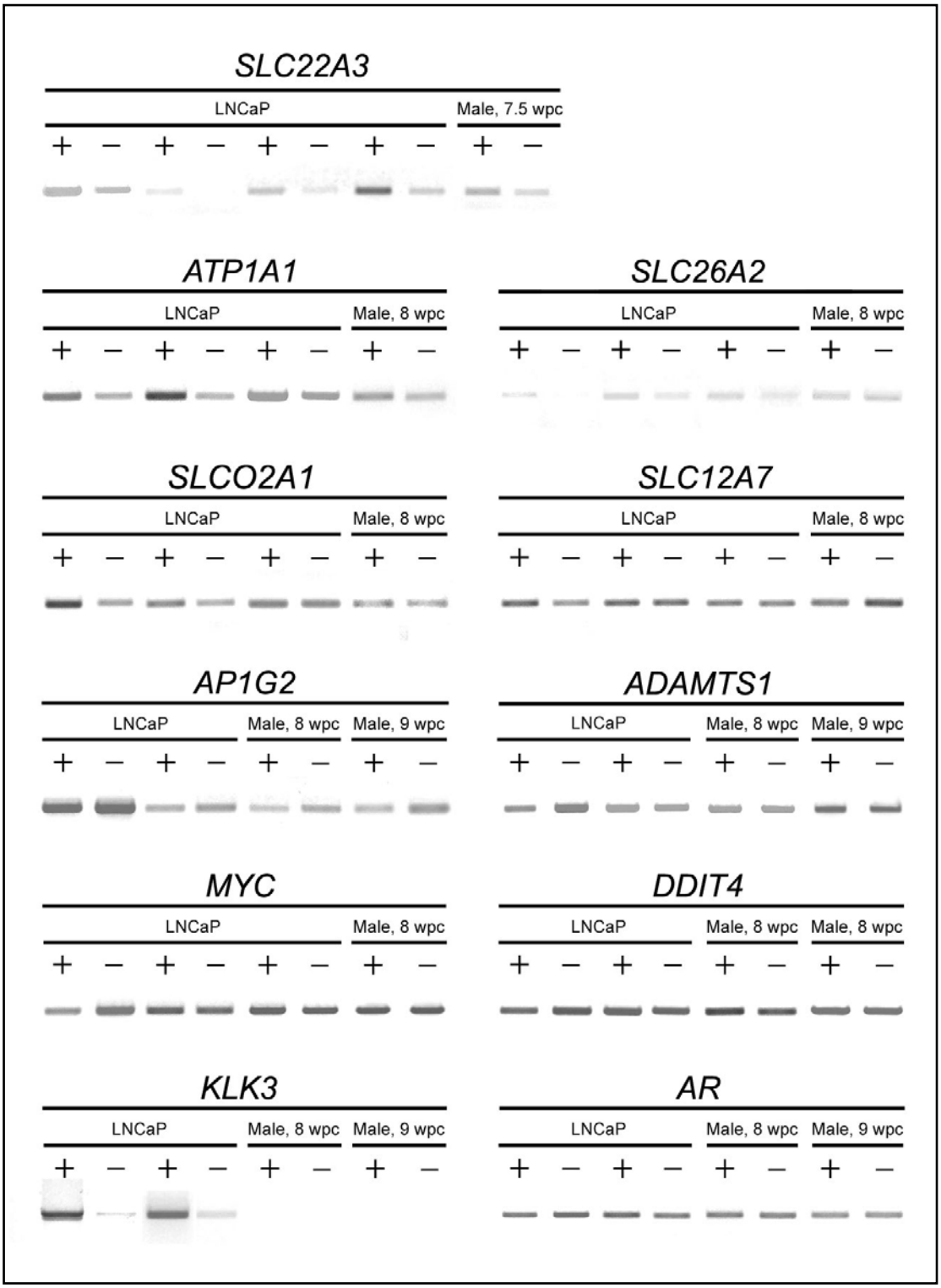

Figure 5.9: RT-PCR analysis of the effect of androgen on expression of putative AR-regulated genes in LNCaP cells and human fetal external genitalia.

RT-PCR analysis was performed to assess the expression of genes previously shown to be regulated by AR. Expression was analysed in cultured LNCaP cells and 7.5-9 wpc 46,XY genital fibroblasts. 28 cycles of PCR used (N.B. the far left pair of bands for SLC22A3 and AP1G2 used 35 cycles, and all reactions for ADAMTS1, MYC, and DDIT4 used 30 cycles). +, cells received overnight 10 nM DHT treatment. –, cells cultured in androgen-free conditions. wpc, weeks post-conception. For full gene names see Table 5.1.

5.3.6 Further analysis of the effect of androgen treatment on AR gene expression

Because the RT-PCR analysis of *AR* expression in the external genitalia revealed its level of expression to be unaffected by androgen treatment, this phenomenon was investigated further owing to previous reports of autoregulation of AR expression by AR (Shan *et al*., 1990; Wiren et. al., 1990; Yeap *et al*., 1999; Quarmby *et al*., 1990), and putative AREs in the coding region of the *AR* gene (Burnstein et. al., 1995; Grad et. al., 1999; Grad *et al*., 2001). To achieve this, AR protein levels in genital fibroblasts and LNCaP cells were analysed using Western blotting, along with further analysis of AR mRNA levels through to use of real-time quantitative PCR (qPCR). In contrast to the previous data, a range of precisely determined treatment times was used for DHT (10 nM) treatments of cell cultures, prior to mRNA or protein extraction. A range of different fetuses (aged 7.5-8.5 wpc) was used for this, including two fetuses where mRNA and protein samples were taken in parallel from the same cell culture.

Results from the qPCR analysis (Fig. 5.10) indicated that there is no significance in *AR* mRNA expression in response to androgen treatments, with 4-48 hr treatments leading to only minimal effects on expression in the three male fetuses tested; hence, concurring with the previous RT-PCR data (Fig. 5.9). In contrast, immunoblot analysis of protein levels (Fig. 5.11) indicated a distinct increase in AR expression following androgen treatment. AR protein levels were increased as a result of short periods of androgen treatment (4-8 hr). In most cases, an apparent decrease towards background levels occurred after longer treatments (8-48 hr). However, two specimens demonstrated increased AR levels at 12h or later. Quantitative comparisons of the relative intensities of the bands observed, showed that in the genital fibroblasts a 4 hr DHT treatment led to an average fold increase in intensity/mm^2 of 1.86 (+/-0.14; n=3) and 1.83 in LNCaP cells, whereas an 8 hr treatment in genital cells showed a 1.69 (+/-0.36; n=3) fold increase. Interestingly, the effect of DHT on AR protein expression was observed in protein samples from both male (46,XY) and female (46,XX) external genitalia. The precise correlation between AR protein levels and length of androgen treatment is not clear, but there appears to be a definite increase in AR protein as a result of short term androgen exposure. These data suggest that AR

expression is upregulated by androgens via post-transcriptional control mechanisms, not as a result of direct gene regulation by ligand- bound AR.

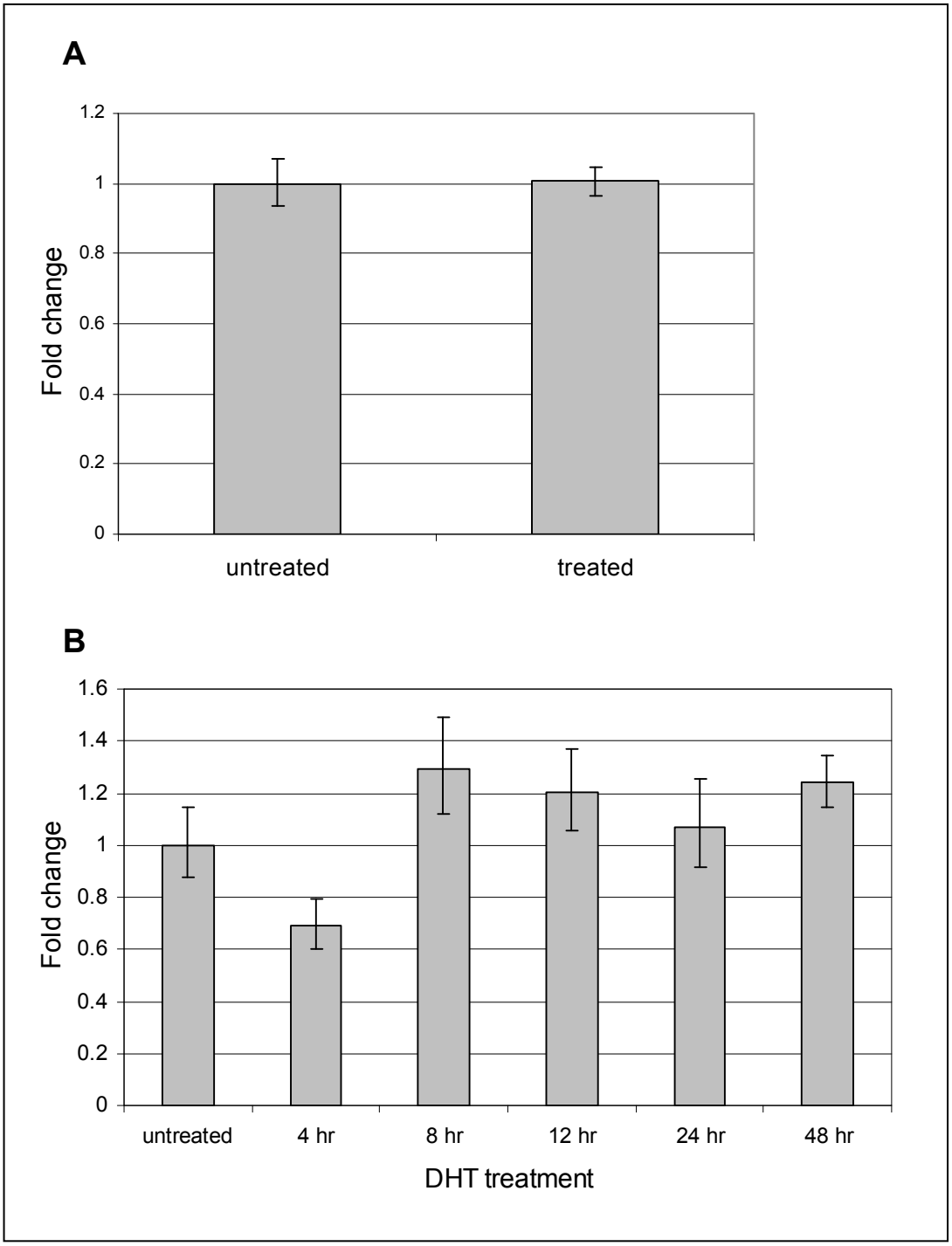

Figure 5.10: qPCR analysis of fold change in AR expression in genital fibroblasts either untreated or treated with DHT for 4, 8, 12, 24 or 48 hr.
A, comparison of expression of AR in cultured 8 wpc male genital fibroblasts following androgen deprivation or treatment with 10 nM DHT for 4 hr (n=3). B, comparison of expression of AR in cultured 8-9 wpc genital fibroblasts following treatment with 10 nM DHT for 4-48 hr (n=2). Data normalized to negative control expression of TATA box-binding protein (TBP). Expression for untreated cells standardized to 1. Error bars show standard deviation.

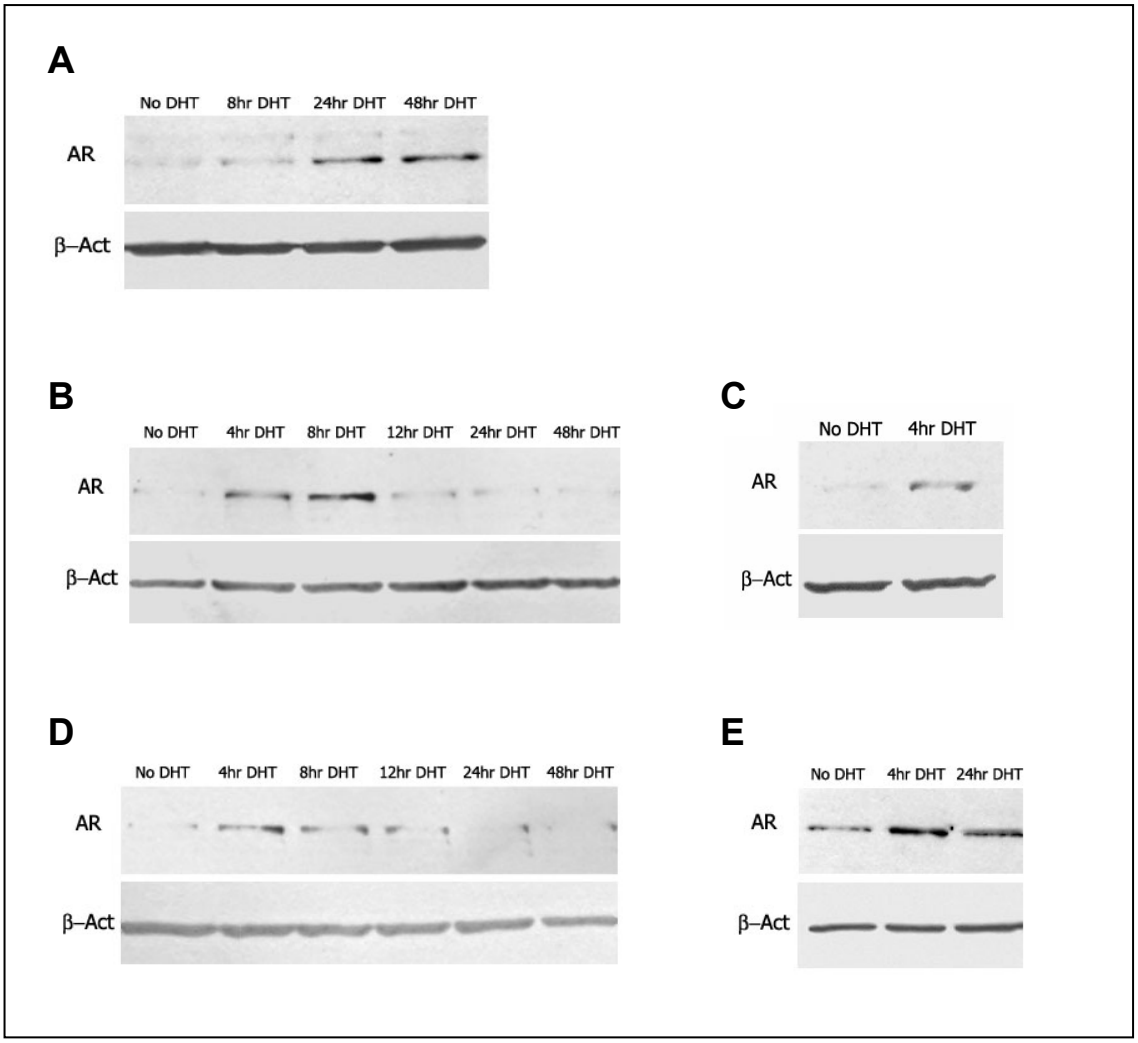

Figure 5.11: immunoblot analysis of the effect of androgen on levels of AR protein in cells of the human fetal external genitalia.
Analysis of AR protein levels (top row) in cells following androgen deprivation or 4-48 hr treatment with 10 nM DHT. A-C, 8 wpc male genital fibroblasts; D, 9 wpc female genital fibroblasts; E, LNCaP cells. Lower rows show protein levels for β-actin (β-Act) negative control.

5.4 Discussion

The results from this chapter have identified a small number of putative AR-regulated genes in the developing human external genitalia, while discounting several others. Moreover, we have shown that many of the putative targets previously found through the study of LNCaP cells seem unlikely to be targets in primary human genital tissues. The work in this chapter has also produced a method for modelling the pathological contribution of adrenal androgens to the development of the DSD in CAH due to CYP21A2 deficiency, and added further

data on the potential influence of the normal adrenal cortex during early development of the female external genitalia. Finally, these data produced new findings regarding the potential autoregulation of AR expression in human external genitalia during the critical window of sexual differentiation.

5.4.1 Development of a culture model of the human fetal external genitalia

The main objective of this chapter was to begin to identify androgen-regulated genes that contribute to the processes of sexual differentiation. Currently, little is known about downstream androgen signalling in primary human cells beyond the liganding of AR by androgen. A handful of AR target genes have been identified (Montgomery *et al*., 1992; Chen *et al*., 2008a), but thus far, none have been demonstrated in normal human external genitalia. Genes that have been highlighted as potential AR targets in primary cells, have typically been found either through *in silico* approaches whereby analyses of the genome have yielded putative targets based on the location of ARE sequences upstream of the transcriptional start site of genes (Devos *et al*., 1997; Burnstein, 2005), or alternatively, gene expression profiling in cancer cell line cultures has been used, where genes have been identified based on their differential expression in androgen treated and androgen deprived cells (Holterhus *et al*., 2003; Massie *et al*., 2007; Bolton *et al*., 2007). Owing to the inherent variation in the ARE consensus sequence and complexities of bioinformatic-based studies, we have initially chosen to pursue the latter more direct approach. However, in contrast to the majority of *in vitro* models used for identification of AR target genes, our work strived to use primary cells from the human fetal external genitalia from the late first trimester period, to directly determine the genes important to sexual differentiation in the primary cells of interest, at the precise time when this process is unfolding.

In order to begin to address the task of investigating downstream androgen signalling pathways in the cells of the external genitalia, a culture model was required. To achieve this, two main obstacles had to be overcome. The first was a need to develop a robust and reliable system for the culture and growth of

primary cells from the fetal external genitalia, whereby cells could be cultured for prolonged time periods with or without androgen, and lead to the generation of plentiful numbers of homogeneous cells for storage and/or multiple analyses. This is particularly important because microarray analysis with the future option of ChIP-Chip or ChIP-Seq requires significant material. Secondly, there was a need for a marker of androgen action in cells given the lack of certainty regarding positive controls for downstream androgen target genes in human external genitalia.

To resolve the first problem, cultures of genital fibroblasts were established from specimens of external genitalia collected from fetuses between 7 to 12 wpc. This cell-type was used as it adequately represented the growth of the developing external genitalia, expressed AR, and after some optimization, was also readily cultured *in vitro*. Large monolayer cultures of fetal genital fibroblasts were consistently grown to confluency, with a homogeneity and consistent morphology in the presence or absence of androgen. The cells obtained from culture could be used for both protein and RNA analyses and were resilient to freeze/thaw cycles, and after prolonged storage cells could also be re-seeded for further rounds of culture with no apparent loss in AR function or significant changes in cell character or morphology.

We were also able to address the problem of concern as to whether the AR was functional. Following treatment with sufficient potent androgen (determined through preliminary experiments), the resulting nuclear translocation of AR in cells, which was readily observable using ICC, provided a facile proxy marker of androgen action. Hence, during prolonged cell culture, aliquots of cells were taken as a part of sub-culturing and their capacity for AR translocation checked to assess cell character and confirm AR expression and apparent function was not affected by the culturing process.

With the combination of a suitable culture model and marker for androgen action, downstream effects of androgen in the differentiating cells of the fetal external genitalia could be investigated. Importantly, the culture model showed little variation across cells from different fetuses regarding the concentration of

androgen required to induce AR nuclear translocation, suggesting relatively limited variability due to the intrinsic well-characterised glutamine repeat polymorphism located in exon 1 of the *AR*. This polymorphism has been shown to associate with functional differences in AR across populations (Lieberman *et al*., 2002; Silva *et al*., 2008), as well as different degrees of virilisation in females with CAH due to CYP21A2 deficiency (Rocha *et al*., 2008).

5.4.2 Effects of androgens on the cells of the human fetal external genitalia

Initially the effects of different concentrations of androgens on activation of AR translocation were studied to gain insight into the threshold concentration required to begin the process of androgen-induced genomic AR signalling. This work demonstrated the critical threshold levels of DHT, testosterone, androstenedione and DHEA required to trigger widespread AR nuclear translocation and thus likely to result in AR-mediated effects on gene transcription. These data identified a scale of potencies for each androgen in terms of there capacity to elicit comprehensive AR translocation, which showed DHT was most competent and DHEA was least effective, only triggering widespread AR translocation at very high concentrations (i.e. androgen potency in this regard was DHT > testosterone > androstenedione > DHEA). An approximately ten-fold difference in potency was found between each androgen on this scale. Importantly, because this relationship between the effectiveness of each androgen was shown in both male and female tissue cultures, and over a range of ages, this would indicate that the key androgen concentrations are important in all fetuses during sexual differentiation, and are able to promote downstream androgen signalling independently of gender or age. Interestingly, the critical concentrations required for abundant AR translocation conformed to the established understanding of the relative potencies of the androgens tested (Koh *et al*., 2001).

Critically, it is not clear whether the relative effects of androgens in the fetal external genitalia determined from our experiments represent the actual capacity of each androgen to elicit AR translocation and regulation of transcription, or

whether intracellular metabolism was converting these to more potent androgens such as DHT. For example 10 nM androstenedione gives rise to 100 pM DHT in cells expressing the correct enzymes for the conversion (Koh, Kanaya & Namiki, 2001). Hence the observed effects of testosterone, androstenedione and DHEA are arguably more likely a product of their conversion within target cells to DHT, rather than direct genomic effects of the added steroid on the cells.

Although the latter consideration is important and the potential steroid metabolising pathways of the external genitalia cells need elucidation, it was apparent that the threshold values determined from our model closely correlate to the concentrations of steroids secreted from the fetal adrenal glands during the critical window of human sexual differentiation (Goto *et al*., 2006). Previous work in our group showed that 1.15 ± 0.10 nmol/l of testosterone and 29.3 ± 2.77 nmol/l androstenedione was secreted from overnight cultures of single 8 wpc fetal adrenal glands (Table 5.3). In the present study we showed that approximately 1 nM testosterone and 10 nM androstenedione was required for abundant AR translocation in cells of the developing external genitalia, either directly or by conversion to DHT. Therefore, it is possible to speculate that the fetal adrenal cortex is capable of producing sufficient androgens to elicit AR-mediated androgen action in the developing external genitalia either directly or via local conversion to DHT. The implications of this are unknown. Because the data on adrenal androgen secretion from Goto *et al*. was based on measurements of normal female adrenal gland secretion (as well as male) this would imply that early female development occurs in the presence of significant amounts of androgen, with the strong potential to mediate effects on tissues expressing AR. This not only controverts the classical view that early female sexual development occurs in an androgen-free environment, but suggests quite counter intuitively that female sexual differentiation takes place amidst an androgenic hormonal milieu. The data also highlight a clear pathway by which aberrant virilised development could proceed, such as that observed in cases of CAH due to CYP21A2 deficiency.

Androgen	Key concentration	Adrenal normal output
DHT	~100 pM	-
Testosterone	~1 nm	1.15 +/- 0.10 nmol/l
Androstenedione	~10 nM	29.3 +/- 2.77 nmol/l

Table 5.3: Summary of concentrations of androgens needed to elicit comprehensive AR translocation in fetal genital fibroblasts, as compared to normal fetal adrenal cortex androgen secretion.
Adrenal output data from Goto et al., 2006

5.4.3 Adrenal androgen output during sexual differentiation and its significance to CAH

Sexual differentiation of the female external genitalia is reliant on the tight regulation of androgen production. In the 46,XX female fetus, overexposure to androgens during this critical period results in virilised genitalia at birth (Achermann and Hughes, 2008). This most commonly occurs in the DSD CAH due to CYP21A2 deficiency (Biglieri *et al*., 1981; Therrell, 2001), which essentially entails unregulated androgen biosynthesis, and an increased availability of androgen. Following on from the discovery here that normal levels of androgen synthesised in the developing adrenal glands correlate to those required for initiation of downstream AR signalling, we wished to directly investigate the potential for the normal adrenal gland to influence AR-mediated transcriptional regulation in the developing external genitalia, especially considering the pathological female virilisation witnessed in CAH due to CYP21A2 deficiency.

Using the culture model once again, we showed that the normal fetal adrenal gland is capable of bringing about comprehensive levels of AR translocation in fetal genital fibroblasts. Conditioned culture media from a normal 8 wpc whole fetal adrenal gland, when applied to cultures of genital fibroblasts from the same fetus, mimicked the effects of potent androgen. In contrast, ovary-conditioned media did not bring about observable AR translocation, indicating that the nominal androgen production of the ovary is less capable of activating AR-mediated pathways.

This paradigm highlights a mechanism by which CAH due to CYP21A2 deficiency could lead to virilisation in female newborns. It would better explain why mutations that restrict adrenal cortisol synthesis and facilitate androgen synthesis, lead to significant virilisation of female tissues. Based on the work of this chapter and data from Goto *et al*., it appears that a surge of either androstenedione or testosterone secretion from the fetal adrenal gland could serve to increase AR signalling and cause the virilisation seen in patients with CAH due to CYP21A2 deficiency. This paradigm clearly reinforces the need for tight regulation of androgen levels in female development, not because the hormonal milieu must remain androgen-free, but because it must remain below a strict upper limit. Whether or not the normal levels of androgens present during female development have a specific function is not yet known, but by acting as a trophic factor, this androgen may explain why initial growth of the genital tubercle/glans during the period of sexual differentiation is visually similar in both males and females.

5.4.4 Analysis of the expression of putative androgen-regulated genes in the human fetal external genitalia

The principle purpose of developing a culture model for the external genitalia was to identify genes regulated by AR. To this end, in this chapter we used RT-PCR analysis to highlight the increased/decreased expression of putative AR target genes in genital fibroblasts treated overnight with DHT. The results of these experiments showed that compared to data from LNCaP cells, there was little change in expression for most of the genes studied following androgen treatment. Differential expression within genital fibroblasts was observed for *SLC22A3*, *ATP1A1* and *AP1G2*. For some genes, previous data from LNCaP cells was reproduced. For instance *KLK3* demonstrated clear androgen-mediated upregulation. This gene is considered a principle marker of AR signalling in LNCaP cells (Montgomery *et al*., 1992), as well as breast cancer cell lines (Magklara *et al*., 2002). However, the RT-PCR data would suggest that many of the targets identified in previous studies using LNCaP cells and other cancer cell lines, do not directly translate into targets in primary cells and may not be

relevant to normal human sexual differentiation. As discussed in previous chapters, this highlights the problem of using model cell lines to investigate biological processes that naturally occur in primary cells; hence why there has been a concerted effort in the present study to use the relevant primary cells in our experiments. Following this initial work to identify AR target genes, there is an obvious need for further experiments in primary cells of the external genitalia to ascertain which AR target genes are important for major human sexual differentiation. This was the aim of the next chapter.

5.4.5 AR is subject to positive feedback as a result of androgen action

Despite no clear indication of an androgen-mediated effect on AR expression during our initial investigation of putative AR targets, there was a desire to further explore the possible autoregulation of AR expression in the developing human external genitalia. Several previous studies have indicated that the expression of the AR gene is influenced by androgen and the activity of AR protein in the nucleus (Quarmby *et al*., 1990b; Shan, Rodriguez & Janne, 1990; Wiren *et al*., 1997; Yeap, Krueger & Leedman, 1999). Primarily, these previous studies have come to this conclusion of autoregulation based on sequence analysis of the *AR* gene that has indicated the presence of possibly one or more ARE sites upstream of the *AR* transcriptional start site.

Using qPCR to perform a more in-depth analysis of *AR* expression, we showed that levels of *AR* mRNA in genital fibroblasts remain unchanged following treatment of cells with androgen for between 4 and 48 hrs. Hence it appears that transcription of the AR gene was not directly regulated by binding of liganded AR in the cells of the external genitalia. However, analysis by immunoblotting of genital fibroblast extract revealed that following androgen treatment AR protein expression showed consistent incremental increases, especially after short-term androgen exposure (0-8 hrs DHT treatment), but also following longer term exposure in some instances (8-48 hrs treatment). This suggests that although expression of AR in the external genitalia is not affected by androgen in terms of gene transcription, some form of post-transcriptional activity, mediated by liganded AR, does occur following androgen exposure. Therefore, this indicates

that androgen has the capacity to indirectly increase AR levels in the fetal human external genitalia, presumably resulting in increased effects on the expression of AR target genes – hence suggesting AR-mediated positive feedback.

How androgen action leads to this increase in AR protein is not yet known. Regulation of AR gene expression by androgen is likely to involve complex sets of pathways, with cell-specific, differential androgen effects occurring at multiple levels of transcription and mRNA turnover. However, it is probable that one of three mechanisms may play a role in achieving this: (i) post-transcriptional modification or stabilisation of *AR* mRNA; (ii) AR protein stabilisation; or (iii) the influence of micro RNAs. Androgen-mediated stabilization of *AR* mRNA has been demonstrated in LNCaP cells (Yeap *et al*., 1999). Interactions between specific sequences within mRNA (cis-acting elements) and cellular RNA-binding proteins (trans-acting factors) as well as ribonuclease activity degrading the mRNA, are factors that determine the rate of mRNA turnover (Peltz *et al*., 1991; McCarthy and Kollmus, 1995). The identity of such cis-elements and trans-acting factors for AR mRNA are yet to be fully elucidated. However, androgen-regulation of RNA binding proteins seems likely, and could influence *AR* mRNA stability. The increased *AR* mRNA in cells would serve to provide more substrate for translation of AR protein, and hence explain why more AR protein was detectable following DHT treatment.

Stabilisation of AR protein represents an alternative hypothesis for the increased detection of AR observed in genital fibroblasts following DHT treatment. As a part of normal AR liganding by androgen, AR undergoes posttranslational modifications including phosphorylation. It has been shown previously in LNCaP cells that low levels of androgen leads to phosphorylation of AR at multiple sites by cyclin-dependent kinases, which serves to enhance protein stability via conformational changes in protein structure (Chen *et al*., 2006). Therefore, in tandem with *de novo* AR protein synthesis, this would bring about an overall increase in the abundance of AR in cells. Interestingly, Chen and colleagues (2006) found this phenomenon only occurred after a minimum of 4 hrs, which is the time point where we also detected significant increases in AR protein. It has also been shown that the process of binding to androgens can

augment AR stability via protein folding and interactions of the N and C terminals, with a possible correlation between the degree of AR stability and the specific androgen bound (Kemppainen *et al*., 1992; Zhou *et al*., 1995).

The final mechanism that may be responsible for the correlation between AR expression and androgen action observed in the current data, is the influence of micro RNA (miRNA). miRNAs are a recently discovered class of naturally-occurring, small non-coding RNAs, associated with performing a myriad of biological functions through regulation of the expression of protein-encoding genes (Takada *et al*., 2009). miRNA is generated by a complex multi-step process involving different RNases, whereby a ~22 bp miRNA duplex is initially created, which following degradation of one strand and the binding of the main miRNA strand to a silencing complex, is able to suppress the expression of genes via post-transcriptional regulation (Shi *et al*., 2008). It has been estimated that over 1000 distinct miRNAs exist in humans, which can each recognize hundreds of different mRNA targets and regulate an array of different cellular processes including proliferation, differentiation, apoptosis, survival, motility and morphogenesis (Lewis *et al*., 2005). It remains incompletely understood what regulates miRNA expression and also what genes are regulated by miRNAs.

Previous work has shown that there are significant differences in the miRNA repertoire between males and females, during sexual differentiation in mouse (Takada *et al*., 2009) and other higher vertebrates (Bannister *et al*., 2009). Furthermore, several recent studies now support a role for the AR in the transcriptional regulation of intronic and exonic miRNA expression in LNCaP cells (Porkka *et al*., 2007; Rokhlin *et al*., 2008), PC3 cells (Louro *et al*., 2007; Lin *et al*., 2008) and other cells lines (Shi *et al*., 2007), where it has been shown to regulate miRNAs including miR-29b, miR-34a, miR34c and miR-125b-2. Regulation of miRNA expression by AR in the external genitalia is yet to be studied in any depth. It has also been demonstrated that miRNAs (for example miR-331-3p) can effect androgen signalling and possibly expression of the AR in cancer cell lines (Epis *et al*., 2009). Taken together, the strong evidence that AR can regulate miRNA expression and support for a role of miRNAs in affecting androgen signalling, as well as the associated sexual dimorphism of the miRNA

repertoire, suggest that downstream transcriptional effects of liganded AR during sexual differentiation may be in part due to the influence of miRNA and, moreover, that this mechanism may also be responsible for autoregulation of AR (see Fig. 5.12).

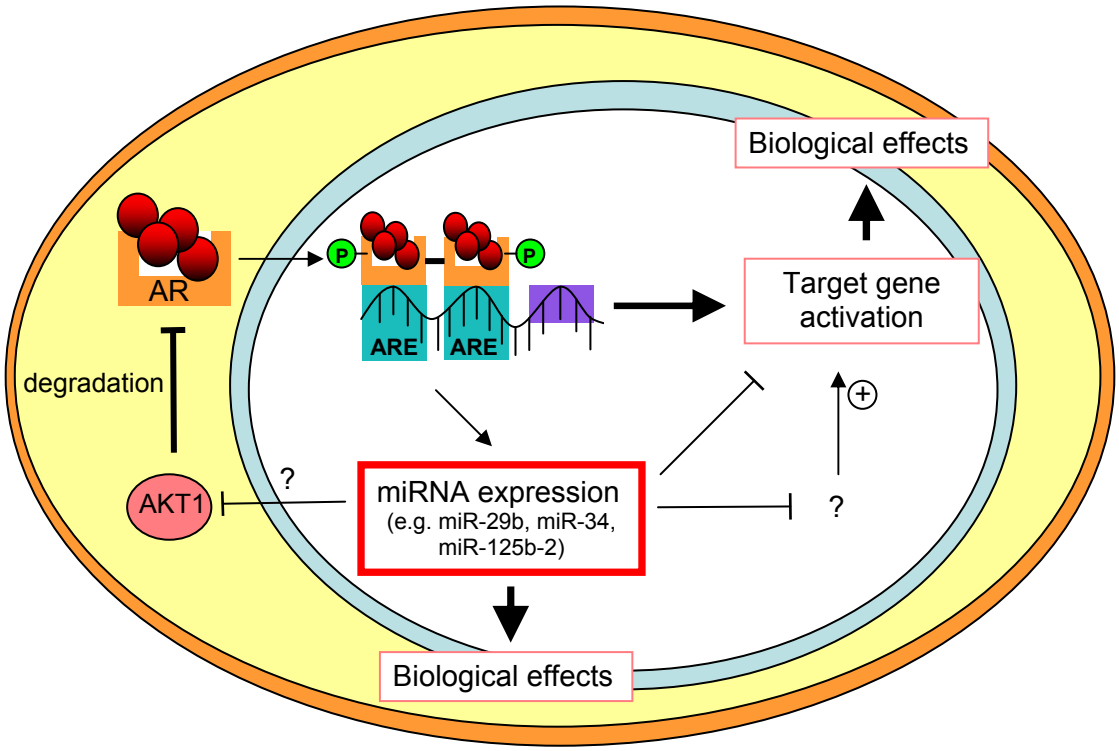

Figure 5.12: Putative miRNA-mediated signalling pathways that could influence downstream androgen signalling and promote positive autoregulation of AR protein levels.
miRNA, microRNA; ARE, androgen response element; AR, androgen receptor; P, phosphate group; AKT1, v-akt murine thymoma viral oncogene homolog 1.

5.4.6 Normal and virilised female sexual differentiation

The data shown in this chapter, combined with some of the findings from the previous chapters, has led to the formulation of a model for the processes underlying virilised female development, as in CAH due to CYP21A2 deficiency, in comparison to normal female development (Fig. 5.13). We have outlined how normal female sexual differentiation probably occurs in the presence of significant adrenal androgen (this chapter and Goto *et al.*, 2006).

Furthermore, we have demonstrated that the cells of the fetal female external genitalia are able to respond to androgen with AR translocation (Chapter 5) and do so during normal development (Chapter 4), which probably leads to at least nominal activation of AR target genes. This may contribute to normal female development. In contrast, we predict that only a marginal increase in adrenal androgen output would increase AR signalling, possibly initiate a positive feedback loop of AR levels, and this amplification of androgen action would result in pathological virilisation of the developing female external genitalia.

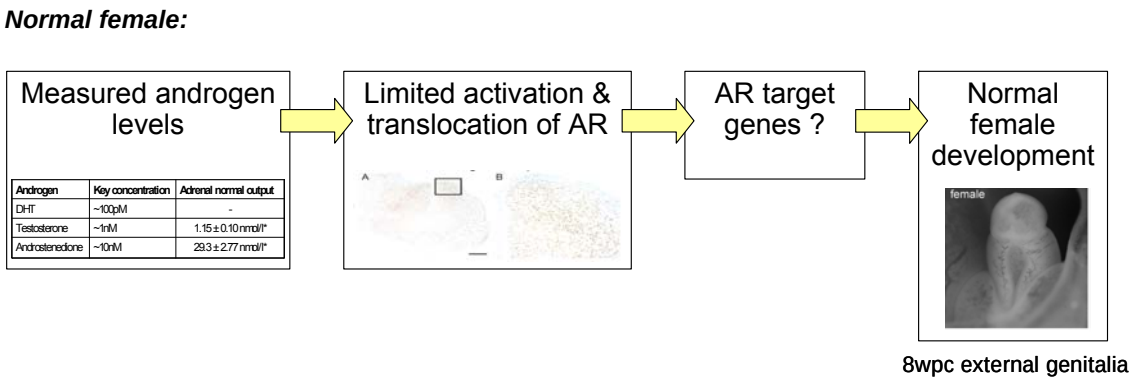

Figure 5.13: A possible route to virilised development in female patients with CAH due to CYP21A2 deficiency, in the context of normal development

Model of external genitalia development in female patients with CAH due to CYP21A2 deficiency in the context of what has been discovered regarding normal development of the female external genitalia in the present study. AR, androgen receptor; DHT, dihydrotestosterone; wpc, weeks post-conception; β-Act, β-actin.

5.4.7 Conclusion

The establishment of a successful culture model for growth and manipulation of genital fibroblasts from the fetal phallus has shown the effect of androgens on AR in male and female cells, and indicated the female adrenal cortex as a putative source of bioactive androgen. Taken together these data imply that there is a fine balance between normal female sexual differentiation and pathological virilisation, and that only a small increase in normal androgen levels such as that which occurs in CAH due to CYP21A2 deficiency, could tip the balance toward virilisation of female external genitalia during development. These findings make the identification of AR target genes in the human external genitalia during sex differentiation particularly important.

Chapter 6: Microarray analysis of AR target genes

6.1 Introduction

Work of the previous chapter established an *in vitro* culture model to replicate the *in vivo* growth of fetal genital fibroblasts and their responses to androgen hormones. This work also included a preliminary analysis of previously identified AR target genes largely based on research in the LNCaP cell line. Following on from this work, which demonstrates poor responsiveness of these candidate AR target genes in human primary cells, there was a clear need to identify the real spectrum of target genes in the established culture model. Utilizing mRNA samples obtained from the culture of genital fibroblasts, this chapter details expression microarray experiments to identify androgen-regulated genes across the whole human genome.

The profiling of gene expression by microarray analysis permits the quantification of the expression of thousands of genes in parallel from a single RNA sample (Dufva, 2009). A variety of methodologies have been developed from the original microarray technique, however, fundamentally, all follow the same basic protocol (outlined in Fig. 6.1). This consists of the use of a glass slide or membrane (the chip) that is spotted or 'arrayed' with DNA oligonucleotide probes representing specific gene coding regions from the target genome. Applied to this is purified cRNA that has been fluorescently- or radioactively-labelled, which after hybridisation leads to the production of signal in the coordinates of the chip containing genes that are expressed in the transcriptome of interest. The chip undergoes laser scanning or autoradiographic imaging to capture the fluorescent or radiographic emission, illustrating which genes are expressed in the sample tested, and their relative expression levels.

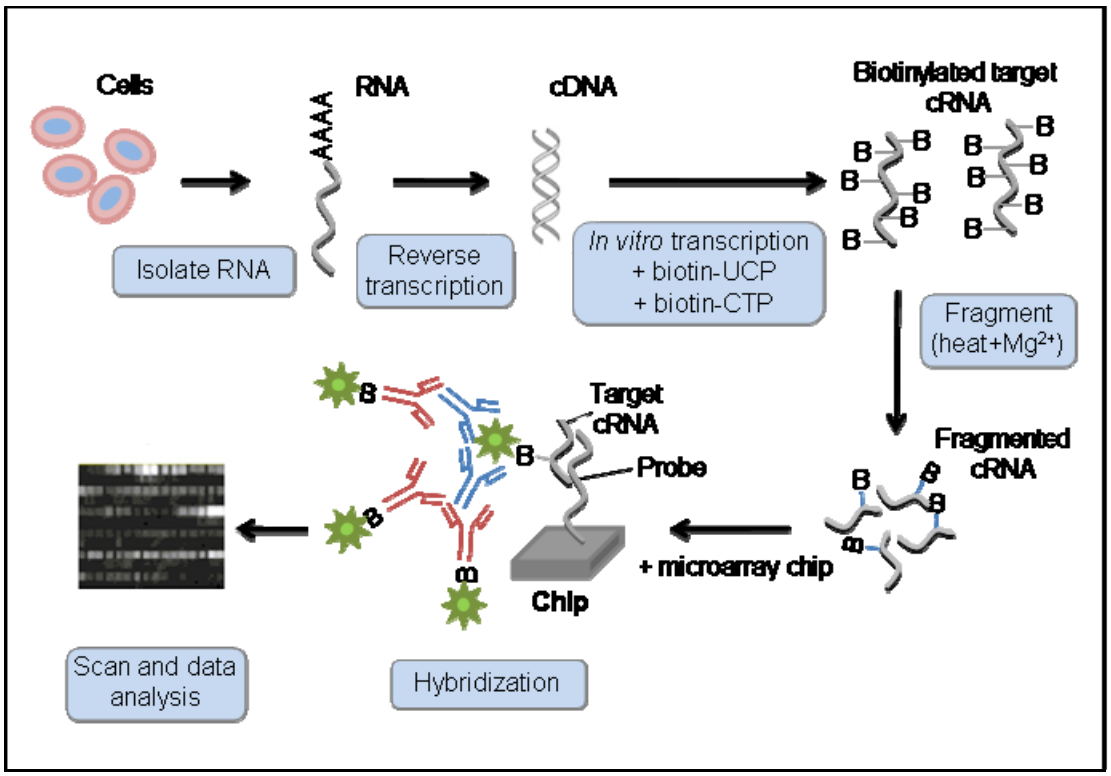

Figure 6.1: Flow diagram illustrating the key steps taken when performing a microarray experiment

The reasons for the use of expression microarray technology to achieve the goals of this chapter were manifold. In principle, the usefulness of the microarray technique stems from its high-throughput nature. This profiling of the whole human genome in a single experiment can allow gene pathways to be deciphered. Therefore, in terms of the current study, the data anticipated would have the potential to identify genetic pathways functioning downstream of AR transcriptional modulation; for example, the relationship between AR activity, and *SOX2* and *CHD7* expression. As outlined in Chapter 4, *SOX2* is expressed in fetal external genitalia and, when mutated, causes genital defects. Furthermore, in the literature there is a known link between *SOX2* and *CHD7* as both are associated with Anophthalmia-Esophageal-Genital (AEG) syndrome, suggesting these genes could function in a single androgen-regulated pathway.

Much previous work has utilised *in silico* analyses to identify the ARE consensus sequence within putative regulatory regions of potential androgen-regulated genes (Schuur *et al*., 1996; Cleutjens *et al*., 1996; Verrijdt *et al*., 1999; Bolton *et al*., 2007). The ARE typically comprises the inverted repeat sequence 5′-

AGAACAnnnTGTTCT-3′ (Roche *et al*., 1992; Beato and Klug, 2000). However, sequence alignment of known AR genomic binding sequences reveals both inverted repeat and direct repeat consensus sequences (Verrijdt *et al*., 2003). This degeneracy of functional AREs, plus divergence from the archetypal *in vitro*-defined binding sequence, makes positive identification of AREs problematic. In short, *in silico* prediction analyses are inadequate and direct biological experiments are needed.

In recent years a number of studies have employed expression microarray technology to identify AR-regulated genes (Waghray *et al*., 2001; Chen *et al*., 2004; Velasco *et al*., 2004; Bolton *et al*., 2007). However, this work has almost entirely been performed in cancer cell lines, most frequently LNCaP prostate tumour cells. Such cell types are fundamentally different from primary cells and may not adequately represent AR signalling pathways in normal human cells involved in sexual differentiation. Indeed, despite most of the work on AR target genes being performed in LNCaP cells, this cell line contains a point mutation in the AR steroid-binding domain (codon 868, Thr to Ala; Veldscholte *et al*., 1992). The consequences of this mutation are not fully understood but it has been shown to effect binding affinity, heat-shock protein interactions, and possibly activation of transcription. This difference between abnormal cancer cell and primary human cells may explain the apparent lack of androgen responsiveness observed in the last chapter (Fig. 5.9).

RNA expression profiling in LNCaP cells has previously been used to describe ~500 transcripts whose expression is altered by androgen treatment (DePrimo *et al*., 2002; Nelson *et al*., 2002). However, there is still a huge deficit of information on AR targets in normal human cells, especially during the important period of sexual differentiation. The primary goal of this chapter is to begin to fill this gap. A greater understanding of the genes transcriptionally regulated by the AR in human development will help to identify candidates for inactivating mutations in syndromes of under-virilisation, for example hypospadias. This could lead to more accurate diagnoses of such genital abnormalities, which are known to be very distressing to both patient and parents.

6.2 Aims

- To culture genital fibroblasts taken from the fetal external genitalia, in order to produce sufficient numbers of cells to be either treated with DHT (+ DHT cells), or ethanol vehicle (-DHT cells).
- To perform comparative mRNA expression profiling microarray experiments using extracted mRNA from the genital fibroblast cultures, to quantify the expression of genes across the human genome in the presence or absence of DHT.
- To analyse the microarray data produced to identify genes showing significantly different levels of expression in response to DHT.
- To validate microarray data and further analyse these putative AR target genes through the use of RT-PCR and qPCR

6.3 Results

6.3.1 Preparation of genital fibroblast cultures for microarray analysis

Using the same culture conditions for the growth of genital fibroblasts optimised in chapter 5, primary fibroblasts from human fetal external genitalia were grown to confluence and passaged twice to generate adequate cell numbers. Cultures were divided (and an aliquot stored) and exposed for 4 h to fresh media containing 10 nM DHT or vehicle. This concentration of DHT was chosen as it caused clear homogeneous translocation of AR to the nucleus (see Fig. 5.2 and 5.3). Four hours was chosen as the optimal exposure for identifying the direct primary target genes of AR action but not those involved in later secondary responses. Following this strictly timed treatment period, cells were taken into the RNA preservation medium, 'RNA Later' and stored, pending total RNA extraction. During the earlier passages of the culture, small aliquots of cells were used for the 'check slide' methodology described previously and monitored for a 'normal' AR translocation response to DHT (performed in at least duplicate cell aliquots). This step gave some confidence that the cell phenotype was that of external genitalia fibroblasts prior to expensive microarray experiments. In addition to collecting genital fibroblast RNA to be used for expression microarray analysis, chromatin was also prepared from the same cultures for

combined chromatin immunoprecipitation and sequencing (ChIP-Seq) analysis. In the future, it is hoped to correlate AR binding sites across the genome with the expression microarray data. Although ChIP-Seq experiments fell outside the remit of the present study, it was prudent to prepare and store these samples alongside the expression microarray material. All of these procedures, including ChIP-Seq preparation, were performed on four cell cultures originating from different fetuses (Table 6.1). The best culture (criteria in Table 6.1) was used for the expression microarray analysis, and future ChIP-Seq analysis. The fetus was male at 8 wpc (Table 6.1, (2)); chosen based on overall quality of cells and preparation, and because it was felt necessary to investigate a male fetus prior to a female, in order to gain more insight into the fundamentals of normal male virilisation pathways, before looking at pathological female virilisation mediated by AR.

	General quality control			DHT treatment	ChIP pellet preparation	
	Cell homogeneity	Overall cell morphology	Nuclear translocation		Procedure	Pellet size
(1) Female; 7.5 wpc	✓✓	✓	✓✓✓	✓✓✓	✓	✓✓✓
(2) Male; 8 wpc	✓✓✓	✓✓✓	✓✓	✓✓	✓✓✓	✓✓✓
(3) Male; 8 wpc	✓✓✓	✓✓	✓✓	✓✓✓	✓✓✓	✓
(4) Female; 9 wpc	✓✓✓	✓✓✓	✓✓	✓✓✓	✓✓	✓✓✓

Table 6.1: Collated information on cell culture attributes and quality of experimental procedures for the preparation of genital fibroblast samples for microarray expression analysis and future ChIP-Seq analysis.
Ticks represent arbitrary scale of success in each regard on a scale of 1 (worst) to 3 (best).

6.3.2 Microarray analysis of changes in gene expression due to androgen exposure of the external genitalia

As mentioned previously, very little is known regarding the genes that are targeted by the AR following its activation and translocation to the nucleus due to androgen exposure. Chapter 5 demonstrated that those few genes that have been suggested as androgen-regulated genes, mainly due to work in cancer cell lines, are likely not to be AR targets in primary cells. Therefore in order to analyse the changes in gene expression in cells of the developing male human external genitalia, gene expression profiles of genital fibroblasts, in the presence or absence of 10nm DHT, were compared by microarray analysis (Fig. 6.1). RNA was isolated from treated and untreated cells in duplicate, and used as templates to make four sets of biotinylated cRNA, each of which were then hybridized to an Affymetrix human genome U113 plus 2.0 array (performed by Dr. Leo Zeef in the Faculty of Life Sciences Core Microarray Facility, University of Manchester). Hybridisation, scanning and quality control steps are described in Chapter 2 (section 2.18.3).

6.3.3 Identification of genes with altered expression due to androgen treatment

Initial analysis of the microarray data took the form of a scatter plot representation of the expression of all probes in androgen deprived versus androgen treated cells (Fig. 6.2). From this it was apparent that most genes analysed showed no response to androgen treatment (e.g. expression was the same in both treated and control cells represented graphically as dots on a 45° line). Further analysis compiled a list of genes that demonstrated substantial changes in expression in response to DHT. A number of parameters were considered when doing this. Mean fold change (+/-SD) in gene expression levels between cells in the presence or absence of DHT was calculated. The two individual values for fold differences were then used to calculate the mean fold changes in expression for each transcript between + and - DHT cells. From the 54,600 probe sets present on the array, 1465 showed a doubling or more of gene expression in the + DHT samples when compared to the – DHT samples, while 1454 probes demonstrated at least a two-fold mean decrease in expression in the + DHT samples when compared to the – DHT samples. From these groups of

probes, a list was compiled that comprised actual genes (rather than hypothesised genes), and excluded those genes for which baseline expression was very low (mean value of less than 0.5 arbitrary units) and/or where SD was greater than 5. The list of upregulated genes selected is shown in Table 6.2, and selected downregulated genes shown in Table 6.3.

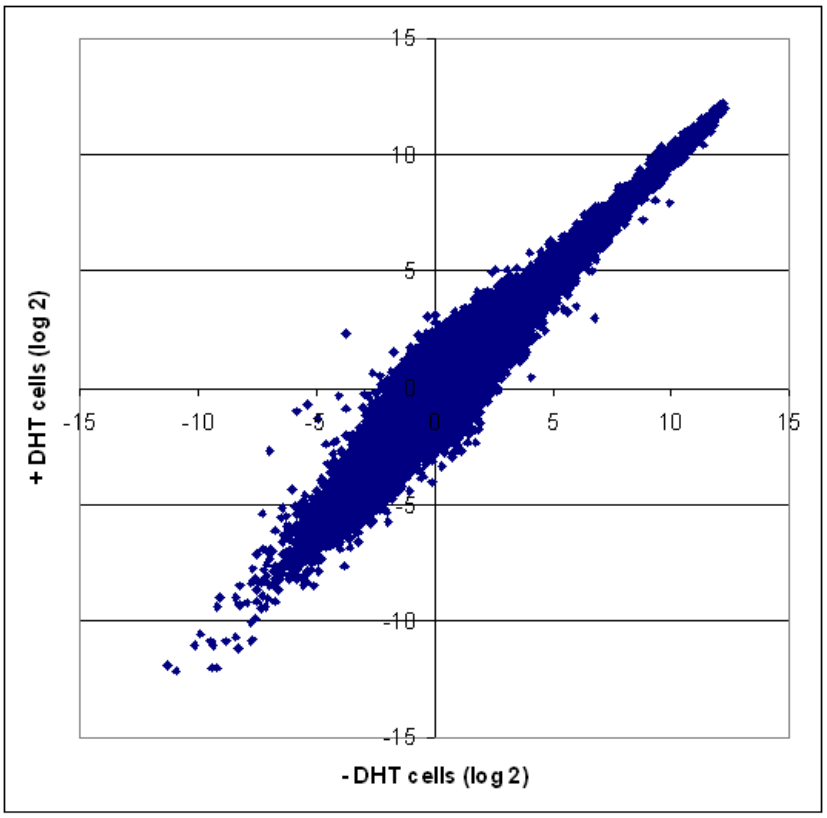

Figure 6.2: Scatter plot representation of total microarray raw data.
Graph illustrating relative mean expression levels of probe sets in cells treated with ethanol vehicle (-DHT) or 10 nM DHT (+DHT) for 4 hrs.

Accession no.	Gene symbol	Array 1 fold increase	Array 2 fold increase	Mean fold increase	SD
NM_001998	*FBLN2*	34.79	11.95	23.37	15.15*
N37057	*LSM4*	12.55	3.37	7.96	4.59
AK022330	*TBX6*	10.23	3.52	6.88	4.74
NM_000765	*CYP3A7*	7.88	5.62	6.75	1.60
NM_000397	*CYBB*	9.79	3.41	6.60	4.51
U09609	*NFKB2*	9.02	3.83	6.43	3.68
AU155094	*ANXA1*	7.24	5.16	6.20	1.47
NM_014790	*JAKMIP2*	8.45	3.53	5.99	3.48
D00943	*MYH6*	7.45	4.11	5.78	2.36
AB085901	*MCF2*	7.63	3.50	5.57	2.91
NM_000733	*CD3E*	3.50	7.61	5.56	2.90
BC012536	*HSD17B12*	6.56	4.52	5.54	1.44
AI421972	*NSD1*	8.06	2.85	5.46	3.69
AI669815	*SOX2*	6.02	4.75	5.38	0.91
AI740544	*ADAMTS16*	5.00	5.71	5.35	0.51
AF427618	*CD300LB*	8.12	2.36	5.24	4.07
AL590118	*SERHL2*	8.70	1.66	5.18	4.98
X16866	*CYP2D6*	7.05	3.27	5.16	2.67
AI732702	*RNASEN*	7.21	3.08	5.15	2.91
AI635379	*XPNPEP3*	8.41	1.77	5.09	4.70
AU155621	*TSGA14*	8.41	1.67	5.04	4.77
AI809325	*GTSF1L*	2.49	7.51	5.00	3.55
AB083211	*ZAP70*	7.95	1.94	4.95	4.26
AW072790	*CNTN1*	7.74	2.02	4.88	4.04
NM_000354	*SERPINA7*	6.75	2.72	4.74	2.86
AW009562	*MARVELD2*	6.67	2.74	4.71	2.79
AI129949	*NRXN3*	5.09	4.29	4.69	0.57
AB044805	*PFKFB2*	4.48	4.82	4.65	0.24
BC029051	*ARSB*	7.62	1.52	4.57	4.31
NM_030785	*RSHL1*	7.59	1.55	4.57	4.27
AV658684	*GADD45B*	7.15	1.92	4.53	3.69
NM_170664	*OTOA*	7.53	1.51	4.52	4.26
AA719797	*LOC441453*	2.36	6.67	4.51	3.05
BF514096	*SYT15*	7.45	1.57	4.51	4.16
BE467577	*UBXD1*	1.99	7.01	4.50	3.55
BC000607	*ANAPC11*	7.37	1.59	4.48	4.09
AB046400	*SERPINB4*	7.33	1.61	4.47	4.04
BC002700	*KRT7*	2.83	6.08	4.46	2.31
AF099740	*ACSL6*	5.53	3.35	4.44	1.54
X65661	*SOX4*	5.70	3.15	4.42	1.81
AI459826	*RANBP5*	4.92	3.88	4.40	0.74
D12625	*NF1*	5.08	3.66	4.37	1.00

Table 6.2: Mean expression data for genes shown to be upregulated by androgen treatment in the microarray analysis.
*List of top 250 transcripts in terms of magnitude of fold increase in expression when comparing expression in androgen-treated cells to that in untreated cells (list excludes hypothesized genes, transcripts with very low expression levels in either sample duplicate (<0.5 arbitrary units) and transcripts with standard deviation >5). SD, standard deviation. *, FBLN2 included due to very large fold increase, despite standard deviation >5.*

Accession no.	Gene symbol	Array 1 fold increase	Array 2 fold increase	Mean fold increase	SD
BF679507	*WTAP*	4.23	4.50	4.37	0.26
AA766264	*IPPK*	5.14	3.59	4.36	1.09
BC000329	*SFN*	5.21	3.45	4.33	1.24
AF339814	*hCG_1795283*	6.19	2.44	4.32	2.64
AL575177	*NOG*	3.80	4.78	4.29	0.69
U40317	*PTPRS*	2.91	5.38	4.15	1.75
BC000134	*SWAP70*	2.91	5.37	4.14	1.74
Y11339	*ST6GALNAC1*	3.88	4.13	4.01	0.18
BF511602	*IPO11*	4.23	3.78	4.00	0.33
AA811540	*BANK1*	6.20	1.68	3.94	3.20
S79281	*RNASE1*	6.24	1.63	3.93	3.27
BE550855	*SH3BGRL2*	4.28	3.54	3.91	0.52
AK023470	*AGXT2L2*	2.96	4.85	3.90	1.34
AF007162	*CRYAB*	5.07	2.74	3.90	1.65
BC041694	*MYO10*	5.26	2.53	3.90	1.92
AI671237	*ATP2A2*	4.98	2.79	3.89	1.56
AU144623	*ASB7*	1.76	5.99	3.87	3.00
NM_022449	*RAB17*	4.81	2.90	3.85	1.36
NM_144990	*SLFNL1*	4.48	2.72	3.60	1.24
AF322648	*IRGQ*	5.16	2.02	3.59	2.22
BC032490	*SPIN3*	4.01	3.13	3.57	0.62
NM_000307	*POU3F4*	5.50	1.63	3.57	2.73
NM_004488	*GP5*	5.08	2.01	3.55	2.16
BC029429	*SOX6*	7.52	2.18	3.51	1.90
NM_024922	*CES3*	4.47	2.53	3.50	1.37
AL120025	*TRIP12*	4.17	2.82	3.50	0.96
AI912696	*MAGEE1*	5.07	1.53	3.30	2.50
AB014532	*PTCD1*	4.36	2.23	3.29	1.50
AF029325	*LAMB4*	4.20	2.39	3.29	1.29
AA918425	*PM20D1*	4.07	2.48	3.28	1.12
R85779	*SPTBN1*	4.80	1.75	3.28	2.16
N51479	*ATXN3*	3.68	2.78	3.23	0.64
BQ027856	*NCOA6*	3.19	3.26	3.23	0.14
AY191416	*ZAR1*	4.71	1.71	3.21	2.12
NM_023107	*FGFR1*	2.41	3.94	3.17	1.09
NM_016180	*SLC45A2*	3.13	3.21	3.17	0.16
BC000585	*SLCO3A1*	2.87	3.46	3.17	0.42
NM_012064	*MIP*	3.45	2.87	3.16	0.41
AI670852	*PTPRB*	4.28	2.04	3.16	1.58
BF196691	*COX5A*	4.57	1.74	3.15	1.99
NM_014179	*HSPC157*	4.28	2.02	3.15	1.60
NM_005856	*RAMP3*	3.19	3.05	3.12	0.10
AB041844	*DIO2*	2.64	3.60	3.12	0.68
BE551763	*MLL3*	3.92	2.29	3.11	1.15
X89657	*ADAM3A*	4.46	1.75	3.10	1.92
AF111799	*ITGA11*	3.93	2.27	3.10	1.17
BF000296	*HS3ST1*	2.25	3.93	3.09	1.19
BC001956	*FNIP1*	4.43	1.74	3.09	1.90
AI683694	*EFCAB4A*	3.07	3.10	3.09	0.21

Table 6.2 continued (part 2)

Accession no.	Gene symbol	Array 1 fold increase	Array 2 fold increase	Mean fold increase	SD
AI806482	*PTPRJ*	3.85	2.30	3.08	1.09
BG334196	*EHBP1L1*	2.94	3.19	3.07	0.17
AU143929	*FANK1*	2.24	3.89	3.06	1.17
BC002510	*RAB6B*	2.27	3.64	2.96	0.98
NM_000742	*CHRNA2*	3.90	2.00	2.95	1.34
NM_020247	*CABC1*	4.07	1.83	2.95	1.58
AK074635	*STBD1*	3.33	2.56	2.95	0.54
NM_002098	*GUCA2A*	1.83	4.06	2.95	1.57
S76476	*NTRK3*	4.02	1.84	2.93	1.54
X62009	*FBN2*	3.39	2.46	2.92	0.65
BE968806	*ATP5S*	3.32	2.51	2.92	0.58
AL157452	*SLC1A2*	1.95	3.86	2.90	1.36
BF059512	*DNER*	3.32	2.48	2.90	0.59
AW515704	*LTBP3*	3.30	2.48	2.89	0.58
AW974143	*UCHL1*	2.20	3.58	2.89	0.98
X06828	*VWF*	3.31	2.47	2.89	0.59
AL162062	*FMNL3*	1.79	3.96	2.88	1.54
AK000847	*GPER*	3.30	2.43	2.87	0.61
BC015665	*LATS1*	3.88	1.85	2.86	1.44
BE856607	*NISCH*	3.89	1.84	2.86	1.44
AI990366	*ARHGEF7*	2.08	3.64	2.86	1.10
NM_024623	*OGFOD2*	2.44	3.26	2.85	0.58
AI269290	*SLC18A2*	1.89	3.73	2.81	1.30
AI634662	*SLC13A3*	3.41	2.21	2.81	0.85
AA724708	*SLC1A4*	2.49	3.12	2.81	0.45
AF284095	*ADRA2A*	1.97	3.64	2.80	1.19
BE220445	*VASH1*	3.54	2.07	2.80	1.05
BE259395	*SNAPC5*	3.74	1.85	2.80	1.33
AI677888	*ORAOV1*	4.00	1.57	2.79	1.71
AK025059	*FBXO42*	3.40	2.16	2.78	0.88
BC011022	*DDX3Y*	2.69	2.83	2.76	0.10
AL567376	*LYPD1*	3.26	2.26	2.76	0.71
AI056852	*SHBG*	3.66	1.85	2.75	1.27
AF182274	*CYP1A2*	3.27	2.24	2.75	0.72
BC020814	*SUGT1L1*	3.49	2.01	2.75	1.05
BE645386	*MPPED2*	2.36	3.13	2.74	0.55
AL389934	*SLC30A6*	3.00	2.48	2.74	0.37
NM_003561	*PLA2G10*	3.86	1.60	2.73	1.60
NM_006862	*TDRKH*	2.79	2.66	2.72	0.10
AW297226	*SIX5*	3.53	1.81	2.67	1.22
AF135025	*KLK12*	3.29	2.04	2.67	0.89
NM_000102	*CYP17A1*	1.70	3.62	2.66	1.36
U18549	*GPR6*	3.35	1.95	2.65	0.99
AA939154	*NAP1L4*	2.81	2.48	2.64	0.24
NM_003740	*KCNK5*	2.68	2.61	2.64	0.06
NM_013268	*LGALS13*	2.07	3.21	2.64	0.81
AY026506	*TTLL2*	2.32	2.96	2.64	0.45
NM_021805	*SIGIRR*	3.53	1.74	2.63	1.27
AL133645	*KRT8P12*	1.81	3.44	2.63	1.15

Table 6.2 continued (part 3)

Accession no.	Gene symbol	Array 1 fold increase	Array 2 fold increase	Mean fold increase	SD
NM_017636	*TRPM4*	3.32	1.92	2.62	0.99
AV700621	*TGFBR1*	3.63	1.60	2.61	1.43
NM_133638	*ADAMTS19*	2.40	2.80	2.60	0.28
AA283642	*MAP6D1*	2.23	2.96	2.60	0.52
BC017084	*XYLT2*	3.55	1.64	2.59	1.36
AI970898	*ACACB*	2.72	2.45	2.58	0.18
AI681419	*hCG_1985469*	3.59	1.53	2.56	1.46
NM_024707	*GEMIN7*	2.03	3.09	2.56	0.75
NM_000258	*MYL3*	2.75	2.37	2.56	0.27
AI968068	*BMP6*	2.14	2.96	2.55	0.58
NM_020041	*SLC2A9*	3.17	1.93	2.55	0.88
AI017382	*ATXN7L1*	1.96	3.14	2.55	0.83
NM_002772	*PRSS7*	2.70	2.33	2.51	0.25
NM_173352	*KRT78*	1.98	3.04	2.51	0.75
AK096918	*MPV17L*	2.82	2.20	2.51	0.44
NM_022148	*CRLF2*	3.39	1.62	2.51	1.26
AW292746	*MYLIP*	2.83	2.18	2.50	0.47
AU144102	*SNRPE*	2.94	2.07	2.50	0.61
AU144243	*CCPG1*	2.64	2.36	2.50	0.20
AL831967	*GATS*	3.46	1.53	2.49	1.36
AA343027	*SEMA3D*	3.13	1.85	2.49	0.91
NM_002920	*RFX4*	3.18	1.81	2.49	0.98
AW014734	*KLF15*	2.51	2.47	2.49	0.03
BC006276	*APOL4*	3.20	1.78	2.49	1.00
BC020924	*STX8*	2.68	2.30	2.49	0.27
NM_005510	*DOM3Z*	1.81	3.17	2.49	0.96
BC039480	*DEPDC4*	2.48	2.49	2.48	0.01
BC005872	*HDAC6*	3.03	1.93	2.48	0.78
AF325460	*CLEC4C*	3.21	1.74	2.48	1.05
NM_016186	*SERPINA10*	3.31	1.64	2.47	1.19
AI628573	*FGFBP3*	2.45	2.49	2.47	0.03
AV703470	*PIAS2*	2.79	2.15	2.47	0.45
AB002356	*MADD*	2.55	2.36	2.46	0.14
BE327650	*GTDC1*	2.03	2.88	2.46	0.59
L32185	*SLC11A1*	3.29	1.61	2.45	1.19
AF078803	*CAMK2B*	3.09	1.81	2.45	0.91
AL832613	*SLC46A1*	1.72	3.18	2.45	1.03
AA731713	*DDN*	2.77	2.12	2.44	0.45
AK026883	*GPR157*	3.12	1.76	2.44	0.96
AF131796	*EXOSC6*	2.62	2.25	2.44	0.27
R06750	*ACOT12*	2.68	2.19	2.43	0.35
NM_025134	*CHD9*	2.09	2.78	2.43	0.48
NM_025247	*ACAD10*	3.24	1.61	2.43	1.15
AI765180	*CPNE8*	1.87	2.98	2.43	0.79
AW769732	*PAX2*	2.10	2.74	2.42	0.45
NM_022975	*FGFR2*	2.54	2.29	2.42	0.18
AA527180	*ELF3*	1.69	3.14	2.41	1.02
NM_002015	*FOXO1*	3.08	1.73	2.41	0.95
NM_000458	*HNF1B*	3.11	1.71	2.41	0.99

Table 6.2 continued (part 4)

Accession no.	Gene symbol	Array 1 fold increase	Array 2 fold increase	Mean fold increase	SD
BC020838	*CLDN20*	2.92	1.89	2.40	0.74
NM_007084	*SOX21*	2.97	1.80	2.39	0.83
NM_000576	*IL1B*	3.05	1.72	2.38	0.95
NM_012139	*SERGEF*	2.81	1.95	2.38	0.61
AI066599	*SNAPC3*	2.09	2.65	2.37	0.40
NM_007117	*TRH*	3.03	1.63	2.33	0.99
AV728846	*RG9MTD3*	2.36	2.29	2.32	0.06
AK092565	*EPHA6*	2.24	2.40	2.32	0.11
AB014594	*DOCK10*	2.19	2.44	2.31	0.17
AI359165	*SERPINE2*	3.10	1.52	2.31	1.12
AW025687	*CYP4F22*	1.85	2.65	2.25	0.57
AA056099	*SUV420H1*	2.09	2.40	2.25	0.21
AL512713	*STOX2*	2.04	2.45	2.24	0.30
BQ944989	*STRAP*	2.75	1.73	2.24	0.72
NM_002010	*FGF9*	1.88	2.59	2.23	0.49
NM_000766	*CYP2A13*	2.27	2.19	2.23	0.06
AI133721	*MEG3*	2.69	1.75	2.22	0.66
BM914560	*MUC20*	2.37	2.03	2.20	0.24
U59479	*PNN*	2.61	1.79	2.20	0.58
AF070577	*OPCML*	2.60	1.79	2.19	0.57
S58722	*DXS542*	2.33	2.02	2.18	0.21
AA911569	*TPM4*	2.62	1.73	2.18	0.62
T65123	*CDH8*	2.08	2.28	2.18	0.14
N25562	*TWF1*	1.81	2.54	2.17	0.52
BC005196	*KLK2*	2.83	1.51	2.17	0.93
NM_021071	*ART4*	2.66	1.68	2.17	0.69
BE672217	*SEZ6L*	1.74	2.58	2.16	0.59
AI675298	*CCNL1*	2.57	1.73	2.15	0.59
AL512755	*SLC37A1*	2.37	1.92	2.15	0.31
BE675173	*TNFSF13*	1.97	2.31	2.14	0.24
BF103605	*RAB3GAP1*	2.65	1.63	2.14	0.72
BC042451	*CACNA1A*	2.64	1.61	2.12	0.72
NM_001671	*ASGR1*	2.45	1.79	2.12	0.47
BF447669	*TRPM6*	2.37	1.87	2.12	0.35
AA687627	*OXCT2*	2.02	2.21	2.11	0.14
BC005924	*PSG3*	1.91	2.30	2.11	0.28
M36653	*POU2F2*	1.99	2.21	2.10	0.16
NM_021044	*DHH*	1.77	2.43	2.10	0.47
BC012503	*MITF*	2.20	1.99	2.09	0.16
NM_022122	*MMP27*	2.60	1.50	2.05	0.78
AK022853	*SLC6A15*	2.47	1.62	2.05	0.59
BF183535	*RAB11FIP3*	2.28	1.79	2.04	0.34
AL122090	*LYRM2*	2.11	1.96	2.04	0.11
X63118	*ESR1*	1.88	2.18	2.03	0.21
AL045306	*NUDT10*	2.08	1.98	2.03	0.07
NM_014207	*CD5*	1.69	2.36	2.02	0.47
AI762621	*GATA6*	2.34	1.70	2.02	0.45
AF027205	*SPINT2*	1.79	2.24	2.02	0.31
BC020604	*TDRD3*	2.09	1.92	2.00	0.15

Table 6.2 continued (final part)

Accession no.	Gene symbol	Array 1 fold decrease	Array 2 fold decrease	Mean fold decrease	SD
NM_015995	*KLF13*	9.65	18.94	14.30	4.78
AI218580	*RORC*	5.55	22.62	14.08	11.09*
M22734	*PDGFRA*	4.41	13.95	9.18	4.89
AW195553	*ADO*	5.72	11.00	8.36	3.73
AW295295	*SLC34A1*	2.97	12.57	7.77	4.91
AC000099	*GRM8*	8.79	6.62	7.70	1.54
BF527050	*SOX8*	7.48	7.70	7.59	0.26
U31931	*CIITA*	10.77	4.27	7.52	4.60
AF123654	*LZTS1*	8.85	4.93	6.89	2.77
U76388	*NR5A1*	2.39	11.22	6.81	4.24
AF333762	*VN1R3*	1.79	8.85	5.32	4.99
NM_152552	*SAMD3*	3.85	6.78	5.31	2.06
NM_002587	*PCDH1*	1.58	4.62	5.30	2.04
NM_004360	*CDH1*	2.07	8.52	5.30	4.57
AF195116	*TINAG*	2.97	7.58	5.27	3.27
AL136808	*TTLL5*	2.76	7.78	5.27	3.55
AL137673	*DR1*	5.20	5.31	5.25	0.07
AA705063	*CALD1*	5.62	4.81	5.21	0.58
NM_000770	*CYP2C8*	1.84	7.48	4.66	3.99
BC034769	*IZUMO1*	1.90	7.37	4.63	3.87
AK024437	*MAPK8IP3*	2.15	7.11	4.63	3.51
X63759	*TNP2*	2.83	6.40	4.62	2.53
AF348077	*TSSK6*	2.53	6.70	4.61	2.96
AW080339	*BRE*	1.91	7.09	4.50	3.66
AL096734	*ISG20L2*	2.27	6.65	4.46	3.10
AF493430	*FOXP2*	2.52	6.40	4.46	2.74
AA348410	*ITPKB*	2.54	6.35	4.45	2.69
AF208159	*SLC12A5*	3.62	5.25	4.44	1.16
AV752499	*OXCT1*	4.96	3.90	4.43	0.75
N72525	*GATA5*	1.57	7.23	4.40	4.00
BF109557	*FBXO10*	1.63	7.01	4.32	3.80
AL034348	*KRT18P50*	1.71	6.93	4.32	3.69
BG420053	*CAPZB*	2.81	5.79	4.30	2.11
AE000660	*TRAV20*	2.15	6.42	4.28	3.03
AW385452	*SHROOM1*	3.54	4.99	4.27	1.03
AW007929	*FOXK1*	1.56	6.96	4.26	3.82
NM_003803	*MYOM1*	1.37	7.13	4.25	4.07
AK026640	*SLC27A5*	2.70	5.79	4.25	2.18
AF334676	*TEKT3*	1.53	6.51	4.02	3.52
AA148535	*PTCHD1*	3.49	4.54	4.01	0.74
H38921	*FRZB*	1.27	6.74	4.01	3.86
AK097594	*FBXW12*	1.69	6.31	4.00	3.27

Table 6.3: Mean expression data for genes shown to be downregulated by androgen treatment in the microarray analysis.
*List of top 250 transcripts in terms of magnitude of fold decrease in expression when comparing expression in androgen-treated cells to that in untreated cells (list excludes hypothesized genes, transcripts with very low expression levels in either sample duplicate (<0.5 arbitrary units) and transcripts with standard deviation >5). SD, standard deviation. *, RORC included due to very large fold increase, despite standard deviation >5.*

Accession no.	Gene symbol	Array 1 fold decrease	Array 2 fold decrease	Mean fold decrease	SD
BC037547	*CDC20B*	2.64	5.35	4.00	1.92
NM_013992	*PAX8*	3.65	4.30	3.98	0.47
U08092	*HNMT*	3.90	4.05	3.98	0.11
AJ001306	*INADL*	1.66	6.26	3.96	3.25
AA601213	*PYGB*	2.24	5.67	3.96	2.42
BC046095	*AKAP12*	2.22	5.70	3.96	2.46
AA565499	*NLRP7*	1.34	6.56	3.95	3.69
BF110551	*GRIA3*	3.17	4.72	3.95	1.10
NM_003053	*SLC18A1*	1.56	6.27	3.92	3.34
Z82214	*SCUBE1*	2.01	5.82	3.91	2.70
NM_022147	*RTP4*	3.46	4.37	3.91	0.64
AL022101	*PRAMEF10*	3.57	4.20	3.88	0.45
AK025002	*ARRDC1*	3.76	4.01	3.88	0.17
NM_012259	*HEY2*	1.48	6.24	3.86	3.37
NM_000061	*BTK*	1.58	6.12	3.85	3.21
NM_000718	*CACNA1B*	3.15	4.55	3.85	0.99
AI380289	*PTBP1*	2.74	4.95	3.85	1.57
AI767388	*SLC5A9*	1.55	6.08	3.82	3.20
NM_004321	*KIF1A*	1.39	6.25	3.82	3.44
AF061812	*KRT16*	3.41	4.19	3.80	0.55
AK026415	*CHN2*	3.35	4.23	3.79	0.62
BF793585	*NRG4*	1.88	5.70	3.79	2.70
NM_003332	*TYROBP*	2.66	4.89	3.77	1.57
AA905508	*SPATA17*	1.57	5.96	3.77	3.10
AL136728	*SLC10A7*	3.00	4.45	3.73	1.03
BF512061	*MPP7*	1.65	5.69	3.67	2.86
AK025044	*SLC26A3*	2.06	5.24	3.65	2.25
NM_005430	*WNT1*	3.48	3.76	3.62	0.20
NM_018635	*HDLBP*	1.31	5.90	3.61	3.24
AF164963	*VENTXP1*	3.14	4.06	3.60	0.65
NM_144639	*UROC1*	2.11	5.08	3.59	2.09
NM_014759	*PHYHIP*	2.42	4.75	3.58	1.64
W67461	*ANGPTL1*	1.35	5.81	3.58	3.15
NM_004752	*GCM2*	2.92	4.22	3.57	0.92
NM_006192	*PAX1*	3.29	3.83	3.56	0.38
NM_006671	*SLC1A7*	2.53	4.57	3.55	1.44
NM_032243	*TXNDC2*	2.32	4.78	3.55	1.74
AF285604	*MOV10L1*	1.52	5.58	3.55	2.87
AL117659	*ATP9A*	1.40	5.68	3.54	3.03
AI831932	*KLF2*	1.33	5.73	3.53	3.11
AF115544	*CDKN2A*	2.79	4.16	3.47	0.98
NM_019844	*SLCO1B3*	1.39	5.53	3.46	2.93
NM_014037	*SLC6A16*	1.93	4.97	3.45	2.15
W72348	*WNT4*	3.04	3.70	3.37	0.47
NM_004277	*SLC25A27*	1.99	4.71	3.35	1.92
AA393479	*CYP19A1*	1.29	5.28	3.28	2.81
AI431730	*ADAMTS9*	2.20	4.33	3.26	1.50
NM_000780	*CYP7A1*	2.15	4.28	3.22	1.50
NM_000384	*APOB*	2.46	3.97	3.21	1.07

Table 6.3 continued (part 2)

Accession no.	Gene symbol	Array 1 fold decrease	Array 2 fold decrease	Mean fold decrease	SD
AA906578	*FBXO41*	2.73	3.69	3.21	0.68
AI970061	*GPR155*	1.99	4.41	3.20	1.71
AL079281	*NPAS3*	2.24	4.14	3.19	1.34
AF137396	*OR51B4*	2.54	3.82	3.18	0.91
N29801	*ADAM22*	1.49	4.86	3.18	2.39
U11701	*LHX2*	1.51	4.79	3.15	2.49
BC032854	*ZRF1*	1.78	4.48	3.13	1.91
AF240697	*DHRS9*	1.95	4.29	3.12	1.65
AW207389	*ADAMTS8*	1.74	4.50	3.12	1.95
BC014430	*MEIS3*	2.17	4.05	3.11	1.33
AA442149	*MAF*	1.44	4.76	3.10	2.35
AA527340	*HOXB6*	1.33	4.87	3.10	2.50
AY014285	*TEX101*	2.06	4.11	3.09	1.46
NM_152453	*TMCO5*	2.00	4.05	3.02	1.44
NM_001240	*CCNT1*	1.94	4.09	3.02	1.53
AF338730	*KCNB2*	1.58	4.43	3.01	2.02
NM_021220	*OVOL2*	1.70	4.30	3.00	1.84
BC031261	*ZPLD1*	1.36	4.63	3.00	2.31
BF514781	*MYC*	1.60	4.38	2.99	1.97
AK022172	*FMO5*	1.47	4.40	2.93	2.08
NM_015839	*FCN2*	1.52	4.33	2.93	1.99
NM_006862	*TDRKH*	1.54	4.28	2.91	1.94
BC040275	*RASGRF1*	2.11	3.67	2.89	1.10
BC028005	*GRASPOS*	3.02	2.76	2.89	0.18
NM_000864	*HTR1D*	2.35	3.41	2.88	0.75
X64977	*OR2K2*	2.51	3.24	2.88	0.52
AL041441	*KCTD19*	3.48	2.27	2.87	0.86
NM_000540	*RYR1*	3.37	2.37	2.87	0.71
AF189270	*PBOV1*	2.56	3.18	2.87	0.44
AB022847	*SLC6A2*	2.90	2.80	2.85	0.17
AF143869	*CARD8*	3.29	2.42	2.85	0.62
BC020765	*SERPINE1*	4.14	1.46	2.80	1.90
NM_139057	*ADAMTS17*	4.01	1.56	2.78	1.73
NM_000594	*TNF*	3.50	2.05	2.77	1.02
L14482	*POU6F1*	1.79	3.66	2.73	1.32
BC042138	*PGPEP1*	3.67	1.78	2.73	1.33
AI797276	*SHE*	1.62	3.82	2.72	1.56
AK021881	*HIF3A*	1.55	3.89	2.72	1.65
NM_002009	*FGF7*	3.47	1.97	2.72	1.06
AF268613	*POU5F1P4*	3.32	2.11	2.72	0.86
NM_000349	*STAR*	2.53	2.90	2.71	0.25
NM_014834	*LRRC37A2*	2.58	2.85	2.71	0.20
AF311856	*SPTBN4*	3.04	2.38	2.71	0.47
AI659683	*ANK1*	3.06	2.37	2.71	0.49
AK025041	*LMAN2*	1.61	3.81	2.71	1.56
AL832766	*GADL1*	3.44	1.97	2.71	1.05
NM_002119	*HLA-DOA*	3.44	1.98	2.71	1.03
BC003111	*PBX2*	3.85	1.56	2.70	1.61
NM_018557	*LRP1B*	2.83	2.57	2.70	0.18

Table 6.3 continued (part 3)

Accession no.	Gene symbol	Array 1 fold decrease	Array 2 fold decrease	Mean fold decrease	SD
H05303	*SEZ6*	2.47	2.93	2.70	0.33
AY039237	*DDX53*	1.89	3.51	2.70	1.15
NM_006543	*HHCM*	1.61	3.78	2.70	1.53
NM_004694	*SLC16A6*	2.42	2.97	2.70	0.40
NM_014420	*DKK4*	2.41	2.98	2.70	0.41
NM_020328	*ACVR1B*	3.75	1.56	2.66	1.56
BC028936	*IKZF2*	1.56	3.74	2.65	1.54
AF329092	*FILIP1L*	2.12	3.17	2.65	0.75
BU680030	*TTC6*	2.96	2.34	2.65	0.44
BC034571	*PLA2G4D*	1.57	3.71	2.64	1.51
AI198586	*LMX1A*	2.68	2.59	2.63	0.06
AF327560	*MFAP3L*	3.48	1.78	2.63	1.20
AI857933	*ITGA6*	2.51	2.75	2.63	0.17
AF019382	*MBL1P1*	2.31	2.95	2.63	0.45
NM_004196	*CDKL1*	2.52	2.74	2.63	0.16
X75363	*KLK15*	2.15	3.11	2.63	0.68
NM_000842	*GRM5*	1.37	3.88	2.63	1.78
BC004936	*SCD5*	3.42	1.83	2.63	1.12
BF726531	*AQP5*	2.83	2.43	2.63	0.28
BF997225	*PAPPA*	3.06	2.19	2.62	0.61
BC028232	*SNORA78*	3.43	1.81	2.62	1.15
Z25428	*MAP3K13*	2.53	2.71	2.62	0.13
AF173154	*HYAL1*	3.07	2.17	2.62	0.64
NM_015848	*KRT76*	2.49	2.66	2.58	0.11
NM_000223	*KRT12*	3.06	2.09	2.57	0.69
NM_017783	*CHD7*	1.84	3.30	2.57	1.03
NM_145027	*KIF6*	1.96	3.18	2.57	0.86
AB055704	*LHX4*	3.33	1.74	2.54	1.12
AA132448	*MUC20*	2.30	2.75	2.53	0.33
AB058895	*GHRHR*	3.31	1.74	2.52	1.10
NM_138454	*NXNL1*	2.47	2.58	2.52	0.08
AA115759	*RDH13*	1.48	3.56	2.52	1.47
AL133109	*ARPP-21*	2.38	2.65	2.51	0.18
NM_004750	*CRLF1*	2.22	2.80	2.51	0.41
NM_019113	*FGF21*	1.64	3.37	2.51	1.23
AF257210	*NPFFR2*	2.94	2.07	2.51	0.62
AW375186	*CLDN23*	2.60	2.40	2.50	0.14
BC040857	*SERPINA12*	2.11	2.89	2.50	0.55
N62814	*NKAIN2*	2.71	2.28	2.49	0.31
NM_138322	*PCSK6*	3.31	1.67	2.49	1.16
AB037776	*IGSF9*	2.57	2.41	2.49	0.11
AL162082	*USP22*	2.47	2.50	2.49	0.03
AY009401	*WNT6*	3.10	1.87	2.49	0.86
AF073299	*SLC9A2*	1.89	3.01	2.45	0.79
BC023578	*TNKS*	1.85	3.06	2.45	0.85
NM_052885	*SLC2A13*	3.22	1.67	2.44	1.09
AI802877	*SLC16A11*	1.59	3.27	2.43	1.19
AK057223	*hCG_2007354*	1.95	2.91	2.43	0.68
AA836116	*SLC15A2*	1.98	2.84	2.41	0.61

Table 6.3 continued (part 4)

Accession no.	Gene symbol	Array 1 fold decrease	Array 2 fold decrease	Mean fold decrease	SD
BE504430	*SOX1*	1.44	3.35	2.39	1.34
U08024	*SULT2A1*	1.40	3.36	2.38	1.39
NM_004115	*FGF14*	1.53	3.23	2.38	1.20
M60316	*BMP7*	1.27	3.48	2.38	1.57
NM_000894	*LHB*	1.75	2.99	2.37	0.88
AA496211	*ARX*	1.39	3.35	2.37	1.39
NM_000771	*CYP2C9*	2.04	2.69	2.36	0.47
NM_020956	*PRX*	2.01	2.72	2.36	0.49
AI394679	*KLK12*	3.11	1.61	2.36	1.06
NM_005630	*SLCO2A1*	2.45	2.26	2.36	0.14
NM_000590	*IL9*	1.55	3.16	2.35	1.13
NM_006588	*SULT1C4*	2.48	2.21	2.35	0.18
AL833335	*ATP1A4*	1.40	3.27	2.34	1.32
NM_005593	*MYF5*	2.34	2.33	2.34	0.18
AL121753	*MMP24*	1.43	3.23	2.33	1.27
AK024128	*SBNO1*	1.30	3.36	2.33	1.46
NM_002423	*MMP7*	2.30	2.35	2.32	0.24
NM_024016	*HOXB8*	2.70	1.92	2.31	0.55
AW192795	*MUC5AC*	1.71	2.90	2.31	0.83
T49766	*MAGIX*	1.67	2.95	2.31	0.91
NM_001231	*CASQ1*	1.57	3.03	2.30	1.03
BF436898	*ETV6*	2.45	2.16	2.30	0.21
AI632972	*TPD52*	2.44	2.16	2.30	0.20
NM_014009	*FOXP3*	1.74	2.85	2.29	0.78
U27331	*FUT6*	1.40	3.16	2.28	1.24
AF325549	*MYLK2*	2.31	2.25	2.28	0.04
NM_152772	*TCP11L2*	1.72	2.84	2.28	0.79
BG055348	*RAB3C*	1.89	2.65	2.27	0.54
NM_000326	*RLBP1*	1.40	3.14	2.27	1.23
AJ296272	*LHX9*	2.09	2.43	2.26	0.24
NM_005123	*NR1H4*	2.73	1.79	2.26	0.66
AF124790	*ESR2*	2.98	1.53	2.25	1.03
NM_006174	*NPY5R*	2.13	2.34	2.24	0.16
NM_004626	*WNT11*	1.85	2.61	2.23	0.54
Y10259	*MC2R*	1.48	2.96	2.22	1.05
NM_003822	*NR5A2*	1.29	3.16	2.22	1.33
AI381544	*SLC25A25*	1.88	2.49	2.19	0.42
AI627943	*SLC12A3*	1.45	2.80	2.13	0.95
AI955614	*CRIM2*	1.43	2.80	2.11	0.96
NM_005906	*MAK*	1.57	2.63	2.10	0.75
AW964006	*CYP3A5*	1.60	2.56	2.08	0.68
NM_006194	*PAX9*	1.99	2.17	2.08	0.13
U22044	*CYP2A7P1*	2.22	1.94	2.08	0.20
R59977	*TADA3L*	1.66	2.49	2.07	0.59
AF026303	*SULT1C2*	2.11	2.02	2.06	0.07
BC012536	*HSD17B12*	2.00	2.11	2.05	0.08
NM_030807	*SLC2A11*	2.03	2.01	2.02	0.11
NM_000125	*ESR1*	1.36	2.66	2.01	0.92
BC039000	*CCNYL2*	1.99	2.02	2.00	0.13

Table 6.3 continued (final part)

6.3.4 Selection of putative androgen-regulated genes for validation of microarray data and for further confirmation as targets of androgen action via the AR

In order to validate the findings of the microarray experiment, a selection of genes were chosen from the preceeding data tables, and their expression in genital fibroblasts analysed using RT-PCR and/or qPCR. RNA samples for this task were obtained from parallel cultures of cells from the same fetus that was studied in the microarray experiment, as well as from cells originating from the other fetuses considered for microarray analysis (Table 6.1). As such, all RNA was derived from cells that received either DHT or vehicle treatment for 4 hrs; just like in the microarray analysis. The subset of genes analysed by RT-PCR was chosen arbitrarily according to the scale of the fold change on microarray in conjunction with known associations (or gene family associations) with sexual differentiation, steroid hormone action or steroidogenesis. The genes analysed by RT-PCR and the details of their selective criteria are shown in Table 6.4 (upregulated genes) and Table 6.5 (downregulated genes).

Gene symbol	Gene name	Mean fold increase on array	Function/relevance
FBLN2	Fibulin 2	23.37 (+/- 15.15)	Organogenesis/expression in developing seminiferous tubules of fetal mouse (Zhang *et al*., 1996); normal testis development in rat (Loveland *et al*., 1998); vas deferens development (Vogel and Hedgecock, 2001).
LSM4	LSM4 homolog, U6 small nuclear RNA associated	7.96 (+/- 4.59)	Important for processing/degradation of mRNA in humans (Ingelfinger *et al*., 2002); essential for early mouse embryonic development (Hirsch *et al*., 2000).
TBX6	T-box 6	6.88 (+/- 4.74)	Mesoderm formation, cellular differentiation, expressed in early human development and in adult testis (Papapetrou *et al*., 1999).
CYP3A7	Cytochrome P450, family 3, subfamily A, polypeptide 7	6.75 (+/- 1.60)	Putative interaction with oestrogen and progesterone receptor, mediates alternative pathway for hormonal physiological effects (Masuyama *et al*., 2003); adrenal androgen production (Goodarzi *et al*., 2008); fetal steroid metabolism (Stevens, 2006).
CYBB	Cytochrome b-245, beta polypeptide	6.60 (+/- 4.51)	Microbial clearance (Leusen *et al*., 1994).
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells2	6.43 (+/- 3.68)	Ubiquitous transcription factor, implicated in prostate cancer tumour growth (Nadiminty *et al*., 2008); putatively androgen regulated (Lessard *et al*., 2007).
SOX2	SRY (sex determining region Y)-box 2	5.38 (+/- 0.91)	Genital abnormalities when mutated, AEG syndrome (Williamson *et al*., 2006).
LOC441453	Similar to olfactory receptor, family 7, subfamily A, member 17	4.51 (+/- 3.05)	Effects on oestrogen receptor (Frasor *et al*., 2006).
SOX6	SRY (sex determining region Y)-box 6	3.51 (+/- 1.90)	Importance in early development, homologue expressed in rat testis and ovary (Narahara *et al*., 2002); role in spermatogenesis (Connor *et al*., 1995); interaction with SOX9 (Lefebvre *et al*., 2007).
PAX2	Paired box 2	2.42 (+/- 0.45)	Importance in early development, Shh/WT1 interaction (Riccomagno *et al*., 2002; Discenza *et al*., 2003); urogenital tract development (Eccles *et al*., 2002).
FGF9	Fibroblast growth factor 9 (glia-activating factor)	2.23 (+/- 0.49)	Importance in sex determination, Sertoli cell function, SOX9 activity (Kim *et al*., 2006); testicular embryogenesis, sex reversal when absent and Shh interaction (Colvin *et al*., 2001).
GATA6	GATA binding protein 6	2.02 (+/- 0.45)	Expressed in urogenital ridge (Morrisey *et al*., 1996); Important regulator of cell differentiation during early development, regulates gonad development and function in mouse and humans (Ketola *et al*., 1999; Laitinen *et al*., 2000).
TDRD3	Tudor domain containing 3	2.00 (+/- 0.15)	DNA/RNA modification (Huang *et al*., 2006); germ cell development (Breitwieser *et al*., 1996).
SOX5	SRY (sex determining region Y)-box 5	n/a	Importance in early development, SOX9 interaction (Lefebvre *et al*., 2007); aromatase regulation (Huang *et al*., 2009); prostate cancer growth (Ma *et al*., 2009).

Table 6.4: Genes shown to be upregulated by androgen treatment in the microarray analysis, chosen for further analysis and validation of data.
SOX5 was included for analysis given its interesting function, potential relevance and possible redundant role with SOX6. +/-, standard deviation.

Gene symbol	Gene name	Mean fold decrease on array	Function/relevance
KLF13	Kruppel-like factor 13	14.30 (+/- 4.78)	Cell differentiation in early development, StAR and CYP11A1 regulation (Natesampillai *et al*., 2008).
RORC	RAR-related orphan receptor C	14.08 (+/- 11.09)	Orphan nuclear hormone receptor, transcriptional regulation in early development (Hirose *et al*., 1994).
PDGFRA	Platelet-derived growth factor receptor, alpha polypeptide	9.18 (+/- 4.89)	Human embryonic differentiation, steroidogenesis modulation and Leydig cell differentiation (O'Shaughnessy *et al*., 2008; Schmahl *et al*., 2008).
SLC34A1	Solute carrier family 34 (sodium phosphate), member 1	7.77 (+/- 4.91)	Important for correct urogenital tract functioning in mouse and humans (Tenenhouse *et al*., 1994; Prie *et al*., 2002); regulation of spermatogenesis through induction of *profilin-III* expression (Braun *et al*., 2002).
SOX8	SRY (sex determining region Y)-box 8	7.59 (+/- 0.26)	Strong expression in fetal mouse testis (Sock *et al*., 2001); role in gonad formation, co-expression and functional redundancy with SOX9, regulation of spermatogenesis (Barrionuevo *et al*., 2009).
PCDH1	Protocadherin 1	5.30 (+/- 2.04)	Gene family implicated in prostate cancer progression, expressed in fetal urogenital tract (Yang *et al*., 2005; Redies *et al*., 2008; Jennbacken *et al*., 2009).
SHROOM1	Shroom family member 1	4.27 (+/- 1.03)	Cytoskeleton formation, egg formation, epithelium morphogenesis (Dye *et al*., 2009; Lee *et al*., 2009a; Lee *et al*., 2009b).
WNT4	Wingless-type MMTV integration site family, member 4	3.37 (+/- 0.47)	Development of mouse urogenital tract (Stark *et al*., 1994); repression of testosterone production and required for mullerian duct formation in mouse (Vainio *et al*., 1999); regulation of DAX1 and gonad development in humans (Jordan *et al*., 2001); WNT6 interaction (Itaranta *et al*., 2002).
LHX2	LIM homeobox 2	3.15 (+/- 2.49)	Stem cell expression and differentiation (Rhee *et al*., 2006); relatively increased expression in ovary compared to testis (Perrett, 2008).
TMCO5	Transmembrane and coiled-coil domains 5	3.02 (+/- 1.44)	Membrane protein with unknown function (Zhang *et al*., 2009).
SERPINA12	Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 12	2.50 (+/- 0.55)	Gut hormone involved in regulation of body weight homeostasis (Caminos *et al*., 2009).
WNT6	Wingless-type MMTV integration site family, member 6	2.49 (+/- 0.86)	WNT4 interaction (Itaranta *et al*., 2002); genital ridge and testis cords expression, putative requirement for maturation of Sertoli cell precursors (Cory *et al*., 2007).
BMP7	Bone morphogenetic protein 7 (osteogenic protein 1)	2.38 (+/- 1.57)	Expressed early in embryogenesis, important for organogenesis, regulation of PAX2 in mouse (Morcillo *et al*., 2006); early mesonephric duct development in mouse, interacts with FGF (Godin *et al*., 1999; Dudley *et al*., 1999).
NPY5R	Neuropeptide Y receptor Y5	2.24 (+/- 0.16)	Proliferation of postnatal neuronal precursor cells (Hansel *et al*., 2001); other NPYs increased expression in ovary relative to testis (Perrett, 2008).

Table 6.5: Genes shown to be downregulated by androgen treatment in the microarray analysis, chosen for further analysis and validation of data.
+/-, standard deviation

6.3.5 Validation of microarray data through the use RT-PCR

Initial RT-PCR analyses of the expression of all the genes selected in Tables 6.5 and 6.6, revealed only minor differences in expression when comparing RNA extracted from untreated and DHT-treated cultured fetal genital fibroblasts. Generally, overall expression of these genes was inconsistent or only evident in one fetus of the three tested, and there was little indication of upregulation or downregulation of expression due to DHT treatment (data not shown). However, four genes did consistently demonstrate strong expression in cultured fetal genital fibroblasts, along with significant differences in levels of expression as a result of DHT treatment. These genes were: *SOX2, CYP3A7*, *KLF13* and *PCDH1*. Further fully optimised RT-PCR reactions indicated *SOX2* and *CYP3A7* gene transcription increased in genital fibroblasts treated with DHT compared to untreated cells (Fig. 6.3, A and B), suggesting androgen-mediated upregulation of these genes, consistent with the microarray data. In contrast, *KLF13* and *PCDH1* expression appeared downregulated by DHT (Fig. 6.3, C and D); once again concordant with the results from the microarray. These patterns of expression for *SOX2, CYP3A7*, *KLF13*, and *PCDH1* were consistent in all the fetuses tested, although generally the effects of androgen treatment appeared slightly less potent in 46,XX cells.

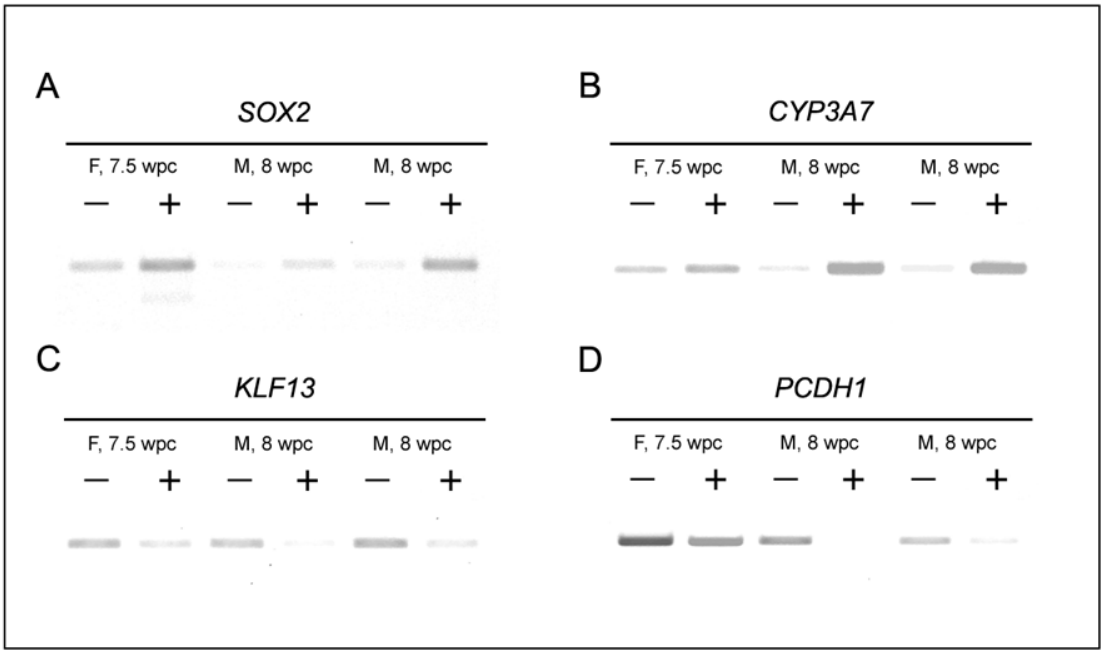

Figure 6.3: RT-PCR analysis of the expression of the putative androgen-regulated genes SOX2, CYP3A7, KLF13 and PCDH1 in fetal human external genitalia.
A and B, genes upregulated by androgen treatment in the microarray data; C and D, genes downregulated by androgen treatment in the microarray data. PCR Cycle no used.: A, 38; B, 38; C, 30; D, 32. –, genital fibroblasts treated with vehicle for 4 hrs; +, genital fibroblasts treated for 4 hrs with 10 nM DHT. F, female fetus; M, male fetus; wpc, weeks post conception. Each reaction was repeated three times and one representative image is shown for each.

6.3.6 Validation of microarray data through the use of qPCR

In addition to the use of RT-PCR, qPCR analysis was also employed to validate the microarray data, and perform a more detailed quantitative study of a small number of genes demonstrated to have good candidature for AR regulation. The qPCR analysis examined the expression of *SOX2* and *CYP3A7* from the previous list of candidate AR target genes, primarily based on the RT-PCR data, but also because of the important associations between *SOX2* and sexual differentiation discussed previously. The expression of *CHD7* (chromodomain helicase DNA binding protein 7) was also investigated, due to the association of this gene and *SOX2* in AEG syndrome. From the array data, *CHD7* expression failed to show a consistent change due to androgen treatment.

By qPCR, *CHD7* expression was unaffected by 10 nM DHT treatment for 4 hrs (Fig. 6.4). In contrast, *SOX2* expression showed an approximately 2-fold increase

in expression in +DHT samples compared to -DHT samples (Fig. 6.5). Similarly, *CYP3A7* expression was upregulated by DHT, demonstrating an approximately 2.5-fold mean increase in expression in response to the 4 hr androgen treatment (Fig. 6.6). For each gene, RNA from two fetuses was tested (including the fetus used for the expression microarray), and a total of four experiments were performed for each gene investigated.

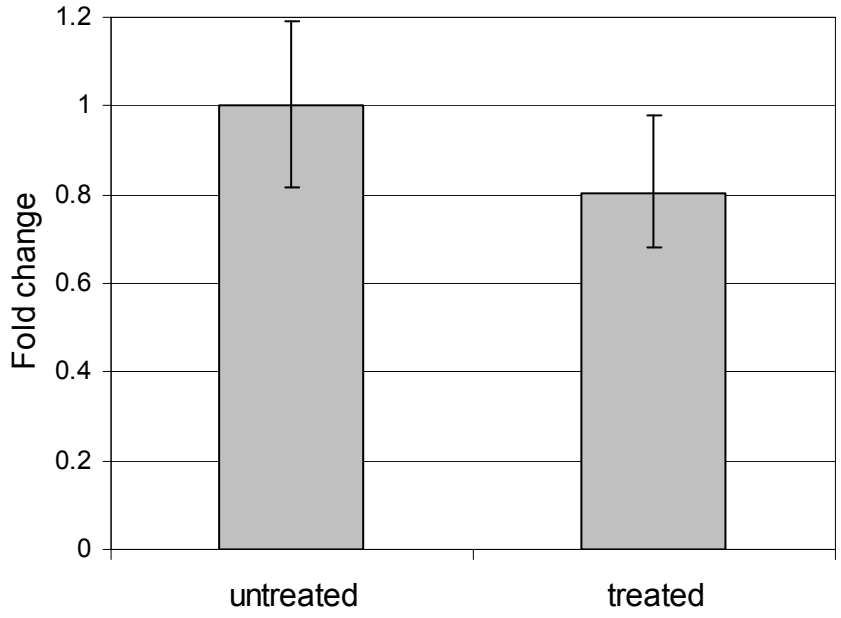

Figure 6.4: Fold change in CHD7 expression by qPCR analysis in genital fibroblasts either untreated, or treated with 10 nM DHT for 4 hrs. *n=2 (two 8 wpc 46,XY male fetuses). Data normalized to negative control expression of TATA box-binding protein (TBP). Expression for untreated cells standardized to 1. Error bars represent standard deviation (SD). Untreated cells SD +/- 0.2; treated cells SD +/- 0.2/0.1.*

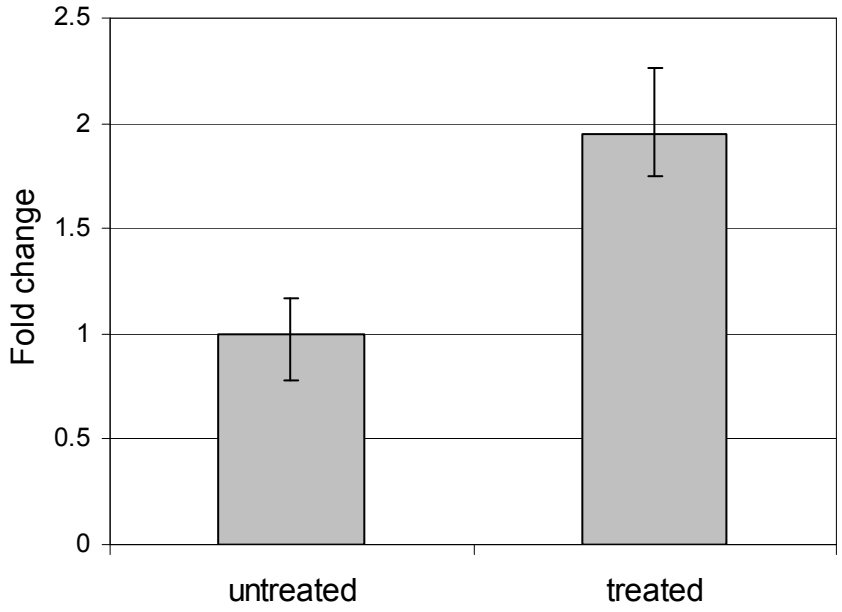

Figure 6.5: Fold change in SOX2 expression by qPCR analysis in genital fibroblasts either untreated, or treated with 10 nM DHT for 4 hrs. *n=2 (two 8 wpc 46,XY male fetuses). Data normalized to negative control expression of TATA box-binding protein (TBP). Expression for untreated cells standardized to 1. Error bars represent standard deviation (SD). Untreated cells SD +/- 0.2; treated cells SD +/- 0.3/0.2.*

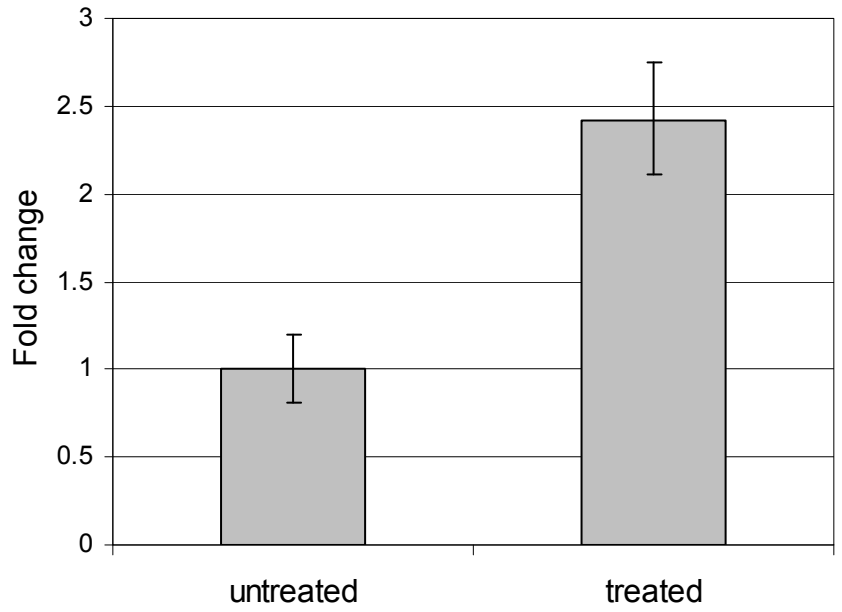

Figure 6.6: Fold change in CYP3A7 expression by qPCR analysis in genital fibroblasts either untreated, or treated with 10 nM DHT for 4 hrs. *n=2 (two 8 wpc 46,XY male fetuses). Data normalized to negative control expression of TATA box-binding protein (TBP). Expression for untreated cells standardized to 1. Error bars represent standard deviation (SD). Untreated cells SD +/- 0.2; treated cells SD +/- 0.3.*

6.4 Discussion

This chapter has produced novel data regarding the effects of androgen on gene expression in human fetal external genitalia at the time of sexual differentiation. Here, we have used mRNA samples from genital fibroblasts grown using the culture model developed in the previous chapter and continued our initial investigation of androgen-regulated genes through the utilization of expression microarray analysis. By permitting a much more detailed, thorough search for androgen-regulated genes spanning the entire human genome, this approach has greatly expanded upon the initial results from the previous chapter and begun to highlight a cohort of interesting genes that may be regulated by the AR. Critically, these data were acquired in the primary cells involved in human sexual differentiation, not cancer cell lines.

6.4.1 Expression microarray analysis

Microarray expression profiling was used primarily for target gene discovery rather than for statistical analysis of AR regulated datasets. The data were shown to be of good quality based on the sound variance analysis and absence of any technical problems. The scatter plot confirmed that most genes were unaffected by androgen treatment, which is what we would expect to find. The strength of the data is also reinforced by the fact that the initial mRNA samples used for the microarray came from cell cultures that were meticulously checked for normal AR functionality after each passage during cell culture, and following final androgen treatment prior to mRNA extraction. At each juncture, AR was found to undergo homogenous nuclear translocation in response to treatment with the same concentration of DHT as used in the microarray.

The microarray data presented in this chapter show that previously androgen-deprived fetal genital fibroblasts grown for 4 hrs in the presence of potent androgen (DHT), have a different expression profile compared to androgen-deprived cells treated for 4 hrs with vehicle (ethanol). Analyses of gene expression revealed androgen-mediated upregulation or downregulation of a number of genes with known associations with early human development and/or sexual differentiation. Interestingly, this included a number of genes from the

SOX gene family, known to have key roles in cell differentiation and organogenesis during early development (Piper *et al*., 2002; Avilion *et al*., 2003; Lefebvre *et al*., 2007; Guth and Wegner, 2008), of which *SOX2* and *SOX9* had already been shown to be expressed in the external genitalia during sexual differentiation (see Chapter 4). In addition, members of the *PAX, GATA* and *WNT* gene families also exhibited modified expression between +DHT and –DHT cells; all of which also have known functions in regulating developmental processes during the embryo and/or fetal stages (Viger *et al*., 2008; Wang *et al*., 2008b; and Chien *et al*., 2009, respectively). The microarray data from this chapter are very exciting and have revealed a large number of putative novel androgen-regulated genes in a primary human cell type, while also potentially discounting many other genes whose expression appeared unaffected in androgen treated cells compared to untreated cells.

6.4.2 Validation of microarray data, and further characterisation of the putative AR target genes CYP3A7, SOX2 and CHD7

In order to verify that the changes in gene expression detected by microarray analysis were authentic, a number of genes were selected for gene expression analyses using alternative methods. For the most part, semi-quantitative RT-PCR was used for this task, owing to the relative straightforwardness of the technique and low cost involved in performing these experiments. Funds were not available for widespread initial analysis by qPCR, which arguably would have been a preferable technique. Although a number of genes tested did not show an effect by androgen on gene expression, considering the relative small number of genes analysed, it was exciting to see that four genes (*SOX2, CYP3A7*, *KLF13* and *PCDH1*) did demonstrate good evidence of androgen-mediated regulation, apparent in more than one fetus and both sexes. These data help prove the validity of the microarray data by clearly illustrating modified expression of these genes in cells treated with androgen via an independent method of analysis. Furthermore, these findings also added to the evidence suggesting these four genes in particular are novel androgen-regulated genes, and represent some of the first known targets of AR action in the human fetus. For the genes tested using RT-PCR that lacked any obvious differences in gene expression between DHT-treated cells and control cells, this is most likely a result of experimental

insensitivity rather than a true lack of androgen dependent effects. It would be interesting to repeat these analyses using qPCR.

In addition to the RT-PCR, a limited amount of qPCR analysis was performed to validate the data from the microarray, and to also further characterise androgen regulation of *SOX2* and *CYP3A7*. Like the microarray data and RT-PCR results, qPCR analysis showed that the expression of both genes was upregulated by androgen; each demonstrating approximately a 2-fold increase in DHT treated cells. The preliminary nature of the work in this chapter and high cost of the qPCR analysis, meant only limited qPCR experiments were performed and there is a clear need for further work in this area. However, taken together, there is clear novel evidence that *SOX2* is a direct target of androgen activated AR.

Hitherto, to our knowledge no putative ARE regulatory sites have been identified for human *SOX2*. To address this apparent inconsistency with the current data on AR regulation of *SOX2*, we performed a preliminary analysis of the sequence of the *SOX2* locus located at 3q26.3-q2 (Stevanovic *et al*., 1994). This revealed the presence of five putative consensus AREs (specifically the sequence nGA/TACAnnnTGTA/TCn) are located within the 1Mb upstream region of the *SOX2* gene (*SOX2* NCBI reference sequence: NC_000003.11). The transcriptional start site of *SOX2* is located at base pair 181,429,722 on chromosome 3, and the five potential AR binding sites were located at 180,600,325 bp; 180,687,014 bp; 180,964,551 bp; 180,985,832 bp; and 181,191,433 bp in the chromosome 3 base sequence (NCBI reference sequence: GRCh37 primary reference assembly). Verification of these putative AREs would further suggest *SOX2* is directly regulated by AR; thus further study of *SOX2* in this context is imperative.

The results from qPCR analysis of *CHD7* expression, performed due to associations with SOX2 in genital tract defects found in AEG syndrome (Williamson *et al*., 2006), indicated that consistent with the microarray data, *CHD7* is not upregulated by DHT. It appears then that *CHD7* is not directly regulated by AR and therefore its interaction with SOX2 may be a result of AR-independent effects of SOX2 on *CHD7* expression, or CHD7 could be upstream

of AR and effect SOX2 function through effects on AR. The latter seems likely, owing to the apparent widespread functions of CHD7 represented by the diverse and highly variable pathological phenotypes associated with mutated *CHD7* in CHARGE syndrome (Sanlaville *et al*., 2006; Vervloed *et al*., 2006). Furthermore, effects on upstream androgen signalling could occur in light of recent evidence from *CHD7* knock-out mice suggesting CHD7 may contribute to regulation of GnRH secretion from the hypothalamus (Bergman *et al*., 2009)

Along with the microarray data, RT-PCR and qPCR analyses both illustrated *CYP3A7* was significantly upregulated, making this gene a likely downstream target of the AR in developing human external genitalia. Its function in this location is unclear, but it is almost certainly involved in steroid hormone metabolism as its encoded gene product fulfils this role in the fetal liver (Komori *et al*., 1990; Paulussen *et al*., 2000). Furthermore, *CYP3A7* is known to be expressed in human placenta; has been shown to contain putative HREs in the 5' flanking region by genomic analysis (Itoh *et al*., 1992); and, through interactions with the pregnane X receptor, may play an important role as an alternative pathway for gonadal hormone chemical effects (Masuyama *et al*., 2003). To further clarify the putative role of *CYP3A7* in androgen signalling studies to elucidate its substrate and gene product are needed, and are envisaged to form part of a collaborative study between the Hanley group and that of Professor Wiebke Arlt (University of Birmingham).

6.4.3 Continuing work on AR target genes

The work presented in this chapter has identified a number of novel putative androgen-regulated genes. These data represent a significant step forward in our understanding of the AR and its regulation of fetal sexual differentiation, and progress in trying to fill the large deficit of knowledge concerning downstream AR signalling in the external genitalia (Robins, 2005; Li and Al-Azzawi, 2009). However, much more work is needed to gain functional insight from the data presented in the chapter. Specifically, a much bigger study is now required, which has been planned, that would encompass more expression microarray experiments to improve the statistical reliability of the current data. This will

facilitate pathway analysis work to determine the relationships between the AR and the multitude of targets identified.

There is also a clear need for ChIP-chip or ChIP-Seq analysis to be performed. This is because, as alluded to earlier, although microarray analysis has the ability to identify androgen-regulated genes, it lacks the potential to distinguish between direct AR mediated events and subsequent downstream events. ChIP-chip or ChIP-Seq on the other hand is able to identify where AR binding occurs in the genome, such that correlations can then be made between AR regulation of a gene and nearby AR binding sites (Park, 2009). This approach goes a long way towards distinguishing direct targets from secondary targets. Such approaches also have the added benefit of producing data clarifying which genomic sequences are bound by AR (i.e. ARE consensus elements) and provide further insight on potential cofactors that have been identified as responsible for contributing to the large number of downstream androgen-regulated transcriptional events (Wang *et al*., 2005; Wang *et al*., 2007b).

ChIP-Seq analyses are planned for the near future, which will be carried out using the samples prepared over the course of the present study. This work is part of a collaborative study with Prof. Myles Brown's group at Harvard University and Professor Andy Sharrocks group at the University of Manchester. Funding for this, as well as further expression microarrays, has recently been secured owing to the preliminary work documented in this chapter. Eventually it is hoped that using these types of further experiments, we will gain an in-depth understanding of AR action in normal human sexual differentiation and in cases of both male under-development and female virilisation.

6.4.4 Conclusion

In this chapter a wealth of data has been produced regarding the identification of genes regulated by the genomic effects of bound AR, which up to now, has remained poorly defined in general and scarcely achieved in primary cells of the human fetal external genitalia. Validation of the expression microarray data using RT-PCR and qPCR techniques has lead to the supposition that the genes

CYP3A7 and *SOX2* may represent novel AR target genes in the developing human external genitalia, which may contribute to sexual differentiation of the human external genitalia. Further work is required, particularly ChIP-chip and ChIP-Seq analyses, to expand upon the initial findings outlined in this chapter, and to more clearly define what genes are directly regulated by AR during human sexual differentiation.

Chapter 7: General discussion

7.1 Overview

As a species, differentiation of the external genitalia so it is consistent with genetic sex is imperative during fetal development, as abnormal genitalia compromises reproductive capacity. In humans, congenital disorders of sex differentiation (DSD) cause major emotional stress to both patients and parents (Warne and Raza, 2008). Congenital adrenal hyperplasia (CAH) is one of the more common congenital endocrinopathies, with CAH due to 21-hydroxylase deficiency accounting for 90-95% of cases, which is equal to approximately 1 in 10,000 live births (Tajima *et al*., 1997; White, 2009). Furthermore, hypospadias is the second most common birth defect of the male genitalia after cryptorchidism, affecting more than 0.5% of male newborns (Fukami *et al*., 2006). The overall prevalence of genital abnormalities at birth is on the rise (Pierik *et al*., 2002; Nelson *et al*., 2007; Nassar *et al*., 2007; Sun *et al*., 2009). Therefore, research into development of the genital tract and the events that underlie fetal virilisation is important. Male under-virilisation and female over-virilisation can not be completely reversed by 'corrective' surgery of the external genitalia. It is a struggle to alleviate the mental and emotional anguish that are a prominent feature of DSD (Brinkmann *et al*., 2007; Warne, 2008). Hence there is a requirement for better fundamental understanding of the basic biology behind virilisation processes to aid in the proper diagnosis and screening of affected neonates, and hopefully eventually enable improved preventative therapeutic strategies, rather than relying on a palliative approach to care.

Previous studies have produced some morphological and gene expression data on differentiation of the external genitalia in humans (Hiort and Holterhus, 2000; Hughes, 2001; Wilson *et al*., 2002), while a great deal more data come from animal studies (Lovell-Badge *et al*., 2002; Glickman *et al*., 2005; Huhtaniemi *et al*., 2006; Basrur, 2006), and the study of androgen signalling in malignancy (Yeap *et al*., 1999; Xing *et al*., 2001; Kung and Evans, 2009; Leveille *et al*., 2009). However, the molecular consequences of androgen action during human sexual differentiation, either in normal male development, or in pathological

virilisation of female development, remain poorly understood. The fundamental aim of the work presented in this thesis was to broaden our limited understanding of what regulatory mechanisms control the production of androgens in the human male fetus, and how the key virilisation signals are propagated to result in the strict dimorphism of the external genitalia observed at birth. The classical view of sexual differentiation is that during the latter stages of the first trimester differentiation of the bipotential external genitalia in males is an active process under the regulation of androgens, while female differentiation represents a period of androgen-free dormancy (Wilson and Davies, 2007). However, data presented here indicate that female development involves androgen signalling.

The present study began by outlining some of the major features of human sexual differentiation and explained how key questions relating to androgen signalling in the early fetus remained unanswered. The work described in Chapters 3 to 6 has hopefully provided some meaningful insight into these issues through assessing what drives early androgen production, how and where this signal is transmitted to individual cells on the external genitalia, and finally how this might be translated into transcriptional events that ultimately determine the morphology of the external genitalia. In this final chapter we attempt to bring together the main findings from previous chapters, highlighting conclusions that can be drawn and indicating future lines of investigation.

7.2 Regulation of testosterone production by the human fetal testis during sexual differentiation

The first objective of the present study was to investigate how testosterone is produced by the fetal testis during sexual differentiation in light of its important role in development of the external genitalia (Warne and Kanumakala, 2002; Sobel *et al*., 2004). Taken as a whole the data presented in Chapter 3 provide a working hypothesis for how testosterone biosynthesis is regulated during the critical window of sexual differentiation between 8-12 wpc. There has previously been uncertainty and mixed opinions regarding what is principally responsible for initiating testosterone production following formation of the fetal testis and what regulates its biosynthesis thereafter (Asby *et al*., 2009). Generally the

candidature of three main factors has been considered: LH from the fetal pituitary, hCG from the placenta, or constitutive regulation by the testes. By characterising the expression and biosynthesis of the first two, along with investigation of the associated signalling pathways, we showed how testosterone production during sexual differentiation is unlikely to be regulated by these extra-testis factors. Hence, the most likely control mechanism is to regulate the initiation and early biosynthesis of testosterone from the fetal testis through constitutive regulation, the details of which were discussed at the end of Chapter 3.

Other interesting features also arose from the work in Chapter 3. For example, we have provided further evidence in the form of immunohistochemistry and testosterone assaying advocating that it is the classical pathway of testosterone production that predominates during early development, rather than the alternative pathway (Auchus, 2004). The steroidogenic enzyme profile of the fetal testis and copious testosterone, and not DHT, argues against a major role for the alternative pathway at this time, consistent with the known *HSD17B3* and *SRD5A2* mutation phenotypes (Can *et al*., 1998). We are one of the first groups to illustrate that the anterior pituitary gland appears active early in the first trimester (Goto *et al*., 2006), however, gonadotrophin secretion appears to remain low until later development. Finally there was a strong indication that despite the apparent importance of LHR owing to clinical data showing that its mutation results in under-virilised male external genitalia, both of its ligands, LH and hCG, as well as the cAMP signalling pathway, do not appear to be necessary for normal testosterone biosynthesis during human sexual differentiation.

7.3 Expression of the AR and SRD5A2 in the developing external genitalia

Following on from the work examining the initial production of testosterone by the male fetal testes, and its regulation, the next logical step in investigating androgen signalling was to study the responsiveness of the target tissue, the external genitalia. The expression of *SRD5A2* was studied to predict where DHT would be formed from testosterone; a function performed by the enzyme 5-alpha-reductase type 2, the gene product of *SRD5A2*. In Chapter 4, spatiotemporal analysis of the expression of the *AR*, along with *SRD5A2*,

demonstrated that both are readily detectable in the external genitalia of developing males and females during the critical window of sexual differentiation. Expression was particularly robust in and around the developing urethra and corpus cavernosa, implying an important role for DHT in stimulating the development of these tissues. Expression was more prominent in older male tissue. Interestingly expression appeared nuclear in both male and female cells, suggesting a liganded and 'active' AR in both sexes at this stage of development. The implications of having a transcriptionally-active AR present during normal female genital development are unclear, but this is certainly in conflict with the traditional views on 'default androgen-free' female genital development. Significantly, these data show that females possess a capability to respond to fetal androgens providing the means by which female genitalia can be virilised during fetal development if androgen levels are excessive. Hence, the data help in understanding the consequences of CAH due to CYP21A2 deficiency in female fetuses.

7.4 Modelling AR receptor activity and the downstream effects of androgen in the external genitalia

To further our understanding of the androgen signalling pathway during development of the external genitalia, the latter half of the study focused on the downstream effects of androgens on fetal genital fibroblasts. Increasing our understanding of the genetic targets of the ligand-activated AR is of great relevance to male syndromes of under-virilisation where identified genes would be candidates for mutation or, possibly, be targets for therapy.

To study the downstream effects of androgen signalling, efforts were made to produce an *in vitro* culture model. The novel model that was devised, presented in Chapter 5, utilized the rapid growth of genital fibroblasts to readily produce monolayer cultures for experimentation. As a corollary, the dynamic nature of AR cellular localisation meant that it was possible to verify that AR responded to androgen treatment. Owing to ligand-mediated nuclear translocation, periodic assessments of the AR spatial expression pattern using IHC enabled us to reliably show that the AR was nuclear following androgen treatment of cells, implying its activity as a transcription factor distinct from the AR in androgen-deprived

control cells. Having this positive control data for an androgenic effect was especially important as previously identified AR targets from cancer cell lines were not corroborated by studies using the human primary cells from the external genitalia. The culture model was shown to be very useful for the study of AR expression and translocation in the fetal genitalia. Cultures were demonstrated to be highly robust and reliable, and could be maintained over a relatively long period of time, with no loss in AR function or significant changes in cell character or morphology. Importantly the model showed that the concentration of androgen required to trigger nuclear translocation did not vary across cells from different fetuses. This suggests that the variability that exists in AR functionality due to the well-characterised glutamine repeat polymorphism found in exon 1 (Lieberman *et al*., 2002; Silva *et al*., 2008; Rocha *et al*., 2008), does not affect translocation. It was also found that cells from this model could withstand freeze/thaw cycles, giving the potential for transportation of cells over large distances and re-seeding elsewhere, without loss of phenotype. Therefore this model represents a useful resource for future work on androgen signalling.

Use of the culture model led to a number of important findings. Our initial culture expansion phase showed AR expression was androgen-independent, and AR readily underwent nuclear translocation in male genital fibroblasts. Interestingly, this was also true of female genital fibroblasts, where the threshold androgen concentrations that caused significant nuclear translocation of AR was approximately the same as for male cells. Furthermore, it was significant that these concentrations required for translocation, correlated well to the group's previous data on concentrations of androgens secreted by the fetal adrenal gland (Goto *et al*., 2006). Hence implying the normal fetal adrenal cortex produces levels of androgens that could be capable of instigating AR transcriptional activity in cells of the external genitalia in developing males and females. Taken together with the co-culture data showing that secretions from the normal fetal adrenal gland, but not the ovary, had the capacity to cause AR translocation, and the data showing the presence of nuclear AR in fixed sections of the female external genitalia, this raises many questions regarding the role of the adrenal cortex in normal female development. Coupled with data that androgen increased protein levels of AR in external genitalia cells, this provides a mechanism with

clear potential to explain the pathological virilisation of female external genitalia observed in patients with CAH due to CYP21A2 deficiency (Berenbaum and Bailey, 2003; Rocha *et al*., 2008); illustrated in Fig. 5.13.

The ultimate application of the culture model was to identify AR target genes. In Chapter 5, very limited initial progress was made through the use of RT-PCR expression profiling, where only a handful of genes demonstrated potential as putative AR target genes in LNCaP cells and, more dubiously, in cultured human genital fibroblasts. What was most apparent from this work was that genes identified in cancer cell lines, frequently did not correlate well with androgen-regulated genes in primary human cells. This obviously has important implications for much of the work previously performed in this field, which has tended to concentrate on identifying AR targets in malignancy (Montgomery *et al*., 1992; Wolf *et al*., 1993; Bolton *et al*., 2007; Jariwala *et al*., 2007; Massie *et al*., 2007), and further emphasizes the deficit of information in our knowledge of downstream effects of androgen during sexual differentiation.

Our initial investigation of AR target genes also produced convincing evidence that the AR is autoregulated in the external genitalia, consistent with the incremental increase in AR detection observed in the external genitalia of males as testicular testosterone secretion rises with increasing age. As transcripts were unaltered we surmised that autoregulation of *AR* occurs most likely post-translationally. The specific mechanism underlying this process remains to be elucidated, but may entail protein stabilization and/or the avoidance of pro-degradative modifications such as SUMOylation (Kemppainen *et al*., 1992; Zhou *et al*., 1995; Chen *et al*., 2006). The phenomenon of *AR* autoregulation has been proposed in some previous studies; where commonly the focus has been computational analysis of the *AR* gene sequence to locate AREs in the *AR* gene (Grad *et al*., 1999; Burnstein, 2005). However we are not aware of a previous instance where autoregulation has been demonstrated directly in the human external genitalia. Significantly, autoregulation was also shown in female cells in the present study. As intimated above for CAH due to CYP21A2 deficiency, in light of our other findings demonstrating nuclear AR in females and the presence of active androgen most likely from the fetal adrenal cortex, we wish to

emphasise that the traditional view of female development occurring as a 'default', in an 'androgen-free' environment, is wholly inaccurate.

The more in-depth expression profiling presented in Chapter 6, achieved by using expression microarray analysis, revealed a multitude of other potential genes hitherto not known to be regulated by the AR in primary human cells. Included were a number of genes from gene families with known importance in developmental processes, such as SOX, PAX, WNT and FOX family members. Furthermore, we were also able to demonstrate through the use of microarray analysis alongside other techniques that *SOX2* expression was be enhanced by androgen most likely as a direct AR target (the effect occurred within 4 h of DHT exposure). Determining the function of SOX2 in the developing external genitalia is highly significant when we consider that we have shown in this study that SOX2 is widely expressed in the fetal external genitalia during sexual differentiation, and that mutations in *SOX2* cause genital abnormalities (Williamson *et al*., 2006). Hence this aspect of our work helps to position SOX2 within the pathways that regulate sexual differentiation.

7.5 Future work

The present study has identified a number of areas where future research is required. Work that needs to be performed includes:

- Fundamental investigation of female sexual differentiation and how virilisation is avoided, despite expression of nuclear *AR* and apparent presence of active androgen; what is the role of the AR in female development? What 'tips the balance' between normal development of female genitals and pathological virilisation found in CAH due to CYP21A2 deficiency?
- Elucidation of the specific mechanism that to regulate testosterone biosynthesis in the testis during first trimester development. There is a need to resolve the seemingly conflicting information on LHR. What factors are important for constitutive androgen production?

- Precisely what are the steroid biosynthesis pathways operating in the human fetal adrenal and testis? We are currently collaborating with Professor Arlt's group at Birmingham University, using radio-labelled steroid precursors in cultures of fetal testis to determine precise pathways, which together with current data, will form the basis of a publication in the near future.
- Determination of how AR levels are autoregulated; in particular, is SUMOylation involved and is this a key factor for maintaining dimorphism between male and female external genitalia?
- Further investigation into the function of SOX2 in male sexual differentiation and its potential role when mutated in the development of hypospadias.
- Continuation of preliminary work on identifying androgen regulated genes. Several more expression microarray analyses are required to clearly define target genes as part of a larger study, which will include ChIP analysis to distinguished direct and secondary AR targets, leading to in-depth androgen signaling pathway analysis. Initial steps towards conducting these experiments have already been taken though collaboration with Professor Myles Brown group at Harvard University and Professor Andrew Sharrocks of the University of Manchester. We will soon be performing ChIP-Seq analysis on the samples described in Chapter 6.

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Appendix: Ethical approval

Documents approving the use of embryonic and fetal material collected from voluntary medical or surgical termination of first trimester pregnancies.

Our Ref: CPW/sta

09 May 2005

Professor I.T. Cameron
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Dear Professor Cameron,

Study title: **The collection of human embryonic and fetal tissue at termination of pregnancy.**
REC reference: **296/00**
Protocol number:
EudraCT number:

Amendment number: **1**
Amendment date: **7th April 2005**

The above amendment was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on Wednesday 27th April 2005.

Ethical opinion

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Notice of Substantial Amendment Form dated 7th April 2005
Annual Progress Report Form dated 7th April 2005

Membership of the Committee

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

Management approval

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects local management approval of the research.

Statement of compliance
The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

REC reference number: 296/00	**Please quote this number on all correspondence**

Yours sincerely,

Catherine Gordon

Mrs Clair Wright
REC Manager

E-mail: clair.wright@nhs.net

Enclosures *List of names and professions of members who were present at the meeting and those who submitted written comments*

Enclosure 1

List of names and professions of members who were present at the meeting and those who submitted written comments

Dr R Patel (Chairman)

Dr H McCarthy (SPR in Haematology)

NHS

Central Office for Research Ethics Committees (COREC)

NOTICE OF SUBSTANTIAL AMENDMENT

For use in the case of all research other than clinical trials of investigational medicinal products (CTIMPs). For substantial amendments to CTIMPs, please use the EU-approved notice of amendment form (Annex 2 to ENTR/CT1) at http://eudract.emea.eu.int/document.html#guidance.

To be completed in typescript by the Chief Investigator and submitted to the Research Ethics Committee that gave a favourable opinion of the research ("the main REC"). In the case of multi-site studies, there is no need to send copies to other RECs unless specifically required by the main REC.

Further guidance is available in section 5 of our Standard Operating Procedures available at www.corec.org.uk/applicants/help/docs/SOPs.doc.

Details of Chief Investigator:

Name:	Professor I.T. Cameron
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Telephone:	023-8079-6044
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Full title of study:	The collection of human embryonic and fetal tissue at termination of pregnancy
Name of main REC:	Southampton & South West Hampshire Research Ethics Committee
REC reference number:	296/00
Date study commenced:	24th October 2000
Protocol reference *(if applicable)*, current version and date:	
Amendment number and date:	

Type of amendment (indicate all that apply in bold)

(a) Amendment to information previously given on the REC application form

Yes *xx*

If yes, please refer to relevant sections of the REC application in the "summary of changes" below.

(b) Amendment to the protocol

xxx ***No***

If yes, please submit either the revised protocol with a new version number and date, highlighting changes in bold, or a document listing the changes and giving both the previous and revised text

(c) Amendment to the information sheet(s) and consent form(s) for participants, or to any other supporting documentation for the study

xxx ***No***

If yes, please submit all revised documents with new version numbers and dates, highlighting new text in bold

Summary of changes

Briefly summarise the main changes proposed in this amendment. Explain the purpose of the changes and their significance for the study.

Supporting scientific information should be given (or enclosed separately) where the amendment significantly alters the research design or methodology, or could otherwise affect the scientific value of the study.

The original application was to establish a tissue resource which therefore did not have a fixed or predetermined end-point but the proposed duration of the project was requested as a "minimum of 5 years", which period will have elapsed towards the end of this year. This tissue bank is proving to be a valuable resource. The obtaining of informed consent has not been problematic (584 to date) and no patients have subsequently withdrawn consent. Approval is therefore sought to extend the project by a further 5 years, to October 2010.

Any other relevant information

Applicants may indicate any specific ethical issues relating to the amendment, on which the opinion of the REC is sought.

List of enclosed documents

None

Declaration

- I confirm that the information in this form is accurate to the best of my knowledge and I take full responsibility for it.
- I consider that it would be reasonable for the proposed amendment to be implemented.

Signature of Chief Investigator: .....

Print name: ...Iain T. Cameron.....

Date of submission: .....

Published work

Rev Endocr Metab Disord (2009) 10:43–49
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The adrenal cortex and sexual differentiation during early human development

Daniel J. Asby · Wiebke Arlt · Neil A. Hanley

Published online: 1 August 2008

Abstract Human sexual differentiation is a critical process whereby a strict dimorphism is established that enables future reproductive success as phenotypic males and females. Significant components of this differentiation pathway unfold during the first three months of gestation when they are sensitive to disruption by abnormal hormonal influences. Excessive exposure of female development to androgens in conditions such as congenital adrenal hyperplasia causes virilization. However, recently we have suggested that female development normally takes place in the presence of low, yet significant, levels of androgen, implying a need for strict regulation to avoid virilization and the potential for a biological role of androgens in females that has not been fully elucidated. Here, we review androgen-dependent male differentiation of the external genitalia in humans, and link this to current understanding of female development and steroidogenesis in the developing adrenal cortex.

Keywords Human · Fetal · Adrenal · Sex differentiation · Androgen

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1 Introduction

This issue of 'Reviews in Endocrine and Metabolic Disorders' focuses on adrenarche, the process whereby the adrenal cortex matures during childhood to secrete androgen precursors alongside glucocorticoids and mineralocorticoids [1, 2]. The trigger for adrenarche remains obscure, but the androgen secretion has visible clinical consequences in terms of pubic hair growth as well as alterations in oil content of the skin. In the search to understand the mechanisms underlying adrenarche, similarity has been noted with the functioning of the adrenal cortex during human development *in utero* [3]. During gestation, especially during the second and early third trimesters, the fetal adrenal gland is remarkable for its production of dehydroepiandrosterone (DHEA) and its sulfated derivative, DHEAS. In fact, it has recently come to light that the human fetal adrenal cortex displays distinctive steroidogenic activity earlier in gestation, during the latter stages of the first trimester [4, 5]. This period, between approximately 8 to 12 weeks post-conception (wpc), is when major sexual differentiation takes place. In this article, we try to complement other recent reviews [6, 7] by considering the physiological and pathological impact of fetal steroidogenesis on differentiation of the external genitalia.

The sexual organs of mammals commonly maintain a strict lifelong dimorphism as an important component of

reproductive success, which, in turn, dictates that it is important for development to unfold correctly. The classical view is that during the latter part of the first trimester male differentiation of the bipotential external genitalia is an active process under the regulation of androgen (Fig.1), while female differentiation reflects a relative period of androgen-free dormancy [8]. When pathological levels of androgen arise in female fetuses, the external genitalia are virilized towards a male phenotype and classified as 46,XX Disordered Sex Differentiation (DSD) [6]. A relatively frequent cause is mutation in the gene *CYP21A2* encoding 21-hydroxlase (CYP21), the commonest form of congenital adrenal hyperplasia (CAH) [9]. CYP21 is an adrenocortical enzyme that catalyzes a crucial step in the pathway from cholesterol to cortisol (and aldosterone) (Fig. 2). This intimately ties the developing adrenal gland to the process of sexual differentiation. We have recently reviewed how the adrenal cortex might produce androgen [6, 7]. Here, we place this phenomenon in the context of human sexual differentiation: both normal and abnormal. By virtue of the virilization seen in CAH, the female external genitalia are clearly capable of responding to a source of androgen. However, although there are some morphological and gene expression data on differentiation of the external genitalia [10, 11] and a wealth of information on androgen signaling in malignancy [12, 13], the molecular consequences of androgen action in humans, either during male development or in pathological virilization of female development, are poorly understood. We set the scene by first discussing androgen production from the testis and how the external genitalia differentiate normally, in preparation for discussing how androgens released from the adrenal cortex might impact on differentiation of the external genitalia both in virilizing DSD and potentially, perhaps counter-intuitively, in normal female development.

2 Androgen production by the developing testis

The under-virilized external genitalia apparent in androgen insensitivity syndrome (AIS, a form of 46,XY DSD) due to inactivating mutations in the androgen receptor (AR), and the phenotypes that arise from mutations in the androgen biosynthetic pathway argue strongly that normal male development relies upon testosterone that is converted into dihydrotestosterone (DHT), which in turn acts as the predominant ligand for AR (DHT is approximately 10-fold more potent than testosterone) (Fig.1) [8]. Other articles in

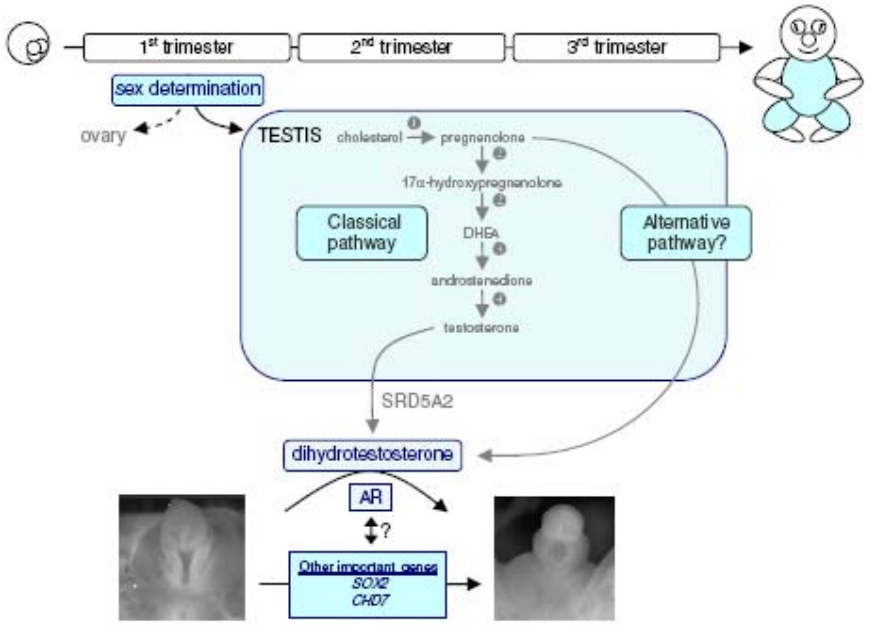

Fig. 1 Diagram of male sex differentiation. Sex determination at 7 wpc generates either a testis or an ovary. In the testis, steroidogenesis commences in the fetal Leydig cells to generate testosterone [19]. It is unclear to what extent the alternative pathway (steroid intermediaries shown in Fig. 2) operates within the human fetal testis [15]. Testosterone is converted to dihydrotestosterone in the external genitalia cells and acts via the AR to elicit male sexual differentiation [8]. The *image* depicting this event shows near complete fusion of the urethral folds. The genes, *SOX2* and *CHD7* are involved in male sexual differentiation but their relationship with AR is unclear. ❶, CYP11A1, P450 side-chain cleavage enzyme; ❷, CYP17, 17α-hydroxylase/17–20-lyase; ❸, HSD3B2, type 2 3β-hydroxysteroid dehydrogenase; ❹, HSD17B3, type 3 17β-hydroxysteroid dehydrogenase. Type 2 5α-reductase (SRD5A2) is shown on the diagram

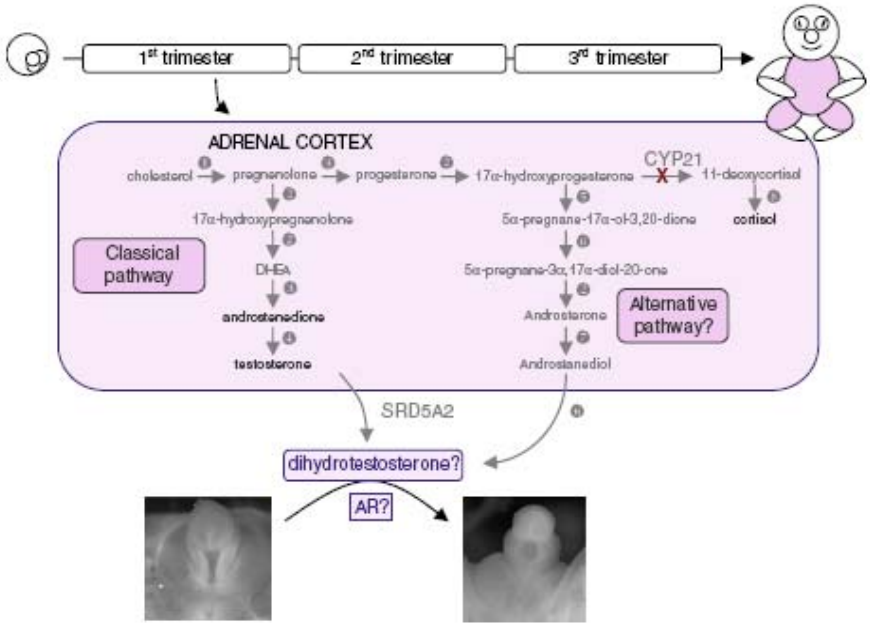

Fig. 2 Diagram of potential virilization during female sex differentiation. In the presence of mutations (*X*) in 21-hydroxylase (CYP21), the fetal adrenal cortex is no longer able to make cortisol and high levels of ACTH stimulate androgen and/or androgen precursor biosynthesis. Steroids shown in *dark text* have been confirmed to be secreted by the fetal adrenal cortex at 8 wpc [4]. It is not yet clear whether the classical or alternative pathway of androgen biosynthesis predominates. It is also reasonably presumed but not proven that female virilization is via DHT acting on AR. ❶, CYP11A1, P450 side-chain cleavage enzyme; ❷, CYP17, 17α-hydroxylase/17–20-lyase; ❸, HSD3B2, type 2 3β-hydroxysteroid dehydrogenase; ❹, HSD17B3, type 3 17β-hydroxysteroid dehydrogenase; ❺, SRD5A, 5α-reductase activity; ❻, HSD3A, 3α-hydroxysteroid dehydrogenase activity; ❼, HSD17, 17β-hydroxysteroid dehydrogenase activity; ❽, CYP11B1, 11β-hydroxylase. Type 2 5α-reductase (SRD5A2) is shown on the diagram

this issue review steroid biosynthesis (both in the testis and in the adrenal cortex). Alongside the classical pathway is the more recently discovered alternative, or 'backdoor' pathway to potent androgen [14–18]. The fact that the fetal testis secretes high amounts of testosterone [19] and that mutations in the enzyme that converts testosterone to DHT, type 2 5α-reductase (SRD5A2) [20], not thought part of the alternative pathway [6, 15], cause profound undervirilization argues that the classical pathway predominates during normal male differentiation in humans. Nevertheless, a contribution 'via the backdoor' cannot be excluded (Fig.1).

We have previously debated what might regulate androgen biosynthesis within the developing testis [6]: the anterior pituitary–gonadal axis, although there is a lack of clear data on its maturity; the potential for other hormones such as human chorionic gonadotrophin (hCG) to substitute for luteinizing hormone (LH), although the placental barrier makes this unlikely; or the possibility of constitutive secretion of androgen from the testis. Here, our purpose is to reinforce the paradigm that testicular androgen secretion during the final four weeks of the first trimester signals to the bipotential external genitalia to cause differentiation irrevocably along a male pathway. Thus, this period should be the focus when it comes to investigating the molecular regulation of normal male development. As a corollary, it becomes feasible to identify and study the mechanisms by which two opposing pathological scenarios arise: male under-virilization syndromes and female virilization due to excessive androgen action.

3 Differentiation of the male external genitalia

3.1 Assembly of bipotential external genitalia

Prior to dimorphic sexual differentiation, the external genitalia are first assembled as a bipotential structure, i.e. with equal capacity for male or female differentiation [8]. The cloacal membrane demarcates the position where hindgut endoderm meets external ectoderm. In the third week of human development mesenchymal cells migrate to either side of the cloacal membrane forming cloacal swellings. Over the following weeks, a septum develops internally that eventually reaches the cloacal membrane, thus forming the perineum. This septum divides the hindgut posteriorly from the anterior urogenital structures. Similarly, by the seventh week of

development, at the surface cloacal membrane, the posterior anal membrane is now physically partitioned from the anterior urogenital membrane. With this segregation of the cloacal membrane into anus and urogenital structures, the anterior cloacal swellings are now termed the urethral folds, which at their superoanterior limit are fused into the genital tubercle. Lateral to the urethral folds are the genital (or labioscrotal) swellings. The genital tubercle is comprised of the glans and the corpora cavernosa. Accordingly, by 7 wpc, the external genitalia are clearly apparent as the bipotential anlage (the left-hand photomicrograph in Fig.1) in preparation for major male sexual differentiation over the remaining weeks of the first trimester [4].

3.2 Male sex differentiation late during the first trimester

Morphologically, the external genitalia undergo a marked change in male fetuses between 8 to 12 wpc. The urethral folds fuse to enclose the urethra, the genital tubercle lengthens into the phallus, and the genital swellings slip posteriorly and approximate in the midline to form a single scrotum comprised of two halves [4, 8]. Significant progress along this pathway is shown in the right-hand photomicrograph of Fig. 1. Kim and colleagues have described the expression profile of SRD5A2 and AR in the structures of the male external genitalia after 12 wpc [10]. AR is expressed in the surface epithelium of the external genitalia, the epithelium of the developing urethra, as well as in the underlying stromal cells, especially those areas condensed as the corpora caverosum. SRD5A2 expression appears similarly located during development [10]. After the end of the first trimester, male development is mainly evidenced by growth and enlargement of pre-existing structures including preparation of the scrotum to receive the descending testes during late gestation. Thus, if male development has not occurred appropriately during relatively early development, the potential to remedy this later is minimal. In addition, the early androgenic milieu is at least partially modified later in development by fetal expression of the enzyme aromatase (CYP19), which converts testosterone to estradiol [21]. This limits the substrate for conversion to DHT, thus moderating the supply of ligand for AR, while only adding minimally to the total pool of estrogens that are already synthesized by the placenta.

Our detailed molecular knowledge of male sexual differentiation largely ends at ligand binding to AR. Some additional information can be gained from the features apparent at birth in infants with known genetic syndromes. Anopthalmia-esophageal-genital syndrome (AEG; OMIM 206900) displays under-development of the external genitalia in males and has recently been shown due to mutations in the gene encoding the SRY box 2 (SOX2) transcription factor [22]. Interestingly, similar genital features can be seen in coloboma–heart anomaly–choanal atresia–retardation–genital and ear anomalies syndrome (CHARGE; OMIM 214800) due to mutations in the chromodomain helicase DNA-binding protein 7 (CHD7) [23]. Both SOX2 and CHD7 are also associated with tracheo-esophageal fistula, although, at present, interactions between the two genes or their encoded proteins are unknown. To return to male external genitalia differentiation, it is also unclear how AR signaling affects SOX2 and/or CHD7, or vice-versa. This is a generalized problem. We know nothing of AR target genes in the primary cells of the differentiating external genitalia at the time of sexual differentiation. Interestingly, studies comparing normal genital fibroblasts with those from individuals with AIS revealed differences in gene expression but these genes were not AR target genes within normal adult genital fibroblasts [24]. These data imply early AR-dependent programming effects leading to the differentiation of distinct cell populations. The same lack of knowledge exists for the proteins that interact with AR in the cells that are undergoing sexual differentiation; available data require extrapolation from other cell-types [25, 26]. Large advances have occurred in recent years regarding understanding the modulation of AR signaling via its interaction with other transcription factors. These experiments have been conducted in cancer cell lines, such as LnCAP [12, 13]. In particular, AR binding motifs frequently occur in regulatory regions of genes alongside those of OCT1 and GATA2 and FOXA2 [13]. Cooperativity of AR with OCT1 and GATA2 has been demonstrated, with additional potential interaction between AR and FOXA2. These newly discovered partner transcription factors, and their wider family members, play important roles in development making it quite likely that they will impact on AR action during human sexual differentiation.

4 Steroidogenesis in the female fetal adrenal cortex

The virilization apparent in CAH due to CYP21 deficiency and the fact that expression of CYP21 is predominantly restricted to the adrenal cortex ties the organ to the process of female sexual differentiation, at least under pathological circumstances [9]. The production of 19-carbon steroids, mainly DHEA and DHEAS, during the second and third trimesters of pregnancy, has been recognized for many years [3]. However, when we researched this recently, we discovered an unappreciated propensity for the normal adrenal cortex to synthesize far more potent androgens during the first trimester of development [4]. To date, we have found no differences between male and female specimens; however, the absence of testicular androgens makes an adrenal source more pertinent during female development.

For *de novo* biosynthesis of androgens or cortisol by the adrenal cortex, a series of enzymes are required to

sequentially modify the starting cholesterol substrate. All of these enzymes and the initial transport protein, Steroid Acute Regulatory (StAR) protein, to ferry cholesterol into the mitochondrion, are present in the fetal adrenocortical cells at approximately 8 wpc [4]. Most strikingly, the key enzyme, type 2 3β-hydroxysteroid dehydrogenase (HSD3B2) was detected in a wave of expression from 8 wpc until the end of the first trimester along with the transcription factor known to regulate its expression, NGFI-B [27]. It seems likely that this presence of HSD3B2 facilitates *de novo* cortisol biosynthesis but it would also be relevant to androgen biosynthesis by either classical or alternative pathways (Fig. 2). In the former, DHEA is converted to androstenedione by HSD3B2; in the latter, HSD3B2 would be a means of providing progesterone from pregnenolone [6, 15]. Another key consideration for both pathways is appropriate 17β-hydroxysteroid dehydrogenase activity either to convert androstenedione to testosterone or androsterone to androstanediol. At present it is unclear whether the classical or the alternative androgen biosynthesis pathway is the most important in the early fetal adrenal cortex, either physiologically or under the pathological conditions of CYP21 deficiency. Experiments following the conversion of labeled steroid precursors upstream or within both biosynthetic pathways are anticipated to provide these answers. Nevertheless, ACTH stimulates the secretion of androstenedione and testosterone by the adrenal cortex during the first trimester with assay data [4] now validated by both gas chromatography and tandem mass spectrometry (data not shown). Overnight *in vitro* culture demonstrated that a single fetal adrenal gland was capable of secreting approximately 1–3 pmol of testosterone. Somewhat artificially excluding other enzymatic modifications, these values would be expected to create high concentrations of potent androgen in female fetuses (in the nmolar range) given the overall body size and small circulating volume. Androstenedione levels were approximately 30-fold higher [4]. The pressing question now is to understand the significance of this androgen secretion during female development. Similarly, the role of cortisol biosynthesis during early human development is far from clear, albeit individuals with adrenal hypoplasia or aplasia are born will no obvious phenotype attributable to this phase of development [28]. At least in part, cortisol is anticipated to regulate androgen biosynthesis by negatively modulating the copious secretion of ACTH by the anterior pituitary. Levels of dexamethasone equivalent to physiological cortisol concentrations suppressed overnight ACTH secretion by approximately 30% [4]. However, there is gathering evidence that differentiation and maturation in other organs is sensitive to prevailing glucocorticoid levels at a time during the first trimester when maternal transfer of cortisol is unlikely to be particularly high (at least the amount anticipated to reach the corticotrophs, otherwise female virilization in CYP21 deficiency would not arise). This period sees the first wave of pancreatic beta cell differentiation and islet formation [29]. In rodents, this developmental process is affected by glucocorticoids as one determinant of postnatal beta cell mass [30].

5 Androgens during normal female differentiation: physiology as well as pathology?

To return to the classical model of human sexual differentiation, the male process is actively mediated by androgens with female differentiation largely perceived as quiescent—the so-called 'default pathway' [8]. Given the emerging data that the normal female fetus synthesizes and is thus exposed to potent androgens, it seems worthwhile revisiting this 'default' concept as it might be an over-simplification. Certainly, appropriate growth, vascularization and innervation of female external genitalia need to be initiated during the first trimester. Interestingly, at 9 wpc when CYP19 expression to convert androgen to estrogen is limited, descriptive studies have demonstrated AR in female external genitalia [11]. After this period, expression in female external genitalia diminishes everywhere except the clitoris, which thus maintains potential androgen-dependent growth. These descriptions are in keeping with clinically extreme female virilization arising from fetal exposure to androgen in the first trimester whereas clitoromegaly remains a risk throughout gestation if androgen levels are excessive. The commonest pathological cause is CAH due to CYP21 deficiency [9], although other causes of excess androgen result in the same effect. Intriguingly, the anlagen of the internal genitalia are resistant to virilization in CYP21 deficiency. This has been attributed to a dose effect, with androgen levels internally being considered inadequate to virilize development. This is perhaps surprising given the proximity of the adrenal gland to the mesonephros; feasibly, the lack of internal virilization may also reflect differential androgen action due to the presence or absence of other AR interacting transcription factors in the female fetus [13]. Putting pathology to one side, the consequences of physiological, yet potent androgen secretion from the female fetal adrenal cortex are particularly unclear. It is not known what regulates AR expression and the downstream consequences of AR action are also unclear. It seems curious for female development, the correct unfolding of which requires strictly limited exposure to androgen action, to express the very receptor that facilitates virilization to occur. With so much our current knowledge derived from scenarios of either pathological excess or deficiency, our understanding of physiologically normal development seems remarkably deficient. It will be important to determine the relative concentrations of androgens present during normal

male and female sexual differentiation and the responses of the target cells in the genitalia. The genetic targets of AR causing virilization of female development are likely to be the same as those that when inactivated mediate male syndromes of under-virilization. It is unknown what level of androgens in females tips the balance from normal differentiation to pathological virilization. Certainly, the effects of physiological androgen concentrations on female development are controversial when one considers neural development, patterning and postnatal behaviour. Furthermore, interactions with other transcription factors make their respective expression profiles during development as important as that of AR in determining androgen action [13]. These data need translating from the study of cancer to human developmental biology along with better understanding of pre-receptor factors, such as the potential for novel steroid biosynthetic pathways that result in potent androgen biosynthesis.

6 Summary

Human development is a fascinating area of biology, the understanding of which brings with it clinical application to congenital disorders and their treatment. Most organs develop in a monomorphic fashion. In contrast, the major impact of hormone signaling on dimorphic sex differentiation creates an enormous complexity where we still know very little about the molecular mechanisms at play.

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