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Exploration of androgen action in the human endometrium

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BSc (Honours), MSc Res

Thesis submitted to the University of Edinburgh for the Degree of

Doctor of Philosophy

December 2014
Declaration

The studies presented in this thesis were the unaided work of the author, except where due acknowledgement is made by reference. The work described in this thesis has not previously been accepted for, or is currently being submitted for another qualification.

Paula Lourenço,

December 2014
Acknowledgements

I acknowledge Dr Elaine Marshall who carried out the hESC DHT experiment for application to microarray study and her assistance with functional analysis of the microarray data (Chapter 3). I acknowledge the assistance of the Finnish DNA Microarray Centre at the Turku Centre for Biotechnology who performed microarray and data analysis.

I would like to thank Hilary Critchley and Philippa Saunders for the opportunity they gave me and for their continued supervision and support. I extend a special thank you to the women who donated tissue samples and the essential support of the research nurses that allowed these studies to be carried out. Thank you to Elaine Marshall, Evan Simpson, Andrew Horne and Kirsty Brown for helpful discussions. Thanks to Sarah, Jacqui, Moira, Alison and Frances for assistance provided at one time or other.

Thanks to my many office and lab mates, you all made it easier. Thank you to Marina Amaral and Shalini Rajagopal for steadfast friendship through happy and difficult times. Thank you to the SFE study group, you put everything in perspective!

Kanimambo to my amazing family especially my parents and Mark.

For Vó Béu, Rita, Florence and Ellen.
Abstract

The endometrium undergoes recurrent cycles of dynamic remodelling, involving breakdown and scarless repair, proliferation and differentiation, including decidualisation of the stroma, during the menstrual cycle. Extensive studies have characterised how the steroid hormones oestrogen and progesterone acting via their nuclear receptors coordinate these remarkable changes. Although a few previous studies have postulated a role for androgens the impact of androgens on endometrial function remains understudied. The studies described in this thesis aimed to 1) identify cellular processes, pathways and networks regulated by androgens in human androgen receptor-positive endometrial stromal cells (hESCs), 2) investigate the potential for regulation and determine the regulation of putative dihydrotestosterone (DHT)-regulated gene expression by androgen in hESCs, 3) investigate the expression and regulation of putative androgen-regulated genes in the human endometrium across the menstrual cycle and in early pregnancy and 4) explore the role of androgens in modulating metformin-induced gene expression associated with decidualisation of hESCs.

Analysis of data from a whole genome array conducted previously in the laboratory using primary hESCs treated with DHT for 2 or 8 hours identified time dependant putative androgen-regulated mRNAs (34 and 268 genes, respectively). Thereafter, all work was completed by the author. Gene ontology and functional based bioinformatic analyses of the putative androgen-regulated gene sets revealed potential androgen regulation of a variety of cell processes, pathways and networks including those associated with gene transcription, signal transduction pathways (such as phosphatidylinositol, oestrogen receptor alpha (ERα) and Wnt signalling), cancer pathways, metabolism, cell cycle, development, apoptosis/survival. In addition, various transcription factors (e.g. AR, c-Myc, SP1, ERα, p53, E2F1, RUNX2, CREB1 and STAT3) were associated with androgen regulation in hESCs. Consensus androgen receptor binding sites were identified in the promoter sequences of 18 genes by transcription factor binding site sequence analysis. Direct DHT regulation of ten of 15 of these genes was validated in endometrial stromal cells using qRTPCR. Of these genes, RGS2, SIK1, and SNCAIP mRNAs were confirmed...
as DHT-regulated in hESCs by use of an AR inhibitor (flutamide) and in addition, were not found to be regulated by oestradiol. Discovery bioinformatics predicted these genes may interact in a gene network involving AR and the cAMP transduction pathway. Expression of the 15 putative androgen-regulated genes was confirmed by qRT PCR in intact human endometrial tissue (13 novel) and 9 of these genes were regulated in association with decidualisation i.e. either in the secretory phase, the time at which decidualisation begins and/or in first trimester decidua. Protein expression of RGS2, SIK1 and Synphilin-1 (encoded by SNCAIP) was confirmed by immunohistochemistry in endometrial tissues and protein expression also appeared greater in decidua. Regulation of putative androgen-regulated gene expression by decidualisation was confirmed in 4 out of 8 genes by employing a model of reduced in vivo decidualisation i.e. decidua from ectopic pregnancies. Regulation of 5 out of 7 genes was confirmed in decidualised hESCs (RGS2, SIK1, SLC6A6, SNCAIP and AXIN2) but expression of these genes was not altered by DHT inclusion during decidualisation. Finally, only a high metformin concentration enhanced hESC decidualisation and putative androgen-regulated gene expression (4 genes) in decidualised hESCs. In comparison, in the presence of DHT, a lower clinically relevant metformin concentration (100µM) did enhance decidualisation marker expression but did not alter expression of putative androgen-regulated genes.

In summary, these studies have revealed new insights into androgen action in the human endometrium. Studies in hESCs 1) predicted the pathways and interacting transcription factor regulatory networks that may be androgen-dependent in this cell type, these were associated with cell differentiation, apoptosis and proliferation, 2) identified novel putative androgen-regulated genes expressed in hESCs and in endometrial tissues, 3) showed putative androgen-regulated genes are regulated by DHT (possibly via AR) in endometrial stromal cells, some of which are also regulated in association with decidualisation and 4) showed that androgens may enhance decidualisation during exposure to the commonly used drug metformin. Collectively, these new findings support a physiological role for androgens in endometrial function and provide a series of new avenues for further studies of the regulation of differentiation and proliferation.
Lay Summary

In humans, the lining of the womb or endometrium undergoes monthly changes in response to female steroid hormones (oestrogen and progesterone). Female steroids drive the growth and transformation of different endometrial cell types including that of the stromal cells that play an important role in the preparation for and maintenance of pregnancy. However, the male steroid hormone testosterone (or androgen) is also present in women and the study of the roles of androgens in the endometrium has until recently been very limited. It is thought that androgens may contribute to the important changes undergone by the stromal cells by binding to a specialised protein receptor, the androgen receptor (AR) and, in this way, lead to changes in the amounts of messenger RNAs (mRNAs) produced from genes in the cells. To understand which genes may be switched on and off by androgen we examined the changes in all the genes of stromal cells taken from the endometrium of women and treated with androgen in the laboratory. Computational study of these mRNA profiles predicted that androgen may control growth and transformation of the stromal cells possibly through binding to the AR and altering mRNA expression patterns. Changes in mRNA concentrations of selected genes due to androgen action were confirmed experimentally in stromal cells. Many of the selected gene mRNAs were reported in endometrial cells or reported as being regulated by androgen for the first time in this thesis. It is possible that other steroid hormones (e.g. progesterone) may also affect these mRNA concentrations but confirmation is still required. Finally, experiments revealed that androgens may also control the action of some commonly used drugs (e.g. metformin) in the endometrium affecting the transformation or decidualisation of the stromal cells. All together, the findings in this thesis provide support for the role of androgens in the endometrium through changes in stromal cell gene expression that may affect endometrial growth and transformation and even contribute to successful pregnancy. This information may provide insight into how either a lack or an excess of androgens may negatively affect endometrial function leading to pregnancy loss. The research findings also highlight the importance of an improved understanding of the actions of androgens and their interactions with, for example, other hormones and drugs.
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List of Abbreviations

A4  Androstenedione
Adiol  Androstenediol
AF  Activation function domain (AF-1 and AF-2)
AKT  protein kinase B / v-akt murine thymoma viral oncogene homolog 1
AMP  adenosine monophosphate
ANOVA  Analysis of variance
AP  Anti-progestin
AR  Androgen receptor
ARE  Androgen response element
ARKO  Androgen receptor knockout
ATP  adenosine triphosphate
BSA  Bovine serum albumin
cAMP  3',5'-cyclic adenosine monophosphate
cDNA  Complementary DNA
CG  Chorionic gonadotrophin
ChIP  Chromatin immunoprecipitation
CREB1  cAMP responsive element binding protein 1
CYP19A1  Aromatase, cytochrome P450 side chain cleavage enzyme
DAB  Diaminobenzidine
DBD  DNA binding domain
DE  Differentially expressed
DHEA  Dehydroepiandrosterone
DHT  Dihydrotestosterone
DMEM  Dulbecco’s Modified Eagles Medium
DMSO  Dimethyl sulfoxide
DNA  deoxyribonucleic acid
E  Notation “times ten raised to the power of”
E2  Oestradiol
ECT  Ectopic pregnancy
EDTA  Ethylenediaminetetraacetic acid
EGF  Epidermal growth factor
ELISA  Enzyme-linked immunosorbent assay
EP  Ectopic pregnancy
ER  Oestrogen receptor
ERα  Oestrogen receptor alpha (ERA) (encoded by ESR1)
ERβ  Oestrogen receptor beta
ERRα  oestrogen-related receptor α
ESC  Endometrial stromal cell
EtOH  Ethanol
FBS  Fetal bovine serum
FDMC  Finnish DNA microarray centre
GDP  Guanosine diphosphate
GO  Gene ontology
GR  Glucocorticoid receptor
GTP  Guanosine triphosphate
hCG  Human chorionic gonadotrophin
HEPES  4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hESC  Human endometrial stromal cell
Chapter 1: Literature review

1.1 Hormonal regulation of the menstrual cycle

The menstrual cycle is regulated by the neuroendocrine pathway constituted by the hypothalamic-pituitary-ovarian axis. In brief, secretion of gonadotrophin releasing hormone (GnRH) from the hypothalamus stimulates the anterior pituitary to produce follicle stimulating hormone (FSH) and luteinising hormone (LH). These gonadotrophins act on ovarian cells stimulating them to produce several factors including the steroid hormones oestrogen and progesterone from the follicles and corpora lutea respectively (the ovarian cycle). Whilst also providing feedback in varying degrees to the hypothalamus and anterior pituitary, the cyclical production of ovarian oestrogen and progesterone regulates the dynamic and recurrent structural and functional changes of the human uterine inner lining, known as the endometrial menstrual cycle (Messinis, 2006).

1.1.1 The menstrual cycle - human endometrial regulation by oestrogen and progesterone

The duration of the standard menstrual cycle is considered to be 28 days although there is much variation among women (Treloar et al., 1967, reviewed by Vitzthum, 2009). There are three main phases in the menstrual cycle, menstruation, the proliferative phase and the secretory phase. The first day of the menstrual cycle is counted from the first day of menstruation.

In the ovarian follicular phase, the oestrogen concentration increases until ovulation occurs. In response to oestrogen, stromal cells of the endometrium undergo rapid proliferation and this stage of the endometrial menstrual cycle is known as proliferative phase (Noyes et al., 1950) (Figure 1.1).
Figure 1.1 The human endometrial menstrual cycle. Illustration of changing ovarian histology, circulatory ovarian hormone levels and endometrial morphology during the endometrial menstrual cycle (menstruation, proliferative and secretory phases) and if pregnancy occurs. The first day of the menstrual cycle is counted from the first day of menstruation. In the proliferative phase oestradiol (E2) promotes endometrial proliferation. After ovulation, at around day 14 (mid-cycle), progesterone (P) levels rise and transform the oestradiol-primed endometrium into secretory tissue (secretory phase). In the absence of a pregnancy, the withdrawal of progesterone (and oestradiol) lead to tissue breakdown and menstruation during which tissue repair and regeneration also occur. If successful implantation occurs (at around or just after the mid-secretory phase) progesterone and oestrogen levels are maintained and the menstrual cycle ceases for the duration of the pregnancy. Figure drawn by Ted Pinner for MRC Human Reproductive Sciences Unit Report (P. Saunders).

An acute rise in peripheral concentrations of LH (the LH surge) triggers ovulation and the development of the corpus luteum. After ovulation, the ovarian luteal phase is characterised by the rise in circulating concentrations of progesterone (secreted from the corpus luteum) and the fall and then rise of oestrogen levels. In the presence of progesterone, the endometrium undergoes differentiation and secretes a range of factors in preparation for implantation of the conceptus (Noyes et al., 1950). This is the secretory phase of the menstrual cycle. During this phase, the expanding stromal compartment differentiates (known as decidualisation) and functional epithelial secretory glands develop. Progesterone levels continue to rise in the presence of a pregnancy but are not maintained in its absence with menstruation ensuing as a consequence. At menstruation, the functional layer of the endometrium is shed, leaving in place the basal layer which acts as a germinal compartment with the
capacity of rapid and non-scarring repair and regeneration of the functional compartment (proliferative phase endometrium) (Noyes et al., 1950, Ferenczy, 1976, Padykula et al., 1989, Jabbour et al., 2006).

1.2 Androgens

1.2.1 Androgens: biosynthesized steroid hormones

Sources of steroids
All steroid hormones, including the sex steroids androgens, oestrogens, and progesterone(s) are derived from cholesterol via an enzyme cascade that regulates sequential transformations of their ring structure and attached side chains (reviewed by Miller and Auchus, 2011). Classically, tissues were defined as being ‘steroidogenic’ because they possessed the capacity to convert cholesterol to pregnenolone and included endocrine tissues such as the adrenals, ovaries and testes. Other tissues have also been reported to have this capacity, for example, placental trophoblast cells, specific cells in the prostate, brain and skin (Thiboutot et al., 2003, Ghayee and Auchus, 2007, Bennett et al., 2012). Another key aspect of steroidogenesis is that in some cases a steroid is produced in one cell or tissue and then is converted to another steroid in a different target tissue or cell (Luu-The and Labrie, 2010). Thus, peripheral tissues (non-endocrine gland) constitute a second source of local or even systemic steroid hormones and include tissues such as the liver, fat, skin, kidneys and brain (Luu-The, 2013).

In summary, sex steroid biosynthesis is regulated in tissues predominantly by the availability of precursor molecules and the expression of active converting enzymes.

Androgen biosynthesis
The key steps in the biosynthesis of the androgens androstenedione (A4), testosterone (T) and dihydrotestosterone (5α-DHT or DHT) are outlined in Figure 1.2 (reviewed by Luu-The and Labrie, 2010, Penning, 2010, Luu-The, 2013).
Figure 1.2 Androgen metabolism. Pathways for androgen biosynthesis from cholesterol to DHT and DHT metabolites including some key converting enzymes. Arrows indicate the direction in which the reactions occur. Shaded boxes indicate enzymes and reactions leading to the synthesis of androgenic metabolites (blue) but also oestrogenic metabolites (grey). A simple overview is shown of the tissues where conversion reactions can occur. However, some reactions may be preferentially carried out in one tissue compared with another. *The initial step requires the transport of cholesterol into the mitochondria by steroidogenic acute regulatory (STAR) protein.

Steroids: DHEA = dehydroepiandrosterone; DHEAS = dehydroepiandrosterone sulphate; 5α-DHT = dihydrotestosterone, DHT; 3α-Adiol = 3α-androstan-3α,17β-diol; 3αHSD = 3α-hydroxysteroid dehydrogenase/Δ4-5-4 isomerase; 3β-Adiol = 5α-androstan-3β,17β-diol; 3βHSD 1,2 = 3β-hydroxysteroid dehydrogenase/Δ4-5-4 isomerases; 17βHSD 1, 2, 5 = 17β-hydroxysteroid dehydrogenase types 1, 2 and 5.

Enzymes: CYP11A1 = cytochrome P450 side chain cleavage enzyme; CYP17A1 = cytochrome P450 17A1, aromatase; STS = steroid sulfatase. Adapted from the reviews by Luu-The & Labrie 2010, Penning 2010, Luu-The 2013.
Briefly, in the biosynthetic pathway, pregnenolone is converted to dehydroepiandosterone (DHEA) via sequential cytochrome P450 C17 (CYP17A1)-dependent enzymatic reactions. DHEA can be transformed to 5-androstenediol (adiol) (by 17β-hydroxysteroid dehydrogenases types 1 and 5, 17βHSD1 and 5) and to androstenedione (A4) (by 3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerases, 3βHSD types 1 and 2). Both adiol and A4 are precursors of testosterone and testosterone itself can be metabolized back to A4. Testosterone is converted to 5α-DHT via 5α-reductases. Testosterone can also be reduced to 5β-DHT by 5β-reductases (not included in Figure 1.2).

From 5α-DHT two metabolites, 3α-androstane-3α, 17β-diol, (3α-adiol) and 5α-androstane-3β, 17β-diol (3β-adiol), can be produced via the activities of 3β-HSD or 3-α-hydroxysteroid dehydrogenase/Δ-5-4 isomerase (3αHSD), 3βHSD and 17βHSD, respectively.

Of clinical importance is the inter-conversion of androgenic and oestrogenic steroids. Notably, the androgens androstenedione and testosterone can be converted by CYP19A1 (aromatase) to the oestrogens, oestrone and oestradiol, respectively.

1.2.2 Androgens in women

The sources and levels of circulating androgens in women

In premenopausal women, the adrenal cortex and the ovaries both contribute significantly to the levels of systemic androgens DHEA, androstenedione and testosterone (Abraham, 1974, Burger, 2002). Some studies have estimated the relative contributions the adrenals, ovaries and peripheral tissues make to these peripheral androgen concentrations. They have reported that the adrenals contribute approximately 50% of all secreted DHEA, with the ovaries contributing about 20% and the peripheral tissues producing about 30% (Longcope, 1986). There is some discrepancy in the estimates regarding the contributions from the adrenals to total DHEA concentrations (from between 50 to 80%). However, there is general agreement that the majority of peripheral DHEA is produced by the adrenals (Abraham, 1974, Longcope, 1986). Androstenedione is produced in equal amounts by both the adrenal glands and ovaries (Abraham, 1974, Longcope, 1986, Piltonen et
al., 2002) although there are variations in ovarian production with the phase of the menstrual cycle, for example higher amounts are produced by the ovary midcycle (Abraham, 1974). Testosterone is produced in equal amounts by both the adrenal glands and ovaries (approximately 25% each) with peripheral tissues contributing the other 50% in the peripheral circulation (Longcope, 1986, Piltonen et al., 2002). DHT is almost exclusively synthesized from testosterone in peripheral androgen target tissues such as muscles (Ito and Horton, 1971, Burger, 2002, and reviewed by Luu-The, 2013, Marchetti and Barth, 2013).

In women with normal ovulatory cycles, the levels of testosterone and androstenedione remain similar during the follicular and luteal phases except for a rise (of ovarian origin) observed mid-cycle at the time of ovulation (Abraham, 1974, Massafra et al., 2000, Salonia et al., 2008) (Table 1.1-A). Androgen levels have been reported to suffer a small decline with age (Goebelsmann et al., 1974, Davison et al., 2005) with the mid-cycle rise in androgens also found to be diminished in older reproductive aged women (43 to 47 years old) compared to younger women (19 to 37 years old) (Mushayandebvu et al., 1996). The steady fall of testosterone (total or free) and androstenedione levels may occur in women between the third and fifth decades with testosterone levels being reduced to less than half after the menopause (Terada et al., 1990, Jo et al., 1993, Davison et al., 2005). However, although a decrease in free testosterone was reported with age, not all studies found that the decrease in circulating total testosterone observed with age was significant (reviewed by Yasui et al., 2012). In addition, local production of steroids, namely androgens, is thought to be crucial after the menopause in peripheral organs/tissues through conversion of DHEA into testosterone (Labrie, 2015). In comparison, oestrogen levels are markedly diminished in women after the menopause compared to early reproductive years, for example, serum oestradiol concentrations are decreased to between 1 and 5% (Terada et al., 1990, Jo et al., 1993, Yasui et al., 2012) although oestrogens may also still be produced in peripheral tissues from DHEA (reviewed by Labrie, 2015). Finally, DHEA is also thought to decline with age although there is much variation between women (reviewed by Labrie, 2015).
Table 1.1 Circulating androgen concentrations in women during the menstrual cycle (A) and during pregnancy (B). Oestradiol and progesterone values are included for comparison.

<table>
<thead>
<tr>
<th></th>
<th>Proliferative phase</th>
<th></th>
<th>Secretory phase</th>
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<tbody>
<tr>
<td></td>
<td>Early</td>
<td>Mid</td>
<td>Late</td>
<td>Early</td>
</tr>
<tr>
<td>Oestradiol</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>51 ±6</td>
<td>80 ±11</td>
<td>231 ±40</td>
<td>116 ±12</td>
</tr>
<tr>
<td>Progesterone</td>
<td>903 ±139</td>
<td>879 ±88</td>
<td>1144 ±178</td>
<td>8851 ±2072</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>1264 ±163</td>
<td>1461 ±101</td>
<td>1599 ±83</td>
<td>1377 ±99</td>
</tr>
<tr>
<td>Free testosterone</td>
<td>2.8 ±0.2</td>
<td>3.4 ±0.24</td>
<td>3.9 ±0.41</td>
<td>3.2 ±0.22</td>
</tr>
<tr>
<td>Testosterone</td>
<td>602 ±46</td>
<td>673 ±39</td>
<td>927 ±160</td>
<td>743 ±87</td>
</tr>
</tbody>
</table>

B - During and post pregnancy

| Testosterone | 14 weeks | 750 |
|             | 18 weeks | 700 |
|             | 22 weeks | 780 |
|             | 26 weeks | 850 |
|             | 30 weeks | 850 |
|             | 34 weeks | 1050 |
|             | 38 weeks | 1000 |
|             | 4 days post-partum | 600 |

All mean concentrations in pg/ml. Testosterone = SHBG bound testosterone. To convert hormone concentrations to units used clinically the following formulae can be used. To convert oestradiol from pg/ml to nmol/L = oestradiol/272.38 or pg/ml to pmol/L = oestradiol/0.27238. To convert progesterone from pg/ml to nmol/L = progesterone/314.46. To convert testosterone from pg/ml to nmol/L = (testosterone in pg/ml)/288.42. Endometrial menstrual cycle data adapted from Massafra et al., 2000 and longitudinal gestational data adapted from Kerlan et al., 1994.

In these studies, the commonly measured systemic androgens (testosterone and androstenedione) should be considered differently due to their distinct ability to elicit androgenic effects upon target tissues or cells (via binding to their target androgen receptor). Androstenedione is considered a much weaker androgen than testosterone as it binds the androgen receptor with much reduced affinity (Chen et al., 2004) (section 1.3.1).
A recent systematic review of reproductive hormones in pregnancy reported that the available data showed testosterone and androstenedione both rise during pregnancy (Kuijper et al., 2013). It is suggested that the increased testosterone during pregnancy is of luteal or placental origin (Harrison and Mansfield, 1980, Castracane et al., 1998, reviewed by Makieva et al., 2014). Small increases in maternal serum testosterone levels are also reported along the course of gestation followed by a decline post-partum (Kerlan et al., 1994) (Table 1.1-B). However, further studies surveying maternal serum testosterone concentrations prior, during and post-pregnancy are still required.

Different assays have been used to measure androgens with varying degrees of accuracy and specificity but recently, advances have been made in the ability to accurately measure androgens (especially low concentrations of total and free testosterone, DHT) using for example, liquid chromatography-tandem mass spectrometry (Ke et al., 2014, Shea et al., 2014, Labrie, 2015). Crucially, these techniques should permit more accurate and specific measurement of androgens both in serum and in peripheral tissues, improving our understanding of their impact upon peripheral tissues. For example, the role of circulating androstenedione as a weak androgen may be reconsidered if it is readily and preferentially converted to testosterone locally.

**Bioavailability of blood testosterone**

Upon secretion into the circulation, all steroids bind proteins (Bradlow, 1988). For example, testosterone can be found bound to sex-hormone binding globulin (SHBG). The amount of unbound testosterone (free testosterone) is dependent on the concentration of SHBG in the blood. The SHGB concentrations present in the blood are produced primarily by the liver (Hammond, 2011) and can be altered by a number of hormonal and metabolic factors. For example, SHBG blood levels can be reduced by inflammation, obesity, menopause, insulin and androgens themselves while concentrations of SHBG in blood can be elevated by oestrogens (including exogenous oestrogens as those contained in the oral contraceptive pill), thyroid hormone and prolonged stress (reviewed by Rosner, 1991, Pugeat et al., 2010, Simo et al., 2015).
Free testosterone passes freely across cell membranes but bound testosterone is unable to do so. In this way, testosterone is made intracellularly available (or unavailable) to bind to the androgen receptor (section 1.3) directly or after conversion to DHT. Thus, the measurement of free testosterone, bound testosterone and SHGB blood concentrations are important clinically. For example, elevated free testosterone and reduced SHGB levels are reported in women in association with clinical manifestations of polycystic ovary syndrome (PCOS) (Hassa et al., 2006, Huang et al., 2010), consistent with the suggestion that in PCOS there is an overall increase in bioavailable androgen. However, the increased bioavailability of androgens (testosterone) and oestrogens (oestradiol) in PCOS may be due to a number of factors including the SHBG suppressive action of insulin (reviewed by Wallace et al., 2013) which is also increased in association with PCOS (Dunaif et al., 1989, Kidson, 1998). While the bioavailability of androgens and oestrogens in PCOS may be dependent upon SHBG levels, SHBG secretion in PCOS patients may itself be affected by the altered endocrine features of PCOS, namely the associated increase in blood concentrations of insulin, androgens and oestrogens. Thus, PCOS is a complex syndrome in which endocrine abnormalities interact in multiple ways with some factors confounding relationships between other factors and leading to the recognition that they cannot be considered in complete isolation from one another.

In a different example, although concentrations of androgens are elevated during normal pregnancy, the amount of SHGB in blood is also increased in what is thought to be part of a poorly understood maternal compensatory mechanism. This mechanism may either protect the fetus from exposure to maternal steroids or conversely, may serve to protect mothers from androgens and other sex steroids originating from the fetus (reviewed by Hammond, 2011).
Chapter 1

1.3 The androgen receptor

1.3.1 The androgen receptor and the nuclear receptor superfamily

In 1988, the gene encoding the human androgen receptor (AR) was located to the X chromosome and subsequent cloning facilitated the identification of the full coding sequence (Chang et al., 1988, Lubahn et al., 1988a, Lubahn et al., 1988b). The androgen receptor, also known as NR3C4 (nuclear receptor subfamily 3, group C, member 4) is a member of the nuclear receptor superfamily of ligand-activated sequence specific transcription factors. AR is grouped within the steroid receptor sub-family which includes the oestrogen receptors alpha and beta (ERα, ERβ) and the progesterone receptor (PR) responsible for mediating the actions of gonadal steroids oestrogen and progesterone respectively (Mangelsdorf et al., 1995) (Figure 1.3). Evolutionary divergence studies suggest that steroid receptors evolved from a common oestrogen-sensitive ER-like ancestral receptor with the first steroid receptor being an ER, followed by the more recent emergence of a separate receptor sub-group constituted by PR, AR, glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) that are activated by other steroid hormones (progesterone, androgens, corticosteroids) (Thornton, 2001). Sequence homology analysis of these steroid receptors revealed that the amino acid sequence of AR shows greater homology to PR (and to GR and MR) than to ERα (ESR1) or ERβ (ESR2) (Robinson-Rechavi et al., 2003). A key factor in the evolution of steroid receptors is the co-evolution of steroid receptors alongside respective ligands. It is suggested that AR and PR evolved after ER through processes of duplication and divergence of the ancestral ER in animals (Eick and Thornton, 2011). However, in many eukaryotes (not only animals) testosterone and progesterone (potential non-aromatised ligands) would have been already present as intermediates in the synthesis of aromatised oestrogens (Figure 1.2) (reviewed by Sladek, 2011). Thus, in the case of AR and PR, the appearance of the ligands may have predated the appearance of the receptors which were then assigned new functions (to bind androgens and progesterone with greater affinity). The co-evolution theory of steroid receptors and converting enzymes involved in steroid synthesis is also the subject of ongoing investigations (reviewed
by Baker, 2011) with a recent study suggesting an important role for these processes in vertebrate evolution (Baker et al., 2015).

<table>
<thead>
<tr>
<th>Steroid Receptor</th>
<th>Endogenous Ligands</th>
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<tbody>
<tr>
<td><strong>N-terminus</strong></td>
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<tr>
<td>NTD</td>
<td>Testosterone and DHT</td>
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<td>DBD</td>
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<td>LBD</td>
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<td>AR</td>
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<td><strong>C-terminus</strong></td>
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Figure 1.3 Steroid receptor sub-family structural organisation. Nuclear receptors contain structural regions: variable N-terminal transactivation domain, NTD (A/B), conserved DNA-binding domain, DBD (C), variable hinge domain (D), the variable C-terminal region containing the ligand-binding-domain sequences, LBD (E). Two AR transactivation domains (AF-1 and AF-2) are localised within the A/B and E regions, respectively. Ligands that bind the receptors with the highest affinity are shown. MR: Mineralocorticoid Receptor. GR: Glucocorticoid receptor. PRA and PRB: Progesterone Receptor isoforms A and B, respectively. The full length translated receptor protein size is shown in amino acid number. Figure adapted from Griekspoor et al., (2007).

Both testosterone and DHT bind with high specificity and affinity to the androgen receptor and affect gene expression (Kemppainen et al., 1992, Pereira De Jesus-Tran et al., 2006). DHT dissociates three times slower from the AR than testosterone, and is considered the more potent androgen resulting in a greater stabilisation of AR and mediation of androgenic effects (Zhou et al., 1995). It is also relevant to note that DHT metabolites (3α-adiol and 3β-adiol, Figure 1.2) have a reduced binding affinity to AR and an increased affinity to ERs thereby leading to oestrogenic effects. For instance, 3β-adiol’s greatest binding affinity is for ERβ (Chen et al., 2013a).
The cross-reactivity between endogenous steroid molecules and AR has been investigated but the affinity and preference with which steroids (besides DHT and testosterone) bind AR is significantly reduced. For example, in order of decreasing binding affinity to AR are 3β-adiol, adiol (DHEA metabolite), oestrone, and oestradiol (Chen et al., 2013a). Conversely, DHT and testosterone have been shown to bind ERα with very low affinity when compared to 3β-adiol, adiol, oestrone and oestradiol (in order of increasing binding affinity to ER) (Chen et al., 2013a). In cell-free assays, the testosterone precursors adiol and A4 were reported to bind AR with reduced affinity compared to DHT and in cell-based transcription assays adiol and A4 elicited AR agonist but also some AR antagonist activity (Chen et al., 2004). Oestradiol and progesterone were also found to bind AR and progesterone increased AR nuclear translocation in cell based luciferase assays, however, binding occurred with a reduced affinity compared to DHT (Kim et al., 2007). Some synthetic steroids also show promiscuous binding to more than one steroid receptor, for example, the synthetic progestin medroxyprogesterone acetate (MPA) was shown to activate both PR and AR in human breast cancer cells (Bentel et al., 1999).

The protein size predicted for AR from the complimentary DNA (cDNA) sequence is approximately 99KDa (Lubahn et al., 1988a, Tilley et al., 1989) and two androgen receptor protein isoforms were first demonstrated in genital skin fibroblasts estimated at 87kDa and 110kDa (Wilson and Mcphaul, 1994). Subsequent in vitro studies in different cell types and in vivo findings from patients carrying certain AR mutations suggest the isoforms may play different functional roles during cell proliferation and development (Liegibel et al., 2003, Barbaro et al., 2007) but their roles remain poorly understood. In humans, the messenger RNA (mRNA) expression of another naturally occurring (normal tissue) 45kDa variant (AR45) has also been reported with greatest expression in the heart, skeletal muscle, uterus, prostate, breast, lung and to a lesser extent in other tissues (Barbaro et al., 2007). Functional in vitro studies indicated the AR45 isoform could bind ligands but inhibited AR transcriptional activity via interactions with the AR amino-terminal transactivation domain under certain conditions (Ahrens-Fath et al., 2005). The role of AR45 has not been further investigated in either normal or abnormal physiologies.
The AR gene has 8 coding exons (Lubahn et al., 1989). The androgen receptor protein shares a conserved structural and functional organisation with other nuclear receptors (Mangelsdorf et al., 1995, Evans, 2005) containing four main structural regions (Figure 1.3). In the receptor family, members have a variable amino (N)-terminal transactivation domain (NTD, region A/B) followed by a DNA binding domain (DBD) located centrally that is the most conserved region (region C). Then a variable hinge domain (region D) is located. Finally, the ligand binding domain (LBD) that confers ligand specificity on the proteins is located at the carboxy (C)-terminus (region E). This LBD domain lacks critical amino acids and a structure capable of binding known ligands in receptors known as “orphans” which is the case in the oestrogen-related receptor α (ERRα) (Mangelsdorf et al., 1995). A fifth domain of unknown function is present in some nuclear receptors such as in ERα (region F).

Also present in nuclear receptors are two transcriptional activation functional (AF) domains. These two domains have also been identified in the AR by mutation and deletion analyses and play key roles in AR transcriptional activity. The activation function domain (AF-1) and AF-2 are harboured within the NTD region A/B and the LBD-region E respectively (reviewed by Gelmann, 2002, Verrijdt et al., 2003, Shen and Coetzee, 2005, Claessens et al., 2008, Aagaard et al., 2011).

The AR has one of the largest NTD of the steroid receptors (Figure 1.3) (Griekspoor et al., 2007). The size of the AR NTD can vary due to reductions or expansions in repeat sequence triplets within the NTD of the AR (section 1.3.5) resulting in altered AR transcriptional activity (reviewed by Gottlieb et al., 2012). There are two repeat polymorphic sites and the longer polyglutamine (CAG) triplet sequences being associated with reduced AR transactivation activity in vitro (Armstrong and Papkoff, 1976).

Extensive sequence analysis, crystallisation studies and domain ‘swopping’ experiments have demonstrated that each domain harbours a distinct activity such as ligand binding, DNA-binding, dimerisation (homo or hetero), protein interactions and transcriptional activation (modulated by the other mentioned activities). For
example, in the AR one of the functions of the AF-1 domain is to carry out multiple protein-protein interactions with the transcriptional machinery resulting in AR folding and also determining further protein-protein interactions important for AR function (reviewed by Mcewan, 2004). A full discussion on the structure and function of the different domains of the AR has been presented in several articles (Gelmann, 2002, Reid et al., 2003, Verrijdt et al., 2003, Shen and Coetzee, 2005, Centenera et al., 2008, Claessens et al., 2008, Bennett et al., 2010, Clinckemalie et al., 2012, Van De Wijngaart et al., 2012).

1.3.2 Androgen receptor mode of action

Genomic action of AR

The activation of AR is usually dependent upon ligand binding although, in some instances, ligand-independent activation can also occur (Huang et al., 2002, Guo et al., 2006). In the absence of ligand most AR can be detected in the cytoplasmic compartment (Georget et al., 1997). Ligand-dependent cytoplasmic-to-nuclear translocation of AR is an important prerequisite for the AR-dependent activation of gene transcription reported in various cell types (Georget et al., 1997, Marcelli et al., 2006, Nakauchi et al., 2007) (Figure 1.4). Briefly, upon activation, intra-domain interactions involving the NTD and the LBD (N-terminal and C-terminal regions, N/C) occur and AR translocates to the nucleus where it can bind specific DNA sequences known as androgen response elements (AREs) in the promoters of target genes. Thus, ligand-bound AR acting in conjunction with transcriptional complexes, can lead to either up- or down-regulation of target gene mRNAs (Verrijdt et al., 2000, Claessens et al., 2001, Verrijdt et al., 2003, Claessens et al., 2008, Denayer et al., 2010) (Figure 1.4). As with other nuclear receptors, for example, with the PR (Vicent et al., 2010), AR binding to DNA has been associated with chromatin remodelling and changes in nucleosome occupancy (He et al., 2010, Andreu-Vieyra et al., 2011, He et al., 2012, Tewari et al., 2012).
Figure 1.4 A simple illustration of the mechanism of action of androgens via androgen receptors upon gene transcription. The androgen testosterone (T) enters the cell mainly by free diffusion where in the cytoplasm they can bind the androgen receptor (AR) directly or after conversion to dihydrotestosterone (DHT) by 5α-reductase activity. The ligand-bound AR translocates to the nucleus where the complex interacts with specific androgen regulatory element (ARE) sites on the DNA and other transcriptional machinery. Numerous co-activators and co-repressors modulate AR transcriptional activity through multiple mechanisms. As a result, gene transcription is altered which may impact upon protein production.

Non-genomic actions of AR

Non-genomic effects of androgens may involve rapid stimulation of intracellular second messenger signal transduction cascades or other signal transduction pathways and have been described over the past decade (reviewed by Falkenstein et al., 2000, Heinlein and Chang, 2002b, Boonyaratanakornkit and Edwards, 2007, Li and Al-Azzawi, 2009, Lang et al., 2013). For example, AR-dependent non-genomic activation of signalling cascades have been reported to be mediated by SRC proto-oncogene, non-receptor tyrosine kinase in various cell types including sertoli cells and vascular endothelial cells (Zhang and Croy, 1996, Cardenas and Pope, 2004, Slomczynska et al., 2006). In this way, non-genomic actions of AR may modulate cellular activity of kinase signalling cascades and other transcription factors ultimately resulting in changes in cell activity. Androgen action may also be
mediated via interactions with the cell membrane and with membrane receptors (reviewed by Boonyaratanakornkit and Edwards, 2007, Foradori et al., 2008).

1.3.3 Modulation of AR-dependent transcriptional activity
The activity of AR can be regulated via transcription, translation, post-translational modifications, ligand binding, dimerisation, ARE sequences, interactions with other transcription factors or co-regulator proteins. Some examples, briefly discussed below, show the complexity of the regulation of androgen-mediated signalling in different cell types.

**Regulation of AR mRNA and protein expression**
In some cells, for example, prostate cells, AR is responsible for cell specific autoregulation of AR gene expression as a result of androgen-bound AR binding to two identified AREs in the AR promoter sequence leading to increased AR mRNA expression (Dai and Burnstein, 1996). Similarly, AR can be regulated at the gene transcription level in cultured human endometrial stromal cells (Cloke et al., 2010). Other transcription factors such as cMyc may also upregulate AR messenger mRNA concentrations (Grad et al., 1999). Translational control of AR protein expression has been reported in different cell types, for example, binding of certain poly(C)-binding proteins to the AR mRNA may inhibit its translation (Yeap et al., 2002, Cloke et al., 2010). Our current knowledge of the regulation of AR in the human endometrium is presented in section 1.7.2.

**AR post-translational modifications**
The ability of AR to regulate transcription of specific genes is also regulated by multiple post-translational modifications to the protein (reviewed by Coffey and Robson, 2012). Examples include phosphorylation (Guo et al., 2006), acetylation (Fu et al., 2003) and sumolyation (Poukka et al., 2000, Callewaert et al., 2004, Cloke et al., 2008) all of which may affect co-factor recruitment.

**Ligand binding, AR Dimerisation and AR domain interactions**
The binding of ligands such as DHT or testosterone to the LBD of AR increases AR protein stability (Kemppainen et al., 1992, Zhou et al., 1995, Yeap et al., 1999), as
already described in section 1.3.1. Before binding to AREs on target gene DNA, dimerisation of androgen bound-AR complexes (testosterone or DHT) is mediated by NTD/C interactions (reviewed by Centenera et al., 2008). Findings from a study utilising quantitative microscopic live cell imaging techniques suggested that AR dimers confer greater binding stability to both high and low affinity ARE sequences whilst AR monomers bind mostly to high affinity ARE sequences (Van Royen et al., 2012).

Adding to the complexity of ligand-activated AR gene promoter interactions are interactions between AR domains (Scheller et al., 1998, and reviewed by Claessens et al., 2008). For example, interactions between specific sequences in the N-terminal transactivation domain (NTD) with those in the C-terminal LBD are induced by agonist binding and can stabilise the hormone-receptor complex (He et al., 1999). This N-C terminal interaction is required for the agonist-dependant transactivation of some genes but not others (He et al., 2000, He et al., 2002). In another example, in silico and in vitro interactions between specific residues in each of the DBD and LBD regions have also been identified to modulate the transactivation capacity of androgen-bound AR (Helsen et al., 2012).

**Androgen response elements**

The DNA sequences of the AREs to which AR binds include palindromic full site AR binding motifs and half site motifs (Verrijdt et al., 2003, Horie-Inoue et al., 2004, Denayer et al., 2010). Genome-wide chromatin immunoprecipitation (ChIP)-based studies have identified the presence of between thousands and tens of thousands of potential AR binding sites in the human genome (Wang et al., 2007b, Jia et al., 2008, Wang et al., 2009, Yu et al., 2010, Takayama et al., 2011, Tewari et al., 2012). It is notable that these may be located at genome sites within or at great distances from the proximal promoters of androgen target genes (Bolton et al., 2007, Lin et al., 2009). Mice with mutations in the Ar gene are also being employed to further elucidate how the sequence and structure of the AR itself confers AR specificity to bind specific ARE sequences (Schauwaers et al., 2007, Sahu et al., 2014).
AR co-regulators
Steroid hormone receptor function is also regulated by the recruitment of additional coregulatory proteins. AR co-regulators may bind to the AR protein to enhance transactivation (co-activators) or reduce transactivation (co-repressors) of target genes (reviewed by Mckenna et al., 1999). Consequently, aberrant expression of co-regulators can also lead to altered AR target gene expression (Chng et al., 2012). A large number of AR co-regulators have been identified (reviewed by Heinlein and Chang, 2002a, Wang et al., 2005, Heemers and Tindall, 2007) and novel co-regulators and the nature of their interaction with AR continue to be the subject of ongoing research (Menon et al., 2010, Jehle et al., 2014). Notably, the expression of AR co-regulators has also been shown to be cell-line and tissue specific and to contribute both to normal physiology and disease (Bebermeier et al., 2006).

Crosstalk between AR and other transcription factors
Interactions between AR and other transcription factors may also modulate the stringency/specificity and magnitude of AR-dependent transcriptional activity by a variety of mechanisms (Adler et al., 1993, reviewed by Aagaard et al., 2011). For example, cooperative crosstalk leading to formation of an active transcriptional complex may involve direct or indirect interactions between AR and other transcription factors that may bind DNA regulatory elements located adjacent to or at great distances from AREs. The ability of AR to bind DNA regulatory elements may also be blocked by DNA occupancy of other TFs. For example, as has been reported with the v-ets avian erythroblastosis virus E26 oncogene homolog (also known as ERG) that may alter AR responsiveness in prostate cancer cells by repressing AR-induced transcription (Chng et al., 2012). Recently it has been proposed that it is the crosstalk between AR with other transcription factors that may contribute to the establishment of androgenic tissue specific transcriptional programmes (Pihlajamaa et al., 2014).

1.3.4 Modulation of AR activity by AR inhibitors/antagonists
Cloning of the AR has facilitated the development of a range of steroid and non-steroidal modulators of AR activity. The AR inhibitors or competitive antagonists
that bind AR in the LBD (in the same way as the agonists testosterone and DHT) have been widely employed in the treatment of steroid-dependent cancers including prostate cancer (Singh et al., 2000). The use of steroidal antagonists such as cyproterone acetate or MPA were found to produce undesirable side-effects due to their binding to other steroid receptors such as GR or PR for which they are weak/partial agonists (Bentel et al., 1999, Honer et al., 2003). Consequently, first generation non-steroidal antiandrogens such as flutamide or bicalutamide were developed as "pure"/AR-specific antagonists that reduce AR-transcriptional activity by competitively binding to the AR within the ligand binding region and blocking the binding of endogenous ligands such as testosterone or DHT (Neri, 1989, Kemppainen et al., 1992). The dynamic nature of the molecular interactions between flutamide and AR have been studied using modern quantitative fluorescence image analysis techniques in living cells including fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP). Findings revealed that flutamide binding to AR was not associated with AR N/C domain interactions or receptor translocation to the nucleus (Van Royen et al., 2013). Additionally, flutamide-bound AR also exhibited reduced DNA binding stability (Farla et al., 2005).

Although first generation non-steroidal antagonists (flutamide and bicalutamide) have been used in clinical androgen deprivation therapies to treat prostate cancer, their low affinity for the AR and reduced effectiveness during long term use especially in the presence of mutant AR (reduced ability to repress androgenic AR activity in castration-resistant prostate cancer) (Taplin et al., 1999, Culig et al., 2001) led to the development of second generation compounds such as enzalutamide (previously known as MDV3100) that blocks androgen binding, binds AR with greater affinity, inhibits AR nuclear translocation and impairs AR binding to DNA (Tran et al., 2009, Helsen et al., 2014). Subsequently, new antagonists have been proposed based on further structure modification of enzalutamide (Voet et al., 2013). In addition, modulation of AR activity is also proposed through compounds such as EPI-001 that bind AR at the NTD (instead of the LBD) and block protein-protein interactions essential for AR transcriptional activity (Andersen et al., 2010) or
through using nucleic acid-based antisense oligonucleotide approaches that target AR expression at the RNA and protein levels (Zhang et al., 2011).

1.3.5 AR mutations
According to the 2012 update of the AR mutation database there have been more than 1000 different AR mutations reported in the literature that are associated with a loss- or a gain-of function (Gottlieb et al., 2012). New mutations are continuously being added to the internet database (http://androgendb.mcgill.ca/). Mutations include reductions or expansions in the trinucleotide tandem repeat sequences located in exon 1 and within the NTD (section 1.3.1), point mutations located within all AR exons, as well as changes in promoters or splicing sites. There are also reports of multiple AR mutations occurring within single individuals and also mutations that lead to a simultaneous gain and loss of AR function (Rajender et al., 2007, Gottlieb et al., 2012). Mutations in the AR gene have been linked with susceptibility to a number of disorders including androgen insensitivity syndrome (in men), cancers (prostate, breast, larynx, liver, testicular) and premature ovarian failure (Gottlieb et al., 2012).

1.3.6 Known roles of androgens and androgen-dependent signalling in women
Insight into the role of androgens in humans has benefited from studies in both humans and animals. In the male, androgens are essential for reproductive function in adulthood as they play a key role in development of the male reproductive tract in the fetus, in spermatogenesis and the maintenance of male secondary sexual characteristics including beard growth (Scott et al., 2007, Welsh et al., 2008, Macleod et al., 2010, Hiort, 2013, Smith and Walker, 2014). In men, androgens also mediate the growth and differentiation of other tissues such as muscle, fat and bone (Leder et al., 2003, De Maddalena et al., 2012, Dubois et al., 2012, Banu, 2013, Hwang et al., 2013) and can provide negative feedback to the central nervous system (reviewed by Veldhuis et al., 2006).

In women, recent findings indicate that androgen/AR signalling may also play essential roles in multiple tissues. For example, it is reported that testosterone has
direct actions upon muscle, bone resorption and growth (Ho et al., 2004, Davis, 2013, Hwang et al., 2013). The role played by androgens in maintaining and regenerating major tissues is evident from observations on aging in men and women where, for example, the decline in muscle mass and strength or bone mass can be triggered by reductions in androgen levels (Lopes et al., 2009, Banu, 2013, Hwang et al., 2013). Interestingly, in women testosterone treatment is used to improve sexual function in postmenopausal women but has also been associated with improved bone, improved body composition, improved cardiovascular function and cognitive function (Davis, 2013).

Besides the direct actions of testosterone, conversion of testosterone to DHT has been shown to modulate the growth of the hair follicle in both men and women (Mauvais-Jarvis, 1986, Inui and Itami, 2013). In addition, metabolism of testosterone to oestradiol in women is suggested to play a role in bone resorption, brain function and behaviour (Simpson, 2002).

Although changes in circulating testosterone have also been associated with ovarian function in particular with folliculogenesis (reviewed by Janse et al., 2012, Lebbe and Woodruff, 2013), the roles played by androgens in the endometrium during the normal menstrual cycle (described in the following section) remain poorly understood. The roles of androgens in pregnancy and parturition related to cervical remodelling and myometrial function have been the subject of a recent review (Makieva et al., 2014).

1.4 Human endometrial tissue histology and function changes during the menstrual cycle

The functional changes in the endometrium mirrors the cyclic structural changes and are primarily regulated by ovarian-derived oestrogen and progesterone.

1.4.1 Endometrial histology

Endometrial histology changes across the cycle at all structural levels. At the gross level, two tissue regions can be distinguished histologically (Noyes et al., 1950, Padykula et al., 1989). The functional layer corresponds to approximately the upper
luminal two-thirds of the endometrium and it is this compartment that is shed during menstruation. The basal layer (or basalis) is between the functional layer and the myometrium. The basal layer is retained after menstrual tissue loss and from it the functional layer develops.

In both compartments, the most abundant cell types are the epithelial and stromal cells. Epithelial cell populations line the uterine glandular structures and form a single layer of cells lining the luminal surface of the endometrium. The *in vitro* study of primary epithelial cells has been limited by difficulties in isolating pure populations and culture time being reduced compared to stromal cells (Mylonas *et al.*, 2000). In addition, the epithelial cells are not a uniform population, for example, some epithelial cells may constitute potential progenitor cells (reviewed by Deane *et al.*, 2013). In another study, the isolation of murine luminal and glandular epithelium by laser-capture microdissection followed by gene profiling revealed distinct molecular signatures for each separate epithelial population (Niklaus and Pollard, 2006). In comparison, the stromal compartment has been better characterised and is more diverse in cellular constitution and function. In addition to the large number of stromal fibroblasts, vascular cells and leukocytes are also present (varying spatially and temporally in number) and all contribute to the development and functional changes in the endometrium (Noyes *et al.*, 1950, Salamonsen and Lathbury, 2000, Salamonsen *et al.*, 2002, Rogers and Abberton, 2003, Dunk *et al.*, 2008).

The histological dating of human endometrial tissue was first described in detail by Noyes and colleagues (1950). The main characteristic features are described below (Noyes *et al.*, 1950, Chandrasekhar *et al.*, 1990, reviewed by Somjen *et al.*, 2004).

In the proliferative phase endometrial thickness varies from between 2mm (post menstruation) to about 5-6mm on each side of the uterus at the time of ovulation (Bakos *et al.*, 1994). The glandular epithelial structures are mainly circular and small in size becoming bigger especially within the functional layer towards the end of the proliferative phase. In comparison, the supporting elongated fibroblast-like stromal cells are present in greater numbers compared to the epithelial cells, and also expand in number over time. The basal arteries are straight and elongate in the direction of
the endometrial surface assuming a corkscrew appearance in the functional layer (termed spiral arteries) (Noyes et al., 1950).

**In the secretory phase** the spiral arteries predominate throughout the functional layer becoming increasingly coiled. At the start of the secretory phase, mitosis, basal vacuolation and secretions are all visible in the glandular epithelium (Noyes et al., 1950). Thereafter, the glands become enlarged (in length and width), appearing tortuous and open to the uterine cavity surface. Predominantly within the functional layer and around the mid-secretory phase, the stromal cells differentiate (termed decidual cells) appearing round, oval or polygonal (Dockery et al., 1990). An infiltration of leukocytes is also clearly visible (Tilley et al., 1989, Pugeat et al., 2010).

### 1.4.2 Menstruation

Menstruation is marked by the degeneration and loss of the functional layer of the endometrium and may last from 1 to 7 days. Demise of the corpus luteum and consequent falls in the concentrations of progesterone (and oestrogen) initiates an inflammatory process leading to the constriction of the spiral arterioles resulting in ischaemia and shedding of the functional layer (reviewed by Maybin and Critchley, 2011). Weakened arterial vessel walls within the functional layer rupture (visible by scanning electron microscopy) (Ludwig and Spornitz, 1991). Thus, blood flows and eventually this flow physically dislodges and helps clear the shed tissue from the uterine cavity.

As the functional layer of the endometrium is shed, healing begins. The straight arterioles maintain blood flow within the residual basal layer and a fibrinous mesh covers the exposed wound-like surface. Re-epithelialisation of the denuded surface begins around the 2\textsuperscript{nd} day of menstruation and is complete after 6 days (Ludwig and Spornitz, 1991).

According to one hypothesis, the endometrium is thought to regenerate from the basal layer (Prianishnikov, 1978, Padykula et al., 1989, Padykula, 1991) via resident or newly niche-induced stem or progenitor cells (Padykula et al., 1989, Chan et al., 2004, Schwab et al., 2005). Other studies suggest the epithelial cells may arise from
proliferation of the remaining epithelial glands (Ferenczy, 1976, Salamonsen, 2003, Chan et al., 2012) and also from differentiation of stromal cells (Baggish et al., 1967, Garry et al., 2009). Notably, recent studies in mice showed that endometrial epithelial tissue regeneration is accomplished, in part, by mesenchymal-to-epithelial transition (MET) both after parturition and after modelled menses (murine model) (Patterson et al., 2013, Cousins et al., 2014).

1.4.3 Proliferative Phase

The processes of tissue regeneration that begin during menstruation continue in the oestrogen-dominant proliferative phase. Endometrial oestrogen receptors ERα and ERβ reach maximal expression in epithelial and stromal cells during the late proliferative phase (Snijders et al., 1992). ERα is the predominant oestrogen receptor in the endometrium and through which endometrial proliferation by oestrogen is stimulated (Ferenczy et al., 1983). The functional layer begins to be reconstituted through stromal cell proliferation promoted by the action of oestradiol (and other growth factors) (Ferenczy et al., 1979, Neulen et al., 1987, Pierro et al., 2001). Studies such as those in the new world primate the common marmoset have reported that oestradiol also stimulates the production of angiogenic factors such as VEGF by endometrial epithelial and stromal cells thereby indirectly promoting angiogenesis to support and maintain the new tissue (Fan et al., 2008, Fraser et al., 2008, reviewed by Koos, 2011). Direct effects of oestrogens upon endometrial endothelial cells may also be possible as oestrogen-dependant gene expression and functional changes were elicited in cultured cells (Krikun et al., 2005, Greaves et al., 2013).

1.4.4 The secretory phase

Progesterone is the dominant hormone of the endometrial secretory phase. The post-ovulatory rise in progesterone is the key initiating factor for human endometrial transformation (or decidualisation) of oestrogen-primed fibroblasts during the secretory phase. Notably, oestrogen stimulates the expression of progesterone receptors in stromal fibroblasts (Lubahn et al., 1989, Cocksedge et al., 2009) and thereafter progesterone exerts both an antiproliferative and remodeling effect, the control of which is essential for the formation of the human decidua independent
from the presence of a conceptus. Subsequently, in the presence of a conceptus and in the case of pregnancy, progesterone is also essential for successful blastocyst implantation, coordinated trophoblast invasion and placenta formation (Glasser and Clark, 1975, Dey et al., 2004, Brosens and Gellersen, 2006, Halasz and Szekeres-Bartho, 2013). Finally, progesterone is also essential for the maintenance of pregnancy (Spencer and Bazer, 2002, Ozlu et al., 2012).

Specifically, progesterone acting via the progesterone receptor drives changes in the endometrial spiral arterioles, stromal, glandular and luminal epithelial cells and these cooperate via a complex paracrine signalling network to regulate the establishment of pregnancy (reviewed by Wetendorf and Demayo, 2012, Vasquez and Demayo, 2013). Initially, the spiral arteries grow and coil and the glandular epithelial cells become engaged in a new secretory programme which includes the production of glycogen, glycoproteins, lipids and mucopolysaccharides (reviewed by Jabbour et al., 2006). Under the action of progesterone the luminal epithelium is also primed for blastocyst apposition, attachment and invasion (Wang and Dey, 2006, Horcajadas et al., 2007a). In addition, the influx of several immune populations include the specialised uterine natural killer cells is also regulated indirectly by progesterone (Sentman et al., 2004, Guo et al., 2012). Also progesterone-driven is the differentiation (or decidualisation) of the endometrial stromal cells (ESCs) (reviewed by Dunn et al., 2003).

**Decidualisation of the endometrial stromal compartment**

Progesterone-dependent decidualisation of the stroma begins in the early- to mid-secretory phase in the cells surrounding the spiral arteries (Bell, 1990). *In vitro* decidualisation of human ESCs is associated with inhibition of proliferation due to cell cycle arrest that may involve the p53 pathway (Tang et al., 2009, Logan et al., 2012). Decidualised stromal cells (or decidual cells) are larger than stromal fibroblasts and they accumulate glycogen, lipids and proteins in the cytoplasm. This nutrient source supports the developing embryo in early pregnancy. A number of products involved in signalling to other endometrial cell types are synthesized *de novo* by the decidualised stromal cells including extracellular matrix components, hormones, peptides, chemokines and growth factors (reviewed by Dey et al., 2004,
Gellersen et al., 2007). For example, decidualised stromal cell-derived factors may regulate growth and differentiation of epithelial cells in humans (Arnold et al., 2001, Blauer et al., 2005). Stromal paracrine signalling may be clarified in greater detail by using murine models (Li et al., 2011). Decidual cells have also been postulated to play an important role in facilitating implantation and trophoblast invasion (reviewed by Oreshkova et al., 2012). For example, in response to trophoblast cues decidualised stromal cells exhibited enhanced motility and invasive capacity (Gellersen et al., 2010).

**Regulation of endometrial stromal decidualisation**

The intracellular concentrations of the second messenger cyclic adenosine monophosphate (cAMP) rise during progesterone-dependent decidualisation with both progestin and cAMP signalling being necessary for decidualisation (Brar et al., 1997, Gellersen and Brosens, 2003). It is postulated that progesterone elevates cAMP levels via protein kinase A (PKA) activation and this was also shown to be required for prolactin expression by endometrial stromal cells (Brar et al., 1997, Telgmann et al., 1997). These findings suggest that both the progesterone and cAMP signalling pathways are necessary for decidualisation where they play important and convergent roles. For example, in cell culture experiments cAMP sensitizes endometrial stromal cells to the actions of progestins (via downregulation of PR expression) (Brosens et al., 1999). In this way, cAMP accelerates progesterone-driven decidualisation of human endometrial stromal cells (hESCs) and enhances the production of decidualisation markers insulin-like growth factor binding protein 1 (IGFBP1) and prolactin (PRL) (Cloke et al., 2008).

Whilst the progesterone pathways and the cAMP-induced PKA pathway initiate decidualisation, decidualisation has been shown to be subsequently driven by other factors and/or pathways. For example, some individual factors include the transcription factors such as forkhead box O1 (FOXO1) (Gellersen and Brosens, 2003, Takano et al., 2007), homeobox A10 and A11 (HOXA10 and 11) (Gui et al., 1999, Lynch et al., 2009), the transcription factor CCAAT enhancer-binding protein β (C/EBPβ) (Ramathal et al., 2011) and AR (Cloke et al., 2008), kinases such as the extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (Lee et al., 2013),

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cytokines such as leukemia inhibitory factor (LIF) (Shuya et al., 2011), interleukin (IL)-11 (Dimitriadis et al., 2002), activin A (Jones et al., 2006) and transforming growth factor β1 (TGFβ1), a negative regulator of decidualisation (Kane et al., 2008, Kane et al., 2010). Examples of signalling pathways found to be involved in regulating decidualisation include the Activin A pathway (Jones et al., 2002, Tierney and Giudice, 2004, Jones et al., 2006), the janus kinase - signal transducer and activator of transcription (JAK-STAT) pathway activated by LIF among other factors (Jabbour et al., 1998, Mak et al., 2002), the SMAD 2/3 pathway (Kim et al., 2005b, Kane et al., 2010), the Wnt pathway (Sonderegger et al., 2010, Duncan et al., 2011), the Notch 1 pathway (Afshar et al., 2012) and the androgens and androgen receptor pathway (Cloke et al., 2008, Cloke and Christian, 2012, Kajihara et al., 2012).

However, decidualisation remains an incompletely understood complex process and there are numerous other genes and/or proteins reported to be altered as a result of decidualisation. Induction of hESC decidualisation is associated with profile changes of cytokines (Popovici et al., 2000), cyclins, mediators of cAMP signalling (Popovici et al., 2000, Takano et al., 2007) and cytoskeletal remodelling proteins (Brar et al., 2001, Paule et al., 2010). Many decidualisation-associated genes have been identified from global gene or protein expression studies of decidualised hESCs although identification has been inconsistent across the studies possibly due to methodological differences (Popovici et al., 2000, Brar et al., 2001, Tierney et al., 2003, Paule et al., 2010, Aghajanova et al., 2011) . In addition, a recent systematic analysis using a text mining and bioinformatic analysis approach has proposed a list of 286 genes involved in human stromal cell decidualisation and associated with, for example, focal adhesion, apoptosis and JAK-STAT signalling factors (Liu and Wang, 2015).

**Markers of decidualisation**

Decidual cells secrete IGFBP1 and PRL (Bell et al., 1991, Tseng and Mazella, 1999) and detection of their mRNAs and secreted proteins have long been used as markers of stromal decidualisation (Bell et al., 1991, Brosens et al., 1999). Besides these most commonly used markers of decidualisation, there are other markers that have been utilised such as relaxin and tissue factor (Bryant-Greenwood et al., 1993,
Lockwood et al., 1993). The cAMP-inducible transcription factor FOXO1 (Gellersen and Brosens, 2003) is another possible marker of decidualisation. Similarly to IGFBP1 and PRL, FOXO1 is also a critical inducer of decidualisation by interacting with the progesterone receptor and regulating decidualisation genes including the upregulation of IGFBP1 and PRL expression (Gellersen and Brosens, 2003, Kim et al., 2005a, Takano et al., 2007). As our understanding of the decidualisation mechanisms increases, the use of alternative markers of decidualisation such as those identified from the global gene or protein expression studies may be considered in future studies.

Currently, the strength of using the decidualisation markers IGFBP1 and/or PRL stems from consistent findings regarding their regulation (by progesterone or cAMP), from our knowledge of their roles during decidualisation in the endometrium and from the availability of specific and suitable assays (mRNA and protein). IGFBP1 expression is regulated by progesterone via the progesterone receptors (Bell et al., 1991, Gao et al., 1999, Gao et al., 2000) and the protein acts in an autocrine and paracrine manner to enhance decidualisation (Matsumoto et al., 2008a). Decidual IGFBP1 may also act to control (inhibit or stimulate) the depth or invasiveness of the trophoblast (Irwin and Giudice, 1998, Gleeson et al., 2001). As such, IGFBP1 may have a pathological role in pre-eclampsia and intrauterine growth restriction (Crossey et al., 2002).

Prolactin expression is regulated by progesterone and cAMP among other factors in decidualised endometrial stromal cells (Zhu et al., 1990, Irwin et al., 1991, Telgmann et al., 1997) and may regulate many functions in implantation (Jabbour and Critchley, 2001). Interestingly, PRL may act in an autocrine manner to decrease expression of proteins associated with decidualisation and thereby negatively regulate the differentiation process (Eyal et al., 2007). Findings from an in vitro study also suggest a role for PRL in trophoblast invasion (Stefanoska et al., 2013). Finally, during pregnancy, decidua-derived PRL regulates the volume of amniotic and fetal extracellular fluid electrolytes (Tseng and Mazella, 1999). Thus a role for PRL in implantation pathologies has been investigated. Animal studies have shown that prolactin deficient mice and prolactin receptor deficient mice lack embryo
implantation (Horseman et al., 1997, Bole-Feysot et al., 1998). In humans, deficient endometrial PRL expression during the window of implantation was associated with unexplained infertility and repeated miscarriages (Garzia et al., 2004).

**Common in vitro hESC decidualisation models**

Progestins (such as progesterone) and cAMP have been used to model *in vitro* the decidualisation of endometrial stromal cells. Other decidualisation protocols utilise oestrogen priming plus progesterone (or another progestin) (Tabanelli et al., 1992). Preference for cAMP-based decidualisation cell models has been based on the ability to recapitulate/mimick the physiological elevation of cAMP observed in the secretory phase endometrium and to allow the endometrial stromal cells to reach the same level of decidualisation after less time of treatment (as determined by decidualisation marker levels).

**1.4.5 Implantation, trophoblast invasion and pregnancy**

The implantation window is the period during the mid-secretory phase characterized by high progesterone and rising levels of oestrogen where the endometrium is functionally most receptive to blastocyst implantation occurring between 5 and 9 days post ovulation (or cycle day 20-24, approximately) (Wilcox et al., 1999). Outside of this window, the endometrium is unreceptive to successful implantation with late implantation being more predisposed to early pregnancy loss (Baird et al., 1991, Wilcox et al., 1999).

In many studies the window of implantation is identified around 7 days post LH serum level surge (which peaks at ovulation) and is characterized by cellular structural and biochemical transformation (Tabibzadeh, 1995, Sarani et al., 1999). For example, microarray analysis of the two major individual cellular compartments (glandular epithelium and stroma isolated through laser capture) showed that the two compartments had distinct transcriptomic signatures at different days of the cycle before the implantation window (LH +2 days) and during the implantation window (LH +7 days) (Evans et al., 2012).
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The coordinated changes in endometrial physiology during the implantation window are essential for normal embryo implantation and trophoblast invasion. The controlled expression of a number of cell adhesion molecules is necessary for successful interactions between the embryo and the endometrium (Donaghay and Lessey, 2007). In addition, adequate trophoblast invasion of the decidua and myometrium with the accompanying spiral artery remodelling are essential for the development of normal pregnancy to establish a low blood pressure, high blood flow supply to the placenta. Factors secreted by the decidua also play a role in regulating placentation (Menkhorst et al., 2012). Finally, it is important to note that further decidualisation of maternal tissues occurs in response to trophoblast invasion and blastocyst-derived factors such as chorionic gonadotrophin (Fazleabas and Strakova, 2002).

**Altered implantation processes and pregnancy disorders**

Reduced or impaired trophoblast invasion and spiral artery transformation have been associated with pre-eclampsia, intrauterine fetal growth restriction, and late sporadic miscarriages (Naicker et al., 2003, Ball et al., 2006, Lyall et al., 2013). In addition, changes in the motile and invasive capacity of endometrial stromal cells are reported to play a role in the impaired reproductive function exhibited in implantation failure, recurrent pregnancy loss, endometriosis and adenomyosis (Weimar et al., 2013).

The decidua at the implantation site becomes the maternal component of the subsequent developing placenta. Impaired development of the placental vasculature has been associated with the obstetric complications of pre-eclampsia and fetal growth restriction (reviewed by Cerdeira and Karumanchi, 2012).

1.5 The applicability of microarrays and to study gene expression in human endometrium

1.5.1 Microarrays and applicability to human endometrium

One approach to identify the genes regulating processes in cells or tissues is to conduct gene expression profiling as developed by Schena and colleagues (Schena et al., 1995). Specifically, gene expression (mRNA) microarray technologies allow us
to measure the expression of thousands of genes simultaneously (Chee et al., 1996, Chen et al., 1998, Duggan et al., 1999).

In humans, genome-wide screening has been successfully employed to look at the developmental processes in normal and dysfunctional endometrial tissues and cells. Indeed, several authors have reviewed the application of the microarray approach to endometrial studies highlighting key issues to be considered for successful applicability and the potential outcomes for the understanding of endometrial biology (White and Salamonsen, 2005, Giudice, 2006a, Sherwin et al., 2006, Horcajadas et al., 2007b, Haouzi et al., 2012, Altmae et al., 2013).

Some examples of the use of microarrays for the characterisation of changes in gene transcription in normal endometrium or endometrial cells include the comparison of whole endometrial biopsies from different menstrual phases (Ponnampalam et al., 2006, Talbi et al., 2006, Tseng et al., 2010) the comparison of individual cell compartments (epithelia *versus* stroma) after laser microdissection separation (Yanaihara et al., 2004, Evans et al., 2012) and comparison of the impact of *in vitro* treatments of cells. Examples of the latter include the comparison of non-decidualised and decidualised hESCs (Aghajanova et al., 2011); comparison of different decidualising signals (Popovici et al., 2000) and comparison of cultured cells after RNA interference knockdown experiments (Cloke et al., 2008).

Several transcriptomic studies have focussed on analysis of tissue or cells recovered during specific menstrual phases. The transcriptomic profile of the implantation window has been especially characterised using microarrays in naturally cycling endometrium (Arnett-Mansfield et al., 2001, Mayr and Montminy, 2001, Hickey et al., 2002, Kao et al., 2002, Altarejos and Montminy, 2011, Evans et al., 2012, Sinreih et al., 2013) and medically treated endometrium (Zoncu et al., 2011, Shimobayashi and Hall, 2014). Notably, these numerous studies have even led to the development of a specific endometrial receptivity array enabling accurate and reliable endometrial dating of receptivity status compared to histologic dating by pathologists (Diaz-Gimeno et al., 2011, Diaz-Gimeno et al., 2013). The endometrial
receptivity array is comprised of 238 genes of which 134 genes form the mRNA signature of the receptive phase.

Examples of studies that employed microarrays to study dysfunctional endometrium using endometrial tissues or cells include the investigation of menstrual complaints (Critchley et al., 2006), investigation of endometriosis (Aghajanova et al., 2010a, Aghajanova et al., 2011), investigation of polycystic ovary syndrome (Kim et al., 2009, Piltonen et al., 2013) and investigation of endometrial cancer (Wong et al., 2007). In a recent study, transcriptomic data was combined with other genomic and proteomic data in order to better categorize endometrial cancer subtypes (Kandoth et al., 2013).

1.5.2 Bioinformatics for the analysis and interpretation of microarray data

Bioinformatic tools are extensively employed in biological and medical research to facilitate the analysis and interpretation of the large amount of biological data such as that generated from microarray transcriptome-wide studies. Firstly, statistical methods are used to pre-process gene expression data (e.g. background correction, normalisation) prior to the identification of significantly differentially expressed genes (Selvaraj and Natarajan, 2011, Altmae et al., 2013). Secondly, the differentially expressed gene list is subjected to functional analyses (reviewed by Werner, 2008, Khatri et al., 2012) such as gene ontology and pathway analyses to identify the biological processes, pathways and networks most associated with a microarray data set. While gene ontology analyses reveal the assigned functional annotations of genes (from a curated database), pathway analyses reveal the relationships between the genes (from a curated database and using specific computational algorithms). Various functional analysis software tools have been used to identify cell processes from data derived from human endometrial tissues or cells, for example, the Kyoto Encyclopaedia of Genes and Genomes, KEGG, (Borghese et al., 2008, Liu et al., 2015) and Metacore (Marshall et al., 2011, Kusunoki et al., 2013).
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The confirmatory and discovery bioinformatic analyses of microarray-derived data can be informative on many levels because: (i) they integrate into their analyses the fact that genes do not work alone but in intricate networks of varied interactions; (ii) they facilitate the interpretation of the data in context of biological processes, pathways or networks; and (iii) they allow us to gain a global perspective on the data (Bessarabova et al., 2012). Finally, the microarray/bioinformatics approach can help to identify novel genes involved in the processes of interest.

The limitations of pathway analysis have been recently reviewed (Khatri et al., 2012, Chowdhury and Sarkar, 2015) and include: the differences between the different databases (pathway nomenclature and boundary), gene annotation inaccuracies, reporting biases in the source literature, differences in statistical analysis methods and missing cell-specific information. Other concerns include the fact that not all known genes have assigned functions (not every gene belongs to a pathway in the database), the information about the interactome is incomplete (and could be biased) and the bioinformatics tools do not model dynamic processes or responses. Finally, the pathways identified are a statistical probability rather than a biological certainty that require experimental proof.

Difficulties associated with the differentially expressed sets of genes identified by microarray analysis include the lack of information about which of the identified genes represent primary and secondary targets. It is thus necessary to identify and confirm primary targets using different methods to validate the microarray results but also to distinguish primary from secondary effects. Finding the primary target that specifically leads to the change in gene expression of a specific secondary target requires knowledge about secondary target known regulation and confirmatory assays. Many types of primary targets can lead to changes in secondary target transcription including transcription factors and microRNAs (miRNAs). In particular, miRNAs can affect gene expression by degrading or repressing mRNA expression and it is estimated that between 30 to 60% of human protein-coding genes with diverse cellular functions may be regulated by miRNAs (Lewis et al., 2005, Friedman et al., 2009).
1.6 Androgens and androgen metabolism in the uterus

The androgens produced in women, as in men, can act systemically as endocrine hormones or when produced locally can act in an intracrine or paracrine manner.

**Serum androgens reach the uterus**

Early studies in rodents established that the endometrium is a target of systemic testosterone. The *in vivo* administration of 3H-labelled testosterone to immature female rats resulted in detection of radiolabelled ligand in the uteri (Giannopoulos, 1971, Giannopoulos, 1973). In an *in vitro* study of whole rat uteri the nuclear uptake of labelled testosterone was also described (Schmidt and Katzenellenbogen, 1979).

1.6.1 Androgen metabolism in the uterus

Local androgen metabolism including *de novo* androgen biosynthesis can play a crucial role in regulating the bioavailability of different ligands (section 1.2.1, Figure 1.2). Cultured uterine tissue slices and endometrial stromal and epithelial cells isolated from non-pregnant and pregnant pigs secreted androstenedione (A4) and testosterone (Franczak, 2008, Franczak and Kotwica, 2010). In humans, testosterone and also precursor androgens that could be directly or indirectly converted to testosterone have been measured in endometrial tissue including androstenediol (adiol), A4 and DHEA (Bonney *et al.*, 1984a, Bonney *et al.*, 1984b, Bonney *et al.*, 1985, Suri, 1997).

Active gene expression, protein expression and the activity of various converting enzymes of the androgen biosynthesis pathway (regulating the production of testosterone, DHT and other steroids, Figure 1.2 and Table 1.2) have been reported in the endometrium. The findings are summarized below and support endometrial capacity for active androgen biosynthesis.
Table 1.2 Summary of key reactions and converting enzymes in androgen synthesis and metabolism.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Converting enzyme/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>DHEA</td>
<td>→ Adiol (T precursor)</td>
<td>• HSD17B1 or AKR1C3</td>
</tr>
<tr>
<td></td>
<td>→ A4 (T precursor)</td>
<td>• HSD3B1</td>
</tr>
<tr>
<td>Adiol (T precursor)</td>
<td>→ T</td>
<td>• HSD3B1</td>
</tr>
<tr>
<td>A4 (T precursor)</td>
<td>→ T</td>
<td>• AKR1C3</td>
</tr>
<tr>
<td></td>
<td>→ Androstanedione (DHT precursor)</td>
<td>• SRD5A1</td>
</tr>
<tr>
<td></td>
<td>→ Oestrone</td>
<td>• CYP19A1</td>
</tr>
<tr>
<td>T</td>
<td>→ DHT</td>
<td>• SRD5A1</td>
</tr>
<tr>
<td></td>
<td>→ Oestradiol</td>
<td>• CYP19A1</td>
</tr>
<tr>
<td>Androstanedione</td>
<td>→ DHT</td>
<td>• AKR1C3</td>
</tr>
<tr>
<td>DHT</td>
<td>→ 3α-Adiol (oestrogenic metabolite)</td>
<td>• HSD3B1</td>
</tr>
<tr>
<td></td>
<td>→ 3β-Adiol (oestrogenic metabolite)</td>
<td>• HSD3B1, HSD17B1, AKR1C4</td>
</tr>
</tbody>
</table>

Substrates/products:
3α-Adiol = 3α-androstane-3α,17β-diol; 3β-Adiol = 5α-androstane-3β,17β-diol; A4 = androstenedione; Adiol = 5-Androstenediol; DHEA = dehydroepiandrosterone; DHT = dihydrotestosterone; T = testosterone.

Enzymes:
AKR1C3 (17βHSD5) = aldo-keto reductase family 1, member C3; AKR1C4 (3αHSD) = aldo-keto reductase family 1, member C4; HSD3B1 (3βHSD) = hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase; HSD17B1 (17βHSD 1) = hydroxysteroid (17-beta) dehydrogenase 1; CYP19A1 (aromatase) = cytochrome P450 family 19 subfamily A member 1.

SRD5A1 (5α reductase) - Conversion of testosterone to DHT; Conversion of androstenedione (A4) to androstanedione (a DHT precursor):
Conversion of testosterone by SRD5A1 to the more potent androgen DHT has been demonstrated using human and rat endometrial tissue homogenates (Rose et al., 1978, Suri, 1997). The conversion of A4 to androstanedione by endometrial tissue explants has also been shown (Jasonni et al., 1982). The expression of SRD5A1 mRNA and protein has been detected in human endometrial epithelial and stromal
cells although less consistently in stromal cells (Ito et al., 2002, Carneiro et al., 2008).

**AKR1C3 (17βHSD5)** - Conversion of A4 to testosterone; Conversion of androstenedione to DHT; Conversion of DHEA to adrostenediol (adiol) (a testosterone precursor):
A4 is converted to testosterone by AKR1C3 in peripheral tissues in women (Luu-The et al., 2001). In the human endometrium, AKR1C3 mRNA is detectable during the menstrual cycle with highest concentrations in the early secretory phase (Catalano et al., 2011). A few studies reported protein expression in the glandular epithelium and sometimes in the luminal epithelium (Pelletier et al., 1999, Ito et al., 2006, Vani et al., 2007, Catalano et al., 2011). In contrast, another study reported positive immunohistochemical staining for AKR1C3 in both the epithelial and stromal cells (Hevir et al., 2011).

**HSD17B1 (17βHSD1)** - Conversion of DHEA to Adiol (a testosterone precursor):
HSD17B1 mRNA expression is low but detectable in human endometrium (Dassen et al., 2007). In the same study, intense protein expression throughout the menstrual cycle was reported in both the epithelial and stromal cells.

**HSD3B1 (3βHSD)** - Conversion of adiol to testosterone; Conversion of DHEA to A4 (a testosterone precursor):
HSD3B1 enzyme activity has been reported in the human endometrium (Collins et al., 1969, Seki et al., 1987, Tang et al., 1993). HSD3B1 mRNA and protein (by Western analysis) was detected in secretory phase endometrium (Rhee et al., 2003). Immunopositive staining for HSD3B1 protein was reported in epithelial cells, with staining being moderate in the proliferative phase and most intense in the secretory phase (Rhee et al., 2003). More recently, cultured primary hESCs have been shown to contain HSD3B1 protein before and after in vitro decidualisation (Gibson et al., 2013).
Conversion of androgens to oestrogens or oestrogenic metabolites (Figure 1.2 and section 1.3.1) effectively deactivates the androgens and may contribute to an enhanced intra- and inter-cellular local oestrogenic environment. There is evidence that supports this activity in the human endometrium.

**HSD3B1 or HSD3B1, HSD17B1, AKR1C4 (3αHSD)** – Conversion of DHT to oestrogenic metabolites 3α-Adiol or 3β-Adiol, respectively:
As described above, HSD3B1 and HSD17B1 activity and/or protein expression has been reported in the human endometrium. AKR1C4 activity and expression have not been reported in endometrial tissues.

**CYP19A1 (aromatase)** - Conversion of androgens to oestrogens:
CYP19A1 is responsible for the conversion of androgens into oestrogens (Simpson et al., 1994), specifically androstenedione to oestrone (E₁) and testosterone to oestradiol. CYP19A1 activity has been shown in endometrial tissue (Tseng et al., 1984). Early studies did not show CYP19A1 activity in hESCs (Bulun et al., 1993) and endometrial mRNA expression in endometrial tissues was reported very low or not detected (Bulun et al., 2003).
A recent study has challenged these findings by demonstrating an increase in CYP19A1 mRNA, protein expression (only detected in the decidualised hESC) and activity in decidualised primary stromal cells compared to non-decidualised fibroblasts (Gibson et al., 2013).
Interestingly, an increase in CYP19A1 protein expression has been reported in the eutopic endometrium (epithelium) of patients with endometriosis compared to women without endometriosis (Hudelist et al., 2007) suggesting altered steroidogenesis. In addition, in women with endometriosis, an increase in CYP19A1 mRNA expression (Dassen et al., 2007) and an increase in CYP19A1 protein expression in either the epithelia or stroma (Velasco et al., 2006, Hudelist et al., 2007) was reported in endometriotic lesions compared to corresponding eutopic endometrium.
1.7 The Androgen Receptor in the uterus

1.7.1 Overview

Early findings in the 1970s suggested the existence of specific receptors that bound androgens in several rodent tissues including the prostate and the uterus (Blondeau et al., 1975, Liao and Liang, 1975, Verhoeven et al., 1975, Schmidt and Katzenellenbogen, 1979). In another study using cultured whole rat uteri, the nuclear uptake of labelled testosterone was inhibited by anti-androgens such as flutamide (Schmidt and Katzenellenbogen, 1979) consistent with a receptor-mediated effect. Finally, in the early 1990s, after the cloning of the AR gene, prediction of its protein sequence and preparation of specific antibodies (section 1.3.1), immunohistochemical localisation studies confirmed that AR was present in a variety of human tissues or major physiological systems including in the central nervous system and in the male and female reproductive tracts (Sar et al., 1990, Takeda et al., 1990, Ruizeveld De Winter et al., 1991). Although such studies did not agree entirely, possibly due to the quality of the anti-AR antibodies available and different methods employed, the wide distribution of AR expression in known and unknown androgen-responsive human tissues (such as the endometrium, at the time) was established. Subsequently, the presence of AR has also been reported in many human fetal and adult non-reproductive tissues including brain, thyroid, thymus, skin, intestine, kidney, fat, bone and the vasculature (Wilson and Mcphaul, 1996, Abu et al., 1997, Joyner et al., 2002, Getov et al., 2008, Sarkey et al., 2008, Wiren et al., 2008, Guerriero, 2009, Kaarbo et al., 2010). The expression of AR has also been detected in many organs in animal species including primates and rodents (Pelletier, 2000, Fix et al., 2004, Unni et al., 2004, Wullschleger et al., 2006, Nguyen et al., 2009, Laplante and Sabatini, 2012, Basualto-Alarcon et al., 2013, Ma et al., 2014).

1.7.2 AR expression in the human endometrium

The androgen receptor has been reported in the human uterus during embryonic development (Wilson and Mcphaul, 1996). In this study, AR protein was detected by Western blot in the uteri of mid-trimester human fetuses.
The Androgen receptor in the endometrium during the menstrual cycle
In normal endometrium from women of reproductive age, the expression of both AR mRNA and protein has been detected most consistently in the stromal compartment although increased expression is variously reported to occur in the proliferative, early secretory or menstrual phases (depending on different authors) (Mertens et al., 2001, Apparao et al., 2002, Milne et al., 2005, Taylor et al., 2005, Critchley and Saunders, 2009, Marshall et al., 2011). The most intense immunoexpression of AR has been reported in the stromal cells of the functional layer in the proliferative phase with maintenance of expression in stromal cells in the basal compartment regardless of cycle stage (Marshall et al., 2011). Reports of AR protein expression in the glandular epithelium have been contradictory. While some authors reported AR expression in glandular epithelial cells throughout the menstrual cycle (Horie et al., 1992), other studies have found AR immunoexpression to be very low or non-detectable with a peak of expression occurring during the late secretory phase coinciding with the time of progesterone withdrawal (Taylor et al., 2005, Marshall et al., 2011). Differences in the staining patterns especially of the endometrial epithelial cells may be attributed to the quality and or specificity of the different anti-AR antibodies used and/or variations in the methods employed.

Regulation of AR expression – a role for oestrogen and progesterone signalling
The regulation of AR expression in the endometrium (mostly in the stroma as detailed above) appears to be somewhat regulated across the menstrual cycle thus implicating regulation by cycling ovarian steroids oestrogen and progesterone. Although this is supported by findings in other cell types, direct experimental evidence in human endometrial cells is limited and should be further investigated.

During the proliferative phase, oestrogen governs endometrial development and in humans, in vitro studies have showed that oestrogens upregulate AR protein expression in human endometrial stromal cells (Apparao et al., 2002). Whilst the effects of oestrogens upon AR expression have not been reported in human endometrial epithelial cells, oestrogens were found to upregulate AR mRNA and protein expression in Ishikawa epithelial adenocarcinoma cells (Lovely et al., 2000,
Apparao et al., 2002). During the secretory phase, progesterone dominates in the endometrium. Progesterone and other synthetic progestins have been reported to reduce AR protein expression in hESCs (Apparao et al., 2002, Cloke et al., 2008). Throughout the menstrual cycle androgens may also regulate the expression of AR as indicated by in vitro observations of human endometrial stromal cells (Cloke et al., 2010). This is further supported by the continued immunodetection of AR in the stromal cells of endometria from post-menopausal women (Zang et al., 2008) although intensity appears reduced compared to that in pre-menopausal women (Horie et al., 1992). In endometria from women with PCOS, AR immunoreexpression is sometimes also elevated in the stromal cells (Apparao et al., 2002) possibly due to many factors including excessive androgens or oestrogens.

Progesterone antagonists including anti-progestins such as mifepristone (RU486) compete with endogenous ligands for the binding to the PR and in some cases they cause a change in receptor conformation resulting in altered DNA binding characteristics (Baulieu and Ulmann, 1986, Catalano et al., 2003). In women (and macaques), administration of progesterone antagonists such as RU486 results in increased AR protein expression in endometrial stromal cells and induced expression in glandular epithelial cells (Slayden et al., 2001, Narvekar et al., 2004, Slayden and Brenner, 2004). Similarly, it is worth noting that in another tissue model where PR plays an important physiological role, the brain of the male rat, neonatal exposure to RU486 can also lead to an increased AR protein level (Forbes-Lorman et al., 2014).

In women and macaques, RU486 suppressed oestrogen-dependent endometrial proliferation and in macaques, the anti-androgen flutamide (in the presence of oestrogen) blocked the anti-proliferative effects of anti-progestins (Slayden and Brenner, 2003). This suggests that the inhibitory effects of anti-progestins on endometrial cell replication may in part be mediated by the AR resulting in endometrial compaction, reduced endometrial weight, mitotic activity and spiral artery degeneration (Slayden and Brenner, 2003).
Studies in other cell types have shown that regulation of AR expression to be complex via several pathways and in a specific manner dependent on cell type, tissue type and developmental stage (Burnstein, 2005). Further complex factors governing the regulation of AR expression and AR activity in human endometrial stromal cells (hESCs) has only recently begun to be unravelled. One study reported that the reduced detection of AR protein in decidualised hESCs is mainly due to regulation at the translational level via, for example, the activity of poly(C)-binding protein-1 (Cloke et al., 2010). On the other hand, it is worth noting, that post-translational modifications such as decreased sumoylation during decidualisation can also contribute to an increase in overall AR activity (Cloke et al., 2008) and thus the responsiveness of endometrial stromal cells to androgen is complex.

**Regulation of AR expression – a role for androgen signalling**

The levels of testosterone remain similar during the proliferative and secretory phases of the menstrual cycle, except for a rise (of ovarian origin) observed mid-cycle at the time of ovulation (Abraham, 1974, Massafr et al., 2000, Salonia et al., 2008) (section 1.2.2). As androgens can also upregulate AR mRNA expression in hESCs (Dai and Burnstein, 1996, Apparao et al., 2002, Cloke et al., 2010) (section 1.3.3) the exposure to moderately stable blood androgen concentrations may contribute to maintain AR expression in hESCs (and consequently androgen signalling) throughout the changing oestrogen and progesterone concentrations during the endometrial menstrual cycle (this remains speculative). Importantly, changes in ovarian steroid and/or in local endometrial steroidogenesis (Gibson et al., 2013, Gibson et al., 2016) may therefore affect the expression of endometrial AR and thereby contribute to endometrial reproductive dysregulation in, for example, women with PCOS (Apparao et al., 2002). Androgens upregulate AR mRNA and protein expression in Ishikawa epithelial adenocarcinoma cells (Lovely et al., 2000, Apparao et al., 2002) but this has not been reported in human endometrial epithelial cells.

After the menopause, AR protein is still detected in endometrial stromal cells (Maia et al., 2001). As the reduction in systemic androgens is comparatively less than the reduction in oestrogens after the menopause (Terada et al., 1990, Jo et al., 1993,
Davison et al., 2005), it could be speculated that androgens may be majorly responsible for maintaining stromal AR expression during this time.

**The androgen receptor in the endometrium during pregnancy**

During pregnancy, decidual AR expression may be reduced (compared to that observed in the proliferative phase) but is still present in stromal cells and some epithelial cells (Tamaya et al., 1985, Milne et al., 2005, Critchley and Saunders, 2009). This may suggest endometrial AR is regulated during pregnancy in a similar manner as during the mid-secretory phase but this has not been reported on to date. It is also interesting to note that AR has been detected in other reproductive tissues during pregnancy. For example, AR is expressed in human uterine myometrial cells and has been shown to be highly expressed in the first half of pregnancy (Liu et al., 2013). AR expression has also been shown in normal placenta (Hsu et al., 2009).

**Speculation on a role for the androgen receptor in the endometrium**

From an evolutionary point of view, the more recent emergence of PR (and AR) in relation to ER (section 1.3.1) (Thornton, 2001) correlates with the appearance of the uterus and especially with the advanced human endometrium and decidual cell where PR plays a critical role during decidualisation and pregnancy (Wagner et al., 2014). Knowing the key role that PR plays in the endometrium, the close homology between AR and PR (Robinson-Rechavi et al., 2003) (section 1.3.1) poses the intriguing question as to the role of AR in the endometrium. The confirmation of the presence and regulation of AR (by PR and ER) in the human endometrium during both the proliferative and secretory phases permits speculation for a significant role for AR and androgen signalling in endometrial physiology.

**1.7.3 AR expression in the uteri of animal models**

The expression of AR has been reported in the uteri of a number of mammalian species including non-human primates such as the rhesus monkey as well as in cows, camels, pigs and rodents (described below).

In ovariectomized rhesus macaques treated with oestradiol and progesterone to produce artificial menstrual cycles the expression of AR in the uterus was detected in
the stromal cells and was maximal during the proliferative phase mirroring human biology (Slayden and Brenner, 2004). In calf uterine tissues a DNA-binding form of AR was also identified (De Boer et al., 1986). Recently, AR protein has also been localized in the uteri of dromedary camels where the greatest AR immunoreactivity was reported in the nuclei of basal stromal cells with weak AR immunostaining also present in epithelial cells (Laplante and Sabatini, 2009).

In pigs, AR mRNA and protein was detected in endometrium from animals with oestradiol/progesterone induced oestrous cycles (Cardenas and Pope, 2003) using quantitative reverse transcriptase PCR (qRTPCR) and immunohistochemistry. After oestrogen alone or oestrogen/progesterone treatment, endometrial epithelial cells in these sows were consistently immunopositive and AR immunostaining of stromal cells varied in intensity. In pregnant endometrium and in the immature uteri of pig fetuses AR mRNA and protein were detected by Northern and Western analysis (Kowalski et al., 2004, reviewed by Pope and Cardenas, 2006, Slomczynska et al., 2006). Northern analysis also revealed AR mRNA expression in the receptive peri-implantation pig uterus (Kowalski et al., 2004). Immunostaining of porcine uterine tissues showed positive staining of stromal and epithelial cells during early pregnancy (Kowalski et al., 2004).

In rat uteri both stromal and epithelial cells were immunopositive for AR (reviewed by Pelletier, 2000). In mouse uteri AR is mainly expressed in the stromal cells (Takeda et al., 1990). The expression of the AR in mice has been exploited to produce AR knockout models (reviewed by Chang et al., 2013) in which the reproductive phenotype has begun to be investigated (section 1.7.5.2). More recently Dart and colleagues (2013) developed a different murine AR transgenic model which allows the visualisation of endogenous AR activity by AR activation of a luciferase reporter construct. Detection of luciferase activity in the uteri of these mice suggests that androgens can act in these tissues directly via AR.
1.8 The *in vivo* study of androgen action and AR signalling in the endometrium

A role for androgens and AR signalling in the endometrium is supported by multiple *in vivo* (and *ex vivo*) findings in both women and animals that include the effects of exogenous androgens, the effects of elevated endogenous androgens associated with pathologies such as polycystic ovary syndrome, altered androgen signalling in endometrial cancer and disruption of AR signalling in AR knockout animal models.

1.8.1 Androgen excess - Exogenous androgens

Long term administration of androgens (testosterone or testosterone-based formulations) to female-to-male transsexuals leads to atrophy of the endometrium (histological appearance/structure and thickness) without stimulation of proliferation according to the reduced expression of one proliferation marker, Ki-67, in glandular and stromal cells (Miller *et al.*, 1986, Mueller *et al.*, 2007, Perrone *et al.*, 2009). The atrophy of the endometrium observed is similar to atrophic menopausal endometrium characterised by a loss of the functional layer with small tubular epithelial glands and fibrous stroma (Perrone *et al.*, 2009). It is unclear if this is due only to the lack of ovarian function and hormones (oestrogen and progesterone) or if the administered androgen also contributes to the absence of growth.

The upregulation of AR nuclear immunostaining of the endometrial stroma has also been reported in transsexual women (Chadha *et al.*, 1994). Notably, in the endometria and other reproductive tissues of androgen-treated female-to-male transsexuals, there is an increase in the expression of kallikreins such as prostate serum antigen (PSA), the expression of which is known to be stimulated by androgens (Gooren and Giltay, 2008).

To increase the understanding of the role of androgens in the endometrium it is clinically relevant to investigate endometrial disorders where androgen levels and/or AR expression or activity is reported to be altered. Examples include polycystic ovary syndrome (Apparao *et al.*, 2002), endometrial cancer (Ito *et al.*, 2002), endometriosis (Carneiro *et al.*, 2008) and fibroids (Leitao *et al.*, 2004). The
association of altered androgen signalling in the endometrium of women with PCOS or with endometrial cancer are specifically discussed below.

### 1.8.2 Excess endogenous androgens – polycystic ovary syndrome

Polycystic ovary syndrome (PCOS) is a complex and common reproductive disorder affecting between 4 to 8% of women in their reproductive years and may be even more prevalent depending on the diagnosis criteria used (Knochenhauer et al., 1998, Lauritsen et al., 2014). According to the criteria established by the Rotterdam consensus (The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004) PCOS is defined by the presence of at least two out of the three following criteria: clinical and/or biochemical signs of hyperandrogenism, oligo- and/or anovulation, and ultrasound confirmation of polycystic ovaries. Signs of elevated androgen levels include hirsutism and acne (PCOS Consensus Workshop 2004). Androgen levels (free testosterone and androstenedione) are elevated between two and four fold in women with PCOS when compared to control populations of normal women such as that included in Table 1.1 (Massafra et al., 2000, Hassa et al., 2006, Huang et al., 2010, O’reilly et al., 2014). Some studies have found that the hyperandrogenism in PCOS women persists after the menopause (Markopoulos et al., 2011, Shah and Bansal, 2014).

Another common feature associated with about 75% of PCOS patients (with and without obesity) is insulin resistance (Dunaif et al., 1989, Kidson, 1998). The interplay between the altered insulin and androgens levels in PCOS involves the pathways of the hypothalamic-pituitary-ovarian axis (section 1.1) resulting in an altered circular metabolic and reproductive physiology (reviews by Duncan, 2014, Dumesic et al., 2015). Briefly, peripheral insulin resistance increases insulin levels which leads to increased ovarian androgen production and increased oestrogen levels (upon conversion by aromatase). Increased bioavailable oestrogen levels (possibly due to reduced SHBG levels) increase LH secretion that subsequently further impacts ovarian androgen secretion which is increased (in parallel with insulin action). The altered physiology negatively affects ovarian function being expressed as altered ovulation and luteal phase (oligo- and/or anovulation) with the consequent
progesterone deficiency potentially impacting fertility at the endometrial level. Whilst this is a simplified overview, it offers an idea of the complexity of the interacting factors involved/observed in PCOS and clearly defines the difficulty faced when dissecting the role of any one factor in isolation, especially in vivo.

For over 50 years, Metformin, an insulin sensitising biguanide, has been commonly used in the medical management of non-insulin dependent type II diabetes mellitus characterised by increased insulin resistance (Lee, 1996). Metformin reverses the insulin resistance developed by many patients with type II diabetes including those with associated PCOS, obesity or hyperlipidemia (reviewed by Melchior and Jaber, 1996). As a result, metformin use has been considered in the treatment of these different disorders where insulin resistance is an important factor and increasingly metformin is being widely used in the treatment of PCOS, although it is not yet licenced for this specific use in the UK (reviewed by Kidson, 1998, Katsiki and Hatzitolios, 2010, Nathan and Sullivan, 2014).

Although metformin action involves diverse effects upon multiple tissues (Lee, 1996), metformin primarily reduces insulin resistance through decreasing hepatic glucose production and increasing the uptake of glucose by other tissues such as skeletal muscle (reviewed by Musi and Goodyear, 2006, Andujar-Plata et al., 2012). In women with PCOS and diabetes or PCOS alone, the outcomes of treatment crucially include a lowering of serum insulin levels that leads to normalisation of androgen levels with resumption of ovulation that may improve infertility (Sung et al., 2014). Reduction of insulin resistance by metformin also helps to prevent the development of diabetes and other associated risks in women with PCOS such as endometrial cancer and cardiovascular diseases (reviewed by Sharma and Nestler, 2006, Carmina, 2012).

At the cellular level, the main mechanism of action of metformin is to decrease the ratio of cellular energy transfer molecules adenosine triphosphate/adenosine monophosphate (ATP/AMP) which triggers phosphorylation and thus activation of one of its cellular targets, adenosine monophosphate-activated protein kinase (AMPK) (reviewed by Andujar-Plata et al., 2012). Active AMPK acts upon targets
belonging to multiple pathways through which it regulates metabolic and cell cycle pathways (reviewed by Hardie et al., 2012).

**PCOS and changes in the endometrial physiology**

Hyperandrogenism in women with PCOS has been associated with altered physiology and function of the endometrium. However, the complex altered physiology of PCOS makes it difficult to dissect the effects of each altered factor present in PCOS that may directly impact the endometrium, namely excessive bioavailable androgens, oestrogens, insulin or progesterone deficiency (deficient progesterone effect).

Similar to the uterine effects of exogenous androgen administered to female-to-male transsexuals, a reduced uterine volume and endometrial thickness has been correlated with a more severe hyperandrogenism in PCOS patients (Panidis et al., 2014). Altered androgen signalling in the endometrium of PCOS women is also supported by the findings that endometrial AR protein expression (detected by Western analysis) is increased (Villavicencio et al., 2006). Other authors reported that in the endometria of women with PCOS, AR protein immunostaining was increased in the glandular and surface epithelium (Apparao et al., 2002, Maliqueo et al., 2003) and sometimes also elevated in the stromal cells of endometria from women with PCOS compared to normal (Apparao et al., 2002). It is important to consider that the altered endometrial physiology in women with PCOS may not be the result of the action of excessive androgens alone but may be due the lack of or altered ovarian hormones and function (eg. progesterone). Thus, as it is difficult to separate the two effects *in vivo*, it remains unclear if the upregulation of endometrial AR associated with PCOS is due to excessive androgens or to a lack of progesterone.

Thus, common confounding effects (insulin or luteal phase progesterone deficiency) cannot be discounted entirely when studying the effects of hyperandrogenism upon the endometrium from women with PCOS (and subsequently any association with miscarriage or cancer). The authors of the endometrial studies from women with PCOS presented below have attempted to circumvent the confounding effects of the luteal phase deficiency by either timing the recovery of endometrial tissue to the
follicular phase alone or to the luteal phase with added progesterone treatment. Meticulous morphological (ultrasonography and/or histology) characterisation of the endometrial tissues and/or measurement of hormone serum concentrations were used to date the PCOS-derived endometrial samples (Maliqueo et al., 2003, Kim et al., 2009, Savaris et al., 2011). However, findings by Usadi and colleagues (2008) suggest the existence of unknown endometrial functional differences that are undetected by these currently available methods (among others).

Transcriptomic analysis of proliferative endometrium from PCOS patients revealed a down-regulation of cell cycle, apoptosis, glycolysis and integrin-mediated cell adhesion pathways (Kim et al., 2009). Changes in endometrial cell survival were demonstrated by altered immunostaining of apoptosis-related proteins in stromal cells and reduced expression of proliferation markers in epithelial cells in endometrial tissue from women with PCOS compared to women without the disorder that were recovered during their follicular phase (Maliqueo et al., 2003, Kim et al., 2009). However, gene profiling studies of the endometria of PCOS patients also support the prevailing hypothesis of the existence of an abnormal progesterone-resistant endometrium (reviewed by Young and Lessey, 2010) characterised by a prolonged proliferative phenotype with changes of several cell pathways including cell cycle and cell survival (reviewed by Aghajanova et al., 2010b, Savaris et al., 2011, reviewed by Li et al., 2014). In one particular study, the endometria of PCOS patients treated with progesterone for 10 days (to surmount any endogenous progesterone deficit and model/mimick mid-secretory endometrium) showed an altered response to progesterone compared to fertile women without PCOS (Savaris et al., 2011). In this study, endometrial expression of progesterone-regulated genes was reduced while expression of proliferation-related genes was elevated in progesterone-treated PCOS women compared to controls demonstrating the progesterone-resistant endometrial response in PCOS.

In hyperandrogenism-associated PCOS, progesterone resistance has been associated with aberrant progesterone receptor isoform expression in the endometrial epithelial and/or stromal cells (reviewed by Aghajanova et al., 2010b, Li et al., 2014). The resultant altered signalling cross-talk between PR and AR or between PR and ER
(leading to modified cell-cell interactions and tissue function) is believed to contribute to the endometrial phenotype exhibited by women with PCOS.

Thus, studies of the endometria from women with PCOS have revealed abnormal changes in endometrial cell proliferation, survival, differentiation and response to progesterone that are implicated in endometrial pathologies such as altered receptivity and cancer (section below). Notably, there is an increased prevalence in infertility and endometrial cancer in women with PCOS and evidence suggests that hyperandrogenism is a contributing factor shared in common (reviewed by Giudice, 2006b).

Infertility in PCOS - androgens and AR

In women, besides anovulation and poor oocyte quality, higher rates of implantation failure and miscarriage also contribute to the infertility associated with PCOS (Tulppala et al., 1993, Clifford et al., 1994, Kodama et al., 1995, Okon et al., 1998, reviewed by Lopes et al., 2011). Underlying these findings may be the impaired decidual trophoblast invasion that has been described in PCOS patients (Palomba et al., 2012). However, the mechanisms involved have not yet been elucidated.

Women with PCOS who are anovulatory exhibit a persistent proliferative endometrial phenotype due to a lack of exposure to “ovulatory” progesterone. Thus, initially, the increased risk of miscarriage and also of late obstetric complications were postulated to be due to altered endometrial responses and impaired placental function (Okon et al., 1998). More recently, gene profiling studies using endometria from women with PCOS have supported the hypothesis of reduced endometrial receptivity associated this disorder. For example, the above mentioned suppression of progesterone-regulated genes during the secretory phase (Savaris et al., 2011) and the abnormal expression of genes associated with receptivity specifically during window of implantation (Yan et al., 2012) have been reported. Pertinently, hyperandrogenism may also be a factor contributing to miscarriage. A correlation between elevated androgen levels and miscarriage has been reported (Tulppala et al., 1993) and interestingly, an elevated testosterone level was identified as a prognostic factor for subsequent miscarriage in women with recurrent miscarriage (Cocksedge
et al., 2008). Although a subsequent study (Cocksedge et al., 2009) suggested the prevalence of PCOS in recurrent miscarriage is lower than previously predicted.

Pregnancy complications some of which may be linked with dysregulation of decidualisation and implantation abnormalities (section 1.4.5) (reviewed by Cha et al., 2012) are also associated with PCOS. Meta-analyses of pregnancy outcomes have also shown a significantly higher association of pregnancy complications in women with PCOS including a higher risk of gestational diabetes, pregnancy-induced hypertension, preeclampsia and preterm birth (Boomsma et al., 2006, Kjerulff et al., 2011). A higher risk of neonatal complications has also been found in women with PCOS such as perinatal mortality (Boomsma et al., 2006).

Finally, with regard to the prevalence of alterations in the AR sequence (section 1.3.5) in infertile women with PCOS, little research has been carried out, findings are contradictory and the functional implications of the AR polymorphisms in the endometrium are unknown. Studies have reported an increased association of infertile women with PCOS with a longer CAG trinucleotide repeat polymorphism when compared to fertile women (Hickey et al., 2002) but also with a shorter CAG repeat polymorphism when compared to infertile ovulatory women (Schuring et al., 2012). The interpretation and relevance of these findings are complicated by the different control populations used in the comparisons, the lack of agreement that a shorter CAG repeat sequence leads to increased AR activity and also by the differential epigenetic effects of X-chromosome inactivation exerted upon AR (reviewed by Baculescu, 2013).

**PCOS and association with endometrial cancer**

PCOS is associated with a three-fold increased risk of developing endometrial cancer (Pillay et al., 2006, systematic review by Chittenden et al., 2009, Fearnley et al., 2010). A systematic review and meta-analysis of current data up to October 2013 confirmed that, when other associated risk factors such as obesity were adjusted for, women of all ages with PCOS had an increased risk of endometrial cancer (odds ratio 2.79) (Barry et al., 2014). When only younger women were considered (less than 54 years old) the risk increased further (odds ratio 4.05) (Barry et al., 2014). As
a result, in women with PCOS endometrial surveillance is considered important in the prevention of endometrial hyperplasia and endometrial cancer (Park et al., 2011, reviewed by Dumesic and Lobo, 2013).

As already mentioned, the study of endometrial tissue from women with PCOS showed altered expression of genes/proteins associated with apoptosis, proliferation and differentiation. Early immunohistochemical evaluation of endometrial tissue from PCOS women also showed altered expression of carcinogenesis-related genes (Pillay et al., 2006). In a recent gene profiling and immunohistologic study in obese PCOS patients, fluorescence-activated cell sorting-isolated proliferative endometrial cell populations showed inflammatory and pro-oncogenic gene and protein expression changes in the stromal and epithelial cells, respectively (Piltonen et al., 2013). This study suggests how distinct changes in each endometrial cell type (epithelial or stromal cells) may affect cell-to-cell interactions and contribute to an environment where hyperplasia may be favoured.

Several factors associated with PCOS have been proposed as contributing to the risk (either alone or in combination) of endometrial cancer in women with this disorder that may underlie the changes reported in the tissues. Some of these factors are common risk factors for other cancers and include hyperandrogenism, the unopposed oestrogen hypothesis (absence of enough progesterone or excess oestrogens originating from for example, conversion from androgens) and insulin resistance (reviewed by Navaratnarajah et al., 2008, Shafiee et al., 2013). However, the authors of a recent review argue for further systems biology-based research to improve our poor understanding of the mechanisms involved in PCOS-associated endometrial cancer (Shafiee et al., 2013).

Hyperandrogenism associated with PCOS was identified as one of the possible contributing factors to the increased risk of endometrial cancer in PCOS patients (reviewed by Shafiee et al., 2013). Management of PCOS with metformin has been found to reverse endometria hyperplasia and thus prevent endometrial cancer (Shen et al., 2008, reviewed by Shafiee et al., 2014). The exact role of metformin in the prevention of endometrial cancer remains unknown but is likely to include multiple
mechanisms including reduction of insulin resistance and normalisation of androgen levels. The contribution of hyperandrogenism and AR signalling to the development of endometrial cancer is discussed below.

1.8.3 Androgen/AR signalling and endometrial cancers

In the UK, endometrial cancer is the fourth most common cancer in women following breast, lung and bowel cancer (Cancer Research UK, UK Cancer Incidence (2011) by Country Summary, January 2014, www.cancerresearchuk.org). Adenocarcinomas originating from the malignant transformation of endometrial glandular epithelial cells (Rose, 1996) are the most prevalent endometrial tumours (estimated at 95%, www.cancerresearchuk.org).

Recently, Gibson and colleagues reviewed the evidence of dysregulated androgen receptor expression, androgen metabolism, and androgen bioavailability in the pathogenesis of endometrial cancer (Gibson et al., 2014). The authors concluded that very few investigations into the expression of AR in endometrial cancer have been carried out. AR positive cells have been detected in tumor carcinoma cells (Horie et al., 1992, Ito et al., 2002) and in either the epithelial cells (Horie et al., 1992) or the stromal cells (Ito et al. 2002) of the surrounding non-transformed endometrial tissues. However, in another study, AR was not always detected in all the endometrial cancer tissues investigated (Sasaki et al., 2000). These disparate results may be explained by findings indicating that as endometrial cancers progress, AR protein levels may decline or be lost (Kato and Seto, 1985, Gibson et al., 2014) especially in the epithelial cells (Gibson et al., 2014). Finally, detection of stromal AR protein in adenocarcinomas of all stages (Gibson et al., 2014) contrasts with the lack of AR protein detected in uterine sarcomas (Koivisto-Korander et al., 2011) of which little is known due to their rarity (Denschlag et al., 2015).

The role of AR in the different cancers in not clear and remains speculative. In the endometrium, it may be speculated that the function of epithelial AR might differ from that of stromal AR. Thus, it could be that the activation of epithelial AR (gain of function) may have a role in epithelial cell transformation (Li et al., 2015b).
leading to endometrial cancer development. While the loss of AR observed in uterine sarcomas may be indicative of a loss of a putative protective function.

In addition, although endometrial cancers are mostly of epithelial origin, the observation that the endometrial stromal cells (undifferentiated or decidualised) inhibit epithelial cell growth in vitro (human primary epithelial cells or adenocarcinoma cells) (Arnold et al., 2001, Arnold et al., 2002) and in vivo (murine model) (Li et al., 2011) in a paracrine manner reinforces the idea that stromal-derived paracrine signalling plays a significant role in the normal endometrial physiology and that its dysregulation may be an additional factor in the development of cancers or implantation failure. Such paracrine cellular crosstalk in both cancer and implantation is thought to be regulated by sex steroids oestrogen and/or progesterone (Hantak et al., 2014, Senol et al., 2015). It is thus also possible to speculate that androgen/AR signalling in endometrial stromal cells may also regulate paracrine signalling that contributes to cancer development or implantation (to date, this has not been studied).

Different AR gene polymorphisms (section 1.3.5) have also been associated with changes in AR activity and disorders such as cancer (Ferro et al., 2002, Gottlieb et al., 2012) (sections 1.3.1 and 1.3.5). The association of AR polymorphisms in endometrial sarcomas is not known. However, the risk of endometrial epithelial cancer has been associated with either one of two AR polymorphic GGC and CAG trinucleotide tandem repeats (Yaron et al., 2001, Sasaki et al., 2003, Mcgrath et al., 2006, Rodriguez et al., 2006, Yang et al., 2009, Ashton et al., 2010, Sasaki et al., 2005) and single nucleotide polymorphisms (Yang et al., 2010).

As mentioned earlier, hyperandrogenism was identified as a contributing factor to the associated increased risk of endometrial cancers observed in women with PCOS. While the direct action of androgens may contribute directly to the development of endometrial cancer, other factors certainly play a role. For example, to date, endometrial cancer has not been reported in female-to-male transsexuals treated with androgens (Van Kesteren et al., 1997, Mueller and Gooren, 2008). Thus, other factors such as excessive oestrogen levels have also been associated with endometrial
cancer (Rose, 1996). Elevated oestrogen levels may arise from the conversion from androgens (in hyperandrogenic individuals) or from altered metabolism (reviewed by Gibson et al., 2014).

1.8.4 Evidence from animal models of a role for androgens in the endometrium

1.8.4.1 Reproductive phenotype of AR knockout transgenic mouse models

The AR gene is located on the X chromosome (Lubahn et al., 1988b) and therefore the male has only a single copy. Due to the key role played by androgens in male sexual development and spermatogenesis, AR dysfunction in AR mutant males leads to infertility. Thus, AR knockout homozygous females cannot be bred for study. The introduction of methods for recombinant DNA Cre/loxP technology has been used to generate transgenic AR-knockout (ARKO) mouse models that circumvent this problem and permit the study of the effects of androgens via AR and the downstream signalling pathways in viable ARKO heterozygous and homozygous female mice. Five global transgenic AR mouse strains have been generated each containing different AR allele sequence segments that are flanked by loxP sites (known as floxed sequences or transgenes) (reviewed by Chang et al., 2013). Upon crossbreeding with a Cre recombinase-expressing mouse strain in which Cre recombinase is under the control of a ubiquitous promoter, the ablation of the floxed AR transgene occurs by Cre-mediated excision in the progeny in utero.

To date, the reproductive phenotypes of female mice have been studied in three of the five ARKO models available and the findings have been the subject of recent reviews (Matsumoto et al., 2008b, Walters et al., 2010, Zhou, 2010, Chang et al., 2013). In these three models, AR protein is ablated when excised in two AR\textsuperscript{flox} strains that carry a floxed exon 1, ARKO\textsuperscript{exon1} (Matsumoto et al., 2003) or a floxed exon 2, ARKO\textsuperscript{exon2} (Yeh et al., 2002). In the third AR\textsuperscript{flox} strain, carrying a floxed exon 3 (a region in the DBD containing the second zinc finger), ARKO\textsuperscript{exon3}, a non-functional AR protein is produced upon Cre-excision, (Notini et al., 2005). In all these global ARKO mouse models, female mice display subfertility, for example, by...
producing reduced number of pups per litter compared to wild-type mice (Yeh et al., 2002, Hu et al., 2004, Shiina et al., 2006, Walters et al., 2007). Interestingly, heterozygote ARKO^{exon3} females also exhibit an age-dependant reduction in fertility (Walters et al., 2007). All together, these observations suggested that although not essential, AR-dependent signalling does contribute to optimal female fertility.

Subfertility in the female ARKO models has been investigated mainly in terms of defects in ovarian but also mammary gland and uterine development and function. All ARKO female mice have a reduced ovulation rate and follicle health due to defective folliculogenesis (Hu et al., 2004, Walters et al., 2007, Cheng et al., 2013). The complete disruption in AR signalling in ARKO^{exon1} females leads to a state of premature ovarian failure (POF) at 40 weeks (Shiina et al., 2006) however this was attenuated in the ARKO^{exon3} females. Findings from the ARKO^{exon3} model also revealed a negative feedback role for AR within hypothalamic neuroendocrine signalling that governs normal ovarian development (Walters et al., 2009, Cheng et al., 2013).

Comparatively, fewer studies have focussed on the effects of AR ablation in the mammary glands and uteri of female ARKO mice. Mammary gland development was impaired in both ARKO^{exon1} and ARKO^{exon2} female mice suggesting that AR is required for normal development during pubertal development, pregnancy and lactation in these models (Yeh et al., 2003, Shiina et al., 2006).

The uteri from ARKO^{exon2} and ARKO^{exon3} model mice have been studied. Uteri appeared macroscopically normal but notably the uterine diameter and endometrial growth was decreased compared to wild-type mice which may in part be due to defective folliculogenesis (Hu et al., 2004, Walters et al., 2007, Walters et al., 2009). There was a difference in the findings between the two ARKO models. While uterine weights did not vary between the ARKO^{exon3} mice and wild-type littermates (Walters et al., 2007, Walters et al., 2009), reduced uterine weights were reported in the ARKO^{exon2} females compared to wild-type mice (Hu et al., 2004).
Uniquely in one of the studies, young ARKO$^{\text{exon2}}$ mice were treated with chorionic gonadotrophin (Hu et al., 2004). The uterine response to treatment was reduced in the ARKO$^{\text{exon2}}$ mice. While the uteri of the wild-type mice responded by increased growth, the uterine weights of ARKO$^{\text{exon2}}$ mice remained reduced. With age, the limited uterine gonadotrophin-response of the ARKO$^{\text{exon2}}$ females decreased further. Histological analysis confirmed that endometrial growth but also cellular transformation was reduced/defective in the ARKO$^{\text{exon2}}$ mice. This suggests a uterine gonadotroph response defect in the AR deficient mice that may impact fertility independent of impaired ovarian development and function (steroidogenesis, i.e. progesterone production).

Taken together, findings from the global ARKO models provide in vivo evidence that AR plays a role in endometrial development and function. Reproductive cell-specific ARKO female mice have been generated but so far, these have been targeted to ovarian cell types alone such as to the granulosa cell and the oocyte (Sen and Hammes, 2010) to dissect the roles of AR in folliculogenesis and ovarian development. Cell-specific ARKO mice where uterine cell types are targeted may help to dissect the molecular mechanisms of the roles of AR in the endometrium and apart from the secondary effects induced by defects in other tissues such as the ovary.

1.8.4.2 Androgen and androgen antagonist affect female endometrial reproductive function in animal models

The studies and observations of the effects of exogenous androgens upon rodent endometrial physiology complement the findings from studies in humans. In an early study, androgens (T, androstenedione or DHT) administered to hypophysectomised rats led to an increase in uterine weight (a surrogate index of uterine growth) suggesting an impact upon endometrial growth (Armstrong and Papkoff, 1976).

In the absence of progesterone, androgen action has been detected in the endometrium suggesting androgen regulation of endometrial apoptosis, growth and differentiation can occur. For example, DHT administration to neonatal rats or ovariectomised adult mice was found to inhibit apoptotic cell death of endometrial
epithelial cells induced after withdrawal of oestradiol treatment (Terada et al., 1990, Jo et al., 1993). Concomitant flutamide administration abolished the antiapoptotic effect of DHT suggesting androgen action occurred via the AR. In another study, changes in genome-wide mRNA expression were documented in the uteri of ovariectomised rats treated with either DHT or oestradiol for 24 hours (Nantermet et al., 2005). Analysis of the findings indicated that DHT modulated the expression of several genes in common with oestradiol with known involvement in several cell processes including cell growth, remodelling, secretion, intracellular signalling pathways and metabolism. However, the order of magnitude of change in transcript abundance elicited by DHT was reduced compared to that elicited by oestradiol. Only a very small proportion of genes (12 out of 503 genes) were distinctly regulated by DHT alone but their identity or known functions were not reported. On the other hand, a large number of genes were regulated by oestradiol alone including a number of growth factor signalling genes implicated in cell proliferation. Interestingly, in a study of ovariectomised pigs, DHT was found to attenuate the proliferative effects of oestrogen in the uterus (Cardenas and Pope, 2004) suggesting an anti-oestrogenic role for androgens via the observed downregulation of oestrogen receptors.

A role for androgens and AR-dependent signalling in the progesterone-dependant decidualisation of stromal cells has also been suggested. In one study, injection of testosterone alone was unable to decidualise the uteri of ovariectomised mice. However, once decidualisation was induced with oestrogen and progesterone, androgens (T or DHT) were able to maintain decidualisation as determined by alkaline phosphatase activity, a decidualisation biomarker (Zhang and Croy, 1996). At the same time, endometrial function was also investigated using inhibition of androgen receptor signalling using the androgen receptor antagonist, flutamide (section 1.3.4). In this study, implantation, fetal development and parturition were all delayed in pregnant rats treated with flutamide (Chandrasekhar et al., 1990). Notably, the authors also reported that flutamide suppressed decidualisation (determined by uterine weight) in ovariectomized, pseudo-pregnant (steroid-treated) rats.
1.9 *In vitro* studies of androgen action upon the human endometrium

The impacts of elevated androgens upon the endometrium have been inferred from studies discussed in section 1.7, for example, from women displaying the effects of exogenous androgens or the effects of PCOS with associated hyperandrogenism. The effects of androgens upon the endometrium have also been studied *in vitro* focussing on the possible roles that androgens may play during the proliferative phase and the secretory phase of the endometrium. The findings are presented in this section.

It is proposed that the mechanism of action of androgens (testosterone and DHT) in hESCs acts via binding to the AR. Thus, a few studies have employed the use of the AR antagonist flutamide (and in one study bicalutamide too) (section 1.3.4) and the findings suggested that the AR mediated the effects elicited by DHT or testosterone (Wen et al., 2006, Ishikawa et al., 2007, Cloke et al., 2008, Marshall et al., 2011, Kajihara et al., 2012) although the mediation through other steroid receptors/effectors cannot not be ruled out. In addition, AR-mediated regulation of gene expression was demonstrated in cAMP/MPA-decidualised hESCs via the knockdown of AR resulting in definite changes in mRNA expression (MPA binds both AR and PR) (Cloke et al., 2008).

1.9.1 Androgen action in the human proliferative phase endometrium

Very few *in vitro* studies on the effect of androgens upon endometrial cells (epithelial and stromal) or explants from normal endometrium have been carried out and their main findings are compiled in Table 1.3. Only three studies employed endometrial tissue exclusively from women in the proliferative phase. Both the types of androgens and the concentrations of androgens employed have varied between studies from supraphysiological concentrations (for example 1, 10 or 100 µM) to physiological or near physiological serum concentrations (10 and 100nM).
Table 1.3 *In vitro* studies on the effect of androgen upon isolated primary human endometrial cell monolayers or upon human endometrial explants from normal endometrium (i.e., cultured in the absence of cAMP and/or progestin).

<table>
<thead>
<tr>
<th>Androgen</th>
<th>Dose</th>
<th>Findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>hESCs</td>
<td>A4</td>
<td>1µM</td>
<td>↑ proliferation</td>
</tr>
<tr>
<td></td>
<td>T, DHT</td>
<td>10µM, 1µM</td>
<td>↑ EGFR protein</td>
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<td></td>
<td>T</td>
<td>100mM</td>
<td>↓ MMP1 protein</td>
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<td></td>
<td>DHT</td>
<td>100nM</td>
<td>↑ ADAMTS-1 mRNA and protein</td>
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<tr>
<td></td>
<td>DHT</td>
<td>10nM</td>
<td>↓ apoptosis ↓ migration</td>
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<td></td>
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<td></td>
<td>10 mRNAs regulated: ↓PMAIP1, SHCBP1, PDGFRL, ACSS2, PPFIBP2, Prune2, Cited2, MMP10, MAOA. ↑CD44</td>
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<tr>
<td>hEECs</td>
<td>A4</td>
<td>1µM</td>
<td>↑ ERβ mRNA, ↓Bcl-2/Bax mRNA ratio</td>
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<tr>
<td></td>
<td>DHT</td>
<td>10µM</td>
<td>↑ EGFR protein</td>
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<tr>
<td></td>
<td>T</td>
<td>1µM</td>
<td>↑ IRS-1, GLUT4 mRNAs</td>
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<tr>
<td></td>
<td>A4</td>
<td>1µM, 100nM</td>
<td>↓ growth ↓Glycoprotein A secretion</td>
</tr>
<tr>
<td></td>
<td>T, DHT</td>
<td>1µM, 100nM</td>
<td>No effect on growth or Glycoprotein A secretion</td>
</tr>
<tr>
<td>hESCs + hEECs</td>
<td>T, DHT</td>
<td>10, 100µM</td>
<td>↓ proliferation</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>N/A</td>
<td>↓ growth</td>
</tr>
<tr>
<td>Explants</td>
<td>A4</td>
<td>1µM</td>
<td>↑ stromal proliferation ↓ stromal apoptosis</td>
</tr>
</tbody>
</table>

*A4 = androstenedione; DHT = 5α-dihydrotestosterone; T = testosterone; hESC = human endometrial stromal cell; hEEC = human endometrial epithelial cell; N/A = androgen dose values not available, authors report using a 1x and 10x expected plasma concentration. The menstrual phase at the time of biopsy of the endometrial tissue employed are denoted by a- Proliferative phase only; b- Early secretory phase only; c- Proliferative and secretory phases; d- Late secretory phase only; e-not available. Arrows denote ↑= increase, ↓ = decrease/inhibition.*
Androgens and endometrial cell glucose metabolism, migration and adhesion
The effects of androgens upon specific gene and protein concentrations (of a variety of cellular functions) have been reported in human endometrial epithelial cells (hEECs) and hESCs. For example, the addition of a high testosterone concentration (1µM) to cultured glandular epithelial cells upregulated mRNA concentrations of the insulin receptor substrate 1 (IRS-1) and solute carrier family 2, facilitated glucose transporter, member 4 (GLUT4) (hEECs in Table 1.3) (Zhang and Liao, 2010). High concentrations of both testosterone and DHT increased epidermal growth factor receptor (EGFR) protein levels in stromal cells (1µM and 10µM androgen) and in glandular epithelial cells (10µM androgen) (Watson et al., 1998).

Other studies employed nearer physiological concentrations of androgen. For example, 100nM DHT upregulated the concentrations of ADAM metallopeptidase with thrombospondin type 1 motif, 1 (ADAMTS-1) mRNA and protein in hESCs (Wen et al., 2006). In another study, 100nM testosterone inhibited the concentrations of cellular and secreted matrix metallopeptidase 1 (MMP1) in hESCs, while in the absence or presence of oestradiol, (Ishikawa et al., 2007). In these last two studies, the addition of flutamide abrogated the effect suggesting the androgen effect was AR-dependent.

Together, these findings suggest that androgens may have an impact on endometrial cell glucose metabolism, migration, adhesion and proliferation when the modulation of these genes/proteins (IRS1, GLUT4, EGFR, ADAMTS-1, MMP1) by androgens is considered together with their known cellular functions.

Androgens and proliferation
Proliferation is a key process during the endometrial proliferative phase (section 1.4.3). Observations from tissues other than the uterus (Li and Al-Azzawi, 2009) as well as breast cancer cell lines (Birrell et al., 1995) and animal models (Cheng et al., 2002, Nantermet et al., 2005) suggest that androgens can have an anti-proliferative effect. Notably, the study of androgen effects upon proliferation or growth in the endometrium were among the first investigations carried out. Two early in vitro studies reported the anti-proliferative effects of androgens (testosterone and DHT) upon human endometrial cells (obtained from disaggregated tissue and containing
both stromal and glandular epithelial cells) (Neulen et al., 1987, Rose et al., 1988). However, mostly higher than normal physiological plasma concentrations were required to elicit the effect (10µM and 100µM). Subsequently, lower concentrations of androgens were used. A study of the effects of androgens upon glandular epithelial cells (hEECs in Table 1.3) also showed that lower concentrations of the androgen androstenedione (1µM and 100nM) inhibited cell growth but androstenedione also inhibited cell secretory activity (though the effects were not observed with the same concentrations of testosterone or DHT) (Tuckerman et al., 2000). The effects of androstenedione upon the cells were inhibited by an anti-androgen suggesting (but not proving) an AR-mediated effect. In contrast, the findings of one study suggested a high concentration of androgen may have a proliferative effect upon stromal cells (hESCs in Table 1.3). Early secretory endometrial explants from women with normal menstruation were incubated with 1µM androstenedione resulting in increased proliferation within the stromal compartment as determined by a single assay only (immunohistochemical staining for proliferation marker Ki-67) (Maliqueo et al., 2004). Isolated endometrial stromal cells also exhibited increased proliferation (in a cell proliferation assay) after androstenedione treatment, while isolated epithelial cells were unaffected (Maliqueo et al., 2004). However, the possible interconversion of androgens and oestrogens and their effects have not been considered in these studies. Thus, it remains unclear if the reported proliferative effects of androstenedione exposure are due to androgenic effects (via activation of AR through binding of A4 or androgenic metabolites such as testosterone or DHT) or due to oestrogenic effects such as those achieved via activation of ER through the binding of the A4 metabolites oestrone or oestradiol. Consequently, there have not been any studies that comprehensively investigated the role of physiological concentrations of non-aromatisable androgen (DHT) upon the proliferation of hESCs and a specific role of androgens in hESC proliferation has not been shown and remains unclear.

**Androgens and apoptosis and cancer development**

Findings from some studies also indicate a role for androgens in association with apoptosis and cancer development.
Early secretory phase endometrial explants from women with normal and regular menstruation incubated with the high concentration of 1µM androstenedione resulted in decreased stromal cell apoptosis (determined by a single assay only, terminal deoxynucleotidyl transferase dUTP nick-end labelling-TUNEL staining) (Maliqueo et al., 2004). However, in the same study, isolated endometrial epithelial and stromal cells responded differently to the same androstenedione treatment. Isolated stromal cells showed no change in apoptotic index (B-cell CLL-Lymphoma 2 / BCL2-associated X protein mRNA ratio, BCL2/BAX) and isolated epithelial cells (hEECs in Table 1.3) exhibited a decreased BCL2/BAX mRNA ratio suggestive of less resistance to apoptotic stimuli and enhanced inducibility to cell death (Maliqueo et al., 2004). It is unclear if these differences are a result of the different assays used or if they highlight inherent differences in response to a high concentration of androstenedione by cultured endometrial explants versus isolated cells. Interestingly, Maliqueo and colleagues (2004) also reported that isolated epithelial cells treated with androstenedione showed an increase in ERβ mRNA concentrations suggesting that androgen action can involve other steroid nuclear receptors, such as ERβ.

One recent study focussed specifically on the role of physiological concentrations of androgens (10nM) during the proliferative stage of the menstrual cycle in the stromal cells, the cell type that has been shown to express the strongest staining for AR (by immunohistochemistry) across the menstrual cycle (Ito et al., 2002, Marshall et al., 2011). Using an in silico-based approach, Marshall and colleagues (2011) identified a small number of mRNAs regulated by DHT (10nM) in stromal cells isolated from proliferative stage endometria. The mRNAs identified encoded proteins have been implicated in the regulation of apoptosis and the development of cancers suggesting that androgens may play a role in these processes within the endometrium. Indeed, by using functional assays in vitro, the authors showed that DHT delayed apoptosis of hESCs and inhibited migration of the cells in a wound assay.

Two studies have reported the effects of androgens upon the human endometrial adenocarcinoma Ishikawa cell line. One study reported that treatment with androstenedione (1nM) inhibited growth and increased apoptosis in Ishikawa cells (Ma and Blenis, 2009). The authors suggested these effects may be mediated by the
protein kinase B/mitogen-activated protein kinase (AKT/MAPK) pathway due to the activation of AKT and MAPK also observed. In another study, treatment of Ishikawa cells with testosterone or DHT (100nM or greater) repressed the mRNA expression of homeobox A10 (HOXA10), a gene essential in endometrial receptivity (Cermik et al., 2003). The effect of testosterone upon HOXA10 gene expression was inhibited by flutamide suggesting AR-dependent signalling. A higher concentration of androgen (1mM) repressed HOXA10 expression in the presence of progesterone or oestradiol (which conversely enhance HOXA10 expression). The authors also showed that endometrial tissues from women with PCOS contained lower HOXA10 mRNA concentrations compared to healthy controls suggesting the reduction in HOXA10 mRNA may be due to PCOS-associated hyperandrogenism. It is interesting to note that HOXA10 mRNA and protein expression has also been reported to be repressed in endometrial cancer (Zhang et al., 2014) the risk of which is greater in women with PCOS (see section 1.7.2).

In summary, these few studies suggest that androgens may impact endometrial proliferation, apoptosis, cancer development, glucose metabolism, migration and adhesion. These processes are complex and involve many genes/proteins and signalling pathways. However, except for one in silico-based study in hESCs (Marshall et al., 2011), the study of the effects of androgens in undifferentiated endometrial cells (not exposed to progesterone and/or cAMP) has focussed on the investigation of altered levels of only a limited number of genes/proteins. Indeed, the study of androgen-dependent global gene expression changes in proliferative phase human endometrial tissues or cells has not been carried out and may help to elucidate the regulation of these cellular processes by androgens.
1.9.2 Androgen action and AR activity during decidualisation in the human endometrium

After some early observations in the 1990s, the study of the role for androgens in the decidualising endometrium has only recently regained attention especially in light of the suggested impact upon fertility and pregnancy outcome. The specific impact of androgens and the AR on the decidualisation process was reviewed for the first time by Cloke and Christian (2012). Androgen signalling has been proposed to modulate decidual cell transformation, marker expression and survival with an impact upon endometrial receptivity. As such, it may be speculated that an excess but also a lack of androgen may have a detrimental impact on decidualisation physiology and endometrial reproductive function (Cloke and Christian, 2012). This idea where an optimum amount is required within certain parameters with extreme amounts (either excess or deficiency) being inadequate or hostile towards a desired effect is termed the Goldilocks principle (Fowler and O'shaughnessy, 2013). This principle has been used to describe the critical androgen balance required during male testes development and ovarian follicular development (Fowler and O'shaughnessy, 2013, Prizant et al., 2014).

Androgens and decidualisation

Studies have reported that androgens (acting via the AR) enhance decidualisation in decidualising hESCs (as determined by measuring the mRNA and protein concentrations of decidualising markers PRL and IGFBP1) (Narukawa et al., 1994, Cloke et al., 2008). Gene profiling of decidualising hESCs (cAMP/MPA) after knockdown of either AR or PR revealed that, not only did AR and PR regulate a set of genes in common, but AR also regulated gene networks independent of those regulated via PR. The genes impacted by AR-knockdown were genes predominantly involved in cytoskeletal organisation, cell motility and cell cycle regulation (Cloke et al., 2008). Recent studies in decidualised hESCs specifically showed androgen increased cytoplasmic organelles and gap junctions (an in vivo marker of decidualisation, Wynn, 1974) further supporting the role of androgens in modulating the morphological and ultrastuctural changes observed in the transformed decidual cell (Kajihara et al., 2013b).
**Androgens and proliferation during decidualisation**

AR knockdown of decidualising hESCs revealed AR involvement in the repression of genes that normally promote cell cycle progression and proliferation (*e.g.* WEE1 G2 checkpoint kinase, nucleosome assembly protein 1-like 1) and that prevent replication (*e.g.* chromatin licensing and DNA replication factor 1, minichromosome maintenance complex component 2 and 4) (Cloke *et al.*, 2008). These microarray findings were supported by a significant increase in proliferation in the AR knockdown-decidualised hESCs that was quantified by a proliferation assay (Cloke *et al.*, 2008) suggesting a role for AR-dependent signalling in curbing proliferation in decidualising hESC. Interestingly, the authors also speculated that these findings may indicate that AR plays a role in protecting the genomic integrity of replicating hESCs but this has not been verified.

**Androgens and inhibition of apoptosis**

Androgen signalling (studied using DHT) during decidualisation also enhanced the concentrations of mitochondrial superoxide dismutase 2 (SOD2) protein which may help to protect against the effects of oxidative stress (increased reactive oxygen species) and lead to reduced apoptosis (Kajihara *et al.*, 2012) in line with and further substantiating the previously postulated anti-apoptotic role for DHT reported in undifferentiated hESCs (Marshall *et al.*, 2011).

ADAMTS-1 mRNA and protein expression was also reported induced by DHT in hESCs with the induction being further increased in the presence of progesterone (Wen *et al.*, 2006). Although it is unknown if ADAMTS-1 affects apoptosis in the endometrium, interestingly, elevated ADAMTS-1 expression has been associated with apoptosis inhibition or promotion of cell survival in some cancers (reviewed by Tan Ide *et al.*, 2013).

Apoptosis was assessed by various methods and reported to be decreased in endometrial samples from ovulatory PCOS women within the window of implantation (Gonzalez *et al.*, 2012, Yan *et al.*, 2012). Apoptosis was reduced in the hESCs isolated from these PCOS patients and in the hESCs isolated from fertile women and treated with androgen (Gonzalez *et al.*, 2012). Subsequently, Gonzalez
and colleagues (2012) also investigated the expression of WT1 in the endometria of PCOS patients as Wilms tumor 1 (WT1) is known to be downregulated by AR in other cell types. In the endometrium, WT1 mRNA and protein are both expressed during the window of implantation and are upregulated by progesterone in hESCs (Makrigiannakis et al., 2001, Anthony et al., 2003). The authors reported WT1 mRNA and protein expression was decreased in secretory endometrium (window of implantation) from women with ovulatory PCOS. Notably, in vitro studies confirmed that DHT downregulated WT1 expression in cAMP-treated hESCs leading to changes in expression of WT1 downstream targets, for example, the increase of the apoptosis inhibitor BCL2 (mRNA and protein) (Gonzalez et al., 2012). The findings suggest that suppression of endometrial apoptosis by excess androgens may occur in part via WT1 repression and may impact on endometrial receptivity.

In summary, there is evidence to suggest a role for AR and androgens in the differentiation of hESCs into decidual cells. Also, as the coordinated events during decidualisation are critical for embryo implantation, the dysregulation of androgen and AR signalling may contribute to fertility outcomes, the mechanisms of which may involve changes in cell survival but these remain poorly understood.

1.10 General conclusions

The oestrogen and progesterone-driven regulation of the profound structural, molecular and functional changes that the endometrium undergoes during the menstrual cycle and pregnancy have been studied extensively.

In comparison, the impact of androgen signalling in the endometrium is remarkably understudied and remains poorly understood. Similar to the presence of other steroids and their receptors in the endometrium, the basic elements required for AR-mediated signalling (androgens and the AR) are present with the AR being most highly expressed by human endometrial stromal cells in the proliferative phase. There is also growing evidence from in vivo and in vitro studies supporting the action of androgens in the endometrium during the menstrual cycle. Roles for androgen signalling in normal endometrial function, include regulation of fertility and abnormal endometrial pathologies. It is reasonable, therefore, to suggest that
androgens may impact endometrial proliferation and differentiation, the control of which, are crucial for endometrial function. Molecular approaches have been used successfully to study a variety of animal and human endometrial molecular changes. However, system-biology approaches have only been employed in two studies to investigate androgen signalling in human endometrial stromal cells. In particular, unbiased genome-wide gene profiling of the effects of androgen action upon human endometrial cells from the proliferative phase has not yet been reported.

In this thesis, genome-wide profiling of hESCs treated with DHT helped to test the hypothesis that *androgens affect gene expression in human endometrial stromal cells to alter proliferation and differentiation*. New findings presented in this thesis support this hypothesis with implications for the understanding of the physiological role of androgens in the endometrium.

### 1.10.1 Aims of the study

The specific aims of this thesis were:

1. To identify cellular processes, pathways and networks regulated by androgens in human endometrial stromal cells (using bioinformatic analyses of transcriptomic data from cells treated with androgen) (addressed in chapter 3)

2. To evaluate the potential for regulation and determine the regulation of putative DHT-regulated gene expression by androgen in human endometrial stromal cells (addressed in chapter 4)

3. To determine the expression and regulation of putative androgen-regulated genes in the human endometrium across the menstrual cycle and in early pregnancy (addressed in chapter 5)

4. To explore if androgens modulate metformin-induced gene expression effects associated with decidualisation of hESCs (preliminary investigations) (addressed in chapter 6)
Chapter 2: General Materials and Methods

2.1 Introduction
This Chapter describes all the general materials and methods employed in this thesis. Specific details and methods, with the relevant cross reference, are included in each chapter’s Materials and Methods section.

2.2 Ethical approval, patient inclusion criteria and tissue collection
All tissue samples were collected under ethical approval obtained from the Lothian Research Ethics Committee (LREC). Uterine tissues were obtained under LREC05/S1103/14, LREC07/S1103/29, LREC10/S1402/59 and first trimester decidual tissues were obtained under LREC04/S1103/20, LREC04/S1104/12 and LREC05/S1103/14. Term placental tissue used as a positive control for immunohistochemical studies was obtained under LREC05/S1103/14. Informed written consent was obtained from all women.

The Materials and Methods section of each results chapter lists the tissue samples employed therein and includes characterisation details for each sample/patient (see section 3.2.1, section 4.2.1 and section 5.2.1). For endometrial tissues, each patient provided information about date of their last menstrual period (LMP) and a day of cycle was calculated. Laboratory analysis of serum oestradiol and progesterone (P4) levels at the time of biopsy was undertaken whenever possible (by the university clinical biosciences assay laboratory or the hospital assay laboratory in some cases) and Dr Alistair Williams (Pathologist) provided standard histological menstrual cycle dating of the endometrial sample (hematoxylin and eosin staining) (Noyes et al., 1950). Consistency of all these details allowed accurate menstrual cycle staging of the endometrial tissue samples.

Endometrium was sampled by suction curette (Pipelle, Laboratoire CCD, Paris, France) from women attending NHS Lothian gynaecological services or collected from the uterus of women undergoing hysterectomy for benign gynaecological
conditions. Women had not received hormonal treatments for three months prior to the tissue collection.

Tissues were collected, dissected and processed by: (a) immersion in RNALater ® (Ambion, Life Technologies, CA, USA) for at least 24 hours at 4ºC then transferred to a fresh 1.5ml tube and stored at -80ºC for subsequent RNA extraction (section 2.7.1); (b) fixation in 4% neutral-buffered formalin (NBF) for 24 hours prior to storage in 70% ethanol for wax embedding (section 2.5.1); (c) incubation in sterile phosphate buffered saline (PBS) for endometrial stromal cell extraction (section 2.3.1).

2.3 Cell culture

2.3.1 Primary human endometrial stromal cell isolation and culture

Human endometrial stromal cells were isolated from endometrial tissue specimens that were washed in PBS and finely diced. Tissue dispersal was achieved by incubation in 2ml PBS containing 0.5mg/ml collagenase and 25µg/ml DNAse (Sigma, Dorset, UK) for 90 minutes at 37°C (Kane et al., 2008). After addition of 2ml of Roswell Park Memorial Institute (RPMI) medium (Sigma, Dorset, UK) the tissue digest was further homogenised by passing through an 18G needle twice. Finally, the cell mixture was filtered through two sized filters (70 and 45µm, BD Biosciences, CA, USA) and the eluate centrifuged for 5 minutes at 800g. After the supernatant was discarded, the pellet was re-suspended in culture maintenance media (see below) and added to a 175cm² flask.

Stromal cells were maintained in RPMI medium containing 10% heat inactivated fetal bovine serum (FBS) supplemented with 2mM L-glutamine, 200U/ml penicillin, 100µg/ml streptomycin, 2.5µg/ml amphotericin B and 4µg/ml Gentamycin. At confluence cells were trypsinized (section 2.3.3) and used in experiments until fifth passage up to which the expression of AR is retained (Dr. Elaine Marshall, unpublished).
Before seeding for experiments, cells were transferred for 2 to 4 days to phenol-free RPMI containing 10% charcoal-stripped serum (section 2.3.4) (Bombail et al., 2010, Marshall et al., 2011, Wilkens et al., 2013), supplemented with 20nM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2mM L-glutamine, 200U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml amphotericin B and 4μg/ml Gentamycin (all reagents from Sigma, Dorset, UK).

### 2.3.2 Culture of immortalised endometrial stromal cell line SHT-290

SHT-290 cells, a gift from Dr Claire Barbier and Dr David Kaufman (University of North Carolina at Chapel Hill, NC, USA) to Professor Philippa Saunders, are human telomerase reverse transcriptase (hTERT)-immortalised human endometrial stromal cells (Barbier et al., 2005). SHT-290 cell cultures were maintained in a 1:1 mixture of M199 basic media (Sigma, Dorset, UK) and Ham F12 media (GIBCO, Life Technologies, USA) supplemented with 5% heat inactivated fetal bovine serum (FBS), 0.1% Mitoplus (BD Biosciences, CA, USA), 1X ABAM (GIBCO, Life Technologies, USA) and 2μg/ml insulin (Sigma, Dorset, UK).

Before experiments, cells were transferred to 10% charcoal-stripped serum medium (phenol-free RPMI containing L-glutamine, 1X MEM-Non-essential amino acid solution, 2mM L-glutamine, 200U/ml penicillin, 100μg/ml streptomycin, 2.5μg/ml amphotericin B (Sigma, Dorset, UK)) for 4 days before seeding into culture wells.

### 2.3.3 Passage and cryopreservation of cells

All cell cultures were maintained in a 37°C humidified air atmosphere supplemented with 5% CO₂. Cell work was carried out in a class II microbiological safety cabinet.

To passage cells, the cell monolayers were washed twice with PBS to remove any serum and incubated for 5 minutes with trypsin (4 ml in a 175cm² flask) (Sigma, Dorset, UK). To fully dislodge the cells gentle agitation of the culture flasks was employed if necessary. Two volumes of cell-appropriate fetal bovine serum (FBS) containing media (standard or charcoal-stripped) was used to neutralize trypsin activity and re-suspend the cells which were then transferred to a 15ml or 50ml
centrifuge tube and centrifuged for 5 minutes at 800g. The supernatant was discarded and the pellet was re-suspended in the required culture media. Cells were then split among fresh flasks or dishes already containing appropriate cell culture media.

To cryo-preserve cells, cell monolayers were trypsinized as described above and cell pellets were gently re-suspended in 3-5ml freezing media (prepared with 1.5ml of heat inactivated fetal bovine serum, 5ml dimethyl sulfoxide, (DMSO) and 20ml complete culture media). Cells were aliquoted in 1ml volumes in cryovials, chilled to -80ºC and stored long term in liquid nitrogen.

2.3.4 Preparation of steroid stripped fetal bovine serum (FBS)
Heat inactivated FBS (Sigma, Dorset, UK) was treated with activated charcoal to remove steroids. About 100-150g of activated charcoal (Sigma, Dorset, UK) was added to one litre of FBS and mixed overnight at 4ºC. To remove the charcoal, the FBS-charcoal solution was centrifuged in 250ml centrifuge bottles for 10 minutes at 8000g. The FBS supernatants were poured into fresh 250ml bottles and centrifuged again as before. Finally, the charcoal-cleared FBS supernatant was sterilised using a 0.22µm vacuum stericup filter (Millipore, MA, USA) and stored in 50ml sterile tubes at -20ºC until use.

2.4 Experimental cell culture conditions
All cells were transferred to their appropriate media containing 10% charcoal-stripped FBS (as detailed in the previous section 2.3) for four days followed by 24 hours serum starvation (before ligand treatments) or decidualisation (serum reduction to 2% plus decidualisation stimulus) described in sections 2.4.1 and 2.4.2, respectively. Cells were seeded for experiments in chamberslides (for immunocytochemistry) (BD Biosciences, CA, USA), 6 well cluster plates (for RNA harvest), 100mm dishes or 165cm2 flasks (for cell protein harvest). The treatments and time of exposure are specified for each experiment in each chapter’s Materials and Methods section.
2.4.1 Dihydrogen-testosterone, Oestradiol and Flutamide

Dihydrogen-testosterone (DHT) and 17β-Oestradiol (Sigma, Dorset, UK) were prepared at a concentration of 10^{-2} M (10mM) and stored at -20°C. DHT was solubilised in ethanol; E_2 was solubilised in dimethyl sulfoxide (DMSO). DHT and E_2 were further diluted from these stocks in PBS to 10^{-5} M for use at final concentrations of 10^{-8}M (10nM) and in the case of DHT in decidualisation experiments 10^{-7}M (Cloke et al., 2008). The concentration of 1 x 10^{-8} M used in experiments was determined based on ligand dose-response studies previously carried out in our laboratory (data not shown). Vehicle stocks of ethanol and DMSO diluted in PBS were also prepared.

The AR antagonist 2-hydroxyflutamide (LKT Laboratories, MN, USA), hereafter referred to as flutamide, was solubilised in methanol to 10^{-2} M (10mM) and used at final concentration of 10^{-5} M (10μM) in experiments. Therefore, the final amount of the vehicle methanol added to cell media was 1μl per 1ml media. Flutamide was added for one hour before the addition of ligands.

For experiments using treatments with DHT or E_2, all cells were grown for four days in their relevant 10% charcoal-stripped serum media then starved for 24 hours in serum-free media before media replenishment with added pre-treatments (for example, flutamide) or treatments (for example, DHT or E_2).

2.4.2 Decidualisation: cAMP and Progesterone

Solubilisation of 3',5'-cyclic adenosine monophosphate (cAMP) (Sigma, Dorset, UK) was performed in water (100mg/ml) and aliquots were stored at -20°C for use at a final concentration of 100μg/ml.

Progesterone (pregn-4-ene-3,20-dione) (Sigma, Dorset, UK) was solubilised in DMSO at a concentration of 10^{-2} M (10mM), stored at -20°C, diluted further in PBS on day of use and added at a final concentration of 10^{-6}M (1μM).

Decidualisation of endometrial stromal cells was induced according to standard protocols (Cloke et al., 2008) for a period of 8 days in RPMI medium containing 2% charcoal-stripped serum plus additives detailed in section 2.3.1 and supplemented
with 1μM (10^{-6} \text{ M}) P_4 and 0.1mg/ml cAMP (Sigma, Dorset, UK). Culture medium with hormonal supplements was changed every two days.

### 2.5 Immuno-histochemistry and -cytochemistry

Immunohistochemistry (IHC) is the detection of antigens carried out on fixed and paraffin wax embedded human tissues. Immunocytochemistry (ICC) is the detection of antigens carried out on fixed in vitro cultured cells. Figure 2.1 gives a general overview of both procedures described in this section.

![Diagram of IHC and ICC procedures](image)

**Figure 2.1 General overview of IHC and ICC procedures.**
2.5.1 Tissue paraffin wax embedding and sectioning (IHC)
Tissues were collected and fixed as described above in section 2.2. After transfer to 70% ethanol the tissues were processed in an automated Leica TP1050 tissue processor (Leica Biosystems, Bucks, UK) and finally embedded in paraffin wax. As required, 5µM thick sections were cut from the pre-chilled wax embedded tissue blocks using a hand operated HM325 MICROM microtome (Carl Zeiss, Gena, Germany). The sections were floated onto a cold demineralised water bath and allowed to lie flat before being transferred to a 50°C (which is about 10°C below the paraffin wax melting point) demineralised water bath with a glass slide. When the sections stretched and smoothed out on the warm water they were mounted onto electrostatically charged glass slides (Leica Biosystems, Bucks, UK). The slides were placed overnight in a 50°C oven to dry and adhere. Once at room temperature the slides were stored protected from dust in dry storage until used.

2.5.2 Cell culture and fixation in chamberslides (ICC)
Falcon chamber slides of 2 or 4 chambers (BD Biosciences, CA, USA) were seeded with cells in the required medium (see above). Cells were allowed to attach overnight and monolayers were fixed when at approximately 70% confluence or after treatments. Cell culture and treatments were carried out as described in sections 2.3 and 2.4.

Fixation of cells began with removal of the culture media and two washes with room temperature PBS (Sigma, Dorset, UK). After addition of -20°C pre-chilled Methanol (0.5ml-1ml per chamber) (Fisher Scientific, Loughborough, UK) the chamber slides were incubated at -20°C for 10 minutes. Subsequently, the chamber slides were washed twice with PBS at room temperature. Finally, fresh PBS (1-2ml per chamber) was added and the chamber slides were stored at 4°C in a sealed environment to prevent dehydration and until used.

2.5.3 IHC and ICC – methods and antibodies
IHC and ICC were performed in a humidified chamber and care was taken to prevent evaporation and specimen dehydration during incubation steps. Incubations and washes were carried out at room temperature unless stated otherwise.
2.5.3.1 Dewaxing and rehydration before IHC (paraffin-embedded tissues)

The sections were dewaxed by immersion in zylene (VWR, Leicestershire, UK) for 5 minutes twice. To rehydrate the tissue the sections were immersed sequentially in 100% ethanol (twice), 95% ethanol, 80% ethanol, 70% ethanol for 20 seconds each step and then finally in tap water for 5 minutes.

2.5.3.2 Antigen retrieval for IHC

Tissue sections were subjected to an antigen retrieval step involving high temperature and change of pH (Shi et al., 1993). Once hydrated (as described in section 2.5.1) the tissue sections were immersed in an appropriate antigen retrieval buffer and placed in a pressure decloaking chamber (Biocare Medical, CA, USA) using the standard protocol of 125ºC for 30 seconds followed by 90ºC for 10 seconds.

One of two different antigen retrieval solutions was employed depending on the antigen. Antigen retrieval for AR detection was carried out in 0.05M glycine / 0.01% EDTA pH 8.0. Antigen retrieval for the detection of all other antigens (RGS2, SIK1 and Synphilin-1) was carried using 0.01M Sodium citrate pH 6.0 (Sigma, Dorset, UK) (Shi et al., 1993, Imam et al., 1995). After the cycle in the decloaking chamber and 10 minutes of cooling in the chamber, the sections were removed and cooled further to room temperature using water.

2.5.3.3 Permeabilisation for ICC

Cells fixed on chamber slides as described in section 2.5.2 were subjected to a permeabilisation step (Marshall et al., 2011). The chamber sides were removed and the slides were placed in PBS (0.14M NaCl / 0.0027M KCl / 0.01M phosphate buffer pH 7.4, Medicago AB, Upssala, Sweden) for 5 minutes. Slides were incubated at room temperature for 20 minutes in permeabilising solution (0.2% IGEPAL / 1% BSA / 10% normal goat serum / PBS) (Sigma, Dorset, UK; Biosera, Boussens, France). Slides were then washed twice for 5 minutes in PBS.
2.5.3.4 Non-specific blocking

To prevent non-specific binding of the primary and secondary antibodies several blocking steps were carried out. For IHC and ICC the endogenous peroxidase block followed either the antigen retrieval or the permeabilisation steps, respectively.

Endogenous peroxidase was blocked by incubation in 3% H$_2$O$_2$ / Methanol (Sigma, Dorset, UK; Fisher Scientific, Loughborough, UK) for 30 minutes at room temperature. The sections were then washed for 5 minutes twice in Tris buffered saline pH 7.4 (TBS, 0.05M Tris-base / 150mM NaCl, Sigma, Dorset, UK).

Incubation for 15 minutes with Avidin, a 5 minute wash with TBS and a 15 minute incubation with Biotin followed (Vector Laboratories, CA, USA). A final 5 minute wash with TBS completed this blocking step.

To block non-specific antibody binding the sections were incubated for 20-30 minutes with Ready-To-Use Serum-Free DakoCytomation Protein Block (Dako, Glostrup, Denmark).

2.5.3.5 Primary antibodies

The blocking serum was removed and incubation with primary antibody followed. Primary antibodies were diluted to optimum concentration detailed in Table 2.1. Most primary antibodies were diluted in REAL antibody diluent (Dako, Glostrup, Denmark).

To reveal any non-specific binding of the primary antibody, negative controls consisted of IgG isotype-matched antibody controls (Table 2.2) incubated at a matched protein concentration as the antigen primary antibody. Secondary antibody controls consisted of omission of the primary antibody.
Table 2.1 Primary antibodies, secondary antibodies and visualisation steps used for IHC and ICC.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody raised in</th>
<th>Source</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Visualisation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>Rabbit</td>
<td>SC-816 Santa Cruz</td>
<td>1:600</td>
<td>anti-rabbit ImmPRESS</td>
<td>DAB</td>
</tr>
<tr>
<td>RGS2</td>
<td>Mouse IgG2a</td>
<td>WH0005997 M1 Sigma</td>
<td>1:300</td>
<td>anti-mouse ImmPRESS</td>
<td>DAB</td>
</tr>
<tr>
<td>SIK1</td>
<td>Rabbit</td>
<td>Ab64428 Abcam</td>
<td>1:100</td>
<td>anti-rabbit ImmPRESS</td>
<td>DAB</td>
</tr>
<tr>
<td></td>
<td>Mouse IgG2a</td>
<td>Ab118317 Abcam</td>
<td>1:600</td>
<td>anti-mouse ImmPRESS</td>
<td>DAB</td>
</tr>
<tr>
<td>Synphilin-1</td>
<td>Rabbit anti-serum</td>
<td>ab6179 Abcam</td>
<td>1:500</td>
<td>anti-rabbit ImmPRESS</td>
<td>DAB</td>
</tr>
</tbody>
</table>

(Primary antibodies: Santa-Cruz, Autogen Bioclear, Wiltshire, UK; Sigma, Dorset, UK; Abcam, MA, USA; Thermo Fisher Scientific, Loughborough, UK. Secondary antibodies and visualisation reagents: Vector Laboratories, CA, USA.)

Table 2.2 IgG isotype negative controls used for IHC and ICC.

<table>
<thead>
<tr>
<th>Antibody isotype</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>Rabbit</td>
<td>SC-2027, Santa-Cruz</td>
</tr>
<tr>
<td>IgG2a</td>
<td>Mouse</td>
<td>M5409, Sigma</td>
</tr>
</tbody>
</table>

(Santa-Cruz, Autogen Bioclear, Wiltshire, UK; Sigma, Dorset, UK)

If an antibody was supplied as whole anti-serum, as was the case for Synphilin-1 (rabbit anti-serum, unknown antibody concentration), then a normal animal serum was used such as normal rabbit serum (Biosera, Boussens, France). The same steps were applied to all the negative controls as for samples with primary antibodies. After incubation overnight at 4°C with the primary antibodies the sections were washed for 5 minutes with TBS (IHC) or TBS / 0.01% Tween 20 (ICC) three times.

2.5.3.6 Secondary antibodies

Figure 2.2 illustrates the steps of the two detection systems used. When a secondary antibody ImmPRESS reagent was used sections were pre-incubated with 2.5% horse serum for 5 minutes (Vector Laboratories, CA, USA) (see general overview in Figure 2.1). The serum was replaced with the ready-to-use secondary antibody.
ImmPRESS reagent (Vector Laboratories, CA, USA) as detailed in Table 2.1. This secondary antibody-polymer complex has a conjugated horse radish peroxidase (HRP).

*When a biotinylated secondary antibody* (Vector Laboratories, CA, USA) was used in ICC the antibody was diluted as detailed in Table 2.1 in 5% BSA / 20% normal goat serum / TBS.

Incubation with all secondary antibodies was carried out for 30 minutes at room temperature followed by three washes with TBS (IHC) or TBS / 0.01% Tween 20 (ICC) for 5 minutes each.

![Diagram of the detection systems used in the IHC and ICC procedures.](image)

**Figure 2.2 Diagram of the detection systems used in the IHC and ICC procedures.** *In Panel A: The polymer-HRP ImmPRESS system and in Panel B: the biotinylated secondary antibody with ABC-HRP system, respectively. (Adapted from Key, 2009)*

### 2.5.3.7 Tertiary step (for biotinylated secondary antibodies)

When a biotinylated secondary antibody was used an additional step was required prior to development (see Figures 2.1 and 2.2). The reagent employed is composed of avidin-biotin-HRP (ABC) complexes that bind the biotin conjugated to the secondary antibody. The sections were incubated for 30 minutes at room temperature with the ready-to-use ABC-Elite Reagent (Vector Laboratories, CA, USA) followed
by three washes with TBS (IHC) or TBS / 0.01% Tween 20 (ICC) for 5 minutes each.

2.5.3.8 Reaction development, counterstaining, dehydration and mounting

The sections were incubated for 5 minutes with working solution of ImmPACT 3, 3-diaminobenzidine (DAB) peroxidase substrate prepared by adding 1 drop of chromogen concentrate per 1ml of diluent (Vector Laboratories, CA, USA). The reaction was terminated by immersion into water. DAB is oxidized indirectly by the action of the peroxidase becoming a visible colour product and precipitating in situ.

After a further 5 minutes wash in water, the sections were counterstained with haematoxylin that stains the nuclei blue. The sections were immersed for 20 seconds in Haematoxylin Harris (Leica Biosystems, Bucks, UK) and immediately washed in water to remove excess stain. To develop the stain, immersion in Scott’s tap water (0.02M potassium hydrogen carbonate / 0.17M magnesium sulphate, Sigma, Dorset, UK) for 30 seconds was followed with immediate washing in water. The sections were dehydrated by sequential immersion in 70% ethanol, 80% ethanol, 95% ethanol, 100% ethanol (twice) each for 20 seconds and then immersion in zylene (VWR, Leicestershire, UK) twice for 5 minutes. Finally, the slides were mounted with pertex (CellPath, Powys, UK) and glass coverslips (Leica Biosystems, Bucks, UK). The slides were allowed to dry and were stored at room temperature.

2.5.4 Light microscopy, immunostaining assessment and photomicroscopy (imaging)

Slides were analysed using light microscopy on an Olympus Provis AX70 microscope (Olympus America Inc, Center Valley, PA, USA). Immunostaining of each entire sample (test, positive and negative control) was evaluated manually for the localisation and distribution of positive immunostaining (visible brown colour product) (not quantitative). Images were captured with an attached AxioCam HRC camera using Axiovert v4.8 software (Carl Zeiss, Gena, Germany). The software embedded calibrated digital scale bars (50μM or 100μM) onto individual images as required and allowed image export as “jpg” files. Images were assembled into montages using Powerpoint 2010 (Microsoft) or Photoshop CS5 (Adobe).
2.6 Western protein analysis

Western protein analysis was carried out on total protein extracts from cultured cell monolayers. Details such as culture conditions, treatments and exposure times are specified in each chapter’s Materials and Methods section.

2.6.1 Whole cell protein extraction

For extraction of total protein from cell monolayers, the media was removed and the cells were washed once with cold PBS to remove serum proteins. Cell lysis buffer was prepared containing 1% Triton X-100, 15mM Hepes-NaOH (pH 7.5), 0.15mM NaCl, 1% Sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 1mM sodium orthovanadate and 10mM Ethylenediaminetetraacetic acid (EDTA) (all reagents from Sigma, Dorset, UK). Cells were lysed in lysis buffer (300µl) supplemented with protease inhibitors (1 tablet per 10ml of lysis buffer, Roche Diagnostics, Mannheim, Germany) and 1nM DHT. The lysed cells were incubated on an agitation platform on ice at 4°C for 15 minutes. Lysed samples were then transferred to 1.5ml tubes and centrifuged at 6000rpm for 10 minutes at 4°C. The supernatant was removed to a new chilled 1.5ml tube and stored at -80°C until used.

2.6.2 Concentration of protein extracts by acetone precipitation

To allow more protein to be loaded onto gels, protein extracts were concentrated when necessary (by one of two methods described below) and quantification was repeated subsequently (as described in section 2.6.3).

Protein sample concentration was carried out using viva spin columns (Vivaspin 500 columns-MW30000, GE Healthcare, Bucks, UK) according to manufacturer’s instructions. Total protein extracts (300-400µl) were loaded onto chilled Vivaspin cartridges and centrifuged at 15000g for 10 to 15 minutes at 4°C. The flow through was discarded. The concentrated sample remaining in the upper cartridge chamber was transferred to a new chilled 1.5ml tube and stored at -80°C. This cartridge-based method does not retain proteins smaller than 30KDa.
The alternate method of acetone precipitation was also used and this allowed the study of proteins smaller than 30KDa. To precipitate the protein extracts, four volumes of acetone (-20ºC pre-chilled) were added to each protein sample (300-400µl). The mixture was vortexed, incubated for 1 hour at -20ºC and then centrifuged at 15000g for 10 minutes at 4ºC. The supernatant was discarded and the protein pellet was allowed to dry very briefly before reconstitution in about 100-130µl (a third of the original volume) with the same cell lysis buffer (described in section 2.6.1).

2.6.3 Protein quantification
Protein was quantified using a modified Lowry assay (Lowry et al., 1951) according to manufacturer’s instructions (BioRad DC Protein Assay method, BioRad, Hercules, CA, USA). Protein standards were prepared by standard serial dilution (ranging from 0.125 to 1.5mg/ml) of Bovine Serum Albumin (BSA) (Sigma, Dorset, UK or Pierce, Thermo Fisher Scientific Inc., IL, USA) using the same lysis buffer described in previous section 2.6.1. Protein samples for assay were also diluted in lysis buffer if required. A mix of Reagent A and Reagent S (20µl of Reagent S was added per 1ml of Reagent A) was prepared immediately before the start of the assay. The assay was performed using a clear 96 well Costar plate (Corning Inc, NY, USA) where 5µl of the protein standards (including a lysis buffer only control) and protein samples was aliquoted in duplicate. To each standard or sample, 25µl of the mixed Reagents A/S was added followed by 200µl of Reagent B. The plate was incubated protected from direct light on an agitation platform for 15 minutes at room temperature. Colour absorbance was measured at 620nm using a plate reader (Multiscan EX, MTX Labsystems Inc., VA, USA). Sample concentrations were calculated from a standard curve (protein standards) generated using Excel (Microsoft).

2.6.4 Western analysis
2.6.4.1 Sample preparation and gel electrophoresis
Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the Novex mini-cell equipment system and the NuPAGE electrophoresis system (Invitrogen, CA, USA). Either 10µl of SeaBlue or SeaBlue
Plus2 pre-stained molecular weight markers (Invitrogen, CA, USA) was included on each gel. The size of these standards ranged between 198 and 3 kDa.

To 0.5 ml centrifuge tubes placed on ice, protein samples (20 μg total protein), 1X NuPAGE Lithium dodecyl sulphate (LDS) sample buffer (26.5 mM Tris HCl, 35.25 mM Tris Base, 0.5% LDS, 2.5% Glycerol, 0.128 mM EDTA, 0.055 mM SERVA Blue G250, 0.044 mM Phenol Red, pH 8.5) and 1 X NuPAGE sample reducing agent (50 mM dithiothreitol) was added (Invitrogen, CA, USA). The remaining volume was made up with protein extract lysis buffer (section 2.6.1). The samples were heated for 10 minutes at 75°C and placed back on ice. Tubes were centrifuged at 1000g for 30 seconds (4°C) and the total volume was loaded onto a 12% NuPAGE Novex BIS-TRIS acrylamide gel (Invitrogen, CA, USA). Electrophoresis was carried out at 200V (constant current) for 55 minutes using 1X NuPAGE MOPS SDS running buffer (2.5 mM MOPS, 2.5 mM Tris Base, 0.005% SDS, 0.05 mM EDTA, pH 7.7) with 500 μl of NuPAGE antioxidant (proprietary reagent) added to the inner tank (Invitrogen, CA, USA).

### 2.6.4.2 Protein transfer and protein detection

After gel electrophoresis, the gel casings were dismantled to expose the gel within. The stacking portion of the gel was discarded. Immobilon-FL membranes (Millipore, MA, USA) were immersed in methanol. Transfer buffer consisted of 25 mM Tris / 0.192 M glycine / 0.1% SDS / 20% methanol (Sigma, Dorset, UK). The membrane, separating gel and transfer buffer-soaked filter paper were assembled on a Trans-blot SD semi-dry transfer cell as per the manufacturer’s instruction (BioRad, CA, USA) so that, when the current was applied, the proteins (that have a net negative charge) migrated and bound the membrane (Figure 2.3). Electroblotting was carried out for 90 minutes at 14 V.
Figure 2.3 View of the semi-dry electroblotting sandwich.

Membranes were subsequently blocked for one hour in Tris buffered saline (TBS) / 5% milk (Sigma, Dorset UK; Marvel skimmed powdered milk, Cadbury’s, UK). Incubation overnight at 4°C in TBS / 5% milk / 0.05% Tween20 (Sigma, Dorset, UK) followed with primary antibodies against the antigen of interest and a control protein raised in a different species (β-tubulin or Lamin-β1) as appropriate (Table 2.3).

Table 2.3 Primary antibodies used in western blotting.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody raised in</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR</td>
<td>Rabbit</td>
<td>ab74272 Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>SIK1</td>
<td>Rabbit</td>
<td>Ab64428 Abcam</td>
<td>1:500</td>
</tr>
<tr>
<td>B-Tubulin</td>
<td>Mouse</td>
<td>T4026 Sigma</td>
<td>1:1000</td>
</tr>
<tr>
<td>Lamin β1</td>
<td>Rabbit</td>
<td>Ab16048 Abcam</td>
<td>1:600</td>
</tr>
</tbody>
</table>

(Abcam, MA, USA; Sigma, Dorset, UK; Cell Signalling, MA, USA)

The following day, membranes were washed three times with TBS for 5 minutes each. Then the membranes were incubated (and protected from light from here onwards) for 1 hour at room temperature with differently-labelled anti-rabbit and anti-mouse secondary antibodies (as detailed in Table 2.4) each diluted 1:10000 in TBS / 5% milk / 0.05% Tween20. Membranes were washed three times for 5 minutes with TBS / 0.05% Tween20 and imaged as described in section 2.6.4.3.
below. This approach permitted simultaneous detection of two antigens with each one being identified by a distinct fluorescence signal.

**Table 2.4 Secondary antibodies used in western blotting.**

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Raised in</th>
<th>Conjugated to</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visualised in green</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-rabbit</td>
<td>Goat</td>
<td>Infra-Red Dye 800</td>
<td>611-132-122</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Rockland</td>
</tr>
<tr>
<td>donkey</td>
<td>Goat</td>
<td>Infra-Red Dye 800</td>
<td>926-32213</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LICOR</td>
</tr>
<tr>
<td><strong>Visualised in red</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-mouse</td>
<td>Goat</td>
<td>alexa fluor 680</td>
<td>A21058</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>donkey</td>
<td>Goat</td>
<td>IR Dye 680</td>
<td>926-68072</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>LICOR</td>
</tr>
</tbody>
</table>

*(Secondary antibodies used at 1:10000: Rockland Immunochemicals, PA, USA; LI-COR Biosciences, NE, USA; Molecular Probes, Invitrogen, CA, USA)*

### 2.6.4.3 Imaging and quantification

Membranes were scanned and the fluorescence of manually designated bands was detected and quantified using the Odyssey Infrared imaging system and Image Studio software (LI-COR Biosciences, NE, USA). Molecular weight markers were always included and these provided an estimate of the size of the bands detected. Protein levels were normalised to either a β-tubulin or Lamin-β1 control and presented as *protein fold expression*. Image montages were prepared using Powerpoint 2010 (Microsoft).
2.7 RNA analysis

All work involving RNA analysis described in this section was carried out using endonuclease-free water and filter-tips (pipetting).

2.7.1 RNA extraction

Total RNA was extracted from cell cultures using RNeasy™ Mini kit columns (Qiagen, Hilden, Germany) or using Tri-Reagent (Sigma, Dorset, UK) followed by the same RNeasy column clean-up.

*RNeasy column extraction:* RNA was extracted using RNeasy Mini kit columns as described in the manufacturers’ manual (Qiagen, Hilden, Germany). Briefly, 350µl of RLT Buffer containing 0.01M β-mercaptoethanol (Sigma, Dorset, UK) was added to each well of a 6 well plate to lyse the cells; the mixture was transferred to a 1.5ml tube and was frozen at -80°C for at least one hour. When thawed, 350µl of 70% ethanol was added, mixed by pipetting and the resultant mixture was added to the top of an RNeasy Mini column. Columns were centrifuged for 30 seconds at 8000g. The columns were washed with 350µl RW1 Buffer and centrifuged for 30 seconds at 8000g. To eliminate any genomic DNA, the column silica-gel membrane was incubated with DNase 1 (80µl of pre-mixed 10µl DNase 1 and 70µl RDD buffer) for 15 minutes at room temperature. The DNase was removed with 350µl RW1 Buffer and centrifugation for 30 seconds at 8000g. A first wash with 500µl of RPE Buffer and 30 seconds centrifugation at 8000g was followed by a second wash with 500µl RPE buffer and centrifugation for 2 minutes at 8000g. After each centrifugation step, the flow-through from each wash was discarded. An additional centrifugation step for 1 minute at 14000g was performed to remove any excess buffer. Finally, elution of the RNA was carried out by addition of 30µl of nuclease free water and 1 minute centrifugation at 8000g. The purified RNA was collected from the lower chamber, transferred to a 1.5ml tube and stored at -80°C.

*TRI-Reagent extraction:* To lyse the cells, 750µl of TRI-Reagent (Sigma, Dorset, UK) was added per well of a 6 well plate and transferred to a 1.5ml tube. Then, to each sample 250µl of chloroform was added, the samples were mixed by inversion 12 times and incubated at room temperature for 10 minutes to allow separation of the
phases. Samples were centrifuged at 13000g for 10 minutes at 4°C. The upper RNA-containing aqueous phase was transferred to a fresh 1.5ml labelled tube to which an equal volume of cold isopropanol (between 300 and 400μl) was added. The samples were mixed twice by inversion, then vortexed and allowed to rest for 15 minutes at room temperature. Centrifugation at 13000g followed for 15 minutes at 4°C. The supernatant was removed and the precipitate was vortexed briefly in 750μl of 75% ethanol then centrifuged again at 13000g for 10 minutes at 4°C. The wash supernatant was removed and the pellet re-suspended in 100μl of nuclease-free water. For column clean-up, 250μl of 100% ethanol was added and the solution was mixed well before transfer to an RNeasy Mini column. The RNeasy column extraction protocol was followed as described above and the purified RNA was stored at -80°C.

RNA from tissue biopsy samples was obtained from frozen RNALater®-immersed (Ambion, Life Technologies, CA, USA) samples (as described in section 2.2) and extracted using RNeasy columns. An approximate 100mg piece of tissue was homogenised (mixer mill MM301, Retsch, Dusseldorf, Germany) in a 2ml microcentrifuge tube with a steel bead and 350μl of Buffer RLT (Qiagen, Hilden, Germany) (containing 0.01M β-mercaptoethanol, Sigma, Dorset, UK). After a quick centrifugation to remove larger cell debris and bead, the RLT supernatant was processed as described above in the RNeasy column extraction protocol. Purified RNA was stored at -80°C.

**2.7.2 RNA quantification and preparation of cDNA**

Total RNA in purified samples was quantified using 1.3μl of each sample on an ND-1000 spectrophotometer zeroed against a nuclease-free water blank (Nanodrop technologies, DE, USA). Purity of the RNA was confirmed by an absorbance ratio of 260nm/280nm of ~2 (lower ratios would indicate DNA contamination).

Reverse transcription of RNA into cDNA was performed using the random-primers-based SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, CA, USA) (Marshall et al., 2011). Reactions of 20μl total volume were set up as follows: 4μl of the 5X VILO Reaction mix, 1μl of 10X SuperScript Enzyme Mix, 400ng of RNA and
nuclease-free water making up volume to 20μl. Control reactions were included in each batch, one with omission of the enzyme mix, another with omission of RNA. All samples were incubated for 10 minutes at 25°C, 60 minutes at 42°C followed by 5 minutes at 85°C.

2.7.3 Quantitative reverse transcriptase PCR (qRT-PCR)
Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is a method by which cDNA is assayed for target gene-specific sequences and provides a quantitative measure of the levels of target transcripts relative to an internal control.

Complimentary DNA samples prepared from total RNA were assayed for the relevant gene sequences using validated primers and probes on a 7900HT Taqman instrument (Applied Biosystems, CA, USA) with a standard real-time PCR protocol: initial step of denaturation at 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

In this quantitative PCR (qPCR) protocol, dual labelled fluorescent probes are used. The reporter dyes used were FAM (6-carboxyfluorescein) or VIC (proprietary, Applied Biosystems, CA, USA). The fluorescent reporter dyes (FAM or VIC) are attached to the 5’ end of a probe but this fluorophore is initially quenched by the close proximity of a non-fluorescent quencher molecule attached to the other end of the probe (3’ end). The probe is designed to anneal to the sequence between two primers.

The steps of each single qPCR are summarised in Figure 2.4. Each qPCR cycle begins with a high temperature double-stranded cDNA denaturing step. Next, the temperature lowers (60°C) permitting the annealing of the primers and the probe to their specific target sequences and serving as a guide for a Taq polymerase-dependent synthesis of complementary DNA. However, when the polymerase reaches the probe it cleaves it (inherent 5’ nuclease activity) resulting in the separation of the reporter dye from the quencher molecule (making the probes used in this qPCR method also known as hydrolysis probes). With the beginning of the next qPCR cycle the process recurs and with each cycle more reporter dye molecules
(with their fluorescence no longer repressed by the quencher) are released and the measured fluorescence signal increases (real-time measurements are recorded). The increase in fluorescent signal is proportional to the amount of amplicon (amplified product) synthesized.

Figure 2.4 Summary of the steps in a single cycle of quantitative PCR. During the annealing step, the primers and hydrolysis probe bind the specific target sequences. Complementary DNA synthesis displaces the probe and cleaves the reporter molecule from the probe. The free reporter fluoresces and is quantified (the signal being proportional to the amplified product). Figure from www.e-loigos.com, accessed April 2013.

2.7.3.1 Primer pair design and reaction validation

Using a web-based programme, gene-specific primers were designed and used in combination with hydrolysis probes of the Universal Probe Library (UPL, www.universalprobelibrary.com, Roche Diagnostics, Mannheim, Germany). The amplicon sequences were chosen to be intron-spanning and to pick up all gene splice variants of each gene. The UPL probes are labelled with reporter FAM and
proprietary quencher molecules. Table 2.5 details the primer pair sequences and UPL probes for all qPCR gene assays performed in this thesis.

Table 2.5 Details of primer pair sequences and UPL hydrolysis probes used in qPCR gene assays.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Left primer</th>
<th>Right primer</th>
<th>UPL probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putative androgen-regulated genes identified by microarray analysis (listed in alphabetical order)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AXIN2</td>
<td>NM_004655</td>
<td>CCACACCTTCTCCATCC</td>
<td>TGCCAGTTCTTTGGCTCTT</td>
<td>36</td>
</tr>
<tr>
<td>CDC25A</td>
<td>NM_001789</td>
<td>CTCGGAGTCACAGAGTCAGG</td>
<td>TTAAGGTTCCTTACTGTCAAC</td>
<td>2</td>
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<tr>
<td>ELK4</td>
<td>NM_001973</td>
<td>CAGTCCTGCTGCTCCCTAA</td>
<td>AATGCCCCATGACTGTCA</td>
<td>20</td>
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<tr>
<td>FOXL2</td>
<td>NM_023067</td>
<td>TCCAAATAAGAGGTTCTGCT</td>
<td>TCTCCAGATAGGAGGATT</td>
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</tr>
<tr>
<td>GADD45G</td>
<td>NM_006705</td>
<td>CAGCCAAGCTCTTGACGAG</td>
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<tr>
<td>ID3</td>
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<td>JMY</td>
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<td>LKB1</td>
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<td>MAP3K8</td>
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<td>CGCAAGAGGCTCTGAGTA</td>
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<td>MGC16121</td>
<td>NR_024607</td>
<td>CAAGCTCCTTGAAAGACCAA</td>
<td>AGATGCTGAGCCCTCAA</td>
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<td>NR4A2</td>
<td>NM_006186</td>
<td>TGAAGAGAGACGAGGAGGAGAC</td>
<td>AAAGCATTGGGAGTCCAG</td>
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<td>PRLR</td>
<td>NM_000949</td>
<td>CTGAGTGCAAGCAGTTAGT</td>
<td>GACAGCTGCTCCTCACCC</td>
<td>19</td>
</tr>
<tr>
<td>RG52</td>
<td>NM_002923</td>
<td>GAAAAGGAAGCCTCCAAAGAGA</td>
<td>TTCTGGGCAAGGTAAGCA</td>
<td>61</td>
</tr>
<tr>
<td>SIK1</td>
<td>NM_173354</td>
<td>CATCCCTCCTTGGTCTGA</td>
<td>GATCGGGCGAGGATGAT</td>
<td>77</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>NM_003043</td>
<td>CTTCAAGCAGCAAGGAGGAAG</td>
<td>CAGGTTTGAGGTAGGCAATG</td>
<td>5</td>
</tr>
<tr>
<td>SNCAIP</td>
<td>NM_005460</td>
<td>ACATTCCCTTGAGCCTACCT</td>
<td>CATTTCCTTTAGTGACTATGCTG</td>
<td>69</td>
</tr>
<tr>
<td>Decidualisation markers</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP1</td>
<td>Nm_000596</td>
<td>AATGGAGTTTTATCAGCACGAGACGAC</td>
<td>GGTAGACGCAGCAGGAGT</td>
<td>58</td>
</tr>
<tr>
<td>PRL</td>
<td>Nm_000948</td>
<td>AAAGGATCGACCAGGAAAG</td>
<td>GCACAGGACAGGGTTCAG</td>
<td>18</td>
</tr>
<tr>
<td>Steroid receptors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>Nm_000044</td>
<td>GCTTGGCTCCTAGGCTCAA</td>
<td>GGTCCATGCAAGGTAAAAATG</td>
<td>14</td>
</tr>
<tr>
<td>ESR1 (ERs)</td>
<td>Nm_000125</td>
<td>AACAGTGGCAGCATTGAAAAAA</td>
<td>TCTCCGGTCTCTCTGATC</td>
<td>69</td>
</tr>
<tr>
<td>PR</td>
<td>Nm_000926</td>
<td>TTTAAGAGGGCAATGGGAAGG</td>
<td>CGAATTTTTATCAAGGTGAC</td>
<td>11</td>
</tr>
<tr>
<td>Steroidogenesis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP19A1</td>
<td>Nm_031226</td>
<td>GAATTTGGAAGTCCTGAGTCT</td>
<td>TCATTATGGAACACTGAGGA</td>
<td>55</td>
</tr>
</tbody>
</table>

(see Appendix A for gene names in full)

Each primer pair and probe reaction was validated. For each gene assay, a curve was generated from a series of doubling dilutions of pooled cDNA template (from 1:10 to
1:160) and the slope of the linear curve was calculated by the SDS v2.3 software (Applied Biosystems, CA, USA). An example of a linear curve is presented in Figure 2.5. Slope values were approximately between -3.2 and -3.4. The slope value allowed the calculation of the PCR amplification efficiency (E = -1 + 10^{(-1/slope)}) which was within the ideal range of 95% and 105% for each gene assay.

![Figure 2.5 The qPCR standard curve plot – A FOXL2 gene assay curve, obtained in this study, is shown here as an example of the curves attained. The linear curve was generated for a series of doubling dilutions of pooled template cDNA (hESCs) by SDS v2.3 software (Applied Biosystems, CA, USA). The slope and $R^2$ values of the linear curve are displayed.](image)

### 2.7.3.2 qRTPCR protocol

Previous studies in our laboratory established the use of 18S as a suitable and stable housekeeping gene for investigations of gene expression in human endometrial tissues and endometrial cultured cells following exposure to steroid hormones including androgens (Marshall *et al.*, 2011).

The qRTPCR reactions were performed on a 384 well plate (Applied Biosystems, CA, USA). Single 10μl reactions (composed of 2.5μl diluted cDNA and 7.5μl reaction mix) were set up separately for each target gene of interest and the control endogenous 18S gene. The cDNA was added first to the plate and then the reaction mix. Sample reactions, standard dilution samples and controls were performed in duplicate.
Complimentary DNA was diluted 1 in 20 with water. In each plate run, pooled template dilutions were prepared for target gene and endogenous control standard curve generation (as in section 2.7.3.1).

The recipe for a single reaction (to detect either a target gene or the endogenous ribosomal 18S control gene) is detailed in the Table 2.6. Prior to use, the pre-mix of the 18S primers and VIC-labelled probe (Applied Biosystems, CA, USA) was prepared by diluting 53µl of each primer supplied (10µM) and 53µl of the probe (40nM) and 241µl nuclease-free water.

Table 2.6 Details of single qPCR reactions to assay target genes of interest and the endogenous ribosomal 18S control gene.

<table>
<thead>
<tr>
<th>Gene of interest</th>
<th>Stock solution</th>
<th>Volume, µl</th>
<th>(final concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X qPCR Supermix</td>
<td>5</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>Left primer (20µM)</td>
<td>0.12</td>
<td>(240nM)</td>
<td></td>
</tr>
<tr>
<td>Right primer (20µM)</td>
<td>0.12</td>
<td>(240nM)</td>
<td></td>
</tr>
<tr>
<td>UPL probe (10µM)</td>
<td>0.12</td>
<td>(120nM)</td>
<td></td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>2.14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted cDNA</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Endogenous control gene</th>
<th>Stock solution</th>
<th>Volume, µl</th>
<th>(final concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X qPCR Supermix</td>
<td>5</td>
<td>1X</td>
<td></td>
</tr>
<tr>
<td>18S primers and probe pre-mix</td>
<td>0.125</td>
<td>(each primer at 66nM) (probe at 16nM)</td>
<td></td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>2.375</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diluted cDNA</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(2X EXPRESS qPCR Supermix - Invitrogen, CA, USA)

2.7.3.3 qRTPCR data acquisition and analysis

Data were acquired within the exponential phase of the PCR amplification (Figure 2.6). From the standard curves generated, absolute gene levels were extrapolated by the SDS v2.3 software (Applied Biosystems, CA, USA). Data were extracted to excel format (Microsoft) where the target gene values were normalised to the endogenous control 18S values. Data were expressed as relative mRNA expression (relative to a pool of the cDNA and then, in some cases, calibrated against vehicle treated controls).
Chapter 2  General Materials and Methods

Figure 2.6 The exponential phase of qPCR amplification – FOXL2 gene assay curves, obtained in this study, are shown here as an example. Data were acquired by setting the amplification threshold within the exponential phase of the PCR amplification – the stage of the reaction where the amount of product doubles with each cycle (indicated by the area within the box). The curves were generated for a series of doubling dilutions of pooled template cDNA (hESCs) by SDS v2.3 software (Applied Biosystems, CA, USA).

2.8 Enzyme-linked immunosorbent assay (ELISA) for insulin-like growth factor binding protein 1 (IGFBP1)

Culture media was harvested from cell experiments and 100µl was assessed for levels of IGFBP1 using the human IGFBP1 ELISA kit (DY871, R&D Inc, Abingdon UK). A sandwich ELISA protocol was followed as detailed by the manufacturer and outlined in Figure 2.7. Reagent diluent (PBS / 5% Tween20, Sigma, Dorset, UK) was used to prepare a 2-fold serial dilutions of IGFBP1 protein for use as standards (from 000 to 15.8 pg/ml). All incubation steps were carried out on a shaking platform. The IGFBP1 capture antibody was prepared to final concentration of 2µg/ml in PBS. A Maxisorp™ flat-bottomed 96 well plate (Nunc, CA, USA) was coated by addition of 100µl per well of the antibody and incubation at room temperature overnight.
Figure 2.7 Diagram of the main steps of the sandwich ELISA protocol for the quantitative detection of IGFBP1 (target protein). Ab: antibody. HRP: horseradish peroxidase. Figure adapted from www.epitomics.com, accessed April 2013.

The next day, wells were aspirated and washed three times with wash buffer (PBS / 0.05% Tween20, Sigma, Dorset, UK). Non-specific binding sites were blocked for 4 hours at room temperature with 300μl of Reagent Diluent and then washed three times with wash buffer. Subsequently, the samples and IGFBP1 assay standards (100μl) were each added to wells in duplicate and incubated overnight at 4°C. The following day, the wells were washed three times with wash buffer and the detection antibody was prepared in 2% Normal Goat Serum / 5% Tween20 / PBS at 0.4μg/ml. Incubation with detection antibody was carried out for 2 hours at room temperature followed by three washes with wash buffer. Streptavidin-Horse radish peroxidase (HRP) was diluted 1 in 200 using reagent diluent as specified by kit instructions. Wells were incubated with Streptavidin-HRP (100μl per well) for 20 minutes at room temperature and protected from light. All further incubation steps were also carried out in the dark. After washing three times with wash buffer, 100μl of the substrate solution was added per well and incubated at room temperature for 20 minutes. Substrate solution was prepared by mixing Reagent A and Reagent B in equal volumes (3,3’,5,5’-tetramethylbenzidine; TMB substrate reagent set, BD BioSciences, CA, USA). The reaction was stopped by addition of 50μl per well of 2N H2SO4. Optical density was determined using a microplate spectrophotometer (ThermoMax microplate reader, Molecular Devices, CA, USA) set at 450nm with
wave correction at 540nm. Concentrations for samples were calculated by extrapolation from the standard curve by Softmax Pro Software (Molecular devices, CA, USA).

2.9 Statistical analysis
Prism 5 for Windows version 5.02, 2008 by Graphpad Software (La Jolla, CA, USA) was used to perform statistical analyses and graph production throughout this thesis. Values are presented as means ± standard error of the mean (SEM). Normally distributed data were assessed for statistical significance using Paired t-test and one-way ANOVA with Bonferroni’s multiple comparison post hoc test. If data did not pass normality testing then non-parametric tests were applied: Wilcoxon test (paired data), Mann-Whitney test (non-paired data), Kruskall-Wallis one-way analysis of variance with Dunn’s multiple comparison post hoc test or two-way ANOVA analysis. P values < 0.05 were considered significant. Statistical significant difference is indicated by a P value of <0.05 (*), P<0.01 (**) or P<0.001 (***).
Chapter 3: Identification of androgen regulated cell processes, pathways and networks in human endometrial stromal cells

3.1 Introduction

The role of androgens in endometrial (cyclic) function is yet to be fully elucidated. As reviewed in Chapter 1, observations from studies in humans and animals support a role for androgens in the human endometrium during the proliferative phase and during decidualisation. Both proliferation and differentiation (decidualisation) are two crucial cellular processes that are controlled by steroid hormones (oestrogen and progesterone) during the normal menstrual cycle. Proliferation in the endometrial stroma appears reduced compared to that in glandular epithelium as determined by immunodetection of the proliferation marker Ki-67 (Mertens et al., 2002). Immunodetection of Ki-67 in the stroma is reported greater in the functional layer than in the basal layer (critically with no significant change throughout the menstrual cycle, i.e. with changing oestrogen concentrations that drive epithelial proliferation) with the detection of Ki-67 being very weak or absent in the basal layer (Shiozawa et al., 1996, Mertens et al., 2002). Androgens, notably testosterone, have been shown to modulate the cellular processes of proliferation and differentiation in a variety of other tissues and cells and have also been extensively studied in males, e.g., in prostate cells and cancer (Gelmann, 2002, Vander Griend et al., 2007) and during male testes development (Yeh et al., 2002, Kerkhofs et al., 2009, Zhou et al., 2011).

Androgen regulation of cellular processes can be achieved through modulation of genomic gene expression via AR transcriptional activity (Verrijdt et al., 2000, Claessens et al., 2001, Verrijdt et al., 2003, Claessens et al., 2008, Denayer et al., 2010). In the human endometrium, AR protein expression has been detected in the stromal cells, with expression being most intense and most widespread in the proliferative phase of the menstrual cycle (Ito et al., 2002, Marshall et al., 2011). Thus, AR expression is present in stromal cells that exhibit low proliferation (compared to the glandular epithelial cells) and also undergo decidualisation.
Chapter 3 Identification of androgen regulated cellular processes and pathways

The presence of AR in hESCs prompts the question - what effects do androgens have upon the hESCs? Several studies have suggested that androgens may alter the behaviour/function of endometrial stromal cells (proliferating/non-decidualised) with impacts on gene expression associated with migration, adhesion, proliferation and apoptosis (Watson et al., 1998, Wen et al., 2006, Marshall et al., 2011) but DHT has only been shown to inhibit apoptosis and migration in functional assays (Marshall et al., 2011). During decidualisation of hESCs, AR-induced genes were reported to be predominantly implicated in cytoskeletal organisation, cell motility and cell cycle regulation in a microarray study (Cloke et al., 2008). Functional assays confirmed that DHT inhibited hESC migration and apoptosis possibly via AR and that lack of AR (AR knockdown) increased proliferation of decidualised hESCs (Cloke et al., 2008, Cloke et al., 2011).

Transcriptomic analysis has been extensively used to study androgen action in a number of tissues and cells, most notably in prostate stromal cells or immortalised prostate epithelial cell lines (Bolton et al., 2007, Tanner et al., 2011) resulting in the identification of many androgen-responsive genes and insight into androgen-regulated cell processes. This gene profiling approach combines the application of genomic microarray technology (genome-wide mRNA expression profiling) and functional gene expression analysis.

In animals and humans, gene profiling of endometrial tissues and cells has been successfully carried out (reviewed in section 1.5) but the specific application of gene profiling to study androgen or AR-mediated actions in the uterus is limited. The effect of DHT upon the endometrial transcriptome has been studied in animals such as rats and mice (Nantermet et al., 2005, Ivanga et al., 2009). In humans, one study used gene profiling to study the effects of the knockdown of AR during decidualisation of isolated hESCs and showed there were AR-induced genes distinct from PR-induced genes (Cloke et al., 2008). However, the effect of androgens on human proliferative phase endometrial tissues or isolated cells and has not been reported using microarrays.
Therefore, in this chapter, bioinformatic functional analyses of mRNA microarray-derived data were employed to dissect the effects of the androgen DHT upon hESCs derived from proliferative endometrium by identifying novel putative androgen-regulated gene lists, cellular processes, pathways and gene networks. Effectively, this chapter is question-led by the analysis of a microarray experiment (What are the effects of DHT upon hESCs?) in order to address the hypothesis:

“Androgens (DHT) affect gene expression in human endometrial stromal cells (isolated from proliferative phase endometrium) to alter proliferation and differentiation”.

Although both the androgens testosterone and DHT bind to the androgen receptor with high affinity to alter gene expression (Kemppainen et al., 1992), testosterone can be converted intracellularly by aromatase to oestradiol in endometrial cells (Tseng et al., 1984, Simpson et al., 1994, Gibson et al., 2013) which can then bind to and activate oestrogen receptors. Thus, in this thesis, the use of non-aromatisable DHT was preferred to testosterone to enable the study of androgen-AR-dependent genomic effects and minimise possible confounding effects due to concomitant oestrogenic signalling. The use of DHT would also permit comparison with previous studies conducted in hESCs (Wen et al., 2006, Marshall et al., 2011).

3.1.1 Aims of Chapter

- To verify AR protein expression in endometrial tissues (in vivo) and in stromal cells (in vitro, in primary hESCs and in an endometrial stromal cell line, SHT-290) by immunohistochemistry

- To demonstrate AR protein expression in both cell models in response to DHT by immunohistochemistry and Western blotting.

- To identify gene sets, cellular processes, pathways and networks regulated by DHT in primary hESCs isolated from proliferative endometrium using multiple bioinformatic analyses of a data set generated by analysis of an in vitro cell experiment previously carried out in our laboratory by Dr Elaine Marshall (post-doctoral research associate).
Chapter 3  Identification of androgen regulated cellular processes and pathways

3.2 Materials and Methods

This section describes all specific methods and outlines the experimental approaches employed in Chapter 3; general methodology was summarised in Chapter 2.

3.2.1 Endometrial biopsies

All endometrial tissues used in this chapter were collected under ethical approval as described in Chapter 2 section 2.2 and processed as described in sections 2.2 and 2.3.1. Table 3.1 summarises the clinical details for the women that donated the samples used in the following investigations. Less strict menstrual staging criteria were applied to samples used for hESC extraction and culture which allowed samples to serve as their own controls i.e., each cell sample was exposed simultaneously to a treatment (for example, DHT) and to a vehicle control.

Table 3.1 Clinical details for women that provided endometrial biopsies used in investigations carried out in Chapter 3 (fixed tissue biopsies and hESC isolations).

<table>
<thead>
<tr>
<th>Sample</th>
<th>LREC</th>
<th>Day of cycle</th>
<th>(E_2)</th>
<th>(P_4)</th>
<th>Histological dating</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>7</td>
<td>88</td>
<td>1.24</td>
<td>Proliferative</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>12</td>
<td>1105</td>
<td>1.91</td>
<td>Proliferative</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>14</td>
<td>1090</td>
<td>6.23</td>
<td>Proliferative</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>16</td>
<td>617.5</td>
<td>8.94</td>
<td>Proliferative</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
<td>21</td>
<td>242</td>
<td>53.1</td>
<td>Mid-secretory</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>23</td>
<td>331</td>
<td>83.50</td>
<td>Mid-secretory</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>n/a</td>
<td>680</td>
<td>45.85</td>
<td>Mid-secretory</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>LREC</th>
<th>Day of cycle</th>
<th>(E_2)</th>
<th>(P_4)</th>
<th>Histological dating</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>A</td>
<td>3</td>
<td>189</td>
<td>5.81</td>
<td>Probably Menstrual</td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>9</td>
<td>142</td>
<td>&lt; 3</td>
<td>Proliferative</td>
</tr>
<tr>
<td>10</td>
<td>C</td>
<td>11</td>
<td>339</td>
<td>&lt; 3</td>
<td>Proliferative</td>
</tr>
<tr>
<td>11</td>
<td>B</td>
<td>13</td>
<td>561</td>
<td>&lt; 3</td>
<td>Proliferative</td>
</tr>
</tbody>
</table>

Circulating levels of \(E_2\): Oestradiol (pmol/L) and \(P_4\): progesterone (nmol/L). LREC approval A) LREC05/S1103/14, B) LREC07/S1103/29 and C) LREC10/S1402/59. Women with benign gynaecological conditions such as caesarean section scar, endometriosis or fibroids less than 3cm diameter were included. All patients presented a history of heavy menstrual bleeding.

3.2.2 AR Immunohistochemistry on endometrial tissue

Endometrial tissue samples (4 proliferative and 3 mid-secretory, Table 3.1) were processed for embedding in paraffin wax as described in section 2.5.1.
Immunohistochemical detection of AR protein and negative controls (incubated with concentration matched rabbit IgG) was carried out on tissue sections and imaged as detailed in sections 2.5.3 and 2.5.4.

3.2.3 AR Immunocytochemistry on cultured hESCs and SHT-290 cells

hESCs extracted from proliferative endometrial samples (section 3.2.1) were cultured in media with 10% charcoal stripped serum-media for 2 to 4 days as described in section 2.3.1. Cells were seeded in chamber slides (0.5 x 10^5 cells per well of 2 well-chamber slides) and allowed to adhere overnight. Cells were serum starved for 24 hours, then were incubated with 10nM DHT or vehicle for 24 hours (section 2.4.1) and finally fixed (section 2.5.2). SHT-290 cells were seeded and treated in the same way (section 2.3.2). Immunocytochemistry for AR protein was carried out as described in sections 2.5.2 and 2.5.3. Negative controls consisted of concentration-matched rabbit IgG. Cells were imaged as described in section 2.5.4.

3.2.4 AR Western analysis of hESCs and SHT-290 cells treated with DHT and flutamide

hESCs isolated from mostly proliferative endometrial samples were cultured in 10% charcoal stripped serum media (section 2.3.1) for 2 to 4 days and were seeded in 100mm dishes (~5x10^5 cells) or 175cm^2 flasks (~15 x 10^5 cells) and allowed to adhere overnight. At 80-90% confluence, cells were serum starved for 24 hours and then treated for a further 24 hours with 10nM DHT or vehicle control (section 2.4.1). The experiment was repeated 3 times, each time with hESCs from 3 different patients.

SHT-290 cells were seeded and treated in the same way using their own media (section 2.3.2) and the experiment was repeated 6 times. Additionally, in three of these experiments, cells were also pre-treated with Flutamide (10 µM) for 1 hour before the addition of DHT or vehicle control (section 2.4.1).

LNCaP cells are an androgen receptor positive human epithelial cell line derived from a prostate adenocarcinoma (Horoszewicz et al., 1983). These cells were used as a positive control for the detection of AR protein. Cells were obtained from the
European Collection of Cell Cultures, reference 89110211 (www.hpacultures.org.uk/collections/ecacc.jsp). LNCaP cells were cultured in 175cm$^2$ flasks until confluent in RPMI medium containing 10% heat inactivated FBS, 1X MEM-Non-essential amino acid solution, 10mM HEPES, 1mM sodium pyruvate, 200U/ml penicillin and 100µg/ml streptomycin (reagents from Sigma, Dorset, UK).

Extraction of total cell proteins from hESCs, SHT-290 and LNCaP cell monolayers was carried out (section 2.6.1) and protein concentrations were quantified (section 2.6.3). Protein extracts (20µg) were subjected to SDS-PAGE separation, western transfer and immuno-detection that allowed quantification of AR and the control β-tubulin as described in section 2.6.4.

3.2.5 Microarray: experiment background, bioinformatic data analyses and gene annotation

3.2.5.1 Microarray experiment background and acknowledgements

*Before the start of this project, a gene expression microarray experiment was performed by a post-doctoral associate in our Laboratory, Dr Elaine Marshall (unpublished).* Endometrial tissues used in this experiment were collected under ethical approval as described in Chapter 2 section 2.2 and processed as described in sections 2.2 and 2.3.1. Endometrial stromal cells were only extracted from proliferative endometrial tissue samples strictly identified through consonant menstrual staging criteria (section 2.2) (Marshall et al., 2011). In brief, human endometrial stromal cells (hESC) were incubated in the presence of 10nM DHT for 2 and 8 hours (n=4 per treatment). The 2 and 8 hour timepoints were chosen based on previous studies using hESCs in our laboratory that showed a change in gene expression in response to DHT at these timepoints (Marshall et al., 2011). Time matched controls were vehicle-treated. RNA was purified, quantified and quality assessed (RNA integrity number above 7.5, 2100 Bioanalyser, Agilent, CA, USA) before being sent to the Finnish DNA Microarray Centre (FDMC), Turku Centre for Biotechnology (TCB) for gene expression analysis using the ‘whole genome’
Illumina Human HT-12 v3 expression beadchip platform (Illumina, Inc., USA). This array contained probes for more than 48000 transcripts.

The FDMC identified differentially expressed (DE, putative androgen-regulated genes) genes from the DHT vs Vehicle comparisons by stringent statistical analysis of the microarray data: Pre-processing consisted of quantile normalisation to remove technical variability between arrays. The differences between the sample groups were calculated by Limma methods designed for analysis of data arising from microarray experiments (Smyth, 2005) and included hierarchical cluster analysis (principle component analysis and heatmap, data not shown). From this statistical testing differentially expressed array targets were filtered based on statistical significance (P values or corrected P values) and fold-changes. Genes with a P value <0.01 were considered to be significantly differentially expressed in each of the 2 hours and 8 hours DHT vs Vehicle comparisons (putative androgen-regulated genes, see Appendix B for full gene lists).

3.2.5.2 Bioinformatic analyses of microarray gene expression – Gene Ontology and Pathway mining

Functional analysis of the microarray-derived putative androgen regulated gene lists (from the DHT vs Vehicle comparisons in hESCs at 2 and 8 hours) was conducted using three separate bioinformatic programs with advice from Dr Elaine Marshall: Pathway Express, GOrilla and Metacore (full details below). Analyses and all interpretation of results were carried out by myself.

Functional analyses consist of analyses of array expression results where functional annotations of the genes are taken in account. Firstly, the putative androgen-regulated genes (identified by the FDMC) were assembled in excel for submission to each software program, such as to include the gene identities in the appropriate format (Gene Symbol was used for GOrilla; RefSeq accession number was used for Pathway Express and Metacore), fold change values or direction of fold change.

Gene ontology (GO) analysis was performed using two software programmes that use distinct gene ontology databases: (i) GOrilla (http://cbl-gorilla.cs.technion.ac.il/) uses the GO terms database (Eden et al., 2009). GOrilla calculates a P value for each
GO term and ranks the terms accordingly. P value calculation depends on the number of genes (enrichment) associated with each GO term. P value threshold was set to < 0.001 to help reduce inherent redundancy in the enriched GO lists. (ii) Pathway-Express (http://vortex.cs.wayne.edu/projects.htm#Pathway-Express), uses the Kyoto encyclopaedia of genes and genomes (KEGG) terms database (Khatri et al., 2005). Pathway Express ranks each pathway by calculation of a probabilistic impact factor that incorporates parameters such as the proportion of differentially regulated genes represented in each pathway.

*Pathway mining* was also conducted with the evidence-based Metacore™ functional analysis software, version 6.13 build 19940 and 43450 (Thomson Reuters, NY, USA, formerly GeneGO Inc, MI, USA) (www.genego.com/metacore.php) that consists of the unique proprietary GeneGO database and specific Metacore algorithms. There are 650 canonical pathways (maps) and 110 cellular and molecular processes (process networks) defined and curated by GeneGO. Lists of the most populated pre-built pathway maps and process networks were generated by Metacore. Hypergeometric-based statistical relevance is calculated as p-value. A lower P value reflects a greater number of genes/proteins that belong to a pathway or process.

Metacore nodes or targets represent the individual genes of a data set or other interconnected genes. Pathway maps define signalling mechanisms (linked interconnected nodes) experimentally validated and accepted by the field of study. Pathway maps represent sections of networks. Networks describe biological function involving interconnecting pathways. Networks are a multidimensional representation of the data containing more information by showing more connections between genes that translate into the multifunctional activities of the proteins.

### 3.2.5.3 Bioinformatic analyses – discovery analyses of microarray-derived putative androgen-regulated genes (in hESCs at 8 hours)

*Application of additional Metacore gene enrichment algorithms to the putative androgen-regulated genes identified from the microarray 8 hours DHT vs Vehicle comparison (8 hours data set):* Two additional Metacore network building algorithms were applied to the 8 hour data set - Analyse Network and Analyse
Chapter 3 Identification of androgen regulated cellular processes and pathways

Transcription Regulation. As these analyses are best applied to larger data sets, they were not applied to the much smaller putative androgen-regulated gene list derived from the microarray 2 hours \textit{DHT vs Vehicle} comparison.

\textit{The Analyse Network algorithm} builds networks ‘on the fly’ (improvised and not yet predefined networks) based on the submitted data set. First this algorithm creates large networks (total nodes of 50) and then breaks them up into partially overlapping multiple sub-networks built with a sub-set of named five data-set genes. Networks and sub-networks are ranked by P value. The P value threshold is calculated for each object (protein) based on the number of interactions it has.

\textit{The Transcription Regulation algorithm} creates sub-networks based on data-set genes but the networks are centred on relationships with transcription factors and are ranked by P value. The legend for nodes and interactions between genes/proteins employed by Metacore are shown in Figure 3.1.
Figure 3.1 Legend for Metacore (GeneGO) networks featuring network objects, (some with added microarray data), object relationships and functional information. Directionality of the interactions between objects is denoted by the arrow. Adapted from GeneGO (www.genego.com/metacore.php).

3.2.6 Statistical analysis

Prism 5 for Windows version 5.02, 2008 by Graphpad Software (La Jolla, CA, USA) was used to perform statistical analyses. For bioinformatics analyses, statistical analysis is detailed specifically in each section above. In culture studies, data is presented as means ± standard error of the mean (SEM) with P < 0.05 considered statistically significant (section 2.9). AR protein levels in primary hESCs and SHT-290 cells were compared with Paired t-test and two-way ANOVA with Bonferroni’s multiple comparison post hoc test, respectively.
3.3 Results

3.3.1 AR protein is strongly expressed in endometrial stromal cells and primarily within the basal layer of the human endometrium

To confirm the presence and localisation of the AR protein in the endometrium immunohistochemistry was carried out on proliferative and mid-secretory phase endometrial tissues (see Figure 1.3 for endometrial tissue composition and structure).

AR was detected in endometrial tissues (Figure 3.2). AR protein expression was most intense in the nuclei of stromal cells of endometrium recovered from the proliferative phase and in the nuclei of stromal cells within the basal compartment of mid-secretory phase endometrium. Some of cells of the stroma did not exhibit positive immunostaining for AR in either the basal or functional layers of endometrial samples from both the proliferative and mid-secretory phase (Figure 3.2).
Proliferative endometrium

Secretory endometrium

Figure 3.2 AR protein was immunolocalised to the stromal cells of proliferative phase (throughout the endometrium) and of mid-secretory phase endometrium (mostly within the basal region). Representative images showing AR protein (stained brown) localised by IHC in human endometrial tissues. The insert figure (labelled *), highlights the presence of AR-negative and AR-positive cells in the stroma. Negative controls were stained with concentration matched Rabbit IgG. B-basal layer of endometrium, M-myometrium, F-functional layer of endometrium, ge-glandular epithelium, se-surface epithelium, st-stroma. Scale bars = 20, 50 or 100µM.
3.3.2 AR protein is expressed and is enhanced by DHT in primary hESCs and in the SHT-290 stromal cell line - establishment of appropriate cell models

To validate the use of primary hESCs and the SHT-290 stromal cell line as appropriate models to study AR-mediated androgen action, the detection (and quantification) of AR in cells incubated in the presence and absence of DHT was carried out on both cell types by immunocytochemistry and Western blotting.

3.3.2.1 AR protein is expressed in primary hESCs and is enhanced by DHT

Positive immunostaining for AR was observed in both the vehicle and DHT-treated hESCs (Figure 3.3). While vehicle-treated cells exhibited diffuse cytoplasmic staining, more intense and nuclear positive staining was observed after DHT treatment.

Figure 3.3 The AR protein is detected in primary hESCs. Cultures were incubated for 24 hours with vehicle (ethanol) or with $10^{-8}$ M DHT before immunostaining. AR protein (stained brown) was localised in both vehicle (cytoplasmic) and DHT-treated cells (mainly nuclear) suggesting AR nuclear translocation in the presence of DHT. Negative controls were incubated with Rabbit IgG. Scale bar = 50µM.
In order to quantify the AR protein levels in response to DHT in primary hESCs, Western analysis of whole cell extracts was performed. Full length AR was detected and quantification of the signals showed AR protein levels were significantly enhanced in a DHT-dependent manner (P<0.05) (Figure 3.4).

Figure 3.4 AR protein levels are significantly enhanced by DHT in cultured primary hESCs by Western analysis. Primary hESCs were cultured for 24 hours with vehicle (ethanol) or with DHT (10^{-8} M) before AR detection by Western blotting. Panel A: Representative fluorescent Western blot image. Lanes (1) SeaBlue marker; (2) vehicle-treated hESCs and (3) DHT-treated hESCs. As indicated by the arrows, AR is visualised in green and β-tubulin control in red. Panel B: Quantification of AR protein. Protein levels were normalised to β-tubulin control and presented as mean ± SEM (paired t-test, n=3). *P<0.05.

3.3.2.2 AR protein is expressed in the SHT-290 stromal cell line and is enhanced by DHT in an AR-dependent manner

To confirm the expression of AR protein in the SHT-290 cell line, cells treated with vehicle or DHT were stained for AR by immunocytochemistry. Positive staining for AR was observed in both the vehicle and DHT-treated cells (Figure 3.5). Vehicle-treated cells exhibited diffuse cytoplasmic staining whilst a more intense and nuclear signal was observed after DHT treatment consistent with ligand binding.
Figure 3.5 The AR protein is expressed and DHT-dependent nuclear translocation is observed in SHT-290 stromal cells. Cultures were incubated for 24 hours with vehicle (ethanol) or with $10^{-8}$ M DHT before immunostaining. AR protein (stained brown) was localised in both vehicle (in the cytoplasm) and DHT-treated cells (staining was weak and diffuse in the cytoplasm but intense in the nucleus). This staining pattern suggests AR nuclear translocation in the presence of DHT. Negative controls were incubated with Rabbit IgG. Scale bar = 50µM.

In order to quantify the AR protein concentrations in response to treatment with DHT, Western analysis of whole cell extracts was carried out. As a positive control for the detection of AR, the androgen-expressing LNCaP cells were included. Quantification showed that AR protein levels were significantly enhanced in a DHT-dependent manner in SHT-290 cells ($P<0.01$) (Figure 3.6).

To determine if the increase in AR protein level in response to DHT was AR-dependant, flutamide was used to inhibit the transcriptional activity of AR and the levels of AR protein were quantified by western analysis. Flutamide was chosen as it has been the AR antagonist commonly used in study the effects of DHT gene

Flutamide negated the DHT-dependent increase of AR protein in SHT-290 cells ($P<0.05$) (Figure 3.6).

\textbf{Figure 3.6 In SHT-290 cells, AR protein levels are enhanced by DHT in an AR-dependent manner.} AR protein was detected in response to DHT treatment in the absence and presence of flutamide (by Western analysis). Cells were pre-treated with flutamide ($10^{-5}$ M) or methanol (MeOH, vehicle) for 1 hour, or not pre-treated. Culture for 24 hours followed with ethanol (EtOH, vehicle for DHT) or with DHT ($10^{-8}$ M). \textbf{Panel A:} Representative fluorescent Western blot image. Lanes (1) SeaBlue plus2 marker; (2 to 7) treated SHT-290 cells; (8) positive control, AR-positive LNCaP cell extract. As indicated by the arrows, AR is visualised in green and β-tubulin control in red (lanes 2-8). \textbf{Panel B:} Quantification of AR protein in SHT-290 cells. Protein levels were normalised to β-tubulin control and presented as mean ± SEM ($n=3$ or 6). * $P<0.05$ and ** $P<0.01$. 

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3.3.3 Microarray analysis identified putative androgen regulated genes, permitting bioinformatic confirmatory and discovery identification of androgen-regulated cell processes, pathways and gene networks in primary hESCs

In this section, in order to attribute biological meaning to the filtered lists of putative androgen-regulated genes (the differentially expressed (DE) gene data sets provided by the FDMC; see section 3.2.5.1 for details), I subjected the data sets to functional bioinformatics analyses (with advice from Dr Elaine Marshall) to identify novel androgen-regulated pathways, cellular processes and gene networks in hESCs (see workflow presented in Figure 3.7).
Chapter 3  

Identification of androgen regulated cellular processes and pathways

**Figure 3.7 Workflow for Microarray data analyses, selection and study of putative DHT-regulated genes (in chapters 3 and 4).** The hESC experiment (in green) was carried out by Dr Elaine Marshall (hESCs treated with DHT or vehicle control for 2 and 8 hours). The Microarray experiment, data preprocessing, normalisation, statistical testing and filtering for Differentially Expressed genes (putative androgen-regulated genes) were performed by the Finnish DNA Microarray Centre, FDMC (shaded white). Functional bioinformatics analyses (advised by Dr Elaine Marshall) and all subsequent steps such as interpretation of the analyses, selection of candidates and their computational investigation were carried out by myself (shaded blue, contained within sections 3.3.3, 4.3.1, 4.3.2 and 4.3.4). The in vitro gene regulation studies were carried out by me (shaded grey, section 4.3.3).

* Beginning of the studies presented in Chapter 4.
Identification of androgen regulated cellular processes and pathways

3.3.3.1 Microarray analysis identified time-dependant putative androgen-regulated genes in hESCs at 2 hours and 8 hours

A summary of the significant differentially expressed (DE) gene sets (putative androgen-regulated genes from the hESC microarray *DHT vs Vehicle* comparisons at 2 and 8 hours) compiled by the FDMC is shown in Table 3.2 (see Appendix B for full DE gene lists). The putative androgen-regulated genes identified from the *DHT vs Vehicle* comparisons at 2 hours were distinct from those genes identified at 8 hours. Changes in expression of 34 genes were identified at the 2 hour timepoint with 35% of these being upregulated and 65% downregulated in response to DHT compared to vehicle controls. At the 8 hour timepoint a larger number of genes were differentially expressed (268) with 61% being upregulated and 39% downregulated. Fold change (FC) values for the DE genes were small, ranging between 1.2 and 2 fold (FC threshold was 1.2 and P value threshold was 0.01). Fold changes for genes outwith the DE gene sets did not exceed 2.029 (data not shown).

**Table 3.2 Summary of differentially expressed microarray targets – the putative androgen-regulated genes in hESCs identified by microarray analysis from DHT vs Vehicle comparisons (at 2 and 8 hours).**

<table>
<thead>
<tr>
<th>Differentially expressed genes (putative androgen-regulated genes)</th>
<th>Up</th>
<th>Down</th>
<th>Total</th>
<th>Fold change (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHT vs Vehicle</td>
<td>12</td>
<td>22</td>
<td>34</td>
<td>1.2 to 1.221</td>
</tr>
<tr>
<td>8 hours</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DHT vs Vehicle</td>
<td>163</td>
<td>105</td>
<td>268</td>
<td>1.2 to 2.029</td>
</tr>
</tbody>
</table>

Fold change threshold was 1.2. P value <0.01. Genes significantly differentially upregulated (Up) or downregulated (Down) by DHT compared with vehicle controls (hESCs). The hESC experiment was conducted by Dr Elaine Marshall and the differentially expressed gene filtering was provided by the FDMC.
3.3.3.2 Gene ontology-based analyses identified androgen-regulated biological processes in hESCs associated with the microarray-derived putative DHT-regulated genes at 8 hours but was less informative at 2 hours (using Pathway Express and Gorilla)

Time-dependent gene expression by androgens had been previously described in hESCs (Marshall et al., 2011) therefore, to identify androgen-regulated processes and pathways in the hESCs, functional analysis was carried out on the distinct putative androgen-regulated gene lists identified from the microarray DHT vs Vehicle comparisons at 2 hours and 8 hours (section 3.3.3.1) using gene ontology-based analyses, namely GOrilla and Pathway Express (carried out with the assistance of Dr. Elaine Marshall).

The significant GO and KEGG processes associated with the putative androgen-regulated gene lists (from the microarray DHT vs Vehicle comparisons at 2 hours and 8 hours) identified by GOrilla and Pathway Express analysis tools are summarised in Table 3.3. Both software packages extracted different cell processes associated with androgen regulation at 2 hours compared with 8 hours.

**Analysis of the androgen-regulated genes identified from the 2 hours DHT vs Vehicle comparison** by GOrilla did not produce any significant associated GO processes and Pathway Express predicted only one potentially androgen-regulated pathway i.e., biosynthesis of unsaturated fatty acids.

**Analysis of the androgen-regulated genes identified from the 8 hours DHT vs Vehicle comparison** by GOrilla produced 14 significant (P<0.001) cell processes potentially regulated by DHT. Six of the 14 processes were associated with regulation of gene transcription (highlighted in bold in Table 3.3). The remaining processes included those associated with metabolic and biosynthetic pathways. Prediction with Pathway Express ranked 43 distinct and more specific pathways and the top 20 pathways included: five signal transduction pathways (phosphatidylinositol signalling, Wnt signalling, calcium signalling, hedgehog signalling, MAPK signalling), two cell communication processes (adherens junction, focal adhesion), seven cancer pathways, 2 endocrine system pathways (melanogenesis and renin-angiotensin system), two immune system pathways and one cellular growth pathway (cell cycle).
The type of biological information extracted by each of the bioinformatics tools (at each timepoint) was distinct from one another. GOrilla produced a list for the 8 hours \textit{DHT vs Vehicle} gene list only constituted of general cell processes (not very specific) with some overlapping categories. On the other hand, because of the greater number of androgen-regulated genes (between 3 and 72) associated with each GOrilla-identified process there was a greater confidence in the results (lower P value). In comparison, KEGG pathway analysis (by Pathway Express) identified more defined (and smaller) pathways but only a limited number of putative androgen-regulated genes (between 1 and 4) were associated with each named KEGG pathway (at either 2 or 8 hours).
Table 3.3 Summary of androgen-regulated cell processes and pathways in hESCs identified by ontology-based analyses of microarray-derived putative androgen-regulated genes (at 2 and 8 hours).

<table>
<thead>
<tr>
<th>GO processes a (GOrilla)</th>
<th>KEGG pathway b (Pathway Express)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P value (P&lt;0.001)</strong></td>
<td>Impact factor rank and pathway</td>
</tr>
<tr>
<td><strong>2 hours DHT</strong></td>
<td></td>
</tr>
<tr>
<td>None identified</td>
<td>4.4 Biosynthesis of unsaturated fatty acids</td>
</tr>
<tr>
<td><strong>8 hours DHT</strong></td>
<td></td>
</tr>
<tr>
<td>regulation of gene transcription</td>
<td>18.2 Phosphatidylinositol signalling</td>
</tr>
<tr>
<td>regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process</td>
<td>10.9 Adherens junction</td>
</tr>
<tr>
<td>regulation of macromolecule biosynthetic process</td>
<td>5.4 Prostate cancer</td>
</tr>
<tr>
<td>regulation of cellular biosynthetic process</td>
<td>5.2 Colorectal cancer</td>
</tr>
<tr>
<td>regulation of nitrogen compound metabolic process</td>
<td>4.3 Non-small cell lung cancer</td>
</tr>
<tr>
<td>regulation of transcription</td>
<td>3.8 Wnt signalling pathway</td>
</tr>
<tr>
<td>regulation of biosynthetic process</td>
<td>3.8 Calcium signalling pathway</td>
</tr>
<tr>
<td>regulation of macromolecule metabolic process</td>
<td>3.7 Focal adhesion</td>
</tr>
<tr>
<td>regulation of primary metabolic process</td>
<td>3.6 Melanogenesis</td>
</tr>
<tr>
<td>regulation of RNA metabolic process</td>
<td>3.3 Cell cycle</td>
</tr>
<tr>
<td>regulation of cellular metabolic process</td>
<td>3.2 T cell receptor signalling</td>
</tr>
<tr>
<td>regulation of transcription, DNA-dependent</td>
<td>3 Endometrial cancer</td>
</tr>
<tr>
<td>regulation metabolic process</td>
<td>3 Acute myeloid leukemia</td>
</tr>
<tr>
<td>mRNA splice site selection</td>
<td>2.9 Pathways in cancer</td>
</tr>
<tr>
<td></td>
<td>2.9 Hedgehog signalling</td>
</tr>
<tr>
<td></td>
<td>2.7 Vibrio cholera infection</td>
</tr>
<tr>
<td></td>
<td>2.7 Renin-angiotensin system</td>
</tr>
<tr>
<td></td>
<td>2.6 B cell receptor signalling</td>
</tr>
<tr>
<td></td>
<td>2.5 MAPK signalling pathway</td>
</tr>
</tbody>
</table>

GO, gene ontology. KEGG, Kyoto encyclopaedia of genes and genomes. Statistically significant processes and pathways ranked top by Gorilla and Pathway Express analyses: a) Gorilla uses GO terms ranked by P value (P<0.001). Between 3 and 72 putative androgen-regulated genes were associated with each listed GO process. b) Pathway Express uses KEGG terms/pathways ranked by impact factor. Between 1 and 4 putative androgen-regulated genes were associated with each KEGG pathway. A higher impact factor reflects a combination of a higher number of associated putative androgen-regulated genes while taking in account the total number of genes in each given pathway.
Chapter 3 Identification of androgen regulated cellular processes and pathways

3.3.3.3 Metacore functional-based analysis identified pathway maps and process networks associated with androgen regulation in hESCs from the microarray-derived putative androgen-regulated genes (at 2 and 8 hours)

Metacore software (GeneGO) is different from other gene ontology-based analysis software (such as the Gorilla and Pathway Express tools, used above in section 3.3.3.2) in that it also takes into account the known relationships between the genes of any given data set. Moreover, Metacore uses GeneGO’s database that is curated manually and separately to GO term databases excluding many inferred or weak annotations (Henderson-Maclennan et al., 2010).

Therefore, to gain further insight into androgen-dependent cell pathways and putative networks in hESCs the relevant biological information was extracted from the microarray-derived putative androgen-regulated genes (2 hour and 8 hour DHT gene data sets) by analysis using the functional analysis tool Metacore (GeneGO). In these analyses, Metacore overlayed the submitted expression data onto pre-established canonical pathways and process networks curated by Metacore/GeneGO.

The Metacore confirmatory data mining procedures resulted in lists of the most populated Metacore/GeneGo curated pre-built Pathway Maps and Process Networks by the data set interrogated (for each 2 and 8 hours DHT timepoint, see Table 3.4 and Table 3.5, respectively). Greater significant results (P<0.05) were obtained from the putative androgen-regulated genes at 8 hours than at 2 hours, consistent with a reduced number of genes in the 2 hour data set.

Androgen-dependent regulation in hESCs (at 2 hours DHT) was associated with some statistically significant pathway maps (P<0.05) (Table 3.4). Pathway maps identified were concerned with metabolism (vitamins, lipids), development (glucocorticoid receptor signalling, myelin-associated glycoprotein signalling), apoptosis/survival (p53-dependent), DNA damage (transcription regulation by BRCA1) and immune responses (cytokine signalling). In the case of associated process networks, only one process network was statistically significant (P<0.05) and it was an apoptosis network.
In contrast, androgen-dependent regulation in hESCs (at 8 hours DHT) was associated with different statistically significant pathway maps (P<0.05) (Table 3.5) including three pathways concerned with transcription (Sin3/NuRD, HIF-1 and ESR1 (ER-alpha)/SP1), four pathway maps concerned with cell cycle and development regulation, one signal transduction pathway (JNK) and two metabolism pathway maps (vitamin and phosphatidylinositol). Statistically significant process networks (P<0.05) identified included two signal transduction (ESR1), three cell cycle, two transcription, two protein processing regulation and one chemotaxis process network.

Overall, Metacore functional analysis revealed androgen-regulation in hESCs was associated with different pathway maps and process networks in a time-dependant way (2 hours compared with 8 hours DHT treatment). Although specific pathways and processes did not overlap over time during androgen treatment, processes linked with, for example, metabolism and apoptotis/survival were identified throughout.

Table 3.4 Androgen-regulated GeneGO pathways and process networks in hESCs identified by Metacore analysis of microarray-derived putative androgen-regulated genes at 2 hours.

<table>
<thead>
<tr>
<th>2 hours DHT</th>
<th>Top GeneGo Pathway Maps</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vitamin K metabolism</td>
<td>0.01542</td>
</tr>
<tr>
<td></td>
<td>Ascorbate metabolism</td>
<td>0.01542</td>
</tr>
<tr>
<td></td>
<td>Development_Glucocorticoid receptor signalling</td>
<td>0.01761</td>
</tr>
<tr>
<td></td>
<td>Apoptosis and survival_p53-dependent apoptosis</td>
<td>0.02125</td>
</tr>
<tr>
<td></td>
<td>DNA damage_Brca1 as a transcription regulator</td>
<td>0.02197</td>
</tr>
<tr>
<td></td>
<td>Immune response_IL-3 activation and signalling pathway</td>
<td>0.0227</td>
</tr>
<tr>
<td></td>
<td>Ascorbate metabolism / Rodent version</td>
<td>0.02487</td>
</tr>
<tr>
<td></td>
<td>Development_MAG-dependent inhibition of neurite outgrowth</td>
<td>0.02704</td>
</tr>
<tr>
<td></td>
<td>Immune response_IL-7 signalling in B lymphocytes</td>
<td>0.03137</td>
</tr>
<tr>
<td></td>
<td>Triacylglycerol metabolism part1</td>
<td>0.04283</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2 hours DHT</th>
<th>Top GeneGo Process Networks</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Apoptosis_Apoptotic mitochondria</td>
<td>0.0437</td>
</tr>
</tbody>
</table>
Table 3.5 Androgen-regulated GeneGO pathways and process networks in hESCs identified by Metacore analysis of microarray-derived putative androgen-regulated genes at 8 hours.

<table>
<thead>
<tr>
<th>Top GeneGo Pathway Maps</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcription_Sin3 and NuRD in transcription regulation</td>
<td>0.001785</td>
</tr>
<tr>
<td>Cell cycle_Regulation of G1/S transition (part 1)</td>
<td>0.001785</td>
</tr>
<tr>
<td>Development_Growth hormone signalling via PI3K/AKT and MAPK cascades</td>
<td>0.002385</td>
</tr>
<tr>
<td>Signal transduction_JNK pathway</td>
<td>0.002553</td>
</tr>
<tr>
<td>Development_ERK5 in cell proliferation and neuronal survival</td>
<td>0.009341</td>
</tr>
<tr>
<td>Transcription_Role of Akt in hypoxia induced HIF1 activation</td>
<td>0.01275</td>
</tr>
<tr>
<td>Transcription_Ligand-dependent activation of the ESR1(ERα)/SP1 pathway</td>
<td>0.01561</td>
</tr>
<tr>
<td>Vitamin B6 metabolism</td>
<td>0.01561</td>
</tr>
<tr>
<td>Cell cycle_Role of APC in cell cycle regulation</td>
<td>0.01765</td>
</tr>
<tr>
<td>Phosphatidylinositol metabolism</td>
<td>0.01801</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Top GeneGo Process Networks</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signal transduction_ESR1(ERα)-nuclear pathway</td>
<td>0.004152</td>
</tr>
<tr>
<td>Signal transduction_ESR1(ERα)-membrane pathway</td>
<td>0.009726</td>
</tr>
<tr>
<td>Cell cycle_G1-S Growth factor regulation</td>
<td>0.009769</td>
</tr>
<tr>
<td>Transcription_mRNA processing</td>
<td>0.01882</td>
</tr>
<tr>
<td>Protein folding_Folding in normal condition</td>
<td>0.02761</td>
</tr>
<tr>
<td>Protein folding_Response to unfolded proteins</td>
<td>0.02874</td>
</tr>
<tr>
<td>Cell cycle_G1-S Interleukin regulation</td>
<td>0.03068</td>
</tr>
<tr>
<td>Cell cycle_G0-G1</td>
<td>0.03092</td>
</tr>
<tr>
<td>Transcription_Chromatin modification</td>
<td>0.03479</td>
</tr>
<tr>
<td>Chemotaxis</td>
<td>0.04297</td>
</tr>
</tbody>
</table>

3.3.3.4 Metacore discovery analysis identified cellular process networks, gene sub-networks and transcription factors associated with androgen regulation in hESCs from the microarray-derived putative androgen-regulated genes at 8 hours

To gain further insight into androgen-regulated gene networks in hESCs, the microarray putative androgen-regulated genes were interrogated with other methods of assessing the connectivity (possible relationships) within a given data set - Analyse Networks and Transcription Factor algorithms (the Metacore enrichment analysis algorithms) – a discovery type of analysis that generates new networks. A workflow for this section is presented in Figure 3.8. The putative 8 hour androgen-regulated gene list was specifically analysed with these algorithms as these are best applied to larger sets of data (with more than 50 genes) and consequently the 2 hour
androgen-regulated data set (comprised of 34 genes as shown in previous Table 3.2) was considered too small.

![Diagram of workflow for section 3.3.3.4](image)

**Figure 3.8 Workflow for section 3.3.3.4 – Analysis of the putative androgen-regulated gene list (derived from the microarray 8 hours DHT vs vehicle comparison) using Metacore discovery algorithms.**

### 3.3.3.4.1 The Metacore Analyse Networks algorithm identified process networks and sub-networks associated with androgen regulation in hESCs (at 8 hours)

The specific new networks built on request by the Analyse Networks algorithm using the 8 hour DE data set are listed in Table 3.6 (with assigned statistical relevancy, $P \leq 3.29 \times 10^{-23}$ and gScore). Between 14 and 21 genes of the 8 hour DE data set were populated in each built network but only the top 3 of the 5 networks had canonical pathways represented within. The known cell processes associated with the built networks included cellular response to stimuli, proliferation/apoptosis (mitochondrial), cell cycle, caspase/peptidase activity and developmental processes.
Table 3.6 The 5 most relevant androgen-regulated networks in hESCs identified by Metacore from the microarray putative androgen-regulated gene list (8 hours DHT) ranked by gScore (Analyze Networks algorithm).

<table>
<thead>
<tr>
<th>Processes</th>
<th>Number of Targets</th>
<th>Number of Pathways</th>
<th>P value</th>
<th>zScore</th>
<th>gScore</th>
</tr>
</thead>
<tbody>
<tr>
<td>response to organic substance, response to chemical stimulus, response to endogenous stimulus</td>
<td>17</td>
<td>31</td>
<td>5.60e-30</td>
<td>40.54</td>
<td>79.29</td>
</tr>
<tr>
<td>Negative regulation of neuroblast proliferation, mitochondrion organisation, apoptotic mitochondrial changes</td>
<td>21</td>
<td>2</td>
<td>1.07e-39</td>
<td>51.28</td>
<td>53.78</td>
</tr>
<tr>
<td>Cell cycle, regulation of cell cycle, cell cycle process</td>
<td>15</td>
<td>5</td>
<td>2.65e-25</td>
<td>34.99</td>
<td>41.24</td>
</tr>
<tr>
<td>Activation of caspase activity, regulation of caspase activity, positive regulation of peptidase activity</td>
<td>15</td>
<td>0</td>
<td>2.65e-25</td>
<td>34.99</td>
<td>34.99</td>
</tr>
<tr>
<td>Negative regulation of biological process, negative regulation of cellular process, developmental process</td>
<td>14</td>
<td>0</td>
<td>3.29e-23</td>
<td>32.63</td>
<td>32.63</td>
</tr>
</tbody>
</table>

Targets are the number of genes from the interrogated data set (8 hour DE gene set) that are populated in the network. Total nodes in each network = 50. Pathways are identified when target genes overlay at the beginning and at the end of a pathway. A high z score reflects a high saturation of the network with genes from the experiment. The gScore is calculated based on the relative number of targets from the DE gene list represented in a network, the zScore, and on the relative saturation of the network with canonical pathways.

Table 3.7 lists the top significant androgen regulated GeneGO sub-networks in hESCs (at 8 hours DHT) ranked by P value (P≤1.67x10^-17). In summary, the cell processes that were over-represented in association with the sub-networks (that may represent novel gene interactions) were cell death, cell differentiation, response to stimuli and regulation of transcription. The cell processes associated with the sub-networks partially overlap with the processes identified for the large Networks (previous Table 3.6).

Interestingly, using only five named genes to construct each sub-network, Metacore was able to connect a minimum of 3 and up to 22 other genes (root nodes) from the original putative androgen-regulated gene data-set to or within each sub-network.
This observation reinforces the connectivity level between the identified putative androgen-regulated genes (Table 3.7). In addition, some of the named genes re-occur in more than one sub-network with distinct associated cell processes showing the interplay between the processes that may be regulated by androgens in hESCs. For example, TPL2/MAP3K8 protein appears in eight different sub-networks with associated processes varying from regulation of transcription, regulation of apoptosis/cell death and response, post-translational protein modification to stimulus/cell differentiation.
Table 3.7 The top 30 androgen-regulated Metacore Process Sub-networks in hESCs built based on sub-sets of five genes from the microarray putative androgen-regulated gene set (8 hours DHT) and ranked by P value. Genes highlighted in blue were further investigated. Total nodes in each network set to 50. Root nodes are the number of genes from the interrogated data set that are populated in the network. GO, gene ontology.

<table>
<thead>
<tr>
<th>Sub-network</th>
<th>GO Processes</th>
<th>Root nodes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ErB2, CAPER, RARalpha, HSP105, VRK3</td>
<td>response to organic substance, response to chemical stimulus, response to stress, response to endogenous stimulus, multi-organism process</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>INPP4B, UTX, VHL, OCRL, MGST</td>
<td>negative regulation of neuroblast proliferation, regulation of protein stability, negative regulation of transcription, positive regulation of histone deacetylation, negative regulation of gene expression</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>SFRS1 (SF2), TGT, CBX7, IRAP, Pinin</td>
<td>negative regulation of cellular metabolic process, response to unfolded protein, negative regulation of biological process, negative regulation of cellular process, negative regulation of metabolic process</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>RARalpha, Suv39H1, FoxL2, ATF-4, TPL2(MAP3K8)</td>
<td>negative regulation of cellular process, negative regulation of biological process, positive regulation of nitrogen compound metabolic process, positive regulation of biological process, regulation of transcription, DNA-dependent</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Geminin, HOXD10, Tankyrase 1, RFX3, WDR5</td>
<td>regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, regulation of nitrogen compound metabolic process, regulation of cellular biosynthetic process, DNA metabolic process, regulation of biosynthetic process</td>
<td>12</td>
</tr>
<tr>
<td>6</td>
<td>GATA-2, RARalpha, FoxL2, CAPER, SFRS1 (SF2)</td>
<td>response to external stimulus, response to stress, response to nutrient levels, multi-organism process, response to extracellular stimulus</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>ATP1A1, FoxL2, NURR1, VHL, HKG(MAP4K4)</td>
<td>protein kinase cascade, post-translational protein modification, positive regulation of molecular function, positive regulation of catalytic activity, protein amino acid phosphorylation</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>A20, SLC6A6, TDG, Suv39H1, ZNF306</td>
<td>Apoptosis, programmed cell death, regulation of apoptosis, regulation of programmed cell death, regulation of cell death</td>
<td>12</td>
</tr>
<tr>
<td>9</td>
<td>FOLC, CX3CR1, HPLC2, CD79B, CRLR</td>
<td>branched chain family amino acid catabolic process, branched chain family amino acid metabolic process, carboxylic acid catabolic process, organic acid catabolic process, o xoacid metabolic process</td>
<td>7</td>
</tr>
<tr>
<td>10</td>
<td>CDC25A, CUGBP1, TRPC5, FoxL2, U1-70K</td>
<td>regulation of cell cycle, interphase of mitotic cell cycle, interphase, positive regulation of cell cycle, senescence</td>
<td>10</td>
</tr>
<tr>
<td>11</td>
<td>ING2, DDx6, CDC25A, QSCN6, RARalpha</td>
<td>regulation of metabolic process, regulation of cellular metabolic process, regulation of biosynthetic process, regulation of biological process, biological regulation</td>
<td>11</td>
</tr>
</tbody>
</table>
### Table 3.7 continued.

<table>
<thead>
<tr>
<th>Sub-network</th>
<th>GO Processes</th>
<th>Root nodes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 TPL2(MAP3K8), Septin 4, NURR1, TGT, Geminin</td>
<td>regulation of apoptosis, regulation of programmed cell death, regulation of cell death, positive regulation of apoptosis, positive regulation of programmed cell death</td>
<td>11</td>
<td>1.67e-17</td>
</tr>
<tr>
<td>13 CDC25A, TPL2(MAP3K8), A20, Suv39H1, ATF-4</td>
<td>post-translational protein modification, regulation of cellular process, biological regulation, protein amino acid phosphorylation, regulation of biological process</td>
<td>11</td>
<td>1.67e-17</td>
</tr>
<tr>
<td>14 FoxL2, ASEF2, Osteopontin, Suv39H1, SFRS1 (SF2)</td>
<td>regulation of developmental process, negative regulation of biological process, negative regulation of cellular process, regulation of apoptosis, regulation of programmed cell death</td>
<td>11</td>
<td>1.67e-17</td>
</tr>
<tr>
<td>15 Osteopontin, A20, TPL2(MAP3K8), HOXD10, HGK(MAP4K4)</td>
<td>positive regulation of cellular process, regulation of developmental process, positive regulation of biological process, regulation of apoptosis, regulation of programmed cell death</td>
<td>11</td>
<td>1.67e-17</td>
</tr>
<tr>
<td>16 STARD5, ISG15, ATF-4, HSP40, Cortactin</td>
<td>multi-organism process, interspecies interaction between organisms, protein kinase cascade, regulation of localisation, positive regulation of biological process</td>
<td>11</td>
<td>1.67e-17</td>
</tr>
<tr>
<td>17 JunB, GATA-2, CBX7, RARalpha, CDC25A</td>
<td>developmental process, response to steroid hormone stimulus, response to chemical stimulus, response to endogenous stimulus, cell differentiation</td>
<td>10</td>
<td>1.42e-15</td>
</tr>
<tr>
<td>18 ATP1A1, Cortactin, HGK(MAP4K4), HSP40, NEFL</td>
<td>localisation, membrane to membrane docking, multi-organism process, response to chemical stimulus, response to stimulus</td>
<td>10</td>
<td>1.42e-15</td>
</tr>
<tr>
<td>19 A20, WDR5, SFRS1 (SF2), Geminin, VHL</td>
<td>biological regulation, regulation of biological process, regulation of cellular process, positive regulation of cellular process, positive regulation of biological process</td>
<td>10</td>
<td>1.42e-15</td>
</tr>
<tr>
<td>20 ErbB2, Cortactin, HOXD10, Hsc20, PAXIP1L</td>
<td>positive regulation of biological process, anatomical structure morphogenesis, multicellular organismal development, positive regulation of cellular process, cell differentiation</td>
<td>9</td>
<td>3.860e-14</td>
</tr>
<tr>
<td>21 RARalpha, GATA-2, TPL2(MAP3K8), ATF-4, TDG</td>
<td>positive regulation of biological process, positive regulation of cellular biosynthetic process, positive regulation of biosynthetic process, positive regulation of transcription, DNA-dependent, positive regulation of transcription</td>
<td>9</td>
<td>1.070e-13</td>
</tr>
<tr>
<td>22 yrdC, PABP2, SSB-2, ELF2, SLC12A6</td>
<td>monoamine transport, catecholamine transport, amine transport, positive regulation of defense response to virus by host, neurotransmitter transport</td>
<td>8</td>
<td>2.890e-12</td>
</tr>
<tr>
<td>23 A20, PNRC2, NURR1, TPL2(MAP3K8), HGK(MAP4K4)</td>
<td>regulation of programmed cell death, regulation of cell death, regulation of apoptosis, negative regulation of programmed cell death, negative regulation of cell death</td>
<td>8</td>
<td>7.090e-12</td>
</tr>
</tbody>
</table>
### Table 3.7 continued.

<table>
<thead>
<tr>
<th>Sub-network</th>
<th>GO Processes</th>
<th>Root nodes</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 TPL2(MAP3K8), GATA-2, Bcl-W, ErbB2, HGK(MAP4K4)</td>
<td>regulation of programmed cell death, regulation of cell death, regulation of apoptosis, apoptosis, programmed cell death</td>
<td>7</td>
<td>4.040e-10</td>
</tr>
<tr>
<td>25 TBXA2R, IRAP, IMPA1, UBR2, Bcl-W</td>
<td>ion homeostasis, positive regulation of phospholipase activity, regulation of phospholipase activity, positive regulation of lipase activity, cell communication</td>
<td>5</td>
<td>7.900e-08</td>
</tr>
<tr>
<td>26 GATA-2, RARalpha, TPL2(MAP3K8), CUGBP1, ASEF2</td>
<td>positive regulation of biological process, response to endogenous stimulus, cell differentiation, response to hormone stimulus, cellular developmental process</td>
<td>5</td>
<td>7.91e-07</td>
</tr>
<tr>
<td>27 TIF1-gamma, Elk-4, Osteopontin, PAXIP1L, WDR5</td>
<td>positive regulation of macromolecule metabolic process, positive regulation of biological process, positive regulation of metabolic process, regulation of developmental process, developmental process</td>
<td>5</td>
<td>7.91e-07</td>
</tr>
<tr>
<td>28 ING2, Tankyrase 1, Septin 4, HGK(MAP4K4), SLC6A6</td>
<td>regulation of programmed cell death, regulation of cell death, regulation of apoptosis, apoptosis, programmed cell death</td>
<td>5</td>
<td>7.91e-07</td>
</tr>
<tr>
<td>29 RARalpha, GATA-2, NURR1, ISG15, SP1</td>
<td>regulation of developmental process, response to endogenous stimulus, positive regulation of nitrogen compound metabolic process, positive regulation of cellular process, positive regulation of biological process</td>
<td>4</td>
<td>2.60e-05</td>
</tr>
<tr>
<td>30 HOXB2, Carbonic anhydrase VB (mitochondrial), DCTD, Cl((^{-})) intracellular, Cl((^{-})) extracellular region</td>
<td>rhombomere 4 development, cranial nerve structural organisation, facial nerve structural organisation, facial nerve morphogenesis, anatomical structure arrangement</td>
<td>3</td>
<td>2.96e-04</td>
</tr>
</tbody>
</table>
3.3.3.4.2 The Metacore Transcription Factor algorithm identified transcription factors associated with androgen regulation in hESCs

Application of the discovery Transcription Factor algorithm (Metacore) to the 8 hour DHT data set identified twenty transcription factors with the most known relationships with the putative androgen-regulated genes (root nodes) \((P \leq 5.79 \times 10^{-22}, \text{Table 3.8})\). The GO processes associated with these relationships are also detailed and reflect the varied roles the TFs are involved in such as proliferation, programmed cell death, differentiation, development and metabolism. The number of putative 8 hour microarray androgen-regulated genes present in each network ranged between 33 and 9 genes. This represents a minimum of between 12.3% and 3.4% of the total number of genes of the data set that have known relationships with each of the TFs identified. For example, approximately 12.3% and 4.9% of genes of the data set have known relationships with the TFs c-Myc and AR, respectively.
Chapter 3

Identification of androgen regulated genes

Table 3.8 Transcription factor-centred networks identified from putative androgen-regulated genes in hESCs (at 8 hours DHT) by Metacore (ranked by p value).

<table>
<thead>
<tr>
<th>Transcription Factor</th>
<th>GO Processes</th>
<th>Root nodes</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 c-Myc</td>
<td>mRNA splice site selection, female pregnancy, regulation of cellular biosynthetic process, decidualisation, chromatin organisation</td>
<td>33</td>
<td>1.14e-81</td>
</tr>
<tr>
<td>2 SP1</td>
<td>positive regulation of biological process, negative regulation of apoptosis, negative regulation of programmed cell death, regulation of multicellular organisinal process</td>
<td>31</td>
<td>1.28e-76</td>
</tr>
<tr>
<td>3 ESR1 (ERα)</td>
<td>negative regulation of transcription, negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, negative regulation of nitrogen compound metabolic process, negative regulation of macromolecule biosynthetic process</td>
<td>20</td>
<td>4.50e-49</td>
</tr>
<tr>
<td>4 p53</td>
<td>positive regulation of cellular process, negative regulation of transcription, regulation of gene expression, positive regulation of macromolecule metabolic process, positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process</td>
<td>20</td>
<td>4.50e-49</td>
</tr>
<tr>
<td>5 E2F1</td>
<td>cellular metabolic process, regulation of cell cycle, primary metabolic process, cell cycle, cellular macromolecule metabolic process</td>
<td>17</td>
<td>1.25e-41</td>
</tr>
<tr>
<td>6 RUNX2</td>
<td>skeletal system development, regulation of RNA metabolic process, regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process, regulation of transcription, DNA-dependent</td>
<td>15</td>
<td>1.10e-36</td>
</tr>
<tr>
<td>7 CREB1</td>
<td>generation of neurons, neurogenesis, nervous system development, regulation of cell morphogenesis involved in differentiation, regulation of anatomical structure morphogenesis</td>
<td>14</td>
<td>3.20e-34</td>
</tr>
<tr>
<td>8 Androgen receptor</td>
<td>negative regulation of cellular biosynthetic process, negative regulation of biological process, regulation of anatomical structure morphogenesis, regulation of transcription, DNA-dependent, regulation of RNA metabolic process</td>
<td>13</td>
<td>9.26e-32</td>
</tr>
<tr>
<td>9 RelA (p65 NF-kB subunit)</td>
<td>negative regulation of apoptosis, generation of neurons, negative regulation of programmed cell death, neurogenesis</td>
<td>13</td>
<td>9.26e-32</td>
</tr>
<tr>
<td>10 STAT3</td>
<td>transcription, cellular macromolecule biosynthetic process, macromolecule biosynthetic process, organ development, nucleic acid metabolic process</td>
<td>13</td>
<td>9.26e-32</td>
</tr>
<tr>
<td>11 EGR1</td>
<td>regulation of cell morphogenesis involved in differentiation, regulation of anatomical structure morphogenesis, regulation of neuron projection development, regulation of cell morphogenesis, regulation of cell projection organisation</td>
<td>13</td>
<td>9.26e-32</td>
</tr>
<tr>
<td>12 GATA-1</td>
<td>organ development, system development, anatomical structure development, negative regulation of collateral sprouting of intact axon in response to injury, multicellular organisinal development</td>
<td>12</td>
<td>2.65e-29</td>
</tr>
<tr>
<td>13 ETS1</td>
<td>regulation of developmental process, regulation of anatomical structure morphogenesis, response to external stimulus, female pregnancy, negative regulation of apoptosis</td>
<td>11</td>
<td>7.51e-27</td>
</tr>
<tr>
<td>14 C/EBPα</td>
<td>cell differentiation, cellular developmental process, placenta development, reproductive developmental processes, response to steroid hormone stimulus</td>
<td>11</td>
<td>7.51e-27</td>
</tr>
<tr>
<td>15 HIF1A</td>
<td>positive regulation of macromolecule metabolic process, regulation of catecholamine metabolic process, positive regulation of cellular metabolic process, positive regulation of transcription, cell migration</td>
<td>11</td>
<td>7.51e-27</td>
</tr>
<tr>
<td>16 SRF</td>
<td>regulation of transcription from RNA polymerase II promoter, positive regulation of transcription, DNA-dependent, positive regulation of RNA metabolic process, positive regulation of transcription</td>
<td>11</td>
<td>7.51e-27</td>
</tr>
<tr>
<td>17 PU.1</td>
<td>response to progesterone stimulus, regulation of developmental process, regulation of angiogenesis, response to external stimulus, response to steroid hormone stimulus</td>
<td>10</td>
<td>2.10e-24</td>
</tr>
<tr>
<td>18 NF-kB</td>
<td>negative regulation of apoptosis, negative regulation of programmed cell death, anti-apoptosis, response to external stimulus</td>
<td>10</td>
<td>2.10e-24</td>
</tr>
<tr>
<td>19 Oct-3/4</td>
<td>response to organic nitrogen, negative regulation of transcription, DNA-dependent, negative regulation of RNA metabolic process</td>
<td>9</td>
<td>5.79e-22</td>
</tr>
<tr>
<td>20 C/EBPβ</td>
<td>placenta development, embryonic placenta development, regulation of RNA metabolic process, cell differentiation, cellular developmental process</td>
<td>9</td>
<td>5.79e-22</td>
</tr>
</tbody>
</table>

Maximal nodes in each network set to 50. Root nodes are the number of genes from the interrogated data set that are present in the network. GO, gene ontology.
Examples of transcription factor-centred networks

To identify the known relationships between the putative androgen regulated genes and the TFs identified in the TF-centred network list (Table 3.8), the networks were depicted visually using Metacore (where the directionality of the interactions is indicated by the arrow head and the interaction is qualified by the colour of the arrow).

Examples of these TF-centred networks (centred on AR, SP1, ESR1-ERα, p53 and CREB1) are found in Figure 3.9, Figure 3.10 and Figure 3.11. In the case of the TFs SP1 and CREB1 (Figures 3.10 and 3.11) the directionality of the direct interactions (between TF and data-set genes) are mostly outwards from the TF to the data set genes showing how the putative DHT-regulated genes are known to be regulated by these factors. Other networks showing this directionality of interactions (from TF to gene) included those centred on c-Myc, E2F1, RelA, STAT3, EGR1, ETS1, HIF1α, PU.1, NFκB and OCT-3/4 (data not shown).

For ESR1 (ERα) and p53 (Figures 3.10 and 3.11) two thirds of the direct interactions point outwards from the TF to the data set genes while the other one third of the interactions are in the opposite direction. For AR (Figure 3.9) and other TFs (GATA1, C/EBPα, SRF and C/EBPβ, data not shown) the number of interactions that point outwards to the data set genes and those that point towards AR (or the TF) are about equal in number. In the case of RUNX2-centred network the interactions mostly pointed from the data set genes towards RUNX2 (data not shown). These results indicate that some of the putative androgen-regulated genes in hESCs (at 8 hours) are not only regulated by these TFs but that some of the genes may also have a known impact upon the TF itself (such as upon ERα, p53, and AR). In some cases, the interactions between the TF and a data set gene were reciprocal, i.e., the putative androgen-regulated genes may themselves interact and regulate TFs. These results suggest that DHT may indirectly impact the listed TFs in hESCs (via the putative-DHT regulated genes).
Figure 3.9 The AR-centred transcription regulation network identified from microarray putative androgen-regulated genes in hESCs (at 8 hours DHT) by Metacore. The Microarray DHT-regulated candidate genes are marked with blue or red circles: Blue circles denote genes upregulated by DHT; Red circles denote genes downregulated by DHT. (see Figure 3.1 for detailed object legend). Genes represented here that were selected for follow up in chapter 4: CDC25A, ELK4.
Figure 3.10 The SP1 and ESR1-centred transcription regulation networks identified from microarray putative androgen-regulated genes in hESCs (at 8 hours DHT) by Metacore. ESR1=ERα. The Microarray DHT-regulated candidate genes are marked with blue or red circles: Blue circles denote genes upregulated by DHT; Red circles denote genes downregulated by DHT. (see Figure 3.1 for detailed object legend). Genes represented here that were selected for follow up in chapter 4: ELK4, SLC6A6, NURR1 (NR4A2), CDC25A, A20 (TNFAIP3) and FOXL2.
Figure 3.11 The p53 and CREB-centred transcription regulation networks identified from microarray putative androgen-regulated genes in hESCs (at 8 hours DHT) by Metacore. The Microarray DHT-regulated candidate genes are marked with blue or red circles: Blue circles denote genes upregulated by DHT; Red circles denote genes downregulated by DHT. (see Figure 3.1 for detailed object legend). Genes represented here that were selected for follow up in chapter 4: SLC6A6, JMY, NURR1 (NR4A2) and TPL2 (MAP3K8).
3.4 Discussion

There are reports that androgens may alter a wide range of cell processes in the human endometrium, but exactly how and the extent to which androgens may regulate processes in individual target cells is unknown. In the current chapter, a study was initiated by undertaking extensive bioinformatic analysis and data mining of array data generated from comparing hESCs incubated with or without DHT.

What is the evidence that endometrial cells in situ and in vitro can be the target of androgen action?

AR protein was immunolocalised to full thickness sections of human endometrium (that extend from the luminal to the endo-myometrial junction regions) and the results were in agreement with expectations (Critchley and Saunders, 2009, Marshall et al., 2011, Cloke and Christian, 2012) (reviewed in Chapter 1). Notably, in the stromal cells of secretory phase endometrium AR immunoexpression was most intense in the basal compartment whereas during the proliferative phase, cells of the stroma were immunopositive both in the functional and basal compartments. It was noted that AR was not detected in some cells of the stroma throughout the endometrium from both the proliferative and mid-secretory phases. It may be speculated that the AR negative cells may represent an unknown subset of stromal cells or other cell types that usually compose the stroma such as leukocytes and vascular cells. Further investigation is required to further characterise both the AR-positive and the AR-negative stromal cells in endometrial tissues, for example, co-staining with AR (and/or other steroid receptors) and specific cell and lineage markers.

Importantly, the presence of AR protein was confirmed in the two endometrial stromal cell models used in these studies (primary hESCs and SHT-290 cells). Upon androgen (DHT) treatment, AR protein concentrations were increased in both cell types, a response previously reported in hESCs (Cloke et al., 2008) and in other androgen responsive cell types, for example, the prostate cancer cell line LNCaP (Yeap et al., 1999). In SHT-290 cells, the upregulation of AR protein by DHT was AR-dependant as suggested by the inhibitory effect of flutamide, an inhibitor of
androgen-induced transcriptional activation (Kemppainen et al., 1992). Although other contributing mechanisms such as, increased mRNA stability as shown in LNCaP cells (Yeap et al., 1999) and/or increased protein stability by DHT/testosterone ligand binding (Kemppainen et al., 1992, Zhou et al., 1995) cannot be discounted, the AR-dependent increase in AR protein is a new finding in SHT-290 cells. Taken together, the expression of AR and responsiveness of the endometrial stromal cell models to DHT confirm them as appropriate models to study the effects of androgens upon endometrial cells.

The intense nuclear AR staining observed in the in vitro cell models was DHT-dependent and mirrored the positive nuclear staining observed in the AR-positive stromal cells in vivo (in endometrial tissue biopsies). Ligand-dependent AR cytoplasmic-to-nuclear translocation has been documented in many cell types (Georget et al., 1997, Marcelli et al., 2006, Nakauchi et al., 2007) and is an important prerequisite for AR-dependent activation of gene transcription via AREs, hence it was important to confirm it occurred in hESCs and SHT-290 cells. It is unknown if the nuclear AR observed in the endometrial tissues is due to activation of the AR by ligand binding alone or if other translocation mechanisms are involved such as activation by other steroids (Kemppainen et al., 1992). It is also unclear how the in vitro primary hESC populations reflect the uncharacterised AR-heterogeneous stromal cell population observed in endometrial tissues or if/how these differences will affect in vitro studies.

**What cellular processes, pathways and networks regulated by DHT were identified from microarray-derived data (hESCs)?**

Microarray analysis of a hESCs+DHT culture experiment identified putative androgen-regulated genes at both the 2 hour and 8 hour timepoints. These timepoints were specifically chosen as the majority of time-dependent regulation of genes by androgens in hESCs (9 genes) had been reported at 2 and/or 8 hours in contrast with only one other gene regulated at 8 and 24 hours (using the same concentration of DHT, 10nM) (in our laboratory, unpublished, Marshall et al., 2011). In a separate study, the expression of only one additional gene, ADAMTS-1, had been reported to
be regulated by androgens (DHT, 100nM) in hESCs at 24, 48 and 72 hours (Wen et al., 2006). The only other three studies that investigated the effect of androgens (A4, Testosterone or DHT) upon hESCs showed changes in protein expression or proliferation after days of exposure (between 1 to 8 days) but did not determine effects upon gene expression (Watson et al., 1998, Maliqueo et al., 2004, Ishikawa et al., 2007).

The fold changes observed in the significantly regulated gene sets (fold change threshold was 1.2, P<0.01) (and also in the whole array data-set, P>0.01) remained small (<2 fold). These small changes in gene expression are comparable to the gene changes reported by microarray analysis when AR has been “knocked down” in decidualising hESCs (Cloke et al., 2008).

Genes were both up- and downregulated by DHT. Both the induction and the repression of gene transcription by androgens has been described previously in hESCs (Marshall et al., 2011) and in immortalised prostate epithelial and stromal cells (Bolton et al., 2007, Tanner et al., 2011).

Notably, there was no overlap between the 2 hour and the 8 hour differentially expressed sets of androgen-regulated genes. The smaller number of putative androgen-regulated genes identified at 2 hours compared with those at 8 hours is suggestive of an early versus a later elicited response. Array data cannot distinguish between primary androgen responsive genes or secondary target genes i.e., those where a primary responsive gene was responsible for the effect upon secondary target genes and further experiments would be required to elucidate this.

Consistent with the roles played by androgens in the regulation of gene transcription, cellular signalling, proliferation, differentiation, development and apoptosis, an enrichment of genes associated with these processes among the microarray-derived putative androgen-regulated genes was identified using bioinformatics tools based on three different ontology databases. Using analysis tools that are based on different ontology databases and that utilise different computational algorithms, analysis was undertaken to enable extraction of informative biological data. At the same time,
comparison of the results also permitted cross-validation of findings. Metacore (using the GeneGO database) was found to be a powerful tool with the power to identify processes even from the small androgen-regulated gene set identified at 2 hours (pathway maps and process networks) and also confirmed enrichment for cellular processes identified independently by analysis with GOrilla or Pathway Express in the putative androgen-regulated gene set at 8 hours. As enrichment analysis is only as informative as the ontology datasets supporting it, the use of different ontology databases added greater confidence to the results. Critically however, the use of different databases cannot overcome the reporting biases in the source literature, for example, the action of androgens/AR has been greatly studied in the prostate and there is missing hESC-specific information. Thus, this tissue bias is an inherent limiting factor of the enrichment analyses carried out.

Figure 3.12 summarises the key cellular processes regulated by androgens in hESCs identified by bioinformatics approaches (Result Tables 3.3, 3.4 and 3.5) and highlights the distinct and overlapping results obtained using the diverse analyses.
### Bioinformatic tools and cellular processes identified:

<table>
<thead>
<tr>
<th></th>
<th>GOrilla</th>
<th>Pathway Express</th>
<th>Metacore</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 hours DHT</strong></td>
<td>biosynthesis of fatty acids</td>
<td>vitamin metabolism, development, apoptosis/survival, immune responses</td>
<td></td>
</tr>
<tr>
<td><strong>8 hours DHT</strong></td>
<td>gene transcription</td>
<td>carbohydrate metabolism</td>
<td>cell cycle, development, signalling</td>
</tr>
<tr>
<td></td>
<td>metabolic and biosynthetic processes</td>
<td></td>
<td>ERα signalling, protein processing, chemotaxis</td>
</tr>
</tbody>
</table>

**Figure 3.12** Summary of unique and overlapping biological cellular processes associated with androgen exposure in hESCs (at 2 and 8 hours) identified by different bioinformatic analyses of microarray-derived putative androgen-regulated genes.

There was no overlap between the array data sets in this thesis and the previously identified androgen-regulated genes that were identified by an *in silico*-based approach (Marshal *et al.*, 2011). This may be due to cell-type bias as the study mentioned was performed using multiple data sets obtained from androgen action mostly in prostate cells (with only one data set being of endometrial origin). However, as already discussed, the biological processes revealed to be androgen regulated in hESCs by the different bioinformatic tools - concur but also complement and extend observations from other studies of the effects of DHT upon hESCs and other cell types.
For example, in the current study, androgen-exposure was also associated with changes in genes associated with apoptosis and cell cycle processes as previously identified by pathway analysis of a different androgen-regulated gene set in hESCs (Marshall et al., 2011). This common finding occurred despite the array dataset in this thesis being distinct from the androgen-regulated genes identified in previous studies using undifferentiated but also decidualised hESCs (i.e. there was no overlap in putative androgen-regulated genes) (Marshall et al., 2011, Cloke et al., 2008). To date, non-aromatisable androgens have not been reported to regulate proliferation in functional assays in undifferentiated hESCs. However, proliferation was found to be upregulated upon AR-knockdown in decidualised hESCs (Cloke et al., 2008) suggesting that AR may contribute to reduce proliferation.

In the current study, androgen-dependent regulation of cell structure and motility was also postulated based on bioinformatics. A previous study by Cloke and colleagues (2008) using decidualised hESCs also claimed expression of genes involved in cytoskeletal organisation and cell motility was AR-dependent. Lipid metabolism has been shown to be altered by androgens in decidualised hESCs (Kajihara et al., 2013b) and in normal and cancer prostate cell lines with possible impacts upon steroidogenesis (Bolton et al., 2007, Vis and Schroder, 2009) but we believe this is the first study to highlight it as AR-dependent in primary, undecidualised hESCs. Enrichment for ESR1 (ERα) signalling in hESCs as a result of androgen exposure (also reported in this thesis) indicates possible cross-talk between the AR and ER signalling networks and is postulated here in hESCs for the first time. This AR-ER cross-talk has been described in uterine and other systems in both normal and abnormal physiologies for instance in luminal breast cancer cells (Need et al., 2012) and in the pig and rat uterus (Kowalski et al., 2004, Nantermet et al., 2005). In the current study, putative ERα binding sites were also identified in several of the target genes.

Additionally, it is interesting to point out some of the other signalling pathways enriched for in the microarray-derived putative androgen-regulated genes in hESCs.
(at 8 hours DHT). For example, the enrichment for signalling pathways such as Wnt and MAPK has also been reported to occur in prostate epithelial cells after 72 hours DHT treatment (Tanner et al., 2011). The Wnt signalling pathway, is believed to be modulated in normal and carcinogenic endometrium (Nei et al., 1999) and has important components such as β-catenin and glycogen synthetase kinase (GSK3β) that not only act as regulators of AR activity (Truica et al., 2000, Terry et al., 2006) but AR itself has been shown capable of signalling through Wnt/B-catenin. Depending on the type of prostate cancer (cell context-dependent) AR-dependent inhibition or enhancement of Wnt signals at the transcriptional level can modulate proliferation (Schweizer et al., 2008).

The newly described putative androgen-dependent effect upon signalling pathways (such as Wnt, MAPK) in non-decidualised hESCs reported in this Chapter contrasts with findings in decidualised hESCs in which such pathways were mainly regulated by PR and not AR (Cloke et al., 2008). Decidualised hESCs play a key role in modulating trophoblast invasion and, in the absence of a pregnancy as progesterone levels decline in the initiation of menstruation (Schatz et al., 1999). Taken together, these observations suggest divergent roles for androgens in the non-decidualised hESC versus in the decidualised hESC.

In addition to the enrichment for specific canonical pathways and networks, novel androgen (DHT)-dependent gene networks and sub-networks were also predicted by Metacore offering possibilities as to how the associated cellular processes (response to stimuli, proliferation/cell cycle, apoptosis, development/differentiation and regulation of transcription) may be differentially regulated by androgens (Result Table 3.6). Additionally, the sub-network analysis suggested how these processes may be interconnected via a number of named putative candidate genes (Result Table 3.7). Specific further experimental work is required to confirm these inferences.

The ten most highly connected transcription factors to the putative androgen-dependent genes were c-Myc, SP1, ERα, p53, E2F1, RUNX2, CREB1, AR, RelA
and STAT3 (Result Table 3.8) one or more of which may be involved in mediating actions of DHT upon hESCs. Each of the TFs was found to connect to between 12.3 and 4.8% of the genes putatively regulated by DHT in hESCs. Interestingly, the TF-centred analysis also revealed that about 95% of the putative DHT-regulated genes in hESCs (255 genes) have never previously been reported as having any relationship with AR (Result Table 3.8). This raises the possibility that if confirmed to be DHT-regulated, some or all the genes may represent entirely novel DHT-regulated genes.

The directionality of the relationships between TFs and putative DHT-regulated genes suggest that while many of putative DHT-regulated genes are known direct targets of the TFs (for example, c-Myc, SP1, E2F1, CREB1, RelA, STAT3) there are a number of candidate genes that also impact the TFs (ERα, p53, RUNX2, AR) themselves. These relationships suggest putative pathways by which DHT may indirectly modulate the activity of specific TFs (namely ERα, p53, RUNX2 and AR) individually or in concert via putative DHT-regulated genes.

The impact of DHT upon hESCs may be inferred from the known individual roles of the TF, for example SP1 is involved in the negative regulation of apoptosis (Table 3.8). If SP1-target genes are also regulated by DHT this may provide a link to the regulation of cell death. SP1-dependent mechanisms in response to steroids such as dexamethasone have also been documented (Mao et al., 2008) as have interactions between SP1 and other steroid receptors such as ERβ (Bartella et al., 2012, Greaves et al., 2013). Expression of candidate genes by DHT may therefore be dependent on mechanisms involving interactions (i.e. complex formation) that alter the binding of AR to DNA (enhancing, stabilising or reducing binding that may affect transcription levels). For example, a previous study has reported that the androgen analog R1881-dependent upregulation of expression of the vascular endothelial growth factor (VEGF) gene was dependent on a SP1 binding site in prostate cancer cells (Eisermann et al., 2013).

There were nine putative androgen-regulated genes identified as being regulated (upregulated or downregulated) by ER in the ESR1-centred network. Only two of
these genes, thromboxane A2 (prostanoid) receptor (TBX2AR) and the cytokine osteopontin (SPP1) have been shown to be expressed (protein) in the human endometrium, with SPP1 being upregulated by decidualisation in hESCs and TBX2AR mRNA upregulated in secretory endometrium (Von Wolff et al., 2004, Catalano et al., 2011). However, their regulation by oestrogen has not been directly demonstrated in the endometrium and they differ from those genes previously reported as oestrogen-regulated in hESCs in vitro (Pole et al., 2005). In addition, only one of the identified ER-regulated genes, AT-rich interaction domain 4A (ARID4A), may be speculatively associated with the regulation of proliferation through its binding to the retinoblastoma protein (Wu et al., 2013). None of the putative androgen-regulated genes (known to be regulated by oestrogen) were commonly known regulators of cell cycle. Other processes associated with the other ER-regulated genes included regulation of development, differentiation, apoptosis (retinoic acid receptor alpha, RARA), migration (spermatogenesis associated 13, ASEF2) and cell cycle arrest and insulin receptor signalling pathway (calcium binding protein 39 like, CAB39L) (functional assignments obtained from gene ontology by NCBI).

Lastly, TF regulatory networks are thought to be cell type-specific allowing different cell types to respond with appropriate cellular fate decisions and gene expression patterns (Jhamb et al., 2011, Neph et al., 2012). Notably, in the current data analysis, some candidate genes were present in more than one TF-centred network for example ELK4 and NR4A2 (NURR1). This may suggest known and novel ways by which the different TFs may be connected in larger networks. Indeed, some TFs associated with the DHT-regulated gene set have been shown to be part of larger genes networks integrating multiple cellular pathways, again offering suggestions as to how the actions of DHT modulate cell processes in hESCs. For example, the TFs c-Myc, SP1, p53, CREB1 and STAT3 were previously implicated together in an interconnected network in prostate cancer cells in response to androgen (Vellaichamy et al., 2010). The TFs c-Myc, ESR1, p53, AR, STAT3, HIF1α, and NFkB identified here were also predicted as putative molecules in a previous network composed of androgen-regulated genes identified as expressed in hESCs in
Chapter 3  Identification of androgen regulated genes

our laboratory (Marshall et al., 2011) However, it is the first time that the remaining identified TFs (E2F1, RUNX2, EGR1, GATA-1, ETS1, C/EBPα, SRF, PU.1, OCT-3/4 and C/EBPβ) have been implicated in interacting networks in hESCs in response to androgens. Comprehensive mapping of TF-networks using a combination of computational and chromatin immunoprecipitation (ChIP)-based approaches should now be exploited to further understand the mechanisms of AR-dependent transcriptional activity in the hESCs.

**Conclusions**
The hypothesis tested by the investigations in this chapter was - *Androgens (DHT) affect gene expression in endometrial stromal cells impacting proliferation and differentiation.* Analysis of the findings presented would lend support to this hypothesis. Sets of putative androgen-regulated genes were identified in hESCs treated with DHT.

Multiple bioinformatic tools were deployed in analysis of microarray data and these provided novel insight into androgen signalling in hESCs highlighting the potential for androgen-dependent modulation of development/differentiation, apoptosis and proliferation/cell cycle progression. Suggestions of how these processes are regulated by androgens in hESCs are proposed through modulation of: specific pathways such as metabolic pathways, specific signalling pathways (eg Wnt), interacting TF regulatory networks. The findings in this chapter add further support for a physiological role for androgens in the human endometrium. However, these findings are derived from analyses of a mRNA microarray experiment that requires validation through, for example, confirmation of the regulation of candidate androgen-regulated genes in hESCs by other methods such as qPCR. The selection of the genes could however, be guided by their functional annotations provided by bioinformatics findings herein and association with processes of interest including development/differentiation, apoptosis and proliferation/cell cycle.
Chapter 4: Identification of androgen regulated genes in human endometrial stromal cells

4.1 Introduction

In the previous chapter, bioinformatics analysis of microarray-derived data identified putative androgen-regulated gene sets, the functional analysis of which provided novel insight into androgen signalling in hESCs. In this chapter, in order to validate the microarray study, a short list of putative DHT-regulated genes (candidate genes) was chosen from the microarray DHT-treated hESC data sets. Androgen regulation of the selected candidate genes was evaluated through analysis of their potential regulation by AR (computational) and through analysis of their regulation by DHT in stromal endometrial cells.

The criteria for the selection of putative androgen-regulated genes were defined and were based on the outcomes of the bioinformatics analyses (e.g. fold change and functional association with the key cell processes revealed by the bioinformatics analyses - proliferation, apoptosis/cell survival, cell differentiation, cell signalling and cell structure; chapter 3). Implementation of the selection criteria to the microarray 8 hour DHT-treated hESC data set would short list candidate androgen-regulated genes to be be assessed computationally for their potential regulation by AR by transcription factor binding site (TFBS) sequence analysis (Marshall et al., 2011). The regulation of the candidate genes by DHT (and possible involvement of AR) could then be evaluated in vitro in stromal endometrial cell models.

Together, these findings could go towards validating the findings from the microarray study and test the hypothesis,

“Putative androgen-regulated genes show potential for regulation by AR and are regulated by DHT in endometrial stromal cells”.
4.1.1 Aims of Chapter

- To select putative DHT-regulated genes in human endometrial stromal cells (assisted by the bioinformatic analysis outcomes) and establish their potential for regulation by androgen receptor using transcription factor binding site sequence analysis.

- To determine if the novel candidate genes are regulated by DHT in endometrial stromal cell models (hESCs and SHT-290 cells) using quantitative polymerase chain reactions (qPCR).

- To evaluate if the candidate DHT-regulated genes are AR-dependent using an AR inhibitor in an endometrial stromal cell model using qPCR.

- To build a novel interaction network featuring AR and the DHT-regulated genes through computational reinterrogation of the Metacore knowledge database.
4.2 Materials and Methods

This section describes all specific methods and outlines the experimental approaches employed in Chapter 4; general methodology was summarised in Chapter 2.

4.2.1 Manual gene annotation

Additionally, manual gene annotation of individual gene candidates and grouping by cellular process was performed using resources such as the National Centre for Biotechnology Information (NCBI) database (www.ncbi.nlm.nih.gov/), the Online Mendelian Inheritance in Man (OMIM) database (www.omim.org), BioGPS (http://biogps.gnf.org), Genecards (www.genecards.org), iHOP (www.ihop-net.org) and the HUGO Gene Nomenclature Committee (www.genenames.org). These resources were chosen based on ease of access (free online access), gene information provided regarding gene/protein name, function, association with disease or resources available in the market (for example, availability of specific antibodies).

4.2.2 Transcription factor binding site sequence analysis

Genomic sequences between 10000bp and 4000bp, upstream and downstream respectively of the transcription start site (TSS), were manually retrieved from the GenBank Sequence Database provided by the NCBI (www.ncbi.nlm.nih.gov/genbank/). These screening parameters were previously established by studies where genomic sequences were interrogated for androgen response elements (AREs) or other steroid receptor binding sequences (Bolton et al., 2007, Marshall et al., 2011). The platform used for the computational identification of predicted TFBSs was Mapper2 available at http://bio.chip.org/mapper (free online access). Mapper combines TRANSFAC® (www.biobase-international.com) and JASPER (http://jaspar.genereg.net/) databases of experimentally-proven binding sites and uses methodology especially developed by Marinescu et al. (2005). M-type models are matrix-derived models (DNA sequence-derived by TRANSFAC) and T-type models are factor-derived models (AR-derived by TRANSFAC and JASPER).

Five Mapper models were used in the interrogation of the sequences for human AREs (Lin et al., 2009, Chen et al., 2010). ARE models and consensus sequences were: M00447 (agtacat.tgttct and agtacatttgttct), M00481 (ggtaCat.gtGttct and
Chapter 4  Identification of androgen regulated genes in endometrial stromal cells

ggtaCatgtGttct), M00962 (tgagaa.g and tgagaacg), T00040 (gtacatt.tGtct), T00042 (ggaaca...tGTtct). These models are probabilistic and the use of a capital letter in the consensus sequences denotes greater probability for that amino acid when in that position in relation to the others in the sequence.

Other TFBS also investigated with the relevant predictive Mapper models were: **PR/GR** (M00960, T05076, T01661, T01660, T00698, T00697, T00696, T00337, T00335, T00334, T00333); **GR** (M00960, M00921, T00334, T05076, T00337, T00333, T00335); **ER-alpha** (M00959, T00264, T00259, T00258, T00263, T00261, T00262); **ER-beta** (M00959, T04651); **ERR-alpha** (M00511, T04849); **SP1** (MA0079) and **CREB1** (MA0018). Models and consensus sequences are detailed at Mapper2 (http://bio.chip.org/mapper).

### 4.2.3 Endometrial biopsies

All endometrial tissues used in this chapter were collected under ethical approval as described in Chapter 2 section 2.2 and processed as described in sections 2.2 and 2.3.1. Table 4.1 summarises the clinical details for the women that donated the samples used in the following investigations. Less strict menstrual staging criteria were applied to samples used for hESC extraction and culture which allowed samples to serve as their own controls i.e., each cell sample was exposed simultaneously to a treatment (for example, DHT) and to a vehicle control.
Chapter 4  Identification of androgen regulated genes in endometrial stromal cells

Table 4.1 Clinical details for women that provided endometrial biopsies used for hESC extraction and culture carried out in section 4.3.3.1

<table>
<thead>
<tr>
<th>Sample</th>
<th>LREC</th>
<th>Day of cycle</th>
<th>E₂</th>
<th>P₄</th>
<th>Histological dating</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>B</td>
<td>7</td>
<td>130</td>
<td>1.66</td>
<td>Proliferative</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>7</td>
<td>1.9*</td>
<td>&lt;1</td>
<td>Weak proliferative</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>7</td>
<td>1.1*</td>
<td>&lt;1</td>
<td>Not available</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
<td>8</td>
<td>325</td>
<td>2.86</td>
<td>Interval phase</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>8</td>
<td>205</td>
<td>2.24</td>
<td>Weak proliferative</td>
</tr>
<tr>
<td>6</td>
<td>A</td>
<td>10</td>
<td>4.2*</td>
<td>1.1</td>
<td>Proliferative</td>
</tr>
<tr>
<td>7</td>
<td>B</td>
<td>13</td>
<td>393.1</td>
<td>3.82</td>
<td>Not available</td>
</tr>
</tbody>
</table>

Circulating levels of E₂: Oestradiol (pmol/L) and P₄: progesterone (nmol/L). LREC approval A) LREC05/S1103/14, B) LREC07/S1103/29 and C) LREC10/S1402/59.

Women with benign gynaecological conditions such as caesarean section scar endometriosis or fibroids less than 3cm diameter were included. All patients presented a history of heavy menstrual bleeding. * The accuracy of the results was checked and the E₂ concentrations for these samples were considered as baseline/low as the values are below the normal threshold of detection of some assays and may not represent actual concentrations.

4.2.4 RNA analysis

Cultured cells (primary hESCs or SHT-290 cells) were harvested at the experimental timepoints following the experimental design outlined in each section below. Timepoints were previously determined by our laboratory (unpublished and Marshall et al., 2011) and by other researchers (Wen et al., 2006). Total cell RNA was extracted, quantified and cDNA was synthesized (sections 2.7.1 and 2.7.2). The concentration of the mRNAs investigated (and endogenous control) were determined by qRT-PCR as described in section 2.7.3 (the primers and probes used are detailed in Table 2.5).

4.2.4.1 Primary hESCs: the effect of DHT upon candidate gene expression

hESCs isolated from proliferative endometrial samples (section 4.2.3) were cultured in 10% charcoal stripped serum media (section 2.3.1.2) for 2 to 4 days and were seeded in 6 well cluster plates (1 x 10⁵ cells/well) and allowed to adhere overnight. At 80-90% confluence, cells were serum starved for 24 hours and incubated with 10nM DHT or vehicle control for 8 or 24 hours (section 2.4.1). Each treatment was performed in duplicate. Each experiment was repeated 7 times, each time with
Chapter 4 Identification of androgen regulated genes in endometrial stromal cells

hESCs isolated from a different endometrial biopsy (Table 4.1). The hESCs were only used in experiments until fifth passage (section 2.3.1).

4.2.4.2 SHT-290 cell line: the effect of DHT upon candidate gene expression

SHT-290 cells were cultured in media with 10% charcoal stripped serum for 2 to 4 days and were seeded in 6 well cluster plates (1 x 10^5 cells/well) as described in section 2.3.2 and allowed to adhere overnight. At 80-90% confluence, cells were serum starved for 24 hours and incubated with 10nM DHT or vehicle control for 8 or 24 hours (section 2.4.1). Each treatment was conducted in duplicate. Each experiment was repeated 5 times.

4.2.4.3 SHT-290 cell line: the effect of flutamide upon DHT-dependent candidate gene expression

In separate experiments to those described in the section above, SHT-290 cells were cultured in media with 10% charcoal stripped serum for 2 to 4 days and were seeded in 6 well cluster plates (1 x 10^5 cells/well) as described in section 2.3.2 and allowed to adhere overnight. At 80-90% confluence cells were serum starved for 24 hours and pre-treated with 10µM flutamide or without (vehicle or no treatment) for 1 hour. The incubation with 10nM DHT or vehicle for 24 hours followed (section 2.4.1). Each treatment was conducted in duplicate. Each experiment was repeated 5 times.

4.2.5 Bioinformatic analysis – discovery analysis applied to a select group of genes regulated by DHT in ESCs

Reinterrogation of the knowledge database and generation of a novel network using genes regulated by DHT in endometrial stromal cells: A new gene list (AR, SIK1, SNCAIP, RGS2 and SLC6A6) was directly uploaded to Metacore and was subjected to pathway analysis. These genes were selected as they were regulated by DHT (based on the results described in sections 3.3.3 and 4.3.3). The network generated was built using the shortest path algorithm (default for network building with 3 to 6 objects) with maximal 3 steps with the same gene and included the canonical pathway overlay parameter (dependent on the possibility of connection of the canonical pathway to the network both at the beginning and at the end of the pathway).
4.2.6 Statistical analysis
Prism 5 for Windows version 5.02, 2008 by Graphpad Software (La Jolla, CA, USA) was used to perform statistical analyses. In culture studies, data is presented as means ± standard error of the mean (SEM) with P < 0.05 considered statistically significant (section 2.9). For qRTPCR analysis of gene expression in cells (primary hESCs and SHT-290 cells) treated with DHT and vehicle Paired t-tests were used. For qRTPCR analysis of gene expression in SHT-290 cells treated with DHT, flutamide and vehicle controls two-way ANOVA with Bonferroni’s multiple comparison post hoc test was used.
4.3 Results

4.3.1 Eighteen candidate genes were selected from the hESC microarray-derived putative androgen-regulated gene list (8 hours DHT) for further study

To validate/evaluate the regulation of putative DHT-regulated genes by DHT in stromal endometrial cells, a short list of genes was established following defined selection criteria. Firstly, candidate genes were chosen from the 8 hour differentially expressed gene list (P<0.01) with a greater than 1.2 fold change (previous Table 3.2). Fold changes at the 2 hour timepoint were considered too modest to allow for robust validation. Secondly, those genes with the highest transcript abundance were considered so as to ensure transcript detection on qRTPCR. Finally, association with a key cell process (proliferation, apoptosis/cell survival, cell differentiation, cell signalling and cell structure) based on manual literature searches and bioinformatic analyses of the microarray gene expression such as identification in the sub-network Table 3.7 (and Figures 3.9, 3.10 and 3.11) was taken into account. Group 1 candidate genes were selected using these criteria.

A second group of possible putative androgen-regulated genes was also selected. As previous studies have reported that microarray analyses may identify only a proportion and not all DHT-regulated genes in some cells (see Bolton et al., 2007), the second group of genes were selected outwith the 8 hour androgen DE gene list (P<0.01). Thus, these Group 2 genes (also termed 'candidate' genes) were selected from the complete 8 hour microarray DHT data set (with P>0.01) while satisfying most of the other criteria of choice with special emphasis given to association with the cell processes mentioned above (previous sections 3.3.3).

Table 4.2 presents the list of eighteen genes putative androgen-regulated selected and summarises the selection criteria applied in the selection of genes in Groups 1 and 2.
## Table 4.2 Details of the eighteen microarray-derived putative DHT-regulated genes in hESCs at 8 hours selected for further study.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Gene name (and aliases)</th>
<th>Fold change (8 hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group 1: Selected from microarray-derived putative androgen-regulated gene list in hESCs at 8 hours DHT</strong>&lt;br&gt;With significant fold change (P&lt;0.01) and with fold change above 1.2 cut-off</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAP3K8</td>
<td>mitogen-activated protein kinase kinase kinase 8 (TPL2)</td>
<td>+ 1.866</td>
</tr>
<tr>
<td>NR4A2</td>
<td>nuclear receptor subfamily 4, group A, member 2 (NURR1)</td>
<td>+ 1.706</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>solute carrier family 6 (neurotransmitter transporter, taurine), member 6 (TAUT)</td>
<td>+ 1.511</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>tumour necrosis factor, alpha-induced protein 3 (A20)</td>
<td>+ 1.508</td>
</tr>
<tr>
<td>JMY</td>
<td>junction-mediating and regulatory protein, p53 cofactor</td>
<td>+ 1.419</td>
</tr>
<tr>
<td>CDC25A</td>
<td>cell division cycle 25A</td>
<td>+ 1.377</td>
</tr>
<tr>
<td>GMNN</td>
<td>geminin, DNA replication inhibitor (GEM)</td>
<td>+ 1.337</td>
</tr>
<tr>
<td>ELK4</td>
<td>ETS-domain protein (SRF accessory protein 1)</td>
<td>+ 1.224</td>
</tr>
<tr>
<td>FOXL2</td>
<td>forkhead box L2 (BPES)</td>
<td>- 1.491</td>
</tr>
<tr>
<td>MGC16121</td>
<td>MIR503 host gene (long non-coding RNA)</td>
<td>- 2.029</td>
</tr>
<tr>
<td><strong>Group 2: Selected based on functional analyses of microarray gene expression (outwith microarray-predicted putative androgen-regulated gene list at 8 hours)</strong>&lt;br&gt;With non-significant fold change (P&gt;0.01)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RGS2</td>
<td>regulator of G-protein signalling 2, 24kDa</td>
<td>+ 1.572</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>synaptosomal-associated protein, 25kDa</td>
<td>+ 1.468</td>
</tr>
<tr>
<td>SNCAIP</td>
<td>synuclein, alpha interacting protein (protein: Synphilin-1)</td>
<td>+ 1.440</td>
</tr>
<tr>
<td>PRLR</td>
<td>prolactin receptor</td>
<td>+ 1.439</td>
</tr>
<tr>
<td>SIK1</td>
<td>salt-inducible kinase 1 (SNF1LK)</td>
<td>+ 1.402</td>
</tr>
<tr>
<td>GADD45G</td>
<td>growth arrest and DNA-damage-inducible, gamma (DDIT-2)</td>
<td>+ 1.399</td>
</tr>
<tr>
<td>AXIN2</td>
<td>axin 2</td>
<td>+ 1.212</td>
</tr>
<tr>
<td>ID3</td>
<td>inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (HEIR-1)</td>
<td>+ 1.018</td>
</tr>
</tbody>
</table>

Candidate genes are listed with the microarray fold change expression values (DHT-treated cells compared to controls).<br>(+) genes upregulated by DHT; (-) genes downregulated by DHT.<br>Criteria of choice: fold change (P<0.01 or P>0.01), transcript abundance and known association with cell processes (proliferation, apoptosis/cell survival, cell differentiation, cell signalling and cell structure).
Chapter 4  Identification of androgen regulated genes in endometrial stromal cells

4.3.2 Eighteen selected candidate genes showed potential regulation by androgen receptor and other transcription factors in a Transcription factor binding site (TFBS) sequence survey

4.3.2.1 AREs were present and located within all eighteen candidate gene sequences surveyed

To investigate the potential regulation of the 18 selected putative DHT-regulated genes (Table 4.2) by AR, genomic sequences were interrogated for human androgen response elements (AREs) following parameters previously established for AREs in hESCs and other cell types (Bolton et al., 2007, Marshall et al., 2011).

AREs were predicted in the gene sequences of all the eighteen genes investigated. Figure 4.1 shows how the AREs were physically distributed along the 10Kb sequences upstream from the Transcription Start Sites (TSS) of all the gene sequences surveyed (Group 1 and Group 2 genes, Table 4.2). For many of the gene sequences tested the AREs were also distributed along the 4Kb sequence downstream from the TSS (exceptions were genes NR4A2, SNAP25, and AXIN2). These findings introduce a potential for regulation by AR for the candidate genes (putative androgen-regulated genes).

Figure 4.1 Graphical representation of the physical distribution of AREs along the sequences of eighteen candidate genes (ordered as in Table 3.9 and 3.10). AREs were predicted in the gene sequences of all the putative androgen-regulated gene sequences surveyed thereby revealing potential regulation by AR. TSS: Transcription Start Site. X-axis unit is equivalent to 100 base pairs (bp) of sequence. Y-axis values (1, 2 or 3) denote the number of TFBSs per 100bp sequence. (-10K) 10Kb sequence upstream of TSS. (+4Kb) 4Kb sequence downstream from TSS. Figure continues on the following pages.
Figure 4.1 - Continued. Graphical representation of the physical distribution of AREs along the sequences of eighteen candidate genes. Figure continues on the following page.
Figure 4.1 -Continued. Graphical representation of the physical distribution of AREs along the sequences of eighteen candidate genes.
4.3.2.2 Sex steroid receptor, steroid-response-associated and CREB1 Response Elements were identified in the candidate gene sequences

To investigate the potential regulation of the 18 selected genes by other sex steroid receptors (PR, GR, ERα, ERβ) and steroid response-associated TFs (ERR-α, SP1), the genomic sequences were surveyed for the relevant TFBSs following parameters previously established for AREs and other steroid receptor binding sequences in hESCs (Marshall et al., 2011).

The cAMP responsive element binding protein 1 (CREB1) is a transcription factor important for prolactin (PRL) expression, endometrial development (Telgmann and Gellersen, 1998, Wang et al., 2007a) and endometrial function for example, during implantation (Kao et al., 2002). As CREB1 was indicated by microarray functional analysis as a Transcription Factor associated with a number of the putative androgen regulated candidate genes (previous Table 3.8), the gene sequences of the 18 selected putative DHT-regulated genes were also surveyed for CREB1 regulatory sequences.

The total number of sex steroid receptors and steroid-response-associated TFBSs identified for each of the 18 genes surveyed are detailed in Table 4.3 (AREs are also included). Most of the TFBS had predicted binding sites in the genomic sequences of all the gene sequences interrogated.
Table 4.3 Summary of the number of Transcription Factor Binding Sites identified for AR, sex steroid, steroid-response-associated (ERR-α, SP1) and CREB1 in the selected eighteen candidate gene sequences (revealed potential regulation by the respective TFs).

<table>
<thead>
<tr>
<th>Gene</th>
<th>AR</th>
<th>PR/GR</th>
<th>GR</th>
<th>ERα</th>
<th>ERβ</th>
<th>ERR-α</th>
<th>SP1</th>
<th>CREB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP3K8</td>
<td>11</td>
<td>2</td>
<td>0</td>
<td>9</td>
<td>7</td>
<td>3</td>
<td>25</td>
<td>16</td>
</tr>
<tr>
<td>NR4A2</td>
<td>2</td>
<td>3</td>
<td>1</td>
<td>5</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>4</td>
<td>2</td>
<td>0</td>
<td>11</td>
<td>7</td>
<td>10</td>
<td>29</td>
<td>9</td>
</tr>
<tr>
<td>TNFAIP3</td>
<td>9</td>
<td>3</td>
<td>1</td>
<td>7</td>
<td>3</td>
<td>3</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>JMY</td>
<td>7</td>
<td>4</td>
<td>0</td>
<td>11</td>
<td>3</td>
<td>0</td>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>CDC25A</td>
<td>12</td>
<td>2</td>
<td>0</td>
<td>21</td>
<td>5</td>
<td>4</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>GMNN</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>9</td>
<td>9</td>
<td>1</td>
<td>25</td>
<td>7</td>
</tr>
<tr>
<td>ELK4*</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FOXL2</td>
<td>15</td>
<td>3</td>
<td>1</td>
<td>6</td>
<td>0</td>
<td>2</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>MGC16121</td>
<td>13</td>
<td>4</td>
<td>1</td>
<td>8</td>
<td>3</td>
<td>1</td>
<td>20</td>
<td>8</td>
</tr>
</tbody>
</table>

Group 1 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>AR</th>
<th>PR/GR</th>
<th>GR</th>
<th>ERα</th>
<th>ERβ</th>
<th>ERR-α</th>
<th>SP1</th>
<th>CREB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS2</td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>8</td>
<td>1</td>
<td>5</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>9</td>
<td>2</td>
<td>3</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>SNCAIP</td>
<td>12</td>
<td>0</td>
<td>2</td>
<td>13</td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>PRLR</td>
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<td>8</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>SIK1</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>24</td>
<td>8</td>
<td>3</td>
<td>24</td>
<td>30</td>
</tr>
<tr>
<td>GADD45G</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>13</td>
<td>7</td>
<td>6</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td>AXIN2</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>9</td>
<td>3</td>
<td>4</td>
<td>26</td>
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<tr>
<td>ID3*</td>
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<td>1</td>
<td>5</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Gene sequences analysed were between 10Kb upstream of TSS and 4Kb downstream from TSS (except for the genes labelled *, where results for only 10Kb upstream of TSS are included). Genes ordered as in Table 4.2.
4.3.3 Candidate genes are regulated by DHT in endometrial stromal cells in vitro

Eighteen candidate genes showed potential for regulation by AR (section 4.3.2). As the candidate genes were chosen based on the functional analysis of gene expression of 8 hour DHT-treated hESCs the expression of 15 putative DHT-regulated genes were investigated for androgen regulation at the same 8 hour timepoint with the aim of validating the microarray results (Genes listed in Table 4.2. Exceptions were TNFAIP3, GMNN and SNAP-25 due to poor qPCR efficiency or gene role association with cell structure). To assess if androgen-modulated gene expression was prolonged, candidate gene regulation by DHT was also investigated at the later timepoint of 24 hours.

Both endometrial stromal cell lines (primary hESCs and SHT-290 cells) were shown to express AR protein and be DHT responsive (previous section 3.3.2). As SHT-290 cells are an immortalised endometrial stromal cell model these cells were also employed to compare DHT-dependent gene expression responses with the primary hESCs.

4.3.3.1 Regulation of seven of fifteen putative androgen-regulated genes (SLC6A6, JMY, RGS2, SNCAIP, SIK1, GADD45G and AXIN2) was validated using primary hESCs

Cells were treated with and without DHT for 8 and 24 hours and the concentrations of mRNAs were determined and are shown in Figures 4.2 (Group 1: MAP3K8, NR4A2, SLC6A6, JMY, CDC25A, ELK4, FOXL2, MGC16121) and in Figure 4.3 (Group 2: RGS2, SNCAIP, PRLR, SIK1, GADD45G, AXIN2, ID3). Genes were grouped according to selection approach described in section 4.3.1 and Table 4.2 to facilitate the comparison of the results. Previous poor reproducibility of the effects of DHT upon candidate gene expression (data not shown) was improved by using fresh reagents and cells.

Group 1 genes - selected from the microarray-derived putative androgen-regulated gene list in hESCs at 8 hours DHT (Figure 4.2): Out of eight Group 1 genes investigated, only mRNAs encoded by two genes (SLC6A6 and JMY) were
significantly changed following treatment with DHT. Only \textit{JMY} was significantly changed by DHT at 8 hours (P<0.05). \textit{SLC6A6} and \textit{JMY} were regulated at 24 hours (P<0.01 and P<0.05, respectively). \textit{SLC6A6} and \textit{JMY} concentrations were downregulated by treatment with DHT but the prediction based on microarray analysis was of upregulation (previous Table 4.2). Levels of six mRNAs were not significantly altered by DHT at either timepoint (\textit{MAP3K8, NR4A2, CDC25A, ELK4, FOXL2, MGC16121}) though a non-significant trend was observed in some cases for example, \textit{MGC16121} and \textit{MAP3K8}.

\textit{Group 2 Genes - Selected based on functional analyses of microarray gene expression (outwith putative androgen-regulated gene list at 8 hours) (Figure 4.3):}

Out of seven Group 2 genes investigated, concentrations of five mRNAs (\textit{RGS2, SNCAIP, SIK1, GADD45G} and \textit{AXIN2}) were significantly regulated by DHT at 8 hours (P<0.05 or P<0.01). Four of these mRNAs were also still altered at 24 hours (\textit{RGS2, SIK1, GADD45G} and \textit{AXIN2}) (P<0.05 or P<0.01). \textit{RGS2} and \textit{SNCAIP} levels were upregulated by DHT. \textit{SIK1, GADD45G} and \textit{AXIN2} levels were downregulated by DHT. Levels of two genes were not significantly altered by DHT at either timepoint (\textit{PRLR} and \textit{ID3}).
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Figure 4.2 In primary hESCs, DHT significantly altered the levels of Group 1 (Table 4.2) putative androgen-regulated genes SLC6A6 and JMY but not MAP3K8, NR4A2, CDC25A, ELK4, FOXL2 or MGC16121 at 8 hours or at both 8 and 24 hours (2 out of 8 genes). Cells were incubated for 8 and 24 hours with vehicle (V) or DHT (10^-8 M). Data are presented as mean ± SEM (paired t-tests, n=7). * P<0.05 and ** P<0.01.
Figure 4.3 In primary hESCs, DHT significantly alters the levels of Group 2 (Table 4.2) putative androgen-regulated genes RGS2, SNCAIP, SIK1, GADD45G, AXIN2 but not PRLR or ID3, at 8 hours and/or 24 hours (5 out of 7 genes). Cells were incubated for 8 and 24 hours with vehicle control (V) or DHT (10⁻⁸ M). Data are presented as mean ± SEM (paired t-tests, n=7). * P<0.05 and ** P<0.01.
4.3.3.2 Expression of seven candidate genes out of fifteen are altered by DHT in SHT-290 cells (SLC6A6, ELK4, MGC16121, RGS2, SNCAIP, SIK1 and ID3)

To investigate if DHT also regulated the candidate genes in the immortalised SHT-290 cells (as in hESCs, previous section 4.3.3.1), the mRNA concentrations of the 15 genes were assayed from cells treated with DHT for 8 and 24 hours. The timepoints were chosen to replicate and allow direct comparison with previous section results obtained in hESCs.

**Group 1 genes - selected from the microarray-derived putative androgen-regulated gene list in hESCs at 8 hours DHT (Figure 4.4):** Out of eight Group 1 genes investigated, three genes (SLC6A6, ELK4 and MGC16121) were significantly regulated by DHT. ELK4 and MGC16121 were significantly regulated by DHT at 8 hours (P<0.05) whilst SLC6A6 was regulated at 24 hours (P<0.05). ELK4 levels were upregulated by DHT as predicted by the microarray analyses (previous Table 4.2). SLC6A6 and MGC16121 gene levels were regulated in opposite manner to that predicted by the microarray. The levels of the remaining five genes were not significantly altered by DHT at either timepoint (MAP3K8, NR4A2, JMY, CDC25A and FOXL2).

**Group 2 Genes - Selected based on functional analyses of microarray gene expression (outwith putative androgen-regulated gene list at 8 hours) (Figure 4.5):** Out of seven Group 2 genes investigated, four genes (RGS2, SNCAIP, SIK1 and ID3) were significantly regulated by DHT. Three of these genes (RGS2, SNCAIP, SIK1) were significantly regulated by DHT at 8 hours (P<0.05 or P<0.01) whilst all four of the genes (RGS2, SNCAIP, SIK1 and ID3) were regulated at 24 hours (P<0.05 or P<0.01). Levels of three genes were not significantly altered by DHT at either timepoint (PRLR, GADD45G and AXIN2).
Figure 4.4 DHT changed mRNA levels significantly in Group 1 (Table 4.2) putative androgen-regulated genes SLC6A6, ELK4 and MGC16121 but not in MAP3K8, NR4A2, JMY, CDC25A or FOXL2 in SHT-290 cells (3 of 8 genes). Cells were incubated for 8 and 24 hours with vehicle control (V) or DHT (10^{-8} M). Data are presented as mean ± SEM (paired t-tests, n=5). * P<0.05.
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Figure 4.5 DHT changed mRNA levels significantly in Group 2 (Table 4.2) putative androgen-regulated genes RGS2, SNCAIP, SIK1 and ID3 but not in PRLR, GADD45G or AXIN2 in SHT-290 cells (4 of 7 genes). Cells were incubated for 8 and 24 hours with vehicle control (V) or DHT (10^{-8} M). Data are presented as mean ± SEM (paired t-tests, n=5). * P<0.05 and ** P<0.01.
Table 4.4 summarises the results obtained for each gene and for each cell type (hESCs or SHT-290 cells). The observed patterns of DHT-dependent upregulation or downregulation (significant and not significant) for the genes SIK1, SNCAIP, RGS2, SLC6A6 and MGC16121 were consistent in both cell models. The DHT-dependent regulation of JMY, GADD45G and AXIN2 observed in primary hESCs (section 4.3.3.1) was not replicated in SHT-290 cells. On the other hand, DHT-regulation of ID3 and ELK4 was uniquely observed in SHT-290 cells.
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Table 4.4 Summary of the candidate genes regulated by DHT in hESCs and SHT-290 cells (regulation predicted by microarray is included).

<table>
<thead>
<tr>
<th>Group 1 genes</th>
<th>The effect of DHT treatment upon gene levels (relative to vehicle control)</th>
<th>qPCR validation of microarray data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microarray prediction</td>
<td>hESCs 8 hours</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>JMY</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>ELK4#</td>
<td>☐</td>
<td>-</td>
</tr>
<tr>
<td>MGC16121</td>
<td>☐</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2 genes</th>
<th>The effect of DHT treatment upon gene levels (relative to vehicle control)</th>
<th>qPCR validation of microarray data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microarray trend (non-significant)</td>
<td>hESCs 8 hours</td>
</tr>
<tr>
<td>RGS2#</td>
<td>☞</td>
<td>☞</td>
</tr>
<tr>
<td>SNCAIP#</td>
<td>☞</td>
<td>☞</td>
</tr>
<tr>
<td>SIK1</td>
<td>☞</td>
<td>☞</td>
</tr>
<tr>
<td>GADD45G</td>
<td>☞</td>
<td>☞</td>
</tr>
<tr>
<td>AXIN2</td>
<td>☞</td>
<td>☞</td>
</tr>
<tr>
<td>ID3</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Genes are grouped as per selection criteria (summarised in Table 4.2).
( ☐ and ☐) genes significantly up or down regulated.
( ☞ and ☞) genes showing non-significant trend in up or down regulation.
( -) no change in gene level.
( ☞) Genes for which the qRT PCR results followed the direction of regulation by DHT predicted by the microarray (significant regulation or trend).
(*) The effect of 24 hours DHT upon expression of these genes was abrogated by flutamide in SHT-290 cells (see following section 4.3.3.3).
4.3.3.3 DHT-induction of candidate genes **RGS2, SNCAIP, SIK1 and ID3** is AR-dependent in SHT-290 cells (using flutamide)

In previous section 4.3.3.2, the levels of seven genes were significantly altered in response to DHT in SHT-290 cells (*ELK4, MGC16121, RGS2, SNCAIP, SIK1 and ID3*). In the case of four of these seven DHT-dependent genes identified in the previous sections (*SLC6A6, RGS2, SNCAIP, SIK1*) the mRNA levels differed significantly with DHT treatment in both hESCs and SHT-290 cells and the mRNA concentrations were greater at 24 hours (see Figure 4.2 and 4.4).

To determine if the effect was AR-dependent, the effect of DHT was investigated in the presence of flutamide (or vehicle control) in separate experiments. Flutamide is a competitive antagonist that binds AR, blocks the binding of endogenous ligands such as testosterone and DHT and inhibits AR transactivation activity (Neri, 1989, Kemppainen *et al.*, 1992, Farla *et al.*, 2005) (as described in chapter 1). Flutamide was chosen as it has been the AR antagonist commonly used in study the effects of DHT gene expression or functional assays carried out in hESCs *in vitro* (Wen *et al.*, 2006, Cloke *et al.*, 2008, Marshall *et al.*, 2011, Kajihara *et al.*, 2012).

Flutamide significantly abrogated the effect of DHT (24 hours) upon the gene expression of **RGS2, SNCAIP, SIK1, ID3** (P<0.05 or P<0.01, Group 2 genes, Figure 4.6) but not **SLC6A6** (Group 1 gene) in SHT-290 cells.
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Figure 4.6 Flutamide abrogated the effect of DHT on the gene expression of candidate genes suggesting AR-dependent gene expression of RGS2, SNCAIP, SIK1, ID3 (Group 2) but not SLC6A6 (Group 1) in SHT-290 cells. Cells were pre-treated with flutamide (10^{-5} M) or vehicle (MeOH, Methanol) for 1 hour. Culture for 24 hours followed without further treatment (untreated), or with ethanol (EtOH, vehicle for DHT) or with DHT (10^{-8} M). Data presented as mean ± SEM (n=5 or n=3). * P<0.05 and ** P<0.01.
4.3.4 The proteins encoded by the DHT-regulated genes RGS2, SNCAIP, SIK1 and SLC6A6 are connected in a common network with AR

RGS2, SNCAIP, SIK1 and SLC6A6 were identified as DHT regulated on the microarray (section 3.3.3 and Table 4.2) and validated in both primary hESCs and immortalised SHT-290 cells (section 4.3.3 and summarised in Table 4.4). To augment the qRTPCR data and explore interactions between the androgen-regulated genes and AR (from the literature), Metacore was used to reinterrogate its knowledge database and generate a new gene interaction network featuring AR and the proteins encoded by RGS2, SIK1, SNCAIP (Synphilin-1) and SLC6A6. This ‘discovery’ computational approach (based on current knowledge) was undertaken to reveal relationships/interactions between AR and the candidate genes, between the candidate genes themselves, and if any of the GeneGO canonical maps may be overlayed onto the new generated network.

Notably, Metacore was able to build a new network of proteins that included all the genes uploaded – AR and the four new genes (Figure 4.7). The network did not indicate any direct interactions between AR and the candidates RGS2, Synphilin-1(encoded by SNCAIP), SIK1 or SLC6A6. However, several indirect links between AR and each of the candidate genes were inferred: (i) AR was linked to each candidate gene indirectly via one other object (protein) alone (the path beginning with AR) for example, AR→SP1/c-Myc→RGS2. Conversely, RGS2 and SIK1 interacted via one other gene/protein leading back to AR, for example, RGS2→STAT3→AR and SIK1→CREB1/p53/SREBP1)→AR; (ii) AR could also be linked in a path to three of the candidate genes (SIK1, RGS2 and Synphilin-1) via 2 or more other objects. These longer pathways were also constituted mainly by TFs except in the case of the AR-to-Synphilin-1(SNCAIP) paths.
Figure 4.7 Gene network built by Metacore with the androgen-regulated genes: AR, RGS2, SIK1, Synphilin-1 (encoded by SNCAIP) and SLC6A6. AR and the candidate genes (highlighted by shaded circles) were directly uploaded to Metacore (GeneGO) and subjected to pathway analysis. The canonical cAMP transduction pathway partially overlays onto the network (blue arrow), from the G-protein alpha-i family via adenylate cyclase, ATP, cyclic AMP through to protein kinase A (PKA). See Figure 3.1 for detailed object legend.

Direct interactions between the candidate genes themselves were not present in the network but indirect connection via one other object/protein were depicted for example, from SIK1 to RGS2 (SIK1→CREB1→RGS2). Overall, SIK1 was connected in longer paths (via 2 or more proteins) to SLC6A6 and Synphilin-1 (SNCAIP). RGS2 was also connected in paths (via 2 objects) to SIK1, SLC6A6 and Synphilin-1 (encoded by SNCAIP).

Many of the nodes linking the proteins of interest (AR and candidate genes) in these paths were transcription factors with a few having other activities especially those in
paths terminating with Synphilin-1 (encoded by \textit{SNCAIP}). It is notable that, 10 out of the 19 previously identified TFs in Table 3.8 (excluding AR) are present in this new network (Figure 4.7): c-Myc, SP1, ESR1 (ER\(\alpha\)), p53, RUNX2, CREB1, STAT3, EGR1, C/EBP\(\alpha\) and HIF1\(\alpha\).

One canonical pathway map partially overlayed onto this built network (Figure 4.7), the cAMP transduction pathway, from the G-protein alpha-i family via adenylate cyclase, ATP, cyclic AMP through to protein kinase A (PKA) (pathway available at Metacore, http://pathwaymaps.com/maps/660/). The proteins encoded by candidate genes \textit{RGS2}, \textit{SIK1}, \textit{SLC6A6} and \textit{AR} all sit directly at either the beginning (RGS2) or the end of the cAMP pathway (SIK1, SLC6A6 and AR).
4.4 Discussion

In this chapter, eighteen candidate/putative androgen-regulated genes were selected from within and from outwith the the 8 hour androgen differentially expressed gene list in hESCs (P<0.01) (Group 1 and Group 2 genes, respectively) using defined criteria (section 4.3.1, Table 4.2) critically informed by pathway bioinformatic analysis (association with proliferation, apoptosis/cell survival, cell differentiation, cell signalling and cell structure, chapter 3). Androgen regulation of the selected candidate genes was evaluated through analysis of their potential regulation by AR (computational) and through analysis of their regulation by DHT in stromal endometrial cell models. Additional pathway analysis was used to explore the relationships/interactions between AR and the newly identified DHT-regulated genes in hESCs upon androgen stimulation.

Do microarray-derived novel putative androgen-regulated genes in hESCs show potential for regulation by AR (using computational TFBS sequence analysis)?

Eighteen putative androgen-regulated genes were subjected to TFBS sequence analysis. The identification of putative AREs in the proximal promoter sequences of all 18 candidate genes (Group 1 and Group 2, Result Figure 4.1) was consistent with the potential for AR-mediated transcriptional regulation of these genes as established previously in that region for other androgen-regulated genes (Bolton et al., 2007, Marshall et al., 2011). Furthermore, confirmation of the presence of DNA sequences consistent with AREs within the candidate gene sequences supported the use of the selection process informed by the bioinformatic pathway analyses (Group 2 genes). Notably, not every potential ARE will have been predicted by the analysis undertaken as the methods are probabilistic (use models based on experimentally proven binding sites where the sequence variety is ascribed varying degrees of certainty or probability) (Marinescu et al., 2005). Of all the eighteen candidate genes surveyed, ELK4 was the only candidate gene previously reported to have AREs and to be directly targeted by AR in prostate cancer cells, LNCaPs (by ChIP analysis) (Makkonen et al., 2008); with identification of putative AREs being a novel finding for the remaining seventeen genes (Table 4.3) in this thesis.
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It is not known if all the AREs identified using the computational analysis represent functional AR binding sites in the candidate genes expressed in hESCs; additionally, TFBS sequence analysis may not discover all relevant AREs as functional AREs may also be found at sites within the genome more distal to the gene than those surveyed (Bolton et al., 2007). To address these two questions other approaches such as genomic wide ChIP analyses and reporter assays using genomic sequences cloned from the target are required (Bolton et al., 2007). Finally, these approaches do not encompass other ways in which AR, a transcription factor, regulates gene transcription such as tethering-mechanisms where protein-protein interactions with other TFs, regulatory or scaffold proteins occur/operate (reviewed by Heinlein and Chang, 2002b, Heemers and Tindall, 2007, Li and Al-Azzawi, 2009). In these cases, the protein-protein interactions would have to be dissected using for example, gene knockdown (inhibition) or immunoprecipitation techniques and confirmed using reporter assays.

The existence of putative binding sites for other steroid receptor (PR, GR, ERα, ERβ) and steroid-response-associated TFBSs (ERR-α and SP-1) in the candidate gene sequences of both Group1 and also Group 2 genes further highlights the potential for complex steroid hormone-dependent regulation of these candidate genes (Result Table 4.3). Notably, in accordance with the TF-centred analysis, multiple SP1 binding sites were identified in several candidate genes sequences. TFBS for TFs such as PR, ERα, ERβ and SP1 also featured in the promoter regions of previously identified androgen-regulated genes in hESCs (Marshall et al., 2011) and this merits further investigation especially as the hESCs are also exposed to oestrogens and progesterone in the physiological setting during the menstrual cycle.

Analysis highlighted the potential that putative DHT-regulated genes may also be regulated by CREB1. With the exception of ELK4 (Group 1) and ID3 (Group 2) all other Group 1 and Group 2 gene sequences contained predicted CREB1 response elements. Notably, CREB1 was found to have the potential to interact with NR4A2 (NURR1) and MAP3K8 genes by Metacore analysis (Result Table 4.3), although to date of the 18 genes investigated only NR4A2 and RGS2 have been reported to be
Chapter 4  

Identification of androgen regulated genes in endometrial stromal cells confirmed as transcriptional targets of CREB1 (Wu et al., 2005, Zhang et al., 2005). We may speculate the regulation of these putative AR/CREB1 target genes may be via direct AR-DNA interactions but it should be noted that androgens can also regulate CREB-mediated gene expression via non-classical AR-DNA independent interactions (reviewed by Walker, 2010) a possibility that cannot be excluded from the DHT-mediated effects in hESCs.

In summary, TFBS sequence analysis revealed that all the eighteen putative androgen-regulated genes sequences surveyed (Group 1 and Group 2 genes) showed potential for AR-mediated transcription. Most of the genes may also be potentially regulated by other steroid receptors (and CREB1).

Are putative androgen-regulated genes regulated by DHT in endometrial stromal cells models (hESCs and SHT-290 cells) in vitro? And if so, is the regulation AR-dependent?

Out of 15 genes investigated, ten microarray-derived putative DHT regulated genes were regulated by DHT in hESCs and in SHT-290 cells (summary in Table 4.4, previous section 4.3.3.2). In primary hESCs, DHT regulated 7 genes (SLC6A6, JMY, RGS2, SNCAIP, SIK1, GADD45G and AXIN2) at 8 hours and/or 24 hours. A smaller proportion of Group 1 genes (2 out of 8 genes) were regulated by DHT compared to a larger proportion of Group 2 genes (5 out of 7 genes): These data illustrate some of the difficulties faced in validating target genes identified by microarray platforms particularly when the fold changes are relatively modest (<2 fold). Non-detection of significant gene expression changes may be due to microarray false positives (although principal component analysis was carried out by the FDMC to correct for this, data not shown) or the variation specifically between the samples used in the different experiments (microarray versus qRTPCR). Notably, the selection of non-microarray predicted genes (Group 2 genes) based on the DHT-dependent biological associations defined in the hESC by the multitude of bioinformatics approaches proved successful in identifying DHT-regulated genes that were validated by qRTPCR. Although the Group 2 DHT-regulated genes cannot be used to validate the microarray differentially-expressed gene data set directly, their regulation by DHT
Identification of androgen regulated genes in endometrial stromal cells

was useful in supporting bioinformatics to analyse pathways and networks regulated by DHT in hESCs.

The regulation of Group 2 genes by DHT (summary in Table 4.4, section 4.3.3.2) shows how the microarray approach did not identify all androgen-regulated genes highlighting one of the caveats of the microarray approach, that not all genes are necessarily identified by microarray analysis (Bolton et al., 2007). The results of one microarray experiment, even if constituted of multiple timepoints do not represent the total dynamic status within cells but only a snapshot. Emphasizing this further, is that out of 12 androgen-regulated genes identified in hESCs by an in silico approach (and confirmed by qPCR) not one of the candidates from that study (Marshall et al., 2011) was identified as a putative androgen-regulated gene by the microarray analysis reported in the previous chapter.

In SHT-290 cells, DHT-regulation of some candidate genes (putative androgen-regulated genes) was also observed (summary in Table 4.4, section 4.3.3.2). Four genes were regulated by DHT in SHT-290 cells as in hESCs and in the same direction (downregulation of SLC6A6 and SIK1; upregulation of RGS2 and SNCAIP). For three of these genes, RGS2, SNCAIP and SIK1, DHT-regulation was AR-dependent (as suggested by inhibition by flutamide, Figure 4.6) indicating these genes may be AR transcriptional targets. For the DHT-regulated candidate mRNAs that were not investigated in the presence of inhibitor, it remains unknown if their regulation is AR-dependent or if indirect effects are responsible such as ER-dependent regulation by oestrogenic-DHT metabolites.

It remains unknown if the DHT-regulated RNAs (in either cell model and even if AR-dependent) constitute primary or secondary targets of DHT action. Regulation of secondary mRNA targets could be due to activity of primary targets affecting transcription or transcript stability, for example, transcription factors or miRNAs.

It was expected that DHT would regulate the same genes in hESCs and SHT-290 cells as SHT-290 cells were derived from hESCs and previously showed unchanged hormone responsiveness compared to primary hESCs (Barbier et al., 2005).
However, there were differences between the two cell models (summarised in Table 4.4, section 4.3.3.2). Regulation of three genes \textit{ELK4}, \textit{MGC16121} and \textit{ID3} were confirmed in SHT-290 cells but not in primary hESCs (though \textit{MGC16121} showed a trend in hESCs). Three other genes, \textit{JMY}, \textit{GADD45G} and \textit{AXIN2} were uniquely regulated by DHT in hESCs indicating that while SHT-290 cells are a good alternative model to test/study DHT- or AR-dependent gene expression, hESCs may be preferred in certain instances.

Differences between the cell models may be due to metabolic differences imposed on the SHT-290 cell line by the telomerase reverse transcriptase (TERT) immortilisation procedure that may favour, slow down or occlude certain processes or pathways in the cells. For example, TERT modulates Wnt signalling (Choi et al., 2008) and may confound understanding of androgen-dependent Wnt signalling predicted by the bioinformatics pathway analysis in this chapter. Thus, study of the candidate gene \textit{AXIN2}, a Wnt pathway negative regulator (both a Wnt feedback product and reliable marker of Wnt activation) (Jho et al., 2002, Lustig et al., 2002) should be preferentially carried out in primary hESCs. Other potential differences in AR transcriptional activity between the primary hESCs and the SHT-290 cell line could be attributed to a distinct cell specific profile of factors that modulate AR activity such as AR post-translational modifications and AR co-regulators. Indeed, the expression of co-regulators has been shown to be cell-line specific (Bebermeier et al., 2006).

DHT was used in the current experiments to limit confounding concurrent oestrogenic signalling (as it cannot be converted to oestradiol by hESC aromatase). However, weak oestrogenic effects cannot be ruled out entirely due to the very weak ER agonist activity of DHT itself and DHT metabolites 3α-diol and 3β-diol (most converting enzymes have been detected in hESCs). Very weak PR agonist activity of DHT has also been reported (Andrieu et al., 2015).

Androgen-dependent expression of candidate genes in both stromal cell models was not only found at 8 hours post DHT treatment but also after 24 hours. Prolonged changes in gene expression by androgens is consistent with previous reports in
prostate cancer cells at 24 hours (Velasco et al., 2004, Bolton et al., 2007, Rajan et al., 2011) and also in the endometrium. For example, ADAMTS-1 (a disintegrin metalloproteinase with thrombospondin type 1 motif, 1) was upregulated by DHT in hESCs after 24 hours treatment and induction was sustained at 48 and 72 hours (Wen et al., 2006).

Of the 10 genes regulated by DHT in both the stromal cell models (summary in Table 4.4, section 4.3.3.2), five genes (SLC6A6, JMY, RGS2, SNCAIP and AXIN2) are novel androgen regulated genes, reported here for the first time. Androgens have previously been reported to regulate the other candidate genes although, as mentioned earlier, direct AR binding has only been shown for ELK4. Specifically, the androgen receptor agonist R1881 has been reported to upregulate the expression of ELK4 in LNCaP cells (Makkonen et al., 2008) and MGC16121 gene levels in an immortalised normal prostate epithelial cell line (HPr-1AR) (Bolton et al., 2007).

DHT can also enhance SIK1 levels during rat cerebral ischaemic injury (Cheng et al., 2011). Androgens upregulated GADD45G in human LNCaP cells, rat ventral prostate cells, murine muscle and the uterus (Jiang and Wang, 2003, Yoshioka et al., 2006, Ivanga et al., 2009). ID3 is reported to be upregulated by testosterone in cultured rat Sertoli cells (Buzzard et al., 2003) and in the ovine foetal ovary after prenatal exposure (Hogg et al., 2011).

Six of the ten DHT-regulated genes (or proteins) SLC6A6, JMY, ELK4, MGC16121, SNCAIP and SIK1 identified in endometrial stromal cells have not been documented as being expressed in endometrial tissues previously. The other four genes RGS2, GADD45G, AXIN2 and ID3 (or proteins) have previously been reported as expressed in murine or human endometrial tissues. Specifically, RGS2 mRNA has been localised to murine decidualised stroma (Huang et al., 2003) and GADD45G mRNA has been both detected and reported as temporally regulated by DHT in the mouse uterus (Ivanga et al., 2009). AXIN2 protein has been detected in human endometrial stromal and epithelial cells (Nguyen et al., 2012) and ID3 protein is not only present in human endometrium (whole tissue, cell type unspecified) but also reported as upregulated in endometrial cancer specimens (Sun et al., 2013).
Chapter 4 Identification of androgen regulated genes in endometrial stromal cells

It is unknown if the DHT-regulated candidate genes are regulated by oestrogen in hESCs. However, their regulation by oestrogen may be expected as the potential for regulation by oestrogen receptors was established by the presence of the respective oestrogen response elements in the gene promoters. In addition, in the endometrial oestrogen-dominant proliferative phase, the hESCs are exposed to both oestrogens and androgens. Thus, the current studies carried out with DHT alone are not fully representative of undifferentiated hESC physiological conditions. It may also be argued that the use of DHT is not physiological (as it is mostly produced intracellularly from testosterone), but the use of DHT is preferred to testosterone to study androgenic responses (with minimal oestrogenic responses) as it is not aromatised to oestradiol.

Regulation of the DHT-regulated candidate genes has been reported by other steroids or factors in various other human or animal cell types. The expression of AXIN2 was upregulated by glucocorticoids in progenitor adipocytes (Naito et al., 2013). cAMP has been shown to regulate RGS2 and SIK1 expression (Pepperl et al., 1998, Xie et al., 2011b, Stewart et al., 2013). Recently, regulation of RGS2, SIK1, GADD45G and AXIN2 expression has been reported by miRNAs (Kim et al., 2013, Zhao et al., 2014, Hommers et al., 2015, Lu et al., 2015, Ren et al., 2016). It is thus also possible that DHT acts upon miRNA expression to effect the downregulation of SIK1, GADD45G and AXIN2 (as secondary targets). Such information may inform future studies as to other regulators of candidate gene mRNAs in hESCs.

The hESCs cells recovered (from proliferative endometrium) and cultured in the experiments herein are thought to be mostly stromal cells from the endometrial functional layer as the pipelle samples endometrium tissue mostly from the functional layer with some possible varying amounts of the basal layer. It may be speculated that the stromal cells from the different endometrial layers may have inherent different characteristics that may be reflected in the findings from mRNA investigations (putative androgen-regulated genes). Notably, there is evidence that suggests AR protein expression can vary between the endometrial basal and the functional compartments (Marshall et al., 2011) (chapter 3). The expression of AR protein is maintained in stromal cells of the basal compartment regardless of cycle
stage with the most intense immunoexpression of AR reported has been reported in the stromal cells of the functional layer in the proliferative phase (Marshall et al., 2011).

*The above new data was used to reinterrogate the bioinformatic databases to reveal that DHT-regulated SLC6A6, RGS2, SNCAIP and SIK1 (in hESCs and SHT-290 cells) are connected in a novel common network with AR:* This network (Result Figure 4.7) includes the TFs cMyc, SP1, p53, CREB1 and STAT3 that feature in an androgen response network in prostate cells (regulating proliferation, (Vellaichamy et al., 2010) and in the endometrial stromal cells, with the exception of CREB1 (Marshall et al., 2011). Other interacting nodes of the generated network such as ERα, C/EBPα, HIF1α and G-protein alpha-q/11 were also integral in the androgen response network in the endometrial stromal cells where the regulation of apoptosis was one of the main associated processes (Marshall et al., 2011).

It was possible for Metacore to overlay one canonical pathway onto the newly generated network (Figure 4.7) and this was identified as the regulation of cAMP signalling. This indicated how androgens may impact the cAMP transduction pathway in hESCs. Androgen effects via nonclassical (AR-DNA independent interactions) have been shown to activate cAMP levels (reviewed by Walker, 2003). However, the current microarray-derived and cell experiment findings suggest a way in which androgens such as DHT may affect cAMP signalling via AR (and in turn, be affected by it): in part by modulation of RGS2 and/or SIK1 mRNA levels situated both at the beginning and end of the signalling pathway, respectively. Depending on cell type, cAMP has been associated with being both pro-apoptotic and anti-apoptotic (reviewed by Insel et al., 2012). Pertinently, cAMP signalling is critically important in decidualisation of endometrial stromal cells (Brar et al., 1997) and there is previous evidence (Cloke et al., 2008) for a role for androgens in decidualisation. Again, the possible regulation of proliferation, differentiation and apoptosis by androgens in hESCs is suggested via the modulation of Protein kinase A (PKA)/cAMP signalling.
Finally, the network could be used to identify other genes (included or excluded from the network) that may also be regulated by androgens in hESCs. Interestingly, GSK3β was also represented in the network, a gene also predicted to be DHT-regulated by the microarray analysis (8 hour putative androgen-regulated gene list, Appendix B). Though not indicated in this particular network, GSK3β is also a known direct target of PKA activity (inhibition via phosphorylation). Another known direct target of PKA is CREB1 (activation via phosphorylation) - an interaction that is also omitted from the network (although PKA and CREB1 are both included in the network). Omission of known interactions from a network highlights the features of the algorithm used to build networks: the networks do not include all interactions known between all the genes of the network but give more emphasis to the interactions between the specific genes used to build the pathway and any canonical map that may overlay onto the built network.

In summary, the new network generated suggests how the direct regulation of candidate genes by androgens (via AR; in the case of RGS2, SNCAIP and SIK1) is a novel finding across the literature and would be a novel addition to this network (and current knowledge). In addition, the network also indicated how androgens may impact the cAMP transduction pathway in hESCs.

Conclusions
The hypothesis tested by the investigations in this chapter was - “Putative androgen-regulated genes show potential for regulation by AR and are regulated by DHT in endometrial stromal cells”. The findings presented would lend support to this hypothesis, although not all mRNAs were confirmed as DHT-regulated in endometrial stromal cells. Confirmation of individual candidate gene regulation by DHT goes some way towards validation of the microarray results although a larger number of putative candidate genes should be investigated. The genes SLC6A6, RGS2, SNCAIP and SIK1 were confirmed as DHT-regulated in hESCs and may be candidates for direct binding by AR. Androgen regulation of these genes (SLC6A6, RGS2, and SNCAIP) has not been previously described and/or are novel for the human endometrium (SLC6A6, SNCAIP and SIK1). Association of these genes with cell processes such as proliferation, differentiation and apoptosis/cell survival and the
PKA/cAMP pathways offers suggestions for pathways/processes that DHT-dependent gene expression may regulate in hESCs. The findings in this chapter add further support for a physiological role for androgens in the human endometrium through the regulation of gene expression in hESCs.
Chapter 5: Evidence for expression of putative androgen-regulated genes in the endometrium and altered expression in association with decidualisation

5.1 Introduction

In the previous chapter, androgen-regulated genes were identified in endometrial stromal cells from a putative androgen-regulated gene list. In this chapter, the expression of these mRNAs in the endometrium and in particular during decidualisation was investigated allowing speculation as to their regulation in the endometrium.

In chapter 4, seven out of fifteen putative androgen-regulated genes investigated (SLC6A6, JMY, RGS2, SNCAIP, SIK1, GADD45G, AXIN2) were confirmed as DHT-regulated in undifferentiated primary hESCs. Although another eight genes were not confirmed as significantly regulated by DHT (MAP3K8, NR4A2, CDC25A, ELK4, FOXL2, MGC16121, PRLR and ID3) their mRNAs were also readily detectable in human endometrial stromal cells. For ten of these genes, this detection in hESCs represented a novel finding. The transcripts and/or protein expression of the other five putative androgen-regulated genes have previously been reported in endometrial cells or tissues. For example, MAP3K8 transcripts are reported to be overexpressed in about 30% of endometrial carcinomas compared to corresponding adjacent normal (non-malignant) endometrial tissue (Aparecida Alves et al., 2006). Expression of the PRLR mRNA and temporal protein expression have previously been described in the human endometrium (Jabour et al., 1998, Jones et al., 1998) with PRLR mRNA and protein demonstrated to be upregulated in secretory endometrium and decidua. AXIN2 (mRNA and protein) has also been identified in human endometrial tissues or cells. Specifically, AXIN2 mRNA concentrations were reported to be reduced in epithelial cells isolated from premenopausal endometrium compared with those cells isolated from postmenopausal endometrium in a study to identify the transcriptional profile of the regenerative premenopausal endometrium (Nguyen et al., 2012). In the same study, AXIN2 protein was detected in both human endometrial stromal and
epithelial cells by fluorescent immunostaining of tissues. ID3 protein was detected by Western analysis in human endometrial tissues and upregulated in endometrial cancer specimens (Sun et al., 2013). FOXL2 mRNA and protein were detected in human endometrial tissues with no change in expression being reported between proliferative and secretory phase endometrium (Governini et al., 2014). This same study reported FOXL2 protein localised to stromal cells and to the cytoplasm of epithelial cells by immunohistochemical staining. Therefore, to date, out of the 15 candidate genes examined in this thesis the detection of mRNA and/or protein of five candidate genes have been reported in the human endometrium, but changes of gene expression across the normal menstrual cycle (in more than one phase of the cycle) have only been described for PRLR and FOXL2.

In addition, the detection of mRNAs from four of these candidate genes (RGS2, PRLR, FOXL2 and GADD45G) has been reported in the uterine tissues of animal models. For examples, RGS2 gene expression has been reported in murine decidualised stromal cells (Huang et al., 2003). PRLR RNA and protein was expressed in the marmoset monkey endometrium (Dalrymple and Jabbour, 2000). PRLR transcripts have also been found to be upregulated in the decidua of the pig, yak and rodent during pregnancy (Trott et al., 2009, Devi et al., 2011, Zi et al., 2012). Expression of the FOXL2 gene was identified and found to be downregulated by progesterone in the bovine uterus (Eozenou et al., 2012). GADD45G was reported in the murine uterus (Ivanga et al., 2009).

It was interesting to note that some of the androgen regulated candidate genes were detectable in the endometria of animal models during pregnancy. Complementing this observation is information suggesting that some of the candidate genes, besides being linked to the cAMP transduction pathway (as presented in Chapter 4, Figure 4.7), may also be regulated by cAMP in other cell types. For example, cAMP has been shown to regulate RGS2 and SIK1 expression (Pepperl et al., 1998, Xie et al., 2011b, Stewart et al., 2013). Together with progesterone, cAMP signalling is pivotal in the differentiation process (decidualisation) of the stromal cells during the secretory phase, embryo implantation and pregnancy (Brar et al., 1997). Thus, based on the bioinformatics analyses performed herein (Chapter 4) and on evidence from
other studies mentioned above there was the suggestion that some of the androgen regulated candidate genes may be expressed, or their expression regulated, in the decidua during pregnancy.

In this chapter, to further investigate the potential roles played by the candidate genes (SLC6A6, JMY, RGS2, SNCAIP, SIK1, GADD45G, AXIN2, MAP3K8, NR4A2, CDC25A, ELK4, FOXL2, MGC16121, PRLR and ID3) in the human endometrium and to reveal if the candidate genes were regulated during the menstrual cycle and/or pregnancy (decidualisation), their expression was investigated in tissue biopsies of proliferative, mid-secretory endometrium and first trimester decidualised endometrium (decidua). This also enabled the gene expression patterns to be compared to the expression pattern of AR in the endometrium. As mentioned before, AR mRNA and protein expression is greatest in proliferative endometrium (stromal cells) and attenuated in secretory endometrium and decidua (stromal cells and some epithelial cells) (Tamaya et al., 1985, Milne et al., 2005, Critchley and Saunders, 2009, Marshall et al., 2011) (section 1.7.2).

Therefore, this chapter addresses the hypothesis: “Putative androgen-regulated genes are expressed and regulated in the human endometrium across the menstrual cycle (proliferative and mid-secretory endometrium) and in early pregnancy (first trimester decidua)”

5.1.1 Aims of Chapter

- To determine the mRNA expression pattern of fifteen putative androgen regulated genes in endometrial and first-trimester decidual tissues.

- To investigate the protein expression of three putative androgen regulated genes in endometrial and first-trimester decidual tissues.

- To investigate if some putative androgen regulated genes might also be induced by oestrogens in hESCs.

- To explore the expression of putative androgen regulated genes in the context of decidualisation.
5.2 Materials and Methods

This section describes all specific methods and outlines the experimental approaches employed in Chapter 5; general methodology has been summarised in Chapter 2.

5.2.1 Endometrial and first trimester decidual tissue biopsies

All endometrial tissues used in this chapter were collected under ethical approval as described in section 2.2 and processed as described in sections 2.2 (tissues) and/or section 2.3.1 (isolation of primary hESCs).

All decidual tissues were collected during surgical procedures, under general anaesthesia and processed as described in section 2.2. First trimester (7 to 12 weeks gestation) decidualised endometrial tissue included: (i) decidua from viable intrauterine pregnancies at the time of surgical termination of pregnancy (STOP) by vacuum aspiration (misoprostol administered about 1 hour before), and (ii) decidua from women undergoing surgical management of ectopic (tubal) pregnancies (non-viable extrauterine pregnancy) (sections 5.2.2.1 and 5.2.2.3; Tables 5.1 and 5.2). The macroscopic procedure employed to isolate decidual tissue from the aspirated products of the STOPs has been shown to be an adequate method to isolate decidua without trophoblast contamination (Horne et al., 2008) based on lack of cytokeratin staining (trophoblast marker) (Proll et al., 1997). The absence of trophoblast in the decidual biopsies was confirmed by histological analysis (hematoxylin and eosin staining) (Dr Alistair Williams, pathologist). Decidual tissues from women with ectopic pregnancies were recovered by suction curette.

The clinical details for the women who donated tissue samples used in the investigations carried out in this chapter are summarised in tables in each relevant section below.

5.2.2 RNA analysis

Tissues were harvested and stored as described in section 2.2. Cultured cells were harvested at the experimental timepoints following the experimental design outlined in each section below. Total cell RNA was extracted from tissues and cultured cells (section 2.7.1). RNA was quantified and cDNA was synthesized (section 2.7.2). The
levels of the genes investigated (and endogenous control) were determined by qRT-PCR as described in section 2.7.3 (the primers and probes used are detailed in Table 2.5).

5.2.2.1 Gene expression in endometrial and first trimester decidual tissues

Table 5.1 summarises the clinical details for the women who donated endometrial and first trimester decidual samples from which RNA was isolated and relative concentrations of mRNAs were quantified from cDNA as described above (section 5.2.2). First experiments were carried out with samples from four patients per group (proliferative endometrium, secretory endometrium, first trimester decidua) but as these samples were finite and became exhausted new samples were prepared and added to the studies.
Table 5.1 Clinical details for women who provided endometrial or first trimester decidual tissues used in RNA investigations (carried out in section 5.3.1)

<table>
<thead>
<tr>
<th>Sample</th>
<th>LREC</th>
<th>Day of cycle/Gestation</th>
<th>E₂</th>
<th>P₄</th>
<th>Histological dating</th>
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</tr>
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<tr>
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<td>n/a</td>
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<tr>
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<td>n/a</td>
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<tr>
<td>2₁</td>
<td>D</td>
<td>10w 4d</td>
<td>n/a</td>
<td>n/a</td>
<td>Decidualised</td>
</tr>
<tr>
<td>2₂</td>
<td>C</td>
<td>10w 6d</td>
<td>n/a</td>
<td>72.91</td>
<td>Decidualised</td>
</tr>
<tr>
<td>2₃</td>
<td>D</td>
<td>10w 2d</td>
<td>n/a</td>
<td>n/a</td>
<td>Decidualised</td>
</tr>
</tbody>
</table>

Circulating levels of E₂: Oestradiol (pmol/L) and P₄: progesterone (nmol/L). n/a: not available. Gestation w: weeks and d: days. LREC approval for endometrial tissues A: LREC17/6/1994 and LREC05/S1103/14, B: LREC07/S1103/29 and decidual tissues C: LREC04/S1103/20 and D: LREC05/S1104/12. Women with benign gynaecological conditions such as heavy menstrual bleeding, endometriosis or fibroids less than 3cm diameter were included. Gestation was confirmed by ultrasound scan. Endometrial tissues sampled from women by pipelle (a) or collected from uterus during hysterectomy (b).

5.2.2.2 Modulation of DHT-regulated candidate genes by oestradiol alone or by oestradiol in the presence of DHT

Endometrial stromal SHT-290 cells (described in section 2.3.2) that have been validated as expressing receptors for the steroids oestrogen, progesterone (Barbier et al., 2005) and androgen (section 3.3.2.2) were cultured in media with 10% charcoal
stripped serum for 2 to 4 days (section 2.3.2). Cells were then seeded in 6 well cluster plates (1 x 10^5 cells/well) as described in section 2.3.2 and allowed to adhere overnight. At 80-90% confluence, cells were serum starved for 24 hours and incubated with 10nM E_2 or 10nM E_2 plus 10nM DHT for 24 hours (section 2.4.1). Treatments with the respective vehicle controls (DMSO or DMSO/Ethanol (EtOH)) were included. Each treatment was performed in duplicate. Each experiment was repeated 4 times (results section 5.3.5).

5.2.2.3 Gene expression determined in vivo during early pregnancy in association with decidualisation: comparison of first trimester decidual tissues from viable intrauterine pregnancies and tubal ectopic pregnancies

To study gene expression in vivo during early pregnancy in association with decidualisation, mRNA levels were investigated in decidua from tubal ectopic pregnancies (EP) and from intrauterine pregnancies (IUP). Decidua from ectopic pregnancies was used as an in vivo model of pregnancy that exhibits variable but overall reduced decidualisation of the endometrium as per assessment of morphological features (reproduced in Figure 5.1) and significantly reduced mRNA levels of the decidualisation markers, insulin-like growth factor binding protein 1 (IGFBP1) and prolactin (PRL) (Horne et al., 2008).

**Figure 5.1** The overall morphological degree of endometrial decidualisation is reduced in ectopic pregnancies compared with viable intrauterine pregnancies. Assessment of the degree of endometrial decidualisation in vivo by morphology (+, ± and -) showed that endometrium from ectopic pregnancies (EP) was less decidualised compared with intrauterine pregnancies (IUP). P<0.05. Figure adapted from Horne et al., 2008.
Table 5.2 summarises the clinical details for the women with viable intrauterine pregnancies or tubal ectopic pregnancies who donated decidual samples from which isolated RNA was used to prepare cDNA and quantify relative mRNA concentrations as previously described above in section 5.2.2. To characterize the pregnancy groups and establish similar characteristics as observed in similar studies (Horne et al., 2008) the levels of serum progesterone and human chorionic gonadotrophin (hCG) were also compared.

Table 5.2 Clinical details for women with IUP and EP that provided decidual tissues used in RNA investigations and detection of AR by IHC (carried out in section 5.3.6)

<table>
<thead>
<tr>
<th>Decidual sample</th>
<th>Gestation</th>
<th>P₄</th>
<th>hCG</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intrauterine pregnancies (IUP)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7w 4d (53)</td>
<td>64.35</td>
<td>31766</td>
</tr>
<tr>
<td>2</td>
<td>8w 3d (59)</td>
<td>78.3</td>
<td>173939</td>
</tr>
<tr>
<td>3</td>
<td>8w 5d (61)</td>
<td>75.07</td>
<td>117633</td>
</tr>
<tr>
<td>4</td>
<td>8w 5d (61)</td>
<td>63.05</td>
<td>94709</td>
</tr>
<tr>
<td>5</td>
<td>9w 1d (64)</td>
<td>70.66</td>
<td>185815</td>
</tr>
<tr>
<td>6</td>
<td>10w 2d (72)</td>
<td>70.63</td>
<td>68605</td>
</tr>
<tr>
<td>7</td>
<td>10w 3d (73)</td>
<td>82.55</td>
<td>116751</td>
</tr>
<tr>
<td>8</td>
<td>10w 6d (76)</td>
<td>72.91</td>
<td>127920</td>
</tr>
<tr>
<td>9</td>
<td>10w 6d (76)</td>
<td>40.99</td>
<td>22772</td>
</tr>
<tr>
<td>10</td>
<td>11w 2d (79)</td>
<td>60.71</td>
<td>93054</td>
</tr>
<tr>
<td><strong>Tubal Ectopic pregnancies (EP)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>&lt; 6w (&lt;42)</td>
<td>9.54</td>
<td>2662</td>
</tr>
<tr>
<td>12</td>
<td>6w 5d (47)</td>
<td>8.8</td>
<td>453</td>
</tr>
<tr>
<td>13</td>
<td>6w 2d (44)</td>
<td>7.06</td>
<td>508</td>
</tr>
<tr>
<td>14</td>
<td>6w 2d (44)</td>
<td>10.64</td>
<td>12161</td>
</tr>
<tr>
<td>15</td>
<td>6w 4d (46)</td>
<td>31.7</td>
<td>10285</td>
</tr>
<tr>
<td>16</td>
<td>7w (49)</td>
<td>57.27</td>
<td>14380</td>
</tr>
</tbody>
</table>

IUP: intrauterine pregnancy EP: ectopic pregnancy. Circulating levels of E₂: Oestradiol (ρmol/L), P₄: progesterone (nmol/L) and hCG: human chorionic gonadotrophin (Units/L). n/a: not available. Gestation w: weeks and d: days (total days). Average gestation for IUP=67.4 days and for EP=45.3 days. Decidual tissues were collected under LREC04/S1103/20 approval. Gestation of IUP was confirmed by ultra-sound scan.
5.2.2.4 Modulation of DHT-regulated candidate genes in decidualised primary hESCs (in the presence or absence of DHT) *in vitro*

hESCs isolated from endometrial samples (as described in section 2.3.1) were seeded in 6 well cluster plates (1 x 10^5 cells/well) in 2% charcoal stripped serum media (section 2.3.1.2) and allowed to adhere overnight. At 80-90% confluence, cells were decidualised by addition of 2% charcoal stripped serum media (section 2.3.1.2) supplemented with 1μM (10^-6 M) P₄ and 0.1mg/ml cAMP (section 2.4.2). In the experiments where addition of DHT was assessed, cells were decidualised as above with the additional 10nM or 100nM of DHT or vehicle controls (section 2.4.1). Culture medium with or without hormonal supplements was changed every two days. Cells and media were harvested after 8 days. Each treatment was carried out in duplicate. Each experiment was repeated 4 to 7 times, each time with hESCs isolated from a different endometrial biopsy (Table 5.3).

<table>
<thead>
<tr>
<th>Sample</th>
<th>LREC</th>
<th>Day of cycle</th>
<th>E₂</th>
<th>P₄</th>
<th>Histological dating</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A</td>
<td>1</td>
<td>8.4*</td>
<td>2.3</td>
<td>Menstrual</td>
</tr>
<tr>
<td>2</td>
<td>A</td>
<td>4</td>
<td>156</td>
<td>&lt;3</td>
<td>Menstrual</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
<td>10</td>
<td>339</td>
<td>&lt;3</td>
<td>Proliferative</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>13</td>
<td>1018</td>
<td>&lt;3</td>
<td>Proliferative</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>15</td>
<td>596.9</td>
<td>88.94</td>
<td>Secretory</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>16</td>
<td>617.5</td>
<td>8.94</td>
<td>Proliferative</td>
</tr>
<tr>
<td>7</td>
<td>A</td>
<td>27</td>
<td>4.2*</td>
<td>1.1</td>
<td>Late secretory</td>
</tr>
</tbody>
</table>

*The accuracy of the results was checked and the E₂ concentrations for these samples were considered as baseline/low as the values are below the normal threshold of detection of some assays and may not represent actual concentrations.*

**Table 5.3 Clinical details for women who provided endometrial tissues used for hESC extraction and in vitro decidualisation.** (The subsequent qRT-PCR and western analysis investigations carried out are described in section 5.3.7 and 5.3.8 and in chapter 6)

Circulating levels of E₂: Oestradiol (pmol/L) and P₄: progesterone (nmol/L). Endometrial tissues were collected under LREC approval A) LREC10/S1402/59 and B) LREC07/S1103/29. Exceptionally for sample 7, serum was sampled several days after tissue biopsy was collected (instead of on the day of sampling). Women with benign gynaecological conditions such as heavy menstrual bleeding or fibroids less than 3cm diameter were included. * The accuracy of the results was checked and the E₂ concentrations for these samples were considered as baseline/low as the values are below the normal threshold of detection of some assays and may not represent actual concentrations.
5.2.3 Immunohistochemical detection of RGS2, Synphilin-1 (encoded by SNCAIP) SIK1 and AR proteins in endometrial and decidual tissues

Immunohistochemical detection of RGS2, SIK1 or SNCAIP was carried out on endometrial and first trimester decidual tissue samples detailed below in Table 5.4 (on the next page).

In chapter 3, immunolocalisation of AR was demonstrated in proliferative and mid-secretory endometrium (sections 3.2.2, 3.3.1 and Figure 3.2). In this chapter, immunohistochemical detection of AR was carried out on first trimester decidual tissue samples (listed in Table 5.2) allowing the comparison of AR expression in decidua from ectopic pregnancies (not reported to date) with that in decidua from viable intrauterine pregnancies (described in section 5.2.2.3).

Human endometrial and decidual tissue samples were embedded in wax as described in section 2.5.1. Immunohistochemical detection of each protein was carried out on tissue sections (Table 2.1 summarises the antibody details and antigen detection steps) and imaged as detailed in sections 2.5.3 and 2.5.4.

Negative controls used are detailed in Table 2.2. RGS2 negative controls consisted of concentration matched mouse IgG. SIK1 was detected with two different antibodies (a rabbit polyclonal and a mouse monoclonal antibody) with negative controls consisting of concentration matched rabbit IgG or mouse IgG, respectively. Synphilin-1 was detected using a whole rabbit anti-serum and negative controls were incubated with normal rabbit serum. AR negative controls were prepared with concentration matched rabbit IgG (section 2.5.3.5).
### Table 5.4 Clinical details for women that provided endometrial or decidual tissues used in immunohistochemical detection of RGS2, Synphilin-1 and SIK1 proteins (carried out in sections 5.3.2, 5.3.3, 5.3.4)

<table>
<thead>
<tr>
<th>Sample</th>
<th>LREC</th>
<th>Day of cycle / Gestation</th>
<th>E₂</th>
<th>P₄</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proliferative endometrium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A</td>
<td>7</td>
<td>88</td>
<td>1.24</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
<td>8</td>
<td>640</td>
<td>1.2</td>
</tr>
<tr>
<td>3</td>
<td>B</td>
<td>12</td>
<td>1105</td>
<td>1.91</td>
</tr>
<tr>
<td>4</td>
<td>A</td>
<td>14</td>
<td>1090</td>
<td>6.23</td>
</tr>
<tr>
<td>5</td>
<td>B</td>
<td>16</td>
<td>617.5</td>
<td>8.94</td>
</tr>
<tr>
<td>6</td>
<td>B</td>
<td>18</td>
<td>6*</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Secretory endometrium</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>B</td>
<td>21</td>
<td>746.5</td>
<td>62.52</td>
</tr>
<tr>
<td>8</td>
<td>A</td>
<td>21</td>
<td>242</td>
<td>53.1</td>
</tr>
<tr>
<td>9</td>
<td>B</td>
<td>22</td>
<td>549</td>
<td>25.47</td>
</tr>
<tr>
<td>10</td>
<td>A</td>
<td>23</td>
<td>331</td>
<td>83.5</td>
</tr>
<tr>
<td>11</td>
<td>B</td>
<td>26</td>
<td>499</td>
<td>28.97</td>
</tr>
<tr>
<td>12</td>
<td>B</td>
<td>28</td>
<td>408.6</td>
<td>31.6</td>
</tr>
<tr>
<td>13</td>
<td>A</td>
<td>n/a</td>
<td>680</td>
<td>45.85</td>
</tr>
<tr>
<td><strong>First trimester decidua</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>C</td>
<td>8w 5d</td>
<td>n/a</td>
<td>75.07</td>
</tr>
<tr>
<td>15</td>
<td>C</td>
<td>9w 1d</td>
<td>n/a</td>
<td>70.66</td>
</tr>
<tr>
<td>16</td>
<td>C</td>
<td>10w 3d</td>
<td>n/a</td>
<td>82.55</td>
</tr>
<tr>
<td>17</td>
<td>C</td>
<td>10w 6d</td>
<td>n/a</td>
<td>72.91</td>
</tr>
<tr>
<td><strong>Anonymous historical tissue samples employed as positive controls</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placenta</td>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tonsil</td>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney</td>
<td>D</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Circulating levels of E₂; Oestradiol (pmol/L) and P₄; progesterone (nmol/L). n/a: not available. LREC approval A) LREC17/6/1994 and LREC05/S1103/14, B) LREC07/S1103/29, C) LREC04/S1103/20 and D) anonymous historical tissue samples. Women with benign gynaecological conditions such as heavy menstrual bleeding or fibroids less than 3cm diameter were included. * The accuracy of the results was checked and the E₂ concentrations for this sample was considered as baseline/low as the values are below the normal threshold of detection of some assays and may not represent actual concentrations.
5.2.4 SIK1 and AR Western analysis
Extraction of total cell protein from hESC and SHT-290 cell monolayers was carried out (section 2.6.1) and protein concentrations were quantified (section 2.6.3). Protein extracts (20µg) were subjected to SDS-PAGE separation, western transfer and immuno-detection permitting quantification of SIK1 or AR proteins relative to the control β-tubulin as detailed in section 2.6.4.

5.2.4.1 SIK1 Western analysis of untreated hESCs and SHT-290 cells treated with DHT and flutamide (anti-androgen)
hESCs isolated from endometrial samples (Table 3.1) were cultured in 10% charcoal stripped serum media (section 2.3.1) for 2 to 3 days and were seeded in 100mm dishes (~5x10⁵ cells) and allowed to adhere overnight. At 90% confluence, cells were harvested for protein. The experiment was repeated 3 times, each time with hESCs from 3 different patients. hESCs were only used in experiments until fifth passage (section 2.3.1).

SHT-290 cells cultured in 10% charcoal stripped serum media (section 2.3.2) for 2 to 4 days and were seeded in 100mm dishes (~5x10⁵ cells) and allowed to adhere overnight. At 80-90% confluence, cells were serum starved for 24 hours and then treated for a further 24 hours with 10nM DHT or vehicle control (section 2.4.1). Cells were also pre-treated with anti-androgen, flutamide (10µM) or vehicle control before the addition of androgen, DHT or DHT vehicle control (section 2.4.1). The experiment was repeated 5 times.

5.2.4.2 AR Western analysis of decidualised hESCs treated with DHT
hESCs isolated from endometrial samples (Table 5.3) were seeded in 100mm dishes (~5x10⁵ cells) in 2% charcoal stripped serum media (section 2.3.1.2) and allowed to adhere overnight. At 80-90% confluence, cells were decidualised as described above in section 5.2.2.4 with the additional 10nM or 100nM of DHT or vehicle controls (section 2.4.1). Cells and media were harvested after 8 days. Each experiment was repeated 3 times, each time with hESCs from a different endometrial biopsy (Table 5.3). hESCs were only used in experiments until fifth passage (section 2.3.1).
5.2.5 Detection of IGFBP1 protein in decidualised hESC culture media by ELISA

Cell culture media from the decidualised hESC experiments described in section 5.2.2.4 was collected at the same time as cell harvest (8 days). Media samples were stored at -80°C until assayed for IGFBP1 following a two-site sandwich ELISA protocol detailed in section 2.8. A standard curve was constructed for each plate, consisting of ten standards added in duplicate, 100µl per well. Media samples to be assayed were added in duplicate, 100µl per well. The remainder of the assay was conducted as described in section 2.8.

5.2.6 Statistical analysis

Prism 5 for Windows version 5.02, 2008 by Graphpad Software (La Jolla, CA, USA) was used to perform statistical analyses. Data is presented as means ± standard error of the mean (SEM) with P<0.05 considered statistically significant (section 2.9). For qRTPCR analysis of gene expression in tissue samples Kruskall-Wallis one-way analysis with Dunn’s multiple comparison post hoc test (endometrium and decidua comparison) and Mann-Whitney test (EP and IUP decidua comparison) were used. For the comparison of serum P₄ and hCG concentrations (from EP and IUP), the Mann-Whitney test was used. For correlation analysis between gene expression in decidual tissues (from EP and IUP) Spearman’s rank correlation coefficients were used. The size of the correlation coefficient was interpreted as defined by Mukaka and colleagues (2012). In culture studies, SIK1 protein levels were compared using two-way ANOVA with Bonferroni’s multiple comparison post hoc test (in SHT-290 cells) and AR protein levels were compared with Paired t-test. IGFBP1 protein secretion levels and qRTPCR analysis of gene expression in decidualised primary hESCs were compared with either Paired t-test, Wilcoxon test or Repeated Measures ANOVA with Bonferroni’s multiple comparison post hoc test (as described in section 2.9).
5.3 Results

5.3.1 The mRNAs of AR and putative androgen-regulated genes are expressed and modulated in human endometrium and first-trimester decidua

To validate the expression of all fifteen putative androgen-regulated candidate genes (NR4A2, SLC6A6, JMY, CDC25A, ELK4, FOXL2, MGC16121, RGS2, SNCAIP, SIK1, GADD45G, MAP3K8, PRLR, AXIN2 and ID3) in the human endometrium, gene patterns of expression were determined by qRTPCR in tissue biopsies collected from women in the proliferative or secretory phases of the menstrual cycle or in the first trimester of pregnancy (viable pregnancies). The expression of the PRLR and FOXL2 genes have previously been reported in human endometrium (Jabbour et al., 1998, Jones et al., 1998, Governini et al., 2014) and was included in the list of fifteen candidate genes for quantification in order to compare and confirm the expression pattern in the tissues employed. Similarly, the levels of AR mRNA were determined to confirm if the gene levels were as previously reported (Cloke et al., 2010).

AR mRNA concentrations were significantly higher in proliferative endometrium than mRNA levels in mid-secretory endometrium (trend) or first trimester decidua (P<0.05) (Figure 5.2).

![Figure 5.2 Tissue AR mRNA is significantly elevated in proliferative endometrium (P) compared to first trimester decidua (DEC). AR concentrations were greater, but not significantly, in proliferative endometrium compared to mid-secretory endometrium (MS). Data presented as mean ±SEM (n=3 for P, n=4 for MS and DEC). *P<0.05.](image-url)
During the course of these studies, some of the tissue samples were exhausted and new samples were prepared from different patients, leading to variable $n$ numbers in the data presented in this section. The results obtained for candidate genes are grouped in separate figures according to their expression patterns (Figures 5.3, 5.4 and 5.5) and are summarised in Table 5.5.

Figure 5.3 shows the candidate genes, the mRNA concentrations of which were lower (significantly or trend) in proliferative endometrium when compared to mid-secretory endometrium and/or first trimester decidua. $RGS2$ and $SIK1$ mRNA were significantly reduced in proliferative endometrium compared to decidua ($P<0.001$). $SLC6A6$ mRNA concentrations were reduced in proliferative endometrium compared to mid-secretory endometrium ($P<0.05$).

A non-significant trend was observed in the patterns of expression of $MAP3K8$, $NR4A2$, $JMY$, $ELK4$, and $PRLR$ mRNAs where the levels in proliferative samples were apparently lower compared to either mid-secretory endometrium and/or decidua.
Chapter 5  
Expression of putative androgen regulated genes in the endometrium

Figure 5.3 Endometrial tissue expression of candidate androgen-regulated genes that show reduced levels in proliferative endometrium when compared to mid-secretory endometrium or decidua (significant or non-significant trend). RGS2 and SIK1 are significantly reduced in proliferative endometrium (P) compared to decidua (DEC). SLC6A6 is significantly reduced in proliferative endometrium compared to mid-secretory endometrium (MS). JMY, ELK4, MAP3K8, NR4A2 and PRLR genes showed similar but not significant expression patterns. Data presented as mean ±SEM. (RGS2, SIK1, SLC6A6: n=8 per group. JMY, ELK4, MAP3K8, PRLR: n=4 per group. NR4A2: n=3 for P, n=4 for MS and DEC). *P<0.05, ***P<0.001.
Figure 5.4 shows the concentrations of mRNAs for candidate genes that were significantly increased in proliferative phase endometrium compared to first trimester decidua. *SNCAIP, AXIN2, MGC16121, ID3, CDC25A* and *FOXL2* mRNAs were significantly elevated in proliferative endometrium compared to decidua (P<0.05 or P<0.01 or P<0.001). *MGC16121* mRNA concentrations was also significantly higher in proliferative endometrium than in mid-secretory endometrium (P<0.05). *SNCAIP* mRNA concentrations in mid-secretory endometrium were significantly higher than the levels in decidua (P<0.05).
Figure 5.4 Endometrial tissue expression of candidate androgen-regulated genes that show higher levels in proliferative endometrium when compared to mid-secretory endometrium or decidua (significant or non-significant trend). The DHT-regulated (in hESCs) SNCAIP, AXIN2 and MGC16121 genes and putative androgen-regulated genes ID3, CDC25A and FOXL2 are significantly elevated in proliferative endometrium (P) compared to decidua (DEC). Additionally, levels of SNCAIP are significantly elevated in mid-secretory endometrium (MS) compared to decidua while MGC16121 levels are significantly higher in proliferative endometrium compared to mid-secretory endometrium. Data presented as mean ±SEM (SNCAIP, MGC16121, ID3: n=8 per group. AXIN2, CDC25A, FOXL2: n=4 per group). *P<0.05, **P<0.01, ***P<0.001.
Figure 5.5 shows the GADD45G gene expression pattern in endometrial and decidual tissues where gene levels did not differ between proliferative, mid-secretory endometrium or decidual tissues.

**Figure 5.5 Endometrial tissue levels of candidate androgen-regulated gene GADD45G is not changed in proliferative endometrium (P) compared with mid-secretory endometrium (MS) or first trimester decidua (DEC). Data presented as mean ±SEM (n=4 per group).**
Table 5.5 Summary of candidate androgen regulated gene expression patterns in endometrial and decidual tissues showing an association with decidualisation. The expression of at least half of the candidate genes was altered in mid-secretory endometrium and/or decidua compared to proliferative endometrium.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mid-secretory endometrium</th>
<th>Decidua</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS2</td>
<td>-</td>
<td>Yes ✷</td>
</tr>
<tr>
<td>SIK1</td>
<td>-</td>
<td>Yes ✷</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>Yes ✷</td>
<td>-</td>
</tr>
<tr>
<td>JMY</td>
<td>-</td>
<td>Trend ✷</td>
</tr>
<tr>
<td>ELK4</td>
<td>Trend ✷</td>
<td>Trend ✷</td>
</tr>
<tr>
<td>MAP3K8</td>
<td>-</td>
<td>Trend ✷</td>
</tr>
<tr>
<td>NR4A2</td>
<td>-</td>
<td>Trend ✷</td>
</tr>
<tr>
<td>PRLR</td>
<td>-</td>
<td>Trend ✷</td>
</tr>
<tr>
<td>SNCAIP</td>
<td>-</td>
<td>Yes ❄</td>
</tr>
<tr>
<td>AXIN2</td>
<td>-</td>
<td>Yes ❄</td>
</tr>
<tr>
<td>MGC16121</td>
<td>Yes ❄</td>
<td>Yes ❄</td>
</tr>
<tr>
<td>ID3</td>
<td>-</td>
<td>Yes ❄</td>
</tr>
<tr>
<td>CDC25A</td>
<td>Trend ✷</td>
<td>Yes ❄</td>
</tr>
<tr>
<td>FOXL2</td>
<td>Trend ✷</td>
<td>Yes ❄</td>
</tr>
<tr>
<td>GADD45G</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(✷ and ❄) significantly up or down regulated.
(звездочка и star) non-significant trend in up or down regulation.
(-) no change in gene level.
5.3.2 RGS2 protein is expressed in human endometrium and first trimester decidua

RGS2 mRNA was upregulated by DHT in undifferentiated hESCs and in an endometrial stromal cell line, SHT-290 (Figures 4.3 and 4.5). Subsequently, in the immediate previous section 5.3.1 (Figure 5.3), RGS2 mRNA concentrations were significantly upregulated in decidual tissues compared to proliferative endometrium. As the RGS2 protein expression pattern has never been reported in these tissues localisation of RGS2 protein in proliferative and mid-secretory endometrium and decidua was investigated.

RGS2 protein was detected in endometrial tissues (Figure 5.6). RGS2 protein immunoeexpression was intense in the epithelial cells of proliferative and mid-secretory phase endometrium. RGS2 staining of the stromal cells showed varying levels of intensity (from no staining to intense staining) in the proliferative and mid-secretory endometrial phase tissues. In decidual tissues, epithelial and decidualised stromal cells exhibited strong RGS2 immunoreactivity. The immunostaining appeared predominantly localised to the cell cytoplasm and in epithelial cells the staining was enhanced at the apical surfaces for example, at the epithelial luminal surface. Cells lining blood vessels, possibly endothelial cells (indicated by an arrow and labelled ec) also exhibited RGS2 immunostaining in all the tissues examined. Positive staining was confirmed in the control human tissue, tonsil. Negative controls were devoid of positive brown staining (proliferative endometrium and decidua).
Figure 5.6 RGS2 protein is expressed in human endometrium and decidua. Representative images of immunohistochemical detection of RGS2 in proliferative (n=6), mid-secretory (n=7) endometrial and decidual tissues (n=4). RGS2 protein is localised to the epithelial and to the stromal cells of proliferative and mid-secretory endometrium although not all stromal cells stain positive. In decidua, RGS2 is expressed in the epithelial and decidualised stromal cells. RGS2 is also detected in endothelial cells in both endometrium and decidua. RGS2 positive staining is present in an RGS2 positive control tissue, the follicular lymphoid region of the tonsil. Negative controls are stained using concentration matched IgG2a antibody. ge-glandular epithelium, st-stroma, dsc-decidualised stromal cells, ec-vessels, endothelial cells (inner lining of blood or lymph vessels). Scale bar = 20µM.
5.3.3 Synphilin-1 (encoded by *SNCAIP* gene) is expressed in human endometrium and first trimester decidua

*SNCAIP* mRNA concentrations were significantly higher in proliferative endometrium compared to decidua tissues (previous section, Figure 5.4). To determine the protein expression pattern (tissue and cell localisation) of Synphilin-1 protein (encoded by *SNCAIP*) immunohistochemistry was performed on proliferative and mid-secretory phase endometrial and first trimester decidual tissues.

Synphilin-1 was detected in the endometrium (shown in Figure 5.7). Synphilin-1 protein expression was intense in most epithelial cells of proliferative and mid-secretory phase endometrium. Weak immunostaining of some of the stromal cells in these tissues was also observed. In contrast, first trimester decidual epithelial and decidualised stromal cells both exhibited strong Synphilin-1 immunoreactivity. In all tissues, immunostaining was predominantly localised to the cell cytoplasm. Positive staining of control tissues (placenta) showed positive signals in the chorionic villi cells comparable to those reported by the commercial Synphilin-1 antibody supplier (Table 2.1). Negative controls were devoid of positive brown staining.
Figure 5.7 Synphilin-1 protein (encoded by SNCAIP) is expressed in human endometrium and first trimester decidua. Representative images showing Synphilin-1 protein (stained brown) localised by IHC in human endometrial tissues. Staining for Synphilin-1 was localised to the cytoplasm. In proliferative (n=6) and mid-secretory phase (n=7) endometria Synphilin-1 was detected mainly in epithelial cells and weakly in some stromal cells. In decidua (n=4), the epithelial and decidualised stromal cells exhibited strong synphilin-1 expression. Placental tissue was included as a control for positive staining. Negative controls were stained using concentration matched rabbit serum. ge—glandular epithelium, st—stroma, dsc—decidualised stromal cells. Scale bar = 20μM.
5.3.4 SIK1 protein is expressed in human endometrium, first trimester decidua and endometrial stromal cells

SIK1 gene levels were significantly upregulated in decidual tissues compared to proliferative endometrium (previous section, Figure 5.3) but the protein expression in these tissues has not been previously described. To determine the protein expression pattern (tissue and cell localisation) of SIK1, detection of SIK1 protein by immunohistochemistry was carried out on proliferative and mid-secretory phase endometrial and first trimester decidual tissues. Immunohistochemical staining for SIK1 was performed with two different antibodies to confirm the expression pattern detected with the first antibody employed (rabbit polyclonal, as this had to be used at a very high concentration).

SIK1 immunoexpression was consistently detected in endometrial tissues (Figure 5.8 and Figure 5.9). The pattern of expression of SIK1 was similar using the two different antibodies. Immunoreactivity was predominantly localised to the cell cytoplasm. SIK1 protein expression was very weak in epithelial and especially in stromal cells of proliferative and mid-secretory endometrium. Strong positive staining for SIK1 was observed in epithelial and decidualised stromal cells in decidual tissues (non-decidualised stroma was negative). Endothelial cells in the endometrial and decidual tissues also stained positive for SIK1. Positive staining of control tissues (placenta and kidney, Figure 5.9) showed comparable signals to those reported by the commercial SIK1 antibody suppliers (Table 2.1). For example, the placental epithelial-like cells (syncytiotrophoblast) and some of the inner chorionic villi cells stained positive. Negative controls employed upon endometrial, decidual or placental tissues were clear of brown staining (examples of primary antibody omitted or IgG-concentration matched negative controls are shown in Figures 5.8 and 5.9, respectively).
Figure 5.8 SIK1 protein is expressed in human endometrium and is upregulated in decidua (using a polyclonal anti-SIK1 antibody). Representative images showing SIK1 protein (stained brown) localised by IHC in human endometrial tissues. Staining was localised to the cytoplasm. Staining for SIK1 is weak in epithelial cells, weak or non-detectable in the stromal cells of proliferative (n=6) and mid-secretory (n=7) endometrium. Strong SIK1 positive staining is observed in the decidualised stromal cells and epithelial cells of the decidua (n=4). Placental tissue was included as a control for positive staining. Negative control was performed with omission of the primary anti-SIK1 antibody. ge-glandular epithelium, st-stroma, dsc-decidualised stromal cells. Scale bar = 20µM.
Figure 5.9 SIK1 protein is expressed in the endometrium and upregulated in decidual stromal cells (using a mouse monoclonal anti-SIK1 antibody). Representative images showing SIK1 protein (stained brown) localised by IHC in human endometrial tissues. Staining was localised to the cytoplasm. Staining for SIK1 is very weak or non-detectable in epithelial cells and stromal cells of proliferative (n=6) and mid-secretory (n=7) endometrium (Panels A and B). Strong SIK1 positive staining is observed in the decidualised stromal cells and some epithelial cells of the decidua, n=4 (Panel C). Placental and kidney tissue were included as controls for positive staining (Panels D-F). Negative controls were stained using concentration concentration matched IgG antibody (Panels G and H). ge-glandular epithelium, st-stroma, dsc-decidualised stromal cells. All scale bars represent 20μM.
SIK1 was only very weakly detected by immunohistochemistry in the proliferative and mid-secretory tissues. To assess if SIK1 protein was present in endometrial stromal cells, Western analysis was carried out on hESCs and SHT-290 cells (endometrial cell line). In addition, to investigate if SIK1 protein concentrations were altered by DHT-dependant activation of AR, SHT-290 cells were treated with DHT (10nM) or vehicle for 24 hours alone or in the presence or absence of flutamide.

As detailed in Figure 5.10, SIK1 protein was detected in both primary hESCs and in SHT-290 cells. Treatment with DHT significantly decreased SIK1 protein concentrations in the SHT-290 cells (P<0.05). Vehicle control treatment (vehicle for flutamide, methanol) also unexpectedly resulted in a reduction in SIK1 protein concentrations.
Figure 5.10 SIK1 protein is detected in cultured endometrial stromal cells (hESCs and SHT-290 cell line) and SIK1 protein levels are reduced by DHT in SHT-290 cells. Panel A: Representative fluorescent Western blot image showing SIK1 is detected in untreated hESCs. Lanes (1) SeaBlue marker; (2) untreated hESCs. SIK1 is visualised in green and β-tubulin control in red. Panels B and C: SIK1 protein was significantly reduced in the SHT-290 cells in response to DHT treatment (by Western analysis). SIK1 levels did not differ in the presence of flutamide when compared to vehicle control. Cells were pre-treated with flutamide (10⁻⁵ M) or methanol (MeOH, vehicle) for 1 hour, or not pre-treated. Culture for 24 hours followed with ethanol (EtOH, vehicle for DHT) or with DHT (10⁻⁸ M). Panel B: Representative fluorescent Western blot image. Lanes (1) SeaBlue plus2 marker; (2 to 7) treated SHT-290 cells as indicated. SIK1 is visualised in green and β-tubulin control in red. Panel C: Quantification of SIK1 protein in SHT-290 cells. Protein levels were normalised to β-tubulin control and presented as mean ± SEM (n=5; with MeOH or flutamide pre-treatment, n=7). * P<0.05.
5.3.5 Oestradiol, alone or in the presence of DHT, may not modulate androgen-regulated $RGS2$, $SNCAIP$, $SIK1$ or $SLC6A6$ genes in SHT-290 cells

Bioinformatic analyses of gene expression of androgen-treated hESCs revealed that androgen-dependent regulation of gene expression may also crosstalk with oestrogen regulated pathways (Table 3.5) as has been reported in breast cancer cells (Need et al., 2012). More specifically, some putative androgen-regulated genes identified in hESCs have previously been shown to be regulated by ERα (Table 3.8 and Figure 3.9), for example, $SLC6A6$ (Han et al., 2003).

Therefore, as part of the characterisation of the regulation of the novel androgen-regulated genes (specifically $SLC6A6$, $RGS2$, $SNCAIP$ and $SIK1$) in endometrial stromal cells, gene modulation by oestrogen was investigated. In addition, it was also investigated if the presence of oestrogen affected (for example, antagonised) the androgen-regulation of the candidate genes. To this end, modulation of the androgen-regulated genes $RGS2$, $SNCAIP$, $SIK1$ and $SLC6A6$ was quantified in SHT-290 endometrial stromal cells treated for 24 hours with either 10nM $E_2$, 10nM $E_2$/10nM DHT or with vehicle controls. The 24 hour timepoint was chosen to allow direct comparison with DHT regulation of the genes previously shown in the SHT-290 cell line (Chapter 4, Figure 4.2 and 4.3).

Figure 5.11 shows that treatment with $E_2$ alone for 24 hours did not alter the mRNA concentrations of $RGS2$, $SNCAIP$, $SIK1$ and $SLC6A6$ when compared with vehicle control. In the presence of DHT, $E_2$ did not appear to inhibit/antagonise the DHT-mediated effect upon gene expression of $SNCAIP$, $SIK1$ or $SLC6A6$, which were confirmed as regulated by DHT ($P<0.05$ or $P<0.01$). It was unclear if $E_2$ attenuated DHT-mediated $RGS2$ gene expression which was upregulated (but not significantly) in the presence of $E_2$ and DHT.
Figure 5.11 Oestradiol did not alter mRNA levels of androgen-regulated genes RGS2, SNCAIP, SIK1 or SLC6A6 in SHT-290 cells. In the presence of oestradiol, DHT-regulation of the SNCAIP, SIK1 and SLC6A6 genes also appeared unchanged. Cells were incubated for 24 hours with vehicle controls (DMSO or EtOH/DMSO) or 10nM E2 or E2/DHT (10nM each). EtOH: vehicle control for DHT, ethanol. Data presented as mean ± SEM (n=4). *P<0.05, **P<0.01.
5.3.6 Some androgen-regulated candidate genes are also regulated in association with decidualisation during early pregnancy (in vivo)

In women, endometrial decidualisation begins during the secretory phase (in response to rising progesterone levels) and is maintained if pregnancy occurs and progesterone levels remain high. Decidualisation is essential for blastocyst implantation, trophoblast invasion and placentation (Loke et al., 1995, Guzeloglu-Kayisli et al., 2007, Salker et al., 2010).

In the previous section 5.3.1, many of the putative androgen-regulated genes identified in hESCs were differentially regulated in proliferative endometrium when compared with decidua of viable intrauterine pregnancies. For example, the androgen-regulated genes RGS2, SIK1 and SLC6A6 were significantly upregulated in decidua and SNCAIP and AXIN2 were significantly downregulated in decidua, compared to proliferative phase endometrium.

In order to further confirm whether the degree of decidualisation was correlated with the expression of some of these genes in endometrial tissues during early pregnancy (first trimester), a further study using tissues from ectopic pregnancies was performed. Ectopic pregnancies allow the study of changes in endometrial tissue that occur in the absence of a “local” viable embryo and trophoblast (Duncan et al., 2011). Decidua from tubal ectopic pregnancies has been previously used as a model that exhibits variable but overall reduced decidualisation of the endometrium compared with decidua of first trimester viable intrauterine pregnancies (Horne et al., 2008) (detailed in section 5.2.2.3 and Figure 5.1).

In this section, the relative concentrations of mRNAs encoded by candidate genes of interest were investigated in decidua from ectopic pregnancies (EP) and from viable intrauterine pregnancies (IUP). In order to facilitate cross-reference with the previous data (shown in Figures 5.2, 5.3 and 5.4) the results of the investigations are presented in the graphs according to expected increasing decidualisation i.e., with EP first on the left followed by IUP.
5.3.6.1 Confirmation of reduced decidualisation in the decidua from ectopic pregnancies compared with first trimester intrauterine pregnancies

Decidualisation is characterised by expression of marker genes insulin-like growth factor binding protein 1 (IGFBP1) and prolactin (PRL) (Bell et al., 1991, Brosens et al., 1999) by the differentiated hESCs. To confirm the samples used were appropriate i.e., that the decidua of ectopic pregnancies exhibited reduced decidualisation as observed before (Horne et al., 2008), the expression patterns of decidualisation marker genes IGFBP1 and PRL were determined in decidua from tubal ectopic pregnancies and pregnancies.

Figure 5.12 shows the decidualisation marker genes, IGFBP1 and PRL, were elevated in decidua from viable intrauterine pregnancies compared to ectopic pregnancies (P<0.05) indicating overall reduced decidualisation in the ectopic pregnancy decidua samples employed in the current study.

*P<0.05.

Figure 5.12 Decidualisation marker gene expression levels in human decidua examined in this thesis are confirmed elevated in viable intrauterine pregnancies compared to ectopic pregnancies. Expression of IGFBP1 and PRL mRNAs is significantly higher in decidua from intrauterine pregnancy (IUP) compared to ectopic pregnancy (EP). Data are presented as mean ±SEM (EP, n=6; IUP, n=10).
5.3.6.2 Circulatory progesterone and human chorionic gonadotrophin levels are reduced in women with ectopic pregnancy compared to viable intrauterine pregnancy

Levels of serum hCG and progesterone have been used clinically to assist the difficult diagnosis of ectopic pregnancy (Cartwright et al., 2009, Seeber, 2012, Van Mello et al., 2012, Feng et al., 2013). Both progesterone and embryo-derived hCG play important roles in the endometrium during implantation and maintenance of pregnancy (Bourdiec et al., 2013, Halasz and Szekeres-Bartho, 2013). For example, these maternal and embryo/placenta-derived factors contribute to the decidualisation of the endometrium by enhancing PRL production or maintaining cAMP stimulation in the tissue (Tseng et al., 1992, Tang and Gurpide, 1993, Brar et al., 1997, Nemansky et al., 1998, Rider, 2002). P₄ and hCG may also affect candidate gene expression directly. For example, hCG has been shown to inhibit mRNA expression of candidate genes ID3 and FOXL2 and enhance concentrations of RGS2 mRNAs in human or animal model ovaries or ovarian cells (Ujioka et al., 2000, Wu et al., 2008, Sridevi and Senthilkumaran, 2011, Agca et al., 2013, Nio-Kobayashi et al., 2013).

Therefore, to complete characterisation of the decidual samples (detailed in section 5.2.2.3), the serum levels of progesterone and hCG from the women that provided the decidual samples were compared.

Figure 5.13 shows serum progesterone and hCG concentrations were significantly elevated in decidua from viable intrauterine pregnancies compared to ectopic pregnancies (P<0.05).
5.3.6.3 Steroid receptor gene levels (AR, PR and ER-α) are reduced in decidua from IUP compared to EP in parallel with increased decidualisation

The mRNA concentrations of the steroid receptors AR, PR and ER-α are reported downregulated in the secretory phase of the menstrual cycle and in decidua (Brosens et al., 1999, Henderson et al., 2003, Bombail et al., 2010, Cloke et al., 2010). However, steroid receptor mRNA concentrations have not been previously reported in the decidua of women with an ectopic pregnancy and therefore mRNA concentrations were compared in decidual tissues from EP and IUP.

AR, PR and ER-α mRNAs were significantly lower in decidua from intrauterine pregnancies when compared to ectopic pregnancies (P<0.01) (Figures 5.14 and 5.15). The reduced steroid receptor expression (AR, PR and ER-α) in decidua from IUP versus EP appear to correlate with the expression of decidualisation marker genes (Figure 5.12) and with the serum progesterone and hCG concentrations (Figure 5.13).
5.3.6.4 AR protein concentrations are higher in ectopic pregnancy decidua than in viable intrauterine pregnancy decidua

As the pattern of AR protein expression pattern has not been previously described in the decidua of ectopic pregnancies, an immunohistochemical study was performed.

More intense positive AR immunostaining was observed in the nuclei of stromal cells in samples recovered from ectopic pregnancies than in the decidua from viable intrauterine pregnancies (Figure 5.16). In EP decidua some epithelial cells were also
immunopositive for AR protein. There was a range of AR staining intensity observed in the stromal cells of the IUP decidual samples with the intensity appearing to be reduced in the stromal cells within the regions of greater stromal decidualisation (compared to less decidualised stromal regions). Negative controls did not demonstrate detectable positive brown staining.
AR expression in decidua from EP

AR expression in decidua from IUP

Negative control (decidua from IUP)

Figure 5.16 AR protein localisation in decidual tissue is strongest in ectopic pregnancies (EP) than in viable intrauterine pregnancies (IUP). AR protein was localised by IHC with anti-AR antibody (EP, n=6; IUP, n=10). Decidua from EP (Panels A and B) show stronger positive AR staining in the stromal cells compared to IUP. In EP decidua some epithelial cells are also positive. There is a range of AR positive staining in the IUP decidual samples (Panels C and D) in the stromal cells: staining is reduced in the regions of greater decidualised cells. Negative controls (Panel E) were stained with concentration matched Rabbit IgG and were devoid of immunostaining (decidua from IUP). ge-glandular epithelium, st-stroma. Scale bar = 50µM.
5.3.6.5 Candidate AR-regulated genes (SNCAIP, AXIN2, ID3 and FOXL2) were differentially modulated in the decidua of IUP and EP

Following confirmation that decidua from viable IUP had higher concentrations of decidualisation markers (section 5.3.6.1) (and increased P₄ and hCG levels, section 5.3.6.2) further evaluation of expression of genes of interest in EP and IUP was undertaken. The relative concentrations of mRNAs encoded by androgen-regulated genes (RGS2, SNCAIP, SIK1, SLC6A6, AXIN2) and putative androgen-regulated genes (ID3, CDC25A, FOXL2) identified in undifferentiated hESCs (chapter 4) were measured in decidua from viable first trimester intrauterine pregnancies and ectopic pregnancies. The results are shown in Figures 5.17, 5.18 and 5.19 and are summarised in Table 5.6.

Expression of RGS2, SIK1 and SLC6A6 did not differ significantly between decidua from viable IUP and EP (Figure 5.17). However, RGS2 tended to be elevated (non-significant) in IUP compared to EP. Expression of SNCAIP and AXIN2 were significantly downregulated in decidua from viable IUP compared to EP (P<0.01 and P<0.001, respectively) (Figure 5.18).
Figure 5.17 Expression of androgen-regulated genes RGS2, SIK1 and SLC6A6 did not differ significantly between decidual tissues of non-viable extrauterine ectopic pregnancies (EP) and viable intrauterine pregnancies (IUP). (EP, n=6; IUP, n=10). Data are presented as mean ±SEM.

Figure 5.18 Expression of androgen-regulated genes SNCAIP and AXIN2 differ significantly in decidual tissues of non-viable extrauterine ectopic pregnancies (EP) and viable intrauterine pregnancies (IUP). SNCAIP and AXIN2 mRNA concentrations were significantly reduced in decidua from IUP compared to EP. (EP, n=6; IUP, n=10). Data are presented as mean ±SEM. **P<0.01, ***P<0.001.
Expression of *ID3* and *FOXL2* were significantly downregulated in decidua from viable IUP compared to EP (*P*<0.01 and *P*<0.001, respectively) (Figure 5.19). Expression of *CDC25A* tended to be downregulated in viable IUP decidua compared to EP decidua (but not significantly).

![Graphs showing expression levels of ID3, CDC25A, and FOXL2 in EP and IUP decidua](image)

*Figure 5.19* Expression of novel endometrial genes *ID3*, *CDC25A* and *FOXL2* differ in decidual tissues from non-viable extrauterine ectopic pregnancies (EP) and viable intrauterine pregnancies (IUP). *CDC25A* mRNA concentrations tended to be decreased while *ID3* and *FOXL2* concentrations were significantly reduced in decidua from IUP compared to EP. *(EP, n=6; IUP, n=10).* Data are presented as mean ±SEM. ***P*<0.01, ***P*<0.001.

Table 5.6 summarises the changes in candidate gene expression in the decidual tissues in association with the degree of decidualisation previously confirmed by the expression of decidualisation marker genes (Figure 5.12).
Table 5.6 Summary of candidate gene expression in association with degree of decidualisation in decidual tissues in vivo. The expression of candidate genes is altered in decidua from viable intrauterine pregnancies (IUP) when compared with ectopic pregnancies (EP) which exhibit reduced degree of decidualisation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Change of decidual gene mRNA levels in IUP (compared to EP) - in association with increased degree of decidualisation:</th>
</tr>
</thead>
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<tr>
<td>RGS2</td>
<td>Trend ↑</td>
</tr>
<tr>
<td>SIK1</td>
<td>-</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>-</td>
</tr>
<tr>
<td>SNCAIP</td>
<td>Yes ↓</td>
</tr>
<tr>
<td>AXIN2</td>
<td>Yes ↓</td>
</tr>
<tr>
<td>ID3</td>
<td>Yes ↓</td>
</tr>
<tr>
<td>CDC25A</td>
<td>Trend ↑</td>
</tr>
<tr>
<td>FOXL2</td>
<td>Yes ↓</td>
</tr>
</tbody>
</table>

(↑ and ↓) significantly up or down regulated.
(♦ and ◊) non-significant trend in up or down regulation.
(-) no change in mRNA concentrations.

5.3.6.6 Expression of candidate AR-regulated genes (SLC6A6, AXIN2, ID3 and FOXL2) are negatively correlated to IGFBP1 expression, a marker of decidualisation, in the decidua of IUP and EP

The association between the mRNA expression of each candidate gene with the degree of decidualisation in the decidual tissues was further assessed by correlating the relative concentrations of candidate gene mRNAs (RGS2, SNCAIP, SIK1, SLC6A6, AXIN2, ID3, CDC25A, FOXL2) with decidualisation marker IGFBP1 mRNA concentrations, in decidua from viable first trimester intrauterine pregnancies and ectopic pregnancies.

Expression of RGS2, SIK1 and SNCAIP and CDC25A did not correlate with IGFBP1 mRNA concentrations (Figure 5.20) in decidua from IUP and EP. However, expression of SLC6A6, AXIN2, ID3 and CDC25A did correlate with IGFBP1 mRNA concentrations in decidua from viable IUP and EP (P<0.05 or P<0.01) (Figure 5.20).
Figure 5.20 Spearman’s rank correlation coefficients (r) and significance for the associations assessed between candidate gene expression and IGFBP1 gene expression, a marker of decidualisation, in decidual tissues from EP and IUP. Significant association was determined between SLC6A6, AXIN2, ID3 and FOXL2 mRNA concentrations and IGFBP1 mRNA concentrations in decidua from IUP and EP. No association was found between RGS2, SIK1, SNCAIP and CDC25A mRNA concentrations and IGFBP1 mRNA concentrations. (EP, n=6; IUP, n=10). ns=not significant. *P<0.05, **P<0.01.
5.3.7 Candidate genes \textit{SLC6A6, RGS2, SIK1, SNCAIP} and \textit{AXIN2} (androgen-regulated in undifferentiated hESCs) are regulated by \textit{in vitro} decidualisation in hESCs

In the previous sections, it was observed that the concentrations of the mRNAs encoded by the androgen-regulated genes \textit{SLC6A6, RGS2, SNCAIP} and \textit{SIK1} were regulated in association with decidualisation in endometrial tissues (Figures 5.3 and 5.4) and decidual tissues (Figures 5.17 and 5.18). The intensity of immunoeexpression of three of the genes (\textit{RGS2, SNCAIP} and \textit{SIK1}) also appeared increased in the decidualised stromal cells in mid-secretory endometrium and/or in decidua (see Figures 5.6 to 5.9).

To investigate the regulation of the candidate genes by decidualisation specifically in hESCs, primary hESCs were decidualised \textit{in vitro} using standard protocols for 8 days (Cloke \textit{et al.}, 2008) i.e., with cAMP alone or with cAMP/progesterone (P$_4$).

\textbf{5.3.7.1 \textit{In vitro} decidualisation of primary hESCs is confirmed by the detection of decidualisation markers \textit{IGFBP1} and \textit{PRL}}

In order to confirm \textit{in vitro} hESC decidualisation the detection of decidualisation markers \textit{IGFBP1} and \textit{PRL} mRNAs and IGFBP1 protein secretion was undertaken.

\textit{IGFBP1} and \textit{PRL} mRNA levels were significantly enhanced in cAMP/P$_4$-decidualised hESCs compared to non-decidualised controls (P<0.01) (Figure 5.21). Decidualisation with cAMP alone enhanced mRNA levels of \textit{IGFBP1} and \textit{PRL} compared to non-decidualised controls (P<0.05). Both decidualisation marker gene levels (\textit{IGFBP1} and \textit{PRL}) were enhanced further by decidualisation with cAMP/P$_4$ than with cAMP alone (respectively, P<0.001 and P<0.01).
Figure 5.21 Decidualisation marker gene mRNAs (IGFBP1 and PRL) are upregulated in primary hESCs cells decidualised with cAMP or cAMP/P₄ compared to non-decidualised cells. Messenger RNA levels of IGFBP1 and PRL were also significantly upregulated by cAMP/P₄ alone compared to non-decidualised cells (non-dec). Cells were decidualised with cAMP or cAMP/P₄ for 8 days. Data are presented relative to non-dec as mean ± SEM (n=4). * P<0.05, ** P<0.01, ***P<0.001, ****P<0.0001.

IGFBP1 protein secretion was readily detected in media from cAMP and cAMP/P₄ decidualised hESCs (Figure 5.22). cAMP/P₄ decidualised cells secreted more IGFBP1 protein compared with cAMP decidualised cells (P<0.05). The levels of IGFBP1 protein secretion were not evaluated in non-decidualised cells.

Figure 5.22 Decidualisation marker IGFBP1 protein is secreted by cAMP and cAMP/P₄-decidualised primary hESCs confirming successful decidualisation. The cAMP/P₄-decidualised hESCs produced the greater amount of IGFBP1 protein. IGFBP1 protein was detected by ELISA in primary hESCs decidualised with cAMP and cAMP/P₄ for 8 days. Data are presented as mean ± SEM (n=4). * P<0.05.
5.3.7.2 Candidate genes *RGS2*, *SIK1*, *SNCAIP*, *SLC6A6* and *AXIN2* mRNAs are altered in hESCs by decidualisation (but not *ID3* or *FOXL2*)

To investigate the regulation of candidate genes specifically in hESCs by decidualisation, candidate gene mRNA concentrations were determined in the decidualised primary hESCs described above (cAMP and cAMP/P$_4$-decidualised) and compared with non-decidualised cells.

Figure 5.23 shows the effect of hESC decidualisation upon mRNA expression of *SLC6A6*, *RGS2*, *SIK1*, *SNCAIP* and *AXIN2*. *SLC6A6*, *RGS2*, *SIK1*, *SNCAIP* and *AXIN2* mRNAs were each significantly upregulated in cAMP/P$_4$-decidualised hESCs compared to non-decidualised controls (P<0.01, P<0.05, P<0.01, P<0.01 and P<0.001, respectively). Similarly, decidualisation of the hESCs with cAMP alone also significantly upregulated the mRNAs of each gene (P<0.01, P<0.05, P<0.01, P<0.01 and P<0.001, respectively). The mRNA concentrations of all the genes investigated did not differ between cAMP and cAMP/P$_4$ decidualised hESCs.
Figure 5.23 Expression of candidate genes SLC6A6, RGS2, SIK1, SNCAIP and AXIN2 in primary hESCs cells is regulated by in vitro decidualisation. The expression levels of SLC6A6, RGS2, SIK1, SNCAIP and AXIN2 were significantly upregulated by cAMP or cAMP/P4 compared to non-decidualised cells (non-dec). The levels of ID3 were unchanged by cAMP or cAMP/P4. Cells were cultured for 8 days with vehicle control, cAMP or cAMP/P4. qRTPCR data are presented relative to non-dec as mean ± SEM (n=4). * P<0.05, ** P<0.01.
Figure 5.24 shows that *ID3* and *FOXL2* mRNA concentrations did not differ in hESCs upon decidualisation compared to non-decidualised cells.

**Figure 5.24** Expression of candidate genes *ID3* and *FOXL2* in primary hESCs cells is not regulated by in vitro decidualisation. The mRNA levels of *ID3* and *FOXL2* were unchanged by 8 days decidualisation with cAMP or cAMP/P₄ compared to non-decidualised cells (non-dec). Cells were cultured for 8 days with vehicle control, cAMP or cAMP/P₄. Data are presented relative to non-dec as mean ± SEM (n=4).
5.3.8 DHT enhances decidualisation markers but does not alter candidate gene expression in decidualising hESCs

To investigate if candidate genes were regulated by DHT during decidualisation, primary hESCs were decidualised for eight days with cAMP/P4 in the presence of DHT or vehicle using standard protocols (Cloke et al., 2008).

5.3.8.1 Secreted decidualisation marker IGFBP1 and IGFBP1 and PRL mRNA concentrations are enhanced by DHT in decidualising hESCs

Decidualisation of hESCs was confirmed by the detection of the secreted decidualisation marker IGFBP1 protein (Figure 5.25). The inclusion of 10nM or 100nM DHT during standard decidualisation of hESCs significantly enhanced IGFBP1 secreted protein compared to vehicle-treated cells (P<0.05).

![Figure 5.25 Decidualisation marker IGFBP1 protein secretion levels are enhanced by the inclusion of DHT during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P4 in the presence of 10nM DHT or 100nM DHT or vehicles for 8 days. Secreted IGFBP1 protein concentrations, determined by ELISA, were greater in the presence of 10nM DHT or 100nM DHT compared to vehicle. Data are presented as mean ± SEM (n=6). *P<0.05.](image)

The inclusion of DHT (10nM or 100nM) during hESC decidualisation significantly enhanced IGFBP1 mRNA concentrations (P<0.05) compared to vehicle-treated hESCs (Figure 5.26). Also in these decidualised hESCs, PRL mRNA concentrations were greater in 10nM DHT-treated cells (P<0.05) but not in 100nM DHT-treated cells compared to vehicle-treated cells.
Figure 5.26 Expression of decidualisation marker genes IGFBP1 and PRL is enhanced by the inclusion of DHT during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄ in the presence of 10nM DHT or 100nM DHT or vehicles for 8 days. IGFBP1 mRNA concentrations were greater in DHT-treated cells (10nM and 100nM) compared to vehicle-treated controls. PRL mRNA concentrations were greater in 10nM DHT-treated cells but not in 100nM DHT-treated cells compared to vehicle. Data are presented relative to vehicle control as mean ± SEM (n=6). *P<0.05.

To evaluate if decidualised hESCs continued to express AR protein during the culture protocols, AR protein was assessed under the same conditions in some of the hESCs employed above. AR protein expression was confirmed in decidualised hESCs and concentrations tended to decrease in decidualised cells compared to non-decidualised cells (Figure 5.27-A). AR protein was detected in hESCs decidualised in the presence of DHT or vehicle (Figure 5.27-B). In the presence of DHT, the decidualised hESCs tended to show increased AR protein levels compared to vehicle-treated decidualised cells but this was not statistically significant.
Figure 5.27 AR protein is expressed in primary hESCs decidualised in vitro. Quantification of AR protein in hESCs was obtained by Western analysis. Panel A: AR protein was present and levels tended to decrease in hESCs after decidualisation (dec) with cAMP/P₄ for 8 days compared to non-decidualised cells (non-dec). Panel B: After 8 days decidualisation with cAMP/P₄, AR protein was present and levels tended to increase with the addition of 100nM DHT (+DHT) compared to vehicle-treated (+V). Protein levels were normalised to β-tubulin control and presented as mean ± SEM (n=3).

5.3.8.2 Candidate gene mRNA concentrations in primary hESCs are unchanged by inclusion of DHT during decidualisation

Figure 5.28 shows the effect of inclusion of DHT upon RGS2, SIK1, SLC6A6, SNCAIP and AXIN2 candidate gene expression during hESC-decidualisation. Candidate gene mRNAs were not altered in primary hESCs by DHT inclusion during decidualisation.
Figure 5.28 Expression of candidate genes RGS2, SIK1, SLC6A6, SNCAIP and AXIN2 is unchanged by inclusion of DHT during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄ in the presence of 10nM DHT or 100nM DHT or vehicle controls for 8 days. Candidate gene mRNA concentrations were unchanged by DHT inclusion compared to vehicle-treated cells during decidualisation. Data are presented relative to vehicle control as mean ± SEM (n=6 except for AXIN2 n=4).
Chapter 5    Expression of putative androgen regulated genes in the endometrium

5.4 Discussion

Investigations carried out in this chapter sought to increase our knowledge about the genes identified in the previous chapter that were putative (microarray-predicted) and shown to be androgen-regulated in undifferentiated hESCs. The detection of most of the mRNAs and proteins encoded by these genes in endometrial tissues (endometrium and first trimester decidua) had not been previously reported. It was postulated that the expression of the 15 candidate genes would be regulated in endometrial and decidual tissues. To investigate this, the mRNA concentrations of all 15 candidate genes were determined. The protein expression of three of the genes (RGS2, SIK1 and Synphilin-1, encoded by SNCAIP) was previously unreported in endometrial tissues.

As a result of the novel gene expression findings in the endometrium, the regulation of some of the genes in association with decidualisation was further explored in ex vivo tissues from first trimester pregnancies and in vitro in hESCs that were decidualised. These last studies focussed on the androgen-regulated genes identified in hESCs in chapter 4 and also included select putative androgen-regulated genes, for example, FOXL2 due to possible regulation by progesterone (Eozenou et al., 2012).

Are the mRNAs of AR and putative androgen-regulated genes expressed in endometrial and first-trimester decidual tissues?

The concentrations of AR mRNA in the endometrial and decidual samples were confirmed as being similar to that reported previously (Cloke et al., 2010). As expected concentrations of AR mRNA were higher in proliferative endometrium compared to secretory endometrium and decidua.

The mRNAs of all the 15 microarray-derived putative androgen-regulated genes were detected in endometrial and decidual tissues. These results supported and extended results in cultured hESCs (see Chapter 4, Figures 4.2 and 4.3) for five of the genes, MAP3K8, PRLR, AXIN2, ID3 and FOXL2, where expression in normal uterine and/or endometrial carcinoma has previously been reported (Jabbour et al., 1998, Jones et al., 1998, Aparecida Alves et al., 2006, Nguyen et al., 2012, Sun et al., 2013, Governini et al., 2014). Notably, this is the first time that ten of the
candidate genes have been detected specifically in human endometrial tissues: NR4A2, SLC6A6, JMY, CDC25A, ELK4, MGC16121, RGS2, SNCAIP, SIK1, GADD45G (Result Figures 5.3, 5.4 and 5.5 with gene expression patterns summarised in Table 5.5).

The current study also suggests, for the first time, the modulation of these genes (except for PRLR and GADD45G) during the human endometrial cycle and pregnancy (decidua). For all but one candidate gene (GADD45G) the mRNA concentrations were altered in proliferative endometrium when compared to decidua (and sometimes mid-secretory endometrium) (significantly or trend). Modulation of PRLR was similar to previously published studies (Jabbour et al., 1998, Jones et al., 1998).

Evidence from the literature (Marshall et al., 2011) and in chapter 3 suggests AR protein expression can vary between the endometrial basal and the functional compartments, for example, in mid-secretory phase endometrium the expression of AR is greater in the basal layer compared to the functional layer. Thus, differences in tissue sampling may affect sample composition and subsequent findings from mRNA investigations (AR and putative androgen-regulated genes). In the current studies, endometrial tissues were recovered by either pipelle suction curette (may exclude part or all of the basal layer) or collected after hysterectomy (may exclude part of the basal layer). Therefore, sampling procedures recovered endometrium from the functional layer with varying amounts of the basal layer. However, both sampling procedures were represented in each proliferative and mid-secretory endometrial tissue group investigated. To clarify any sampling bias, further investigations could include greater number of samples with stratification according to sampling approach, utilise tissues that are all sampled in the same way or employ laser capture microdissection from full thickness sections of human endometrium to isolate cells from specific endometrial regions.
Chapter 5  
Expression of putative androgen regulated genes in the endometrium

*Are the RGS2, Synphilin-1 and SIK1 proteins expressed and regulated in endometrial tissues? What is the expression by tissue, cell type and cell localisation pattern?*

Intense immunostaining for RGS2 protein was detected in both the stromal and epithelial cells of endometrial and decidual tissues (Result Figure 5.6) contrasting with the quantitative mRNA expression pattern where *RGS2* mRNA was elevated in the decidua compared to the proliferative endometrium (Result Figure 5.3). Interestingly, the endothelial cells exhibited positive immunoreactivity for RGS2. The mechanisms of *RGS2* transcript regulation and protein translation regulation (for example protein stability/tturnover) are not known in endometrial cells (including in endometrial endothelial or smooth muscle vascular cells) and would require further investigation in order to elucidate the current findings.

RGS2 is thought to play a role in regulating blood pressure homeostasis by acting as a negative regulator of vasoconstrictor action (mediated by G-protein α-q signalling) (Grant *et al.*, 2000, Sun *et al.*, 2005). Evidence suggesting that hypertension may be caused by abnormalities in RGS2 has been obtained using the *rgs2* knock-out mice (Heximer *et al.*, 2003) and studies in cardiovascular diseases (reviewed by Tsang *et al.*, 2010). For example, one *RGS2* single nucleotide polymorphism (SNP), rs4606, was associated with hypertension and reduced RGS2 gene expression in skin fibroblasts and peripheral blood mononuclear cells from hypertensive patients (Semplicini *et al.*, 2006). Subsequently, it was suggested this same *RGS2* SNP may affect the risk and progression of preeclampsia (Kvehaugen *et al.*, 2013), a pregnancy disorder characterised by onset of hypertension and proteinuria after 20 weeks of gestation (2002). In the current study, *RGS2* mRNA was upregulated by androgens in hESCs (Chapter 4) but it is not known if androgens modulate RGS2 expression specifically in endothelial cells or in smooth muscle vascular cells.

Synphilin-1 protein (encoded by *SNCAIP*) was detected in proliferative and secretory phase endometrium (weakly in stromal cells and stronger in epithelial cells) and strongly in both cell types in decidual tissues (Result Figure 5.7). The overall Synphilin-1 protein expression appears to contrast with the quantitative mRNA
expression pattern where *SNCAIP* mRNA was downregulated in the decidua compared to the proliferative or mid-secretory endometrium (Result Figure 5.4). From studies in other cell types, it has been noted that *SLC6A6* transcriptional and translational regulation is complex (reviewed by Han et al., 2006). Further studies are required to fully explain the apparent discordance between mRNA and protein expression patterns observed in the human endometrium.

SIK1 protein expression in tissues from proliferative and mid-secretory phase endometrium (very weak) and decidua (strong) (Result Figures 5.8 and 5.9) partially correlated with the mRNA expression pattern (Result Figure 5.3) where decidua showed the highest SIK1 mRNA and strongest protein expression. Unexpectedly, SIK1 protein was very weakly detected (or not at all) in the stromal cells of endometrial tissues but strongly detected in the decidua. However, SIK1 was detected by Western analysis in cultured primary hESCs and SHT-290 cells (Result Figure 5.10). Notably, SIK1 protein was reduced in SHT-290 cells upon androgen treatment (correlating with mRNA studies, chapter 4) but it remains unknown if this was AR-dependent. The AR protein was detected by western blot in these same samples (chapter 3, Figure 3.6).

In summary, RGS2, Synphilin-1 and SIK1 proteins were detected in human endometrial tissues in the current study for the first time. Protein expression patterns by immunohistochemistry did not always correlate with the mRNA expression patterns (by qRTPCR). With regards to protein expression in the stromal cells of the proliferative endometrium where AR immunoexpression is most intense, only RGS2 was strongly expressed in the stromal cells which correlates with the idea of *RGS2* mRNA upregulation by DHT (chapter 4). Immunostaining of the Synphilin-1 and SIK1 proteins in the stromal cells was weak or very weak, respectively. Reduced SIK1 protein expression in the stromal cells correlates well with the idea that DHT represses SIK1 mRNA (chapter 4). Expression levels of all three proteins were strong in the decidual stroma where AR has been immunolocalised, however the expression of the three proteins in decidua may be mainly the result of induction during decidualisation (see below). Protein expression was also observed in endometrial epithelial and endothelial cells thus future studies could include the
study of whether androgens affect these cell types or if they alter the expression of these specific genes or proteins in these cell types.

**Does oestrogen regulate DHT-regulated RGS2, SNCAIP, SIK1 or SLC6A6 mRNA concentrations in SHT-290 cells?**

Regulation by E₂ was investigated to characterise further the regulation of the novel four androgen-regulated genes identified in endometrial stromal cells (SLC6A6, RGS2, SNCAIP, SIK1). Androgen-dependent genomic regulation is reported to exhibit crosstalk with oestrogen regulated pathways in the human endometrium (see Chapter 3; Table 3.5), in the uteri of animal models (Kowalski et al., 2004, Nantermet et al., 2005) and in breast cancer cells (Need et al., 2012). Some androgen-regulated genes identified in hESCs have previously been shown to be regulated by ERα (Table 3.8 and Figure 3.9). Additionally, putative oestrogen receptor binding sites were identified in the gene sequences of 18 putative androgen-regulated genes identified in hESCs (Table 4.3).

Unexpectedly, the current study suggests that oestradiol does not regulate SLC6A6, RGS2, SNCAIP or SIK1 in SHT-290 endometrial stromal cells (Result Figure 5.11). Although SHT-290 cells have been shown to express oestrogen receptors and be responsive to oestrogen (Barbier et al., 2005) it would have been preferable to confirm the change in expression of a known oestrogen-regulated gene in stromal cells which could have been used as positive control to confirm oestrogen signalling and rule out the possibility that E₂ may have expired. Examples of possible positive controls include the expression of cell-derived factor 1 (SDF-1/CXCL12) or VEGF (Tsutsumi et al., 2011, Okada et al., 2011).

To date, only SLC6A6 mRNA had previously been shown to be regulated (upregulated) by E₂ and this was in ER-positive MCF-7 human breast cancer cells (Han et al., 2003). It was hypothesized that oestrogen may affect (for example, antagonise) androgen-regulation of the genes. However, the results suggest that oestrogen may not antagonize the DHT-dependent effect upon the expression of SNCAIP, SIK1, SLC6A6 (and probably RGS2), i.e., DHT-dependent gene expression was maintained even in the presence of E₂ in endometrial cells. As the ligand
dependant effects on gene expression were modest, any additive or synergistic effects (between E₂ and DHT) could not be determined from the current observations and cannot be discounted.

The effects of E₂ upon gene expression were investigated at only one timepoint i.e., at 24 hours (determined by the androgen observations) and further studies should consider a range of timepoints e.g., at 2, 4, 8 and 24 hours. Finally, although the SHT-290 cells are ER-positive (Barbier et al., 2005) results should ideally be confirmed in primary hESCs.

*Are the mRNAs of candidate genes modulated in decidua by increasing decidualisation or by contributing trophoblast-derived factors in vivo? (Are the mRNAs of candidate genes differentially modulated in decidua of ectopic pregnancy, a pregnancy model characterised by reduced decidualisation compared to viable intrauterine pregnancies?)*

The decidua from EP was used as it is characterised by reduced decidualisation when compared to IUP (Horne et al., 2008, Duncan et al., 2011). This allowed the study of the *in vivo* regulation of candidate genes (androgen-regulated and putative androgen-regulated genes, Chapter 4, Table 4.2) in the decidua of early pregnancy. Characterisation of the samples and patients used in the current study confirmed robust decidualisation in the decidua from IUP which was attenuated in EP (determined by decidualisation marker Igfbp1 and PRL gene levels, Result Figure 5.12). These results were in agreement with previous reports (Horne et al., 2008, Duncan et al., 2011).

Gene expression analysis of steroid receptors AR, PR and ERα confirmed mRNAs encoded by these genes were reduced in decidua of IUP compared to EP (Result figures 5.14 and 5.15) as expected in accordance with the greater degree of decidualisation observed in the viable IUP decidua (Figure 5.1) (Brosens et al., 1999, Henderson et al., 2003, Bombail et al., 2010, Cloke et al., 2010). The expression of PR and ERα mRNAs has not previously been reported in decidua from ectopic pregnancies. The difference in AR mRNA expression in the decidua of the pregnancy models observed in the current study validates the microarray prediction reported by
Duncan and colleagues (2011) carried out on a similar but distinct set of decidual samples. Also as expected, decidual AR protein appeared reduced in the decidual stromal cells of IUP compared to EP (Result Figure 5.16) and in association with greater decidualisation (Figure 5.1). Although AR protein expression has been studied in first trimester decidua (Horie et al., 1992, Milne et al., 2005, Critchley and Saunders, 2009, Cloke et al., 2010), the expression of AR protein in the decidua of ectopic pregnancies is reported here for the first time.

Maternal and trophoblast-derived circulatory endocrine factors such as progesterone and hCG may affect decidual degree of decidualisation or gene expression. P_4 and hCG levels were greater in women with IUP compared to EP (Result Figure 5.13) indicating that this difference should be taken in account when interpreting gene expression results. Pertinently, the direction of regulation of RGS2, ID3 and FOXL2 in the decidua of the pregnancy models was found to be in accordance not only with decidualisation (Result summary Table 5.6) but also with the previously reported effect of chorionic gonadotrophin upon respective gene expression in human or animal model ovaries or ovarian cells (Ujioka et al., 2000, Wu et al., 2008, Sridevi and Senthilkumaran, 2011, Agca et al., 2013, Nio-Kobayashi et al., 2013). Thus, it is possible that hCG contributes to the expression of the RGS2, ID3 and FOXL2 genes in the endometrium. It is not known if SIK1, SLC6A6, SNCAIP, AXIN2 or CDC25A are regulated by hCG. It should be highlighted that the understanding of a specific role of hCG in decidualisation, signalling via the LH/hCG receptor (Bernardini et al., 1995, Evans and Salamonsen, 2013) is limited to a few studies (reviewed by Perrier D'hauterive et al., 2007). There are reports of the inhibition or no effect of hCG upon decidualisation markers (Kasahara et al., 2001, Licht et al., 2002) but also, contrastingly, reported hCG stimulatory modulation of other receptivity markers (Fluhr et al., 2008, Afshar et al., 2012, Bourdiec et al., 2012, Bourdiec et al., 2013, Tapia-Pizarro et al., 2013, Yang et al., 2013). On the other hand, the role of progesterone in regulating decidualisation and decidualisation marker expression was considered especially as it has been more extensively studied and established. Thus, the surrogate decidualisation marker IGFBPI was used to determine the degree of decidualisation in these decidual tissues and correlation analysis supported the
association between degree of decidualisation (\textit{IGFBP1} expression) and the expression of 4 of the 8 genes (\textit{SLC6A6, AXIN2, ID3, FOXL2}) in decidual tissues from IUP and EP (Result Figure 5.20).

\textit{SLC6A6, RGS2} and \textit{SIK1} mRNA concentrations in the decidua from IUP and EP did not significantly differ (Result Figure 5.17) even though these mRNAs were significantly upregulated in decidua and/or mid-secretory endometrium compared to proliferative phase endometrium (comparison Table 5.7 below). Furthermore, no association was found between \textit{RGS2} or \textit{SIK1} mRNA expression when compared to \textit{IGFBP1} expression, a marker of decidualisation, in the decidual tissues from both IUP and EP (Result Figure 5.20). However, in these same tissues, a moderate negative correlation was found between \textit{SLC6A6} and \textit{IGFBP1} mRNA expression supporting an association between \textit{SLC6A6} expression and decidualisation. These apparently disparate observations may indicate that \textit{SLC6A6, RGS2} and \textit{SIK1} mRNAs may be regulated by factors other than, or in addition to, decidualisation, P$_4$ or hCG.

In contrast, \textit{SNCAIP} and \textit{AXIN2} mRNA concentrations were significantly downregulated in decidua from IUP compared to EP (Result Figure 5.18) confirming previously published microarray expression data (supporting information in Duncan \textit{et al.}, 2011). These findings correlate with the gene downregulation observed in IUP decidua compared to proliferative phase endometrium and reinforces the observation that \textit{SNCAIP} and \textit{AXIN2} mRNA downregulation occurs in association with decidualisation. However, correlation analysis revealed no association between \textit{SNCAIP} mRNA expression and the expression of the decidualisation marker \textit{IGFBP1}, in the decidual tissues from both IUP and EP (Result Figure 5.20). In these same tissues, a moderate negative association was found between \textit{AXIN2} and \textit{IGFBP1} mRNA expression suggesting \textit{AXIN2} mRNA expression is associated with decidualisation. Interestingly, \textit{AXIN2} was upregulated in the human endometrium after progesterone withdrawal induced by treatment with the anti-progestin Mifepristone (RU486) (Catalano \textit{et al.}, 2007). Together with the current findings, this suggests that \textit{AXIN2}, a member and negative regulator of the Wnt signalling pathway, is downregulated by progesterone in the human endometrium.
The expression of a further three genes (putative androgen-regulated in hESCs, chapter 4) were downregulated or tended to be reduced in decidua from IUP compared to EP (ID3, FOXL2 or CDC25A, respectively. Result Figure 5.19). This downregulation of gene expression was in accordance with the reduction observed in decidua when compared with proliferative endometrium and once again was observed in association with decidualisation. Correlation analysis confirmed a moderate negative correlation between ID3 or FOXL2 mRNA expression and decidualisation (as decidualisation marker IGFBP1 mRNA expression) in decidual tissues from IUP and EP (Result Figure 5.20), whilst no association was found between CDC25A and IGFBP1 mRNA expression. Progesterone has been implicated in the reduction of FOXL2 protein expression observed in both the stroma and glands of the bovine uterus (Eozenou et al., 2012). However, chorionic gonadotrophin may also be implicated as it also downregulated FOXL2 in a microarray study of rat ovarian follicles (Agca et al., 2013).

There may be other factors that are different between the decidua from IUP and EP that may affect decidualisation and the gene expression findings. For instance, in the case of the IUP decidual tissues used, a synthetic prostaglandin E₁ (PGE₁) analogue, misoprostol, was administered to women prior to surgery for cervical priming (Tang et al., 2007). Prostaglandins such as prostaglandin E₂ have been implicated in the decidualisation process during which the expression of prostaglandin E receptors EP2, EP3 and EP4 (that can bind and transduce prostaglandin signalling) are also upregulated (reviewed by Salleh, 2014). Although the role of PGE₁ during decidualisation remains unknown, treatment with a PGE₁ analogue (gemprost) has been shown to downregulate endometrial stromal PR expression in first trimester decidua, even after only 1 hour treatment (Hill et al., 1990, Milne et al., 2005). Thus, it is important to note that in the current studies, a possible decidual downregulation of PR as a result of misoprostol treatment may contribute to differences in mRNA expression (namely PR, decidualisation marker genes or candidate genes) detected between IUP and EP decidual tissues.

In summary, studies of decidua from IUP and EP confirmed that most of the candidate genes investigated (RGS2, SNCAIP, AXIN2, ID3, CDC25A, FOXL2)
followed a pattern of expression in the decidua (*in vivo*) in association with degree of decidualisation (summarised in Table 5.7 below). The exceptions were *SIK1* and *SLC6A6* where other unknown factors or even very early decidualisation signalling (that arises in mid-secretory endometrium) may explain the later lack of change in gene expression obtained from the comparison of the EP and IUP decidual models of decidualisation.

**Table 5.7 Candidate gene mRNA concentrations are associated with *in vivo* degree of decidualisation of the decidua during early pregnancy.** Gene expression levels in endometrial tissues showed candidate mRNA levels were altered in association with decidualisation that occurs in the absence of pregnancy or within the first trimester of pregnancy (left side of the table). Comparison of mRNA concentrations in decidua from decidualisation models (viable IUP vs EP) confirmed association of most mRNA concentrations (marked with *) with degree of decidualisation in vivo (right side of the table). Decidualisation is greater in decidua from IUP compared to EP.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mid-secretory endometrium</th>
<th>Decidua (IUP)</th>
<th>Change in mRNA concentrations (compared to proliferative endometrium)</th>
<th>Change in mRNA concentrations in decidua from IUP (compared to EP)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RGS2</em></td>
<td>-</td>
<td>↑</td>
<td></td>
<td>Trend ♀</td>
</tr>
<tr>
<td><em>SIK1</em></td>
<td>-</td>
<td>↑</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><em>SLC6A6</em></td>
<td>↑</td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td><em>SNCAIP</em></td>
<td>-</td>
<td>↓</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td><em>AXIN2</em></td>
<td>-</td>
<td>↓</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td><em>ID3</em></td>
<td>-</td>
<td>↓</td>
<td></td>
<td>↓</td>
</tr>
<tr>
<td><em>CDC25A</em></td>
<td>-</td>
<td>↓</td>
<td></td>
<td>Trend ♀</td>
</tr>
<tr>
<td><em>FOXL2</em></td>
<td>-</td>
<td>↓</td>
<td></td>
<td>↓</td>
</tr>
</tbody>
</table>


(♀ and ♂) significantly up or down regulated.

(♀ and ♂) non-significant trend in up or down regulation.

(-) no change in mRNA concentrations.
Are the mRNAs of candidate genes RGS2, SNCAIP, SIK1, SLC6A6, AXIN2 or ID3, FOXL2 (androgen-regulated or putative androgen regulated in undifferentiated hESCs, respectively) modulated by decidualisation of hESCs in vitro?

Analysis of candidate gene expression in decidualised hESCs (confirmed by upregulated IGFBP1 and PRL gene and IGFBP1 protein marker levels, Result Figures 5.21 and 5.22) suggests that the genes SLC6A6, RGS2, SIK1, SNCAIP and AXIN2 (regulated by DHT in undifferentiated hESCs) are upregulated upon decidualisation in vitro (Result Figure 5.23). When comparing decidualisation of hESCs with cAMP or with cAMP/P4 it appears that cAMP is the main factor responsible for the regulation of these genes. Of these five genes, only transcription of SLC6A6 has been previously shown to be induced by cAMP in some cells (Han et al., 2000, Han et al., 2006). To date, progesterone is not known to regulate any of these five genes.

Regulation of these genes by decidualisation in hESCs may contribute to the altered gene regulation noted in the endometrial tissues in association with decidualisation, for example, SLC6A6, RGS2 and SIK1 (see below Summary Table 5.8 for comparison). However, in some instances, the direction of regulation was in opposite directions (SNCAIP and AXIN2). Synphilin-1 protein (encoded by SNCAIP) was observed in epithelial cells in the current study and AXIN2 protein also has been reported in human endometrial epithelial cells (Nguyen et al., 2012). This observation may support the suggestion that the regulation of SNCAIP and AXIN2 in the epithelial cell compartment may be mostly responsible for the pattern of gene expression observed in the endometrial tissues and decidua.

The putative androgen-regulated genes ID3 and FOXL2 were not regulated in decidualised hESCs (Result Figure 5.24) in contrast to the suggested inhibition observed in decidual tissues in association with decidualisation (see below Summary Table 5.8). This may suggest that although not regulated in hESCs by decidualisation, ID3 and FOXL2 may be modulated instead by hCG itself as already mentioned. Modulation of these genes in other endometrial cell types may also
contribute to the expression levels observed in endometrial tissues versus in hESCs alone.

Table 5.8 Candidate gene mRNA concentrations in primary hESCs are regulated by decidualisation in vitro in the same or contrary direction to that observed in tissues in vivo (endometrium and decidua). Comparison of gene mRNAs regulated by decidualisation in primary hESCs in vitro (right side of the table) with gene regulation observed in tissues in association with decidualisation that occurs before or after pregnancy (left side of the table): Gene mRNAs were regulated in hESCs in the same direction by decidualisation (RGS2, SIK1 and SLC6A6 marked with *), in the opposite direction by decidualisation (SNCAIP and AXIN2) or not regulated by decidualisation (ID3 and FOXL2).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Change in mRNA concentrations in tissues (compared to proliferative endometrium)</th>
<th>Change in mRNA concentrations in in vitro-decidualised hESCs (compared to non-decidualised)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mid-secretory endometrium</td>
<td>Decidua (IUP)</td>
</tr>
<tr>
<td>RGS2 *</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>SIK1 *</td>
<td>-</td>
<td>↑</td>
</tr>
<tr>
<td>SLC6A6 *</td>
<td>↑</td>
<td>-</td>
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<tr>
<td>SNCAIP</td>
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<tr>
<td>AXIN2</td>
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<td>↓</td>
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<tr>
<td>ID3</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>FOXL2</td>
<td>-</td>
<td>↓</td>
</tr>
</tbody>
</table>

First trimester decidua from IUP: viable intrauterine pregnancy (↑ and ↓) significantly up or down regulated. (-) no change in mRNA concentrations.

Finally, when comparing gene expression between tissues and cells, discrepancies in gene expression could also be due to differences in the conditions the cells find themselves, for example, altered physical growth conditions or absent crosstalk between cell types in the in vitro decidualised hESC model (Chen et al., 2013b).
Are the mRNAs of candidate androgen-regulated genes RGS2, SNCAIP, SIK1, SLC6A6, AXIN2 or ID3, FOXL2 modulated by addition of DHT during decidualisation of hESCs in vitro?

The addition of androgen in decidualising hESC cultures enhanced the expression of decidualisation markers IGFBP1 protein and IGFBP1 and PRL mRNA (Figures 5.25 and 5.26) as has been shown before (Cloke et al., 2008, Kajihara et al., 2012). The extent of change in decidualisation marker expression appeared reduced compared to that reported before (Cloke et al., 2008) which may be explained by differences in the methods of analysis used. In addition, the hESCs used in these experiments were isolated from different cycle stages (Table 5.3) which may contribute to variation in the results obtained. However, all hESCs were subjected to the same maintenance culture conditions over days/weeks and subsequently all responded similarly to decidualisation induction as denoted by decidualisation marker expression. The presence of AR protein was confirmed in the cultured decidualised hESCs (Result Figure 5.27) and AR protein levels appeared reduced in decidualised cells compared to non-decidualised cells. The addition of DHT during decidualisation tended to increase AR expression (Results Figure 5.27B) in agreement with previous findings where DHT partially antagonised the decidualisation-dependent (cAMP-progesterone) reduction of AR in decidualised cells (Cloke et al., 2008).

Analysis of candidate gene expression showed that SLC6A6, RGS2, SIK1, SNCAIP and AXIN2 mRNAs were not altered by the addition of androgen during 8 day decidualisation of hESCs (Result Figure 5.28). Although changes in cell number as a result of DHT treatment were not quantified specifically, differences were not detected between the RNA concentrations recovered from cells exposed to the different treatments. Thus, the addition of androgen did not regulate the expression of these genes during decidualisation although expression of these genes was regulated by decidualisation alone. This may be consistent with previous findings by Cloke and colleagues (2008) indicating that in decidualising hESCs, while there are genes regulated in common by AR and PR, there are also genes regulated uniquely by either AR or PR. However, these results should be considered as preliminary.
because the effects of androgen upon gene expression were not studied in the absence of progesterone (e.g. in androgen + cAMP decidualised hESCs).

**Conclusions**

With the exception of PRLR and FOXL2, the expression of thirteen putative androgen-regulated genes identified in hESCs in undifferentiated cells in chapter 3 were found to be expressed and for the first time, regulation of expression is described in endometrial tissues. The expression of at least half of these genes was altered in association with decidualisation (RGS2, SIK1, SLC6A6, SNCAIP, AXIN2, MGC16121, ID3, CDC25A and FOXL2). Protein expression of RGS2, SIK1 and Synphilin-1 (encoded by SNCAIP) was confirmed for the first time in endometrial tissues and decidua where protein expression appeared greater. Although not quantified, the patterns of protein expression did not always seem to follow the respective pattern of gene expression suggesting, for example, unknown translational control regulation.

Further confirmation of regulation of four of the genes (SNCAIP, AXIN2, FOXL2 and ID3) by decidualisation was observed in decidua of in vivo models of early pregnancy (first trimester decidua from viable IUP and non-viable EP) that differ with respect to the degree of decidualisation (established by levels of decidualisation marker genes). The specific impact of in vitro decidualisation upon the hESCs showed that the five genes RGS2, SIK1, SLC6A6, SNCAIP and AXIN2 (regulated by androgen in undifferentiated hESCs, previous chapter 4) were upregulated by decidualisation. Interestingly, upregulation of SNCAIP and AXIN2 in decidualised hESCs conflicted with the downregulation observed in decidual tissues when compared to proliferative tissues. This possibly indicates these genes are present in both the stromal and the epithelial cells of the endometrium. FOXL2 and ID3 were not regulated in hESCs by in vitro decidualisation in contrast with the findings in the decidual tissues. Gene regulation by hCG or again, gene regulation in AR positive epithelial cells may explain this difference but would need to be investigated.

Finally, while androgen enhanced decidualisation marker expression in cAMP-progesterone decidualised hESCs, androgen was unable to alter the considerable
decidualisation-dependent mRNA expression of candidate genes *RGS2, SIK1, SLC6A6, SNCAIP* or *AXIN2*. Further studies are required for a more complete understanding of the regulation of these genes by androgens in the context of physiological oestrogen and progesterone or exposure to exogenous compounds such as commonly used drugs.
Chapter 6: Studies on the action of androgen and metformin on hESC decidualisation and gene expression

6.1 Introduction

As introduced in chapter 1 and indicated by results in chapters 3, 4 and 5, androgens can affect gene expression in both the undifferentiated and the decidualised hESCs. Specific findings from the microarray bioinformatics analysis of androgen action in hESCs (chapter 3) suggest the occurrence of for example, androgen regulation of growth hormone signalling via the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) pathway (Table 3.5) and androgen action crosstalk with other signalling pathways such as with cAMP/PKA/CREB1, a critical regulating pathway during decidualisation (section 3.3.3). These same pathways are specifically inhibited and activated during hESC decidualisation, respectively (Telgmann et al., 1997, Mayr and Montminy, 2001, Yoshino et al., 2003, Altarejos and Montminy, 2011, Fabi and Asselin, 2014).

Metformin can affect many diverse cellular processes (Viollet et al., 2012). Notably, metformin has been shown to regulate proliferation but also, recently, differentiation of some progenitor cell or cancer cell types (Cufi et al., 2010, Vazquez-Martin et al., 2010, Wang et al., 2012a, Fatt et al., 2015, Li et al., 2015a, Liu et al., 2016). Interestingly, the same pathways identified above (PI3K/AKT and cAMP/PKA/CREB1) have also been reported modulated by metformin in various cells (Thomson et al., 2008, Cantrell et al., 2010, Kim et al., 2011, Rice et al., 2013, Brown et al., 2013) and in particular, the inhibition of PI3K/AKT by metformin was demonstrated in decidualised primary hESCs (Ferreira et al., 2014b).

All together, these findings suggested that both androgen and metformin action may impact hESCs, that signalling may occur through common pathways and that androgen and metformin signalling may interact. Studies in this chapter focussed on decidualisation of hESCs as it is a key transformation required for reproductive success. During progesterone-driven hESC decidualisation, dynamic and complex cellular reprogramming occurs involving numerous signalling pathways (reviewed...
by Gellersen and Brosens, 2014), through which, for example, proliferation is curbed (e.g. via cell cycle arrest) and the balance is facilitated in favour of cellular differentiation (Das, 2009, Logan et al., 2012, Tang et al., 2012).

Although the decidualisation process is not yet fully understood, dysregulation of decidualisation, due to a lack of adequate ovarian stimulation or inherent endometrial defects, has been implicated in defective receptivity, implantation and trophoblast invasion that contribute to pathologies such as pregnancy loss (miscarriage and recurrent pregnancy loss) and placental insufficiency resulting in intrauterine growth restriction and/or pre-eclampsia (reviewed by Cha et al., 2012).

Women with PCOS have an increased risk of miscarriage even after taking in account poor oocyte quality (Tulppala et al., 1993, Clifford et al., 1994, Kodama et al., 1995, Okon et al., 1998, reviewed by Lopes et al., 2011, Kamalanathan et al., 2013) (section 1.8.2) and findings suggest that impaired trophoblast invasion as a result of impaired decidualisation may contribute to the associated higher rates of implantation failure and miscarriage (Palomba et al., 2012). Management of PCOS with metformin seems to reduce the risk of pregnancy loss (even in the presence of diabetes, obesity or during assisted reproduction) (Glueck et al., 2001, Glueck et al., 2002, Jakubowicz et al., 2002, Khattab et al., 2006, Nawaz et al., 2008, Sohrabvand et al., 2009, Nawaz and Rizvi, 2010, Zhao et al., 2010, De Leo et al., 2011, Lautatzis et al., 2013, Zheng et al., 2013, Al-Biate, 2015, Feng et al., 2015) although not all research findings agree (Palomba et al., 2009, Liu et al., 2014, Tso et al., 2014, Chiswick et al., 2015, Huang et al., 2015).

Besides the indirect beneficial effects of metformin in PCOS patients of a return of androgens to normal concentrations, improved insulin resistance, induction of ovarian function (timely progesterone production) and consequent improved fertility (systematic review by Tang et al., 2012, meta-analyses by Xiao et al., 2012, Misso et al., 2013, Sivalingam et al., 2014), direct effects of metformin upon endometrial cells and endometrial function, namely hESC decidualisation, may also occur and are understudied.
Chapter 6  Androgen and metformin action in decidualised hESCs

The effects of metformin upon hESC decidualisation? Modulation by androgen?
Two studies have reported that metformin reduced decidualisation marker expression (IGFBP1 mRNA and PRL protein secretion) in decidualised hESCs although it should be noted that a high dose of metformin (1mM) and an uncommonly used hESC-decidualisation model induced by oestradiol/progesterone/epidermal growth factor (EGF) were used (Capp et al., 2011, Germeyer et al., 2011). Thus, these findings in the above model suggest that high doses of metformin may inhibit decidualisation. However, the effect of metformin (at either high or low doses) upon the degree of decidualisation in the more commonly used cAMP/progesterone decidualising hESC model (employed in chapter 5) has not been investigated (in the presence or absence of DHT).

On the other hand, we alternatively speculated that metformin action might directly facilitate hESC decidualisation and help explain, at least in part, the association of metformin treatment with a reduced risk of miscarriage in women with PCOS. If this alternative hypothesis was found to be true we further speculated that, as androgens have been shown to enhance hESC decidualisation (Narukawa et al., 1994, Cloke et al., 2008) and as there is a possibility of crosstalk between androgen and metformin signalling in hESCs, then the concomitant treatment of hESCs with androgen and metformin might also increase decidualisation.

For this preliminary study of the effects of metformin upon decidualisation (in the absence and presence of DHT), alternative decidualisation endpoints were considered based on previous findings in this thesis and observations from the literature. Firstly, assessment of the degree of decidualisation was carried out directly through decidualisation marker expression. Secondly, the expression of PR and CYP19A1 was also assessed. This was considered as changes in PR expression might impact hESC responsiveness to the progesterone decidualisation stimulus whereas changes in CYP19A1 expression, which is induced during hESC decidualisation (Gibson et al., 2013), might lead to increased local production of oestradiol converted from testosterone, thereby altering oestrogen/progesterone balance and causing resistance to progesterone in the cells. In this way, altered PR or CYP19A1 expression might impact directly on decidualisation and even reveal possible
mechanisms of action of DHT and/or metformin in decidualising hESC. To date, it is not known if metformin regulates steroid receptor or aromatase expression in decidualising hESCs and it is not known if androgen will modulate such metformin action. In other steroid responsive cell types including granulosa cells, androgens and metformin have each been shown to regulate \( CYP19A1 \) gene and protein expression with associated changes in steroid metabolism (Henderson et al., 1990, Henderson and Wolf, 1991, Takemura et al., 2007, Brown et al., 2010, Samarajeewa et al., 2011, Rice et al., 2013, Samarajeewa et al., 2013, Fuhrmeister et al., 2014). Thirdly, as the putative androgen-regulated \( SIK1, RGS2, SNCAIP, AXIN2 \) and \( SLC6A6 \) and \( ID3 \) mRNAs were also found to be upregulated in association with decidualisation (chapter 5) and if metformin affects decidualisation then it was postulated that the expression of these decidualisation-regulated genes may be subsequently affected. It was also unknown if the presence of androgen would modulate any metformin action.

Thus, the following hypothesis was proposed and addressed in this chapter: *In decidualising hESCs, metformin alters the degree of decidualisation and gene expression associated with decidualisation. The added presence of DHT modulates this metformin action.*

### 6.1.1 Aims of Chapter

- To investigate in the cAMP/P₄-decidualised hESC model the effect of metformin upon decidualisation - as determined by expression of decidualisation markers, steroid receptors, the steroid enzyme \( CYP19A1 \), and candidate androgen-regulated genes.

- To investigate if DHT, in the alternate cAMP/P₄/DHT-decidualised hESC model, affects the action of metformin upon decidualisation - as determined by the expression of decidualisation markers, steroid receptors, the steroid enzyme \( CYP19A1 \), and candidate androgen-regulated genes.
6.2 Materials and Methods

This section describes all specific methods and outlines the experimental approaches employed in Chapter 6; general methodology has been summarised in Chapter 2.

6.2.1 Endometrial tissue biopsies

All endometrial tissues used in this chapter were collected under ethical approval as described in section 2.2. The collection of endometrial tissues and the isolation of primary hESCs were carried out as described in sections 2.2 and 2.3.1. The clinical details for the women who donated tissue samples used in the investigations carried out in this chapter are summarised in the Table 5.3.

6.2.2 Decidualisation of primary hESCs with metformin in the absence and presence of DHT

Primary hESCs isolated from endometrial samples (as described in section 2.3.1) were seeded in 6 well cluster plates (1 x 10^5 cells/well) in 2% charcoal stripped serum media (section 2.3.1.2) and allowed to adhere overnight. At 80-90% confluence, cells were decidualised using a standard protocol (Cloke et al., 2008) by addition of 2% charcoal stripped serum media (section 2.3.1.2) supplemented with 1µM (10^-6 M) P_4 and 0.1mg/ml cAMP (section 2.4.2).

Solubilisation of metformin (Sigma, Dorset, UK) was performed in water (100mM) and aliquots were stored at -20°C. Metformin was further diluted in water as necessary on day of use for a final concentration of 100µM or 1000µM in the cell culture media. The 100µM metformin concentration is in the range concentrations measured in mouse and human plasma during therapeutic treatment of diabetes or other insulin resistance-associated disorders such as PCOS (Robert et al., 2003).

In the experiments where addition of metformin was assessed, cells were decidualised as above with the additional 100µM or 1000µM of metformin or without metformin (control). In the experiments where addition of DHT and/or metformin was assessed, cells were decidualised as above plus with 100nM DHT (Cloke et al., 2008) (section 2.4.1) and either 100µM Metformin or vehicle control.
In all experiments, culture media with hormonal/metformin supplements or controls were changed every two days. Cells and media were harvested after 8 days. Each treatment was carried out in duplicate. Each experiment was repeated 4 to 7 times, each time with hESCs isolated from a different endometrial biopsy (Table 5.3).

6.2.3 Detection of IGFBP1 protein in decidualised hESC culture media by ELISA

Cell culture media from the decidualised hESC experiments described in section 6.2.2 was collected at the same time as cell harvest (8 days). Media samples were stored at -80°C until assayed for IGFBP1 following a two-site sandwich ELISA protocol detailed in section 2.8. A standard curve was constructed for each plate, consisting of ten standards added in duplicate, 100µl per well. Media samples to be assayed were added in duplicate, 100µl per well. The remainder of the assay was conducted as described in section 2.8.

6.2.4 RNA analysis

Cultured hESCs from the experiments described in section 6.2.2 were harvested after 8 days and total cell RNA was extracted (section 2.7.1). RNA was quantified and cDNA was synthesized (section 2.7.2). The levels of the genes investigated (and endogenous control) were determined by qRT-PCR as described in section 2.7.3 (the primers and probes used are detailed in Table 2.5).

6.2.5 Statistical analysis

Prism 5 for Windows version 5.02, 2008 by Graphpad Software (La Jolla, CA, USA) was used to perform statistical analyses. Data is presented as means ± standard error of the mean (SEM) with P<0.05 considered statistically significant (section 2.9). IGFBP1 protein secretion levels and qRT-PCR analysis of gene expression in decidualised primary hESCs were compared by one-way ANOVA with Bonferroni’s multiple comparison post hoc test or by Wilcoxon test (as described in section 2.9).
6.3 Results

6.3.1 In the absence of DHT, a high dose of metformin is required to enhance hESC-decidualisation, and alter steroid enzyme CYP19A1 and candidate androgen-regulated SIK1, RGS2, SNCAIP and AXIN2 gene mRNA concentrations in decidualising hESCs

In order to investigate if decidualisation, and gene expression (steroid receptors, CYP19A1 steroid enzyme and candidate androgen-regulated genes) were regulated by metformin inclusion during decidualisation, primary hESCs were decidualised for 8 days with cAMP/ P4 (Cloke et al., 2008) in the absence and presence of metformin (100 or 1000µM).

6.3.1.1 A high dose of metformin tends to increase decidualisation marker IGFBP1 secreted protein levels and enhances IGFBP1 mRNA but not PRL mRNA concentrations in decidualising hESCs

To evaluate decidualisation in hESCs decidualised in the presence of metformin the expression of IGFBP1 and PRL mRNAs and the production of IGFBP1 protein were measured in a small pilot experiment.

Figure 6.1 shows that IGFBP1 mRNA concentrations were significantly increased in 1000µM metformin-treated decidualising hESCs (P<0.05) but not in 100µM metformin-treated cells compared to the controls. PRL mRNA concentrations were unchanged by treatment of decidualising hESCs with either dose of metformin (100 or 1000µM) compared to the controls.
Figure 6.1 Expression of decidualisation marker IGFBP1 mRNA, but not PRL, is increased by the inclusion of a high dose of metformin during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄ in the absence or presence of 100µM or 1000µM metformin for 8 days. IGFBP1 mRNA concentrations were greater in 1000µM metformin-treated cells compared to control. PRL mRNA concentrations were unchanged by metformin. Data are presented as mean ± SEM (n=4). * P<0.05.

Figure 6.2 shows that the inclusion of metformin (100 or 1000µM) during hESC decidualisation did not significantly alter IGFBP1 protein secretion compared to controls.

Figure 6.2 Secreted decidualisation marker IGFBP1 protein tends to increase (but not significantly) with the inclusion of a high dose of metformin during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄ in the absence or presence of 100µM or 1000µM metformin for 8 days. Secreted IGFBP1 protein concentrations, determined by ELISA, tended to be greater in the presence of 1000µM metformin compared to control. Data are presented as mean ± SEM (n=4).
6.3.1.2 Metformin inclusion during decidualisation does not significantly affect PR, AR or ERα mRNA concentrations

The effect of metformin upon steroid receptor gene expression during hESC decidualisation was investigated in the same metformin-treated decidualising hESCs (section above).

The mRNA expression of the steroid receptors PR, AR and ERα was unchanged by metformin inclusion during hESC decidualisation compared with controls (Figure 6.3).

Figure 6.3 Steroid receptor gene expression levels are unchanged by the inclusion of metformin during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄ in the absence or presence of 100µM or 1000µM metformin for 8 days. PR, AR and ERα (ER-A) mRNA concentrations were unchanged by metformin inclusion during primary hESC-decidualisation compared to control. Data are presented as mean ± SEM (n=4).
6.3.1.3 A high dose of metformin increases steroid enzyme \textit{CYP19A1} mRNA concentrations in decidualising hESCs

\textit{CYP19A1} gene mRNAs were significantly upregulated by 1000µM metformin (P<0.05) but not by 100µM metformin during hESC decidualisation compared to controls (Figure 6.4).

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure6_4.png}
\caption{Steroid enzyme \textit{CYP19A1} mRNA concentrations are increased by the inclusion of a high dose of metformin during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄ in the absence or presence of 100µM or 1000µM metformin for 8 days. \textit{CYP19A1} mRNA concentrations were significantly increased by 1000µM metformin but not by 100µM metformin compared to control during primary hESC-decidualisation. Data are presented as mean ± SEM (n=4). *P<0.05.}
\end{figure}

6.3.1.4 Candidate androgen-regulated genes \textit{SIK1}, \textit{SNCAIP} and \textit{RGS2} mRNA concentrations are altered by a high dose of metformin but not \textit{SLC6A6} or \textit{ID3} in decidualised hESCs

The effect of inclusion of metformin upon candidate androgen-regulated \textit{SIK1}, \textit{RGS2}, \textit{SNCAIP}, \textit{AXIN2}, \textit{SLC6A6} and \textit{ID3} gene expression during hESC decidualisation is shown in Figure 6.5.

During hESC decidualisation, \textit{SIK1} mRNA concentrations were unchanged by 100µM metformin but were significantly reduced by 1000µM metformin compared to controls (P<0.05). \textit{RGS2}, \textit{SNCAIP} and \textit{AXIN2} mRNA concentrations were unchanged by 100µM metformin but were significantly increased by 1000µM metformin compared to controls (P<0.01, P<0.05, P<0.05 respectively). \textit{SLC6A6} and \textit{ID3} gene mRNA concentrations were not altered by metformin inclusion during cAMP/P₄ hESC decidualisation.
Figure 6.5 Expression of candidate androgen-regulated genes SIK1, RGS2, SNCAIP and AXIN2 (but not SLC6A6 or ID3) is significantly altered by the inclusion of a high dose of metformin during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P4 in the absence or presence of 100µM or 1000µM metformin for 8 days. SIK1 mRNA concentrations were significantly reduced and SNCAIP, RGS2 and AXIN2 mRNA concentrations were significantly elevated by 1000µM metformin but not by 100µM metformin compared to control during hESC-decidualisation. SLC6A6 and ID3 mRNA concentrations were unchanged by metformin inclusion during hESC-decidualisation compared to control. Data are presented as mean ± SEM (n=4). *P<0.05, **P<0.01.
6.3.2 In the presence of DHT, a ‘physiological’ lower dose of metformin may be sufficient to enhance hESC-decidualisation, but does not alter steroid receptor, steroid enzyme CYP19A1 or candidate gene expression in decidualising hESCs

In order to investigate the effects of sustained-exposure to a tolerable ‘physiological’ metformin dose (Robert et al., 2003) upon decidualisation markers, CYP19A1 steroid enzyme, steroid receptors and candidate androgen-regulated gene expression in the standard in vitro hESC-decidualising model containing DHT (Cloke et al., 2008), primary hESCs were decidualised for 8 days with cAMP/P₄/100nM DHT in the absence and presence of 100µM metformin.

6.3.2.1 In the presence of DHT, a ‘physiological’ low dose of metformin enhances concentrations of secreted IGFBP1 protein and mRNA in decidualising hESCs but PRL mRNA concentrations were unchanged

To investigate if metformin altered the degree of decidualisation of hESCs decidualised in the presence of DHT, the expression of IGFBP1 and PRL mRNAs and the production of IGFBP1 protein were determined in cAMP/P₄/DHT decidualised cells treated with metformin or control.

In this cAMP/P₄/DHT decidualised hESC model, 100µM metformin treatment significantly increased IGFBP1 mRNA concentrations (P<0.05) and IGFBP1 secreted protein concentrations (P<0.05) compared to control (Figures 6.6 and 6.7).

PRL mRNA concentrations were unchanged by 100µM metformin compared to control in cAMP/P₄/DHT-decidualised hESCs (Figure 6.7).
Figure 6.6 mRNA expression of decidualisation marker IGFBP1 but not of PRL is increased by metformin when in the presence of DHT during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄/DHT(100nM) in the absence or presence of 100µM metformin for 8 days. IGFBP1 mRNA levels were significantly elevated by metformin compared to the control. PRL mRNA concentrations were unchanged by metformin compared to the control. Data are presented as mean ± SEM (n=6). *P<0.05.

Figure 6.7 Secreted decidualisation marker IGFBP1 protein is increased by metformin in the presence of DHT during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄/DHT(100nM) in the absence or presence of 100µM metformin for 8 days. Secreted IGFBP1 protein concentrations, determined by ELISA, were significantly increased by metformin compared to the control. (Data are presented as mean ± SEM (n=7). *P<0.05.
6.3.2.2 In the presence of DHT, a ‘physiological’ low dose of metformin does not alter steroid receptor PR, AR, or ER-A mRNA expression in decidualising hESCs

Figure 6.8 shows PR, AR and ER-α mRNA concentrations were unchanged by metformin treatment compared to control in cAMP/P₄/DHT-decidualised hESCs.

**Figure 6.8 Steroid receptor gene expression is not altered by metformin when in the presence of DHT during hESC-decidualisation.** Primary hESCs were decidualised with cAMP/P₄/DHT(100nM) in the absence or presence of 100µM metformin for 8 days. PR, AR and ERα (ER-A) mRNA concentrations were unchanged by metformin compared to the control. Data are presented as mean ± SEM (n=6).

6.3.2.3 In the presence of DHT, a ‘physiological’ low dose of metformin does not alter steroid enzyme CYP19A1 mRNA expression in decidualising hESCs

Figure 6.9 shows CYP19A1 mRNA concentrations were not changed by metformin treatment compared to control in cAMP/P₄/DHT-decidualised hESCs.
Figure 6.9 Expression of steroid enzyme CYP19A1 mRNA is unchanged by metformin when in the presence of DHT during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄/DHT(100nM) in the absence or presence of 100µM metformin for 8 days. CYP19A1 mRNA concentrations were not altered by metformin compared to the control. Data are presented as mean ± SEM (n=4).

6.3.2.4 In the presence of DHT, a 'physiological' low dose of metformin does not alter candidate androgen-regulated gene mRNA expression in decidualising hESCs

The effect of metformin upon candidate androgen-regulated SIK1, RGS2, SNCAIP, AXIN2, SLC6A6 and ID3 gene expression was determined in cAMP/P₄/DHT-decidualised hESCs.

Figure 6.10 shows SIK1, RGS2, SNCAIP, AXIN2, SLC6A6 and ID3 mRNA concentrations were not significantly changed by metformin treatment compared to control in cAMP/P₄/DHT-decidualised hESCs.
Figure 6.10 Expression of candidate androgen-regulated gene mRNAs is unchanged by metformin when in the presence of DHT during hESC-decidualisation. Primary hESCs were decidualised with cAMP/P₄/DHT(100nM) in the absence or presence of 100µM metformin for 8 days. SIK1, RGS2, SNCAIP, AXIN2, SLC6A6 and ID3 gene mRNA concentrations were not significantly altered by metformin compared to the control. SIK1 mRNA concentrations tended to be increased by metformin treatment compared to the control. Data are presented as mean ± SEM (n=4).
6.4 Discussion

Combined findings from chapters 3, 4 and 5 and the literature regarding studies using androgens or metformin (section 6.1) suggest that both may affect gene expression and cell processes such as growth and differentiation. In the endometrium, the regulation of such processes are key to endometrial reproductive function especially during hESC decidualisation. However, while androgens have been shown to enhance hESC decidualisation (Cloke et al., 2008 and in chapter 5), metformin action has not yet been investigated in the commonly used cAMP/P₄ induced decidualised hESC model. Thus, investigations carried out in this chapter sought to explore the impact of metformin upon the expression of decidualisation markers and other decidualisation-associated genes (including putative androgen-regulated genes) in cAMP/P₄ but also in cAMP/P₄/DHT decidualised hESCs where the role of androgen was explored as a possible modulator of metformin action.

In the studies current studies, the dose of 100µM metformin was chosen as it represents the ‘physiological’ concentrations close to that detected in human plasma and in some tissues as a result of doses typically administered in diabetes and PCOS patients (Robert et al., 2003, reviewed by Graham et al., 2011). Also, metformin doses of 100µM or lower have been reported sufficient to elicit, for example, differentiation, anti-tumour effects (reduced proliferation) or changes in gene expression such as CYP19A1 expression (Samarajeewa et al., 2011, Xie et al., 2011a, Fatt et al., 2015). The higher dose of 1000µM metformin was also used. While the higher dose of metformin may not be considered to represent a physiologically tolerable concentration of metformin in human blood (even being reportedly linked with some deaths) (Gambaro et al., 2007, systematic review by Dell'aglio et al., 2009, Cantrell et al., 2012, Bonsignore et al., 2014), this concentration of metformin has been used in the only other investigations carried out in decidualising hESCs alternatively stimulated with progesterone/oestradiol/EGF or progesterone/oestradiol decidualisation (Capp et al., 2011, Germeyer et al., 2011, Ferreira et al., 2014a, Ferreira et al., 2014b).
Is the expression of decidualisation markers and steroid receptors modulated by metformin in cAMP/P₄ decidualised hESCs and does the presence of DHT alter metformin action?

In the absence of DHT, only the higher metformin dose (1000µM) significantly increased IGFBP1 mRNA concentrations. In the presence of DHT however, the lower dose of metformin (100µM) significantly increased the expression of IGFBP1 protein secretion and mRNA. PRL mRNA expression was not altered by metformin in either the absence or presence of DHT. These novel but preliminary findings, summarised in Table 6.1, suggest that DHT enhances the action of physiological doses of metformin (100µM) (section 6.3.1) in decidualising hESCs with respect to the expression of the decidualisation marker IGFBP1 thus supporting the hypothesis proposed in this chapter that androgen and metformin may act in concert to alter (increase) hESC decidualisation.

The mechanism by which metformin enhances decidualisation in the model used remains to be elucidated. This may occur, for example, via inhibition of AKT (diminished phosphorylation) as hESC decidualisation has been associated with an inhibition of AKT and subsequent inhibition of the mTOR pathway (Yoshino et al., 2003, Fabi and Asselin, 2014). The finding that metformin inhibits AKT phosphorylation in oestradiol/progesterone decidualised hESCs (Ferreira et al., 2014b) goes some way to support the hypothesis. It should also be considered that the decidualisation model used in the present study included incubation of cells with cAMP and progesterone. Cellular cAMP can be converted to AMP by phosphodiesterases (Vezzosi and Bertherat, 2011). Upon metformin treatment, the levels of AMP may be even further enhanced as metformin has been reported to inhibit AMP deaminase, responsible for AMP breakdown (Ouyang et al., 2011, Vytla and Ochs, 2013). Thus, in the cell model, high cellular AMP levels may be maintained for longer leading to sustained AMPK activation and subsequent mTOR inhibition (Andujar-Plata et al., 2012). Further study is required to confirm this is the case in this cAMP/P₄ decidualisation cell model and if this mechanism contributes to the increase in decidualisation marker expression reported in the present study.
Table 6.1 Summary of the effects of metformin upon hESC-decidualisation markers and the expression of various other genes (steroid receptors, CYP19A1 and putative androgen-regulated genes) in the absence and presence of DHT in decidualising hESCs. In the absence of DHT, only the higher metformin dose (1000µM) altered the expression of IGFBP1 protein and mRNA, the expression of CYP19A1 mRNA and the expression of candidate androgen-regulated SIK1, RGS2, SNCAIP and AXIN2 genes. While in the absence of 100nM DHT, the ‘more physiological’ lower dose of metformin (100µM) did not affect the expression of the decidualisation markers or other genes investigated. However, in the presence of DHT, the same lower dose of metformin (100µM) did significantly increase the expression of IGFBP1 protein secretion and mRNA but not the expression of the other genes investigated.

<table>
<thead>
<tr>
<th>Metformin</th>
<th>Change in mRNA (or secreted protein) concentrations compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gene</td>
<td>100 µM</td>
</tr>
<tr>
<td>Decidualisation markers</td>
<td></td>
</tr>
<tr>
<td>IGFBP1 (protein)</td>
<td>- Trend ‡</td>
</tr>
<tr>
<td>IGFBP1 mRNA</td>
<td>-</td>
</tr>
<tr>
<td>PRL mRNA</td>
<td>-</td>
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<tr>
<td>Steroid receptors</td>
<td></td>
</tr>
<tr>
<td>PR</td>
<td>-</td>
</tr>
<tr>
<td>AR</td>
<td>-</td>
</tr>
<tr>
<td>ERα</td>
<td>-</td>
</tr>
<tr>
<td>Steroid enzyme</td>
<td>CYP19A1</td>
</tr>
<tr>
<td>Candidate androgen regulated genes</td>
<td>SIK1</td>
</tr>
<tr>
<td>RGS2</td>
<td>-</td>
</tr>
<tr>
<td>SNCAIP</td>
<td>-</td>
</tr>
<tr>
<td>AXIN2</td>
<td>-</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>-</td>
</tr>
<tr>
<td>ID3</td>
<td>-</td>
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</tbody>
</table>

(† and ‡) significantly up or down regulated.
(‡ and §) non-significant trend in up or down regulation.
(-) no change in mRNA concentrations.
Contrary to the present findings, it is interesting to note that, the same high metformin dose (1000µM) reduced decidualisation marker expression (IGFBP1 mRNA and PRL protein secretion) has been reported in a different and uncommonly used hESC-decidualisation model in which cells are incubated with oestradiol/progesterone/EGF (Capp et al., 2011, Germeyer et al., 2011). The differences that may account for the contradictory results observed between the two decidualisation models include (but are not limited to) the actions of cAMP, EGF, oestradiol and DHT but these require further study.

These preliminary findings may go towards elucidating mechanisms by which metformin administration in women with PCOS may enhance endometrial function and lead to, for example, improved establishment and maintenance of pregnancy, independently, and in addition to, its contribution to improved ovarian function (normalisation of steroid production e.g., androgens and ovulation induction) (meta-analysis by Lautatzis et al., 2013, systematic review and meta-analysis by Palomba et al., 2014) (section 1.8.2). Enhancement of decidualisation in the endometrium due to local metformin action may oppose the prolonged proliferative phenotype and abnormal progesterone-resistant endometrium described in patients with PCOS (review by Young and Lessey, 2010, Savaris et al., 2011).

With regards to the regulation of steroid receptor mRNA expression by metformin in the presence or in the absence of DHT, changes in PR, ERα or AR expression were not observed in the current studies involving decidualised hESCs. The impact of metformin on the expression of these mRNAs has not been reported to date and the current data suggest that metformin action in decidualised hESCs does not involve modulation of steroid receptor mRNA expression (whether in the absence or presence of DHT). Further study is required to characterise steroid receptor protein expression. However, these findings may suggest that changes in endometrial receptor expression including the observed reduction of abnormally high AR expression in PCOS patients treated with metformin (Apparao et al., 2002, Ito-Yamaguchi et al., 2015) may occur mainly in an indirect manner via effects upon ovarian function. In the case of AR expression, the normalisation of ovarian
progesterone production drives decidualisation that underlies the repression of endometrial AR protein expression (Cloke et al., 2008, Cloke et al., 2010).

**Is the expression of decidualisation-modulated genes (CYP19A1 and putative androgen-regulated genes) affected by metformin in cAMP/P₄ decidualised hESCs and does the presence of DHT alter metformin action?**

Incubation of decidualising hESCs with the higher metformin dose (1000µM) enhanced the expression of 5 out of seven genes (CYP19A1, SIK1, RGS2, SNCAIP and AXIN2 mRNAs) (summarised in Table 6.1). Of the five genes regulated by metformin in decidualising hESCs, only CYP19A1 expression has previously been shown to be regulated by metformin in various cell types where, in contrast, expression was inhibited (Takemura et al., 2007, Brown et al., 2010, Samarajeewa et al., 2011, Rice et al., 2013, Fuhrmeister et al., 2014).

It is interesting to note that CYP19A1, RGS2, SNCAIP and AXIN2 gene mRNA concentrations were upregulated by metformin in these experiments. This is consistent with upregulation by decidualisation stimulus compared to non-decidualised cells (as in chapter 5 and in Gibson et al., 2013). The impact of CYP19A1 mRNA upregulation by metformin, if followed by increased aromatase activity, could contribute to local steroid availability/balance through increased oestradiol production and impact decidualisation and subsequent implantation or immune and vascular function during early pregnancy (Gibson et al., 2013, Gibson et al., 2015). Thus, this finding raises the possibility that metformin administration in women may have an impact upon steroidogenesis in hESCs besides an impact upon ovarian steroidogenesis where the regulation of the expression of enzymes involved such as CYP19A1 has been shown (granulosa cells) (Rice et al., 2013, Fuhrmeister et al., 2014).

Of the candidate genes investigated, only the expression of RGS2 mRNA by metformin (Table 6.5) was regulated in the same direction by metformin or decidualisation in both the decidualised hESC in vitro experiments (Table 5.8) and the decidual tissues (Table 5.5). As in the previous chapter 5, the regulation of
SNCAIP and AXIN2 mRNAs in cultured hESCs by metformin (Table 6.1) (and in association with hESC decidualisation, Table 5.8) was in the opposite direction to that observed by decidualisation in decidual tissues (Table 5.5 and Table 5.7). These findings highlight once again the differences between gene expression in cells in vitro compared to in intact tissues that are composed of other cell types besides the stromal cells and exposed to other possible regulating factors such as oestradiol (absent from the cell culture models). In contrast, the expression of SIK1 and SLC6A6 mRNAs upon metformin treatment did not follow the pattern of regulation observed by decidualisation alone compared to non-decidualised cells in culture (chapter 5). As these genes are regulated by decidualisation it is not known if their modulation by metformin alters the role of the decidual cell within the endometrium. In order to elucidate the mechanisms involved further study into the regulation of the expression of these genes by metformin is also required.

In the presence of DHT however, the lower dose of metformin (100µM) was not able to alter the expression of any of the genes during hESC decidualisation. It remains unknown if the higher concentration of metformin (1000µM) would have an effect. However, the lack of DHT-dependent modulation of metformin regulation of such genes during hESC decidualisation is in line with previous findings presented in chapter 5.

These in vitro cell studies are difficult to carry out due to variability and availability of suitable tissues, however they are helpful to clarify the ovarian versus endometrial effects of metformin in the endometrium especially with a view to understand the role of metformin during decidualisation and early pregnancy in vivo. The results described in this chapter remain preliminary and require further study including further repeat studies (to increase sample size) and reduce the effects of variability between cells recovered from different women. In addition, findings from other in vitro hESC decidualisation studies report varying inductive and repressive dynamic expression patterns of gene expression associated with decidualisation over the inductive timecourse (Brar et al., 2001, Gibson et al., 2016) thus supporting the examination of gene expression at multiple time intervals prior to the single day 8 timepoint used in the current study chapter (not carried out due to lack of time).
The effect of DHT (independent of metformin) upon CYP19A1 mRNA expression in cAMP/P₄ decidualised hESCs was not determined in the current studies due to lack of time. The regulation of CYP19A1 mRNA expression in humans is cell specific and androgens (testosterone and/or DHT) have been reported to regulate CYP19A1 expression in other human and animal cells although in some instances the effect of testosterone was not necessarily mediated by AR (Wada et al., 1988, Henderson et al., 1990, Henderson and Wolf, 1991, Bourguiba et al., 2003, Eriksen et al., 2014). Therefore, the effect of DHT upon CYP19A1 mRNA expression warrants further investigation in decidualising hESCs (not reported to date).

It would also be informative to extend these decidualisation studies with DHT and/or metformin to other more complete decidualisation cell models that include physiological concentrations of oestradiol (cAMP/P₄/E₂ or P₄/E₂). These models could be further combined with the use of specific steroid receptor or pathway inhibitors to clarify the impact of androgen signalling in the complex regulation of decidualisation and associated gene expression including that of the the novel androgen and decidualisation-regulated genes identified in chapters 4 and 5. Metformin may regulate CYP19A1 mRNA expression in decidualised hESCs and a recent study has showed how the expression and activity of enzymes that regulate androgen biosynthesis follow a changing pattern across the hESC decidualisation timecourse (Gibson et al., 2016). Thus, in these other decidualisation models, intracrinology investigations of decidualising hESCs treated with DHT and metformin should also be included to assess changes in steroid balance. On the other hand, supraphysiological concentrations of oestradiol or DHT could also be used to model steroidal imbalance.

**Conclusions**

The studies in this chapter present preliminary evidence in support of an interaction between androgen and metformin signalling affecting hESC decidualisation. The combined examination of the findings from the previous chapters and from the published literature suggested that androgen and metformin might regulate pathways
in common with a possible impact upon hESC decidualisation and associated gene expression.

Exploratory studies showed that a high metformin concentration increased hESC decidualisation, the expression of CYP19A1 (potentially affecting local cellular oestrogen availability/steroid balance) and putative androgen-regulated gene expression (4 genes) in decidualised hESCs. However, in a decidualising model containing DHT, a lower clinically relevant metformin concentration (100µM) increased decidualisation marker expression but did not alter expression of CYP19A1 or putative androgen-regulated genes. These novel findings suggest that androgens may increase decidualisation during exposure to the commonly used drug metformin and that this may occur through androgen signalling sensitizing the decidualised cells to metformin action.

These in vitro studies suggest metformin administration may regulate hESC decidualisation directly and independently of an impact on the ovary and that local androgen signalling may interact with metformin action in the endometrium. These findings may contribute to the establishment of more complex models (proposed by Banerjee et al., 2013) to understand metformin action in the endometrium especially during implantation. Further studies involving, for example, specific inhibitors of specific targets are required to elucidate the pathways involved.
Chapter 7: General discussion

7.1 Introduction

The human endometrium undergoes recurrent cycles of dynamic remodelling during the menstrual cycle that involve changes in cellular proliferation and differentiation, including differentiation (decidualisation) of the stroma, a key transformation for the establishment of pregnancy. Extensive studies have characterised how the steroid hormones oestrogen and progesterone acting via their nuclear receptors coordinate these transformations during the oestrogen-dominated proliferative phase and the progesterone-dominant secretory phase of endometrial function. The basic elements required for AR-mediated signalling including circulating androgens and the AR, are also present in the endometrium during the menstrual cycle. An anti-proliferative effect of elevated androgens upon the endometrium has been inferred from, for example, women displaying the effects of exogenous androgens or elevated endogenous androgens such as that associated with PCOS (Miller et al., 1986, Maliqueo et al., 2003, Mueller et al., 2007, Kim et al., 2009, Perrone et al., 2009). A few previous studies carried out on human endometrial primary cells (stromal or epithelial cells) and tissue explants have postulated a role for androgens during the endometrial proliferative phase (Neulen et al., 1987, Rose et al., 1988, Watson et al., 1998, Tuckerman et al., 2000, Maliqueo et al., 2004, Wen et al., 2006, Ishikawa et al., 2007, Zhang and Liao, 2010, Marshall et al., 2011) and the secretory phase (Narukawa et al., 1994, Wen et al., 2006, Cloke et al., 2008, Gonzalez et al., 2012, Kajihara et al., 2012). With the exception of two studies, one from a group in London (Cloke et al., 2008) and the other from our own laboratory group (Marshall et al., 2011) all these studies have reported the regulation of single genes or proteins by androgens and, in some cases, have involved the use of supraphysiological concentrations of androgens and/or incubation with aromatizable androgens (testosterone or androstenedione).

Thus, the impact of androgens on endometrial function remains understudied. The current studies set out to address this knowledge gap by exploring androgen action (at physiological concentrations) in human androgen receptor-positive endometrial
stromal cells (hESCs). The stromal cells were the primary focus of these investigations as AR immunoexpression is maintained in stromal cells of the basal compartment of the endometrium regardless of cycle stage with maximal expression reported in the stromal cells of the functional layer in the proliferative phase (Marshall et al., 2011). The studies carried out sought to test the hypothesis that androgens affect gene expression in human endometrial stromal cells to alter proliferation and differentiation. Experimental treatments were all carried out using the non-aromatizable androgen DHT.

The aims of the studies described in this thesis were:

1. To identify cellular processes, pathways and networks regulated by DHT in hESCs using microarray-derived data

2. To evaluate if the microarray-derived genes were regulated by DHT in endometrial stromal cell models

3. To examine the expression and regulation of putative androgen-regulated genes in the human endometrium across the menstrual cycle and in early pregnancy

4. To explore if DHT modulates metformin-induced gene expression associated with decidualisation of hESCs

7.2 Key findings of this thesis

Analysis of data from an unpublished whole genome array conducted previously in the laboratory by Dr Elaine Marshall (post-doctoral research associate) using primary hESCs isolated from the proliferative phase and treated with the non-aromatisable androgen DHT for 2 or 8 hours identified time-dependant putative androgen-regulated mRNAs (34 and 268 genes, respectively). These data sets provided the starting point for the studies completed in this thesis and we believe they constitute the first analysis of genome-wide gene profiling of the effects of androgen action upon human endometrial stromal cells isolated from the proliferative phase.
Cellular processes, pathways and networks are regulated by DHT in hESCs

Studies described in chapter 3 revealed the potential for androgen regulation of cell processes, pathways and networks associated with gene transcription, signal transduction pathways (such as phosphatidylinositol, ERα, Wnt and MAPK signalling), cancer pathways, cell metabolism, cell cycle, development and apoptosis/survival. Some of the predicted findings were consistent with previous reports of androgen-dependent regulation of apoptosis, proliferation and motility in hESCs (undifferentiated or decidualised hESCs) (Marshall et al., 2011, Kajihara et al., 2012). Notably however, the majority of the androgen-regulated processes and pathways identified as a result of the bioinformatic analyses are novel for undifferentiated hESCs. These included potential for regulation of lipid metabolism as well as ERα-, Wnt- and PI3K/AKT-dependent signalling which are all pathways that are reported to be modulated or potentially modulated in endometrial stromal cells or tissues during the menstrual cycle (Catalano et al., 2007, Catalano et al., 2011, reviews by Critchley and Saunders, 2009, Sonderegger et al., 2010, Fabi and Asselin, 2014). Notably, signalling pathways such as those involving Wnts, MAPK, PI3K/AKT were reported to be regulated in decidualised hESCs by PR and not AR in a previous gene profiling study that used a model based on siRNA-dependent knock down of receptors (Cloke et al., 2008).

Studies in other cell types have demonstrated that maximal androgen action involving the AR also requires recruitment of other co-factors including a number of transcription factors (reviewed by Aagaard et al., 2011). Transcription factor-centred analysis was therefore carried out with the resultant identification of twenty transcription factors (TFs) that may be involved in androgen regulated gene expression in hESCs. Half of these TFs have previously been reported in networks in association with androgen signalling, for example, in prostate cancer cells (Vellaichamy et al., 2010) and in hESCs (Marshall et al., 2011). The remaining TFs were identified in networks in response to androgens acting in hESCs for the first time and interestingly, some of these TFs are already known to be expressed in endometrial stromal cells where they may play a role in the regulation of proliferation and/or differentiation (decidualisation), notably for example, ETS1,
C/EBPβ and E2F1 (Kessler et al., 2006, Wang et al., 2012b, Tamura et al., 2014). In addition, in cAMP-decidualised hESCs, binding and functional cooperation between C/EBPβ and transcription factor forkhead box O1 (FOXO1), a regulator of hESC decidualisation, has been reported (Christian et al., 2002). Some of these transcription factors have been found to interact directly with AR in other cell types. For example, AR binds RUNX2 and alters RUNX2 binding to DNA and transcriptional activity in a mechanism that is DHT-dependant in ostoblastic and prostate cancer cells (Baniwal et al., 2009, Van Der Deen et al., 2010). Finally, it has been proposed that TF networks involving AR may be key to the establishment of androgenic tissue specific transcriptional programmes (Pihlajamaa et al., 2014). Aptly, the current analysis hints at a novel and wider gene regulatory network including AR that may be involved during DHT modulation of gene expression in hESCs. Although bioinformatic analysis predicted the involvement of TFs in addition to AR in DHT-regulated gene networks in the hESCs, the interactions between them and mechanisms involved require confirmation and further investigation.

Bioinformatic analysis based on proprietary and public knowledge (Metacore, GeneGO) revealed that the majority (95%) of the putative androgen-regulated genes (or gene products) identified in hESCs have not previously been reported to be directly connected with AR in a pathway, by being either directly upstream or downstream of AR regulated gene expression. This is probably not surprising as this is the first report of a genome-wide study of androgen action carried out specifically in undifferentiated hESCs and this could account for the gap in the literature regarding androgen action in these cells as to date most genomic datasets have been generated using male cell types, immortalized cell lines or cancer cell lines. The unbiased nature of the microarray approach may also partly explain why the set of androgen-regulated genes identified in this study were different from the genes identified in an *in silico*-based analysis that included data sets derived from androgen-treated prostate cancer epithelial cells (Marshall et al., 2011). In summary, these data highlight how androgen action may be cell context-dependent and the relevance of using primary hESCs in the microarray experiment. Speculatively, upon
confirmation, downstream AR interactors identified in hESCs may constitute novel androgen regulated genes with implications for endometrial function.

_Microarray-derived putative androgen-regulated genes show potential for regulation by AR and are regulated by DHT in endometrial stromal cell models_

Eighteen putative androgen-regulated genes were selected for TFBS sequence analysis from the DHT-treated hESC microarray dataset generated using cells treated for 8 hours. Consensus androgen receptor binding sites were identified in the gene promoter region (10Kb sequence upstream and 4Kb sequence downstream of the transcription start site) of all of 18 putative androgen-regulated gene sequences surveyed providing evidence of the potential for AR-mediated transcriptional regulation of these genes in hESCs. A single candidate gene, _ELK4_, was previously reported to be targeted by AR in prostate cancer cells (Makkonen _et al._, 2008) but for the remaining 17 genes the identification of a consensus androgen receptor binding site was reported here for the first time. A very similar promoter analysis approach was also used to identify consensus AREs in a different set of candidate androgen-regulated genes identified in hESCs (Marshall _et al._, 2011).

As already mentioned, gene regulation involves complexes of TFs and co-factors. TFBS sequence analysis also revealed the potential for complex regulation of the candidate androgen-regulated genes by other steroid receptors (PR, GR, ERα, ERβ), steroid-response-associated transcription factors (ERR-α, SP-1) and CREB1 based on the identification of their respective regulatory elements within the same region of DNA as the AREs. These factors have all been reported to be expressed in hESCs (Bamberger _et al._, 2001, Tang _et al._, 2002, Bombail _et al._, 2010, review by Gibson and Saunders, 2012, Kawarabayashi _et al._, 2012). Thus, these data allow us to speculate that some androgen-regulated genes may also be regulated by other steroids or CREB1 either independently or in combination with AR.

DHT-dependent regulation of 10 of 15 of these genes was validated in undifferentiated hESCs and in SHT-290 endometrial stromal cells. For nine of these genes, regulation of expression by androgen has not previously been reported. Four of the genes were regulated by DHT in both hESCs and SHT-290 cells.
(downregulation of $SLC6A6$ and $SIK1$; upregulation of $RGS2$ and $SNCAIP$). Studies employing the AR-inhibitor flutamide suggested that the DHT-dependent regulation of $SIK1$, $RGS2$ and $SNCAIP$ but not $SLC6A6$ was AR-dependent. Taken together, the results obtained provide evidence of 10 androgen-regulated genes in undifferentiated hESCs and show that expression may be mediated by AR. Further studies using alternative assay systems are required to confirm the direct involvement of AR-mediated DNA binding.

Modest predicted gene changes (<2 fold) were a feature of the microarray-derived data sets used in these studies. This may result in challenges with regards to confirmation/validation by qRTPCR. For example, it has been estimated that only 66% of genes with a fold change less than 1.6 are confirmed (Wurmbach et al., 2001). This may partly account for a number of genes that were not validated by qRTPCR and highlights differences in results obtained using microarray platforms and qRTPCR assays.

Although the immortalised SHT-290 stromal cell line was initially considered a suitable alternative model to study androgen-dependent gene expression (Barbier et al., 2005), differences in the expression of some candidate genes were found in SHT-290 cells compared to the primary hESCs. This highlights challenges of using immortalized cells as model systems and supports the use of primary hESCs, in particular when investigating certain pathways such as Wnt signalling which has been reported to be altered in similarly TERT-immortalised cells (Choi et al., 2008). Thus, this finding supports the use of hESCs in the microarray experiment.

Computational analysis revealed that DHT-regulated $SIK1$, $RGS2$, $SNCAIP$ and $SLC6A6$ are connected in a common network with AR in undifferentiated hESCs. This network includes transcription factors that feature in androgen response networks in prostate cells and in endometrial stromal cells associated with regulation of proliferation and apoptosis, respectively (Vellaichamy et al., 2010, Marshall et al., 2011). Notably, the PKA/cAMP canonical pathway was also represented in this network. Interestingly, to date, the involvement of androgen/AR signalling with cAMP signalling has only been suggested in decidualising hESCs (Narukawa et al.,
1994, Cloke *et al.*, 2008, Kajihara *et al.*, 2013b) a transformation process in which cAMP signalling is involved (Brar *et al.*, 1997). In addition, these findings suggested once again, that putative androgen-regulated genes may be regulated by other steroids or during hESC decidualisation. In the study by Cloke and colleagues (2008), only a small number of genes that were found to be regulated in common by AR and PR using a strategy involving knockdown of either receptor in decidualising hESCs. While a very different experiment in design, it would have been interesting to compare results but unfortunately, this data set was not published. ERα also integrated this concerted AR and androgen-regulated gene-based network. However, evidence for regulation of the 4 putative androgen-regulated genes (*SIK1, RGS2, SNCAIP* and *SLC6A6*) by oestradiol (in the absence or presence of DHT) was not detected suggesting that AR-ER crosstalk may not be involved in their regulation in endometrial stromal cells. Further studies are required to clarify these preliminary observations.

Together, these data further support the central hypothesis of this thesis and offer suggestions as to how androgen-dependent regulation of proliferation, differentiation and apoptosis may occur in hESCs. Information from the literature on the known roles played by some of the candidate gene products in other cell types allows speculation as to the putative functional impact that their regulation by androgen may have in hESCs (assuming protein expression follows that of mRNA). For example, the reduction in *SIK1* by DHT may constitute a mechanism of androgen-dependent gene repression (Grosse *et al.*, 2012) in hESCs via an increase in HDAC activity (Berdeaux *et al.*, 2007, Van Der Linden *et al.*, 2007). The reduction of *SIK1* and increase of *SNCAIP* by DHT may each act as pro-survival signals (Giaime *et al.*, 2006, Cheng *et al.*, 2009) and contribute to the reduced apoptosis previously documented in hESCs treated with DHT (Marshall *et al.*, 2011). The increased expression of *RGS2* by DHT may play a role in curtailing cell proliferation (Cao *et al.*, 2006) and be involved during the process of implantation (Huang *et al.*, 2003).
Putative androgen-regulated genes are expressed and regulated in the human endometrium across the menstrual cycle and in early pregnancy

As data from isolated hESCs may not reflect the reality in intact primary tissue it was important to extend the studies. Thus, the expression of the 15 putative androgen-regulated genes was confirmed using human endometrial tissue homogenates adding support for the validation of the hESC-based microarray study. The results obtained provide the first evidence of the regulation of expression of 13 of these genes across the endometrial cycle (proliferative and secretory phases) and the first report of detection of 10 of the genes in endometrial tissues. The expression of 9 of these genes were altered in association with decidualisation either in the secretory phase, the time at which decidualisation begins, and/or in first trimester decidua compared with the proliferative phase. Protein expression patterns of 3 of the genes, in endometrial tissues (RGS2, SIK1 and Synphilin-1, encoded by SNCAIP) were also consistent with regulation in association with decidualisation in first trimester decidua. Although the primary focus of the current studies was on androgen action, these findings were consistent with complex regulation of the candidate genes in intact endometrial tissue. This prompted a new direction for study towards the examination of expression of putative androgen-regulated genes in decidualised cells.

Confirmation that 4 out of 8 genes were also modulated by decidualisation (SNCAIP, AXIN2, FOXL2 and ID3 mRNAs were downregulated) was achieved comparing the decidual tissues from a model of reduced in vivo decidualisation (i.e. decidua from ectopic pregnancies) with that of viable first-trimester pregnancies. In these decidual tissues, a negative association between the expression of the marker of decidualisation IGFBP1 and the expression of three of these genes (AXIN2, FOXL2 and ID3 mRNAs) in decidual tissues was also confirmed. The upregulation of 5 out of 7 genes in vitro using a decidualisation stimulus (cAMP or cAMP/P4) was also confirmed using primary hESCs (RGS2, SIK1, SLC6A6, SNCAIP and AXIN2). Unexpectedly, the direction of regulation of SNCAIP and AXIN2 observed in tissues was opposite to that observed in hESCs decidualised in vitro. We can speculate that in the decidual tissues this may be due to the epithelial cell compartment contributing
majorly to the measured mRNA concentrations or the \textit{in vitro} hESC culture being an imperfect decidualisation model. Interestingly, the regulation of \textit{FOXL2} by progesterone (Eozenou \textit{et al.}, 2012) and \textit{RGS2} and \textit{SIK1} by cAMP (Pepperl \textit{et al.}, 1998, Xie \textit{et al.}, 2011b, Stewart \textit{et al.}, 2013) has been reported in other steroid-responsive tissues and cells such as bovine endometrium, rat PC12 adrenal medulla neuroendocrine tumour cells, primary rat aortic vascular smooth muscle cells and mouse primary and C2C12 mouse myoblast cells. These studies supported an emerging secondary hypothesis i.e. that some of these putative androgen-regulated genes may also be regulated by decidualisation.

Differences in gene expression observed between the intact decidualised tissues and the cultured decidualised hESCs may be indicative, for example, of contributions from both stromal and epithelial cells in the intact tissues. Indeed, studies on protein expression of two of the genes (RGS2 and Synphilin-1, encoded by \textit{SNCAIP}) revealed immunoreactivity in epithelial cells as well as in stromal cells. In addition, it is important to note that the hESC cell model lacks critical cues resultant from stromal-epithelial interactions (reviewed by Cunha \textit{et al.}, 2004, Wetendorf and Demayo, 2012, Chen \textit{et al.}, 2013b) and the effects of other decidualisation-contributing factors present in the tissue such as hCG in the decidua (Bourdieu \textit{et al.}, 2013).

Preliminary studies also indicated that while androgen enhanced decidualisation of cAMP/P$_4$-decidualised hESCs (as previously reported by Cloke \textit{et al.}, 2008, Kajihara \textit{et al.}, 2012), addition of DHT had no discernible additional impact on decidualisation-dependent mRNA expression of RGS2, SIK1, SLC6A6, SNCAIP and AXIN2. In one study, only a few genes (of unknown identity) were reportedly regulated in common by the knockdown of AR or PR in cAMP/P$_4$ decidualising hESCs (Cloke \textit{et al.}, 2010). Speculatively, in terms of the expression of the genes studied in our experiments, the findings hint at cAMP/P$_4$-induced decidualisation acting as the predominant signal when in the presence of androgen in hESCs. It is unclear if this is due to decreased AR protein expression in cAMP/P$_4$-decidualised hESCs (Cloke \textit{et al.}, 2010), as the amount of AR does not necessarily correlate with receptor activity. For example, reduced post-translational sumoylation can enhance
cell responsiveness to androgen hormone signalling (Cloke et al., 2008). It is not known if pretreatment with androgens before exposure to the decidualisation stimuli would result in a different pattern of gene expression as these might involve the establishment of different AR target gene promoter complex/chromatin occupation and arrangements.

**DHT modulates metformin-induced gene expression associated with decidualisation of hESCs**

Findings from the current studies and from the published literature led to a further secondary hypothesis i.e. that androgens and the commonly used drug metformin may regulate cellular pathways critical in the regulation of proliferation but also differentiation, with a possible impact upon hESC decidualisation and associated gene expression. Preliminary investigations in cAMP/P<sub>1</sub>-decidualised hESCs (chapter 6) showed that DHT may increase hESC decidualisation (but not the expression of putative androgen-regulated genes) during exposure to a low, clinically relevant dose of metformin (100µM). These findings suggest that DHT might be able to sensitize decidualising hESCs to metformin action. Furthermore, these data recommend the inclusion of androgen during the modelling of endometrial function in vitro (e.g. in order to study decidualisation) as it would ordinarily contribute to the process.

These findings partially support the hypothesis that androgens regulate differentiation in hESCs, in this case during exposure to metformin. The common pathways involved remain unknown but we speculate that downregulation of the PI3K/AKT pathway could be involved and this warrants further investigation as it may play a role in regulating the balance between proliferation and differentiation. For example, it has been shown that decidualised stromal cells from ovarian endometriomas compared to eutopic decidualised ESCs from women without endometriosis exhibit a blunted response to decidualisation (reduced IGFBP1 mRNA expression) (Yin et al., 2012). The authors proposed this may be due to an increased activation of the PI3K/AKT pathway as indicated by increased AKT phosphorylation observed in endometriotic cells and tissues. While the inhibition of AKT phosphorylation activity has been reported in the decidualisation process, its role
remains understudied but may involve effects on downstream targets that lead to reduced proliferation including the inhibition of mTOR or the inhibition of phosphorylation (and activation) of FOXO1 (Yin et al., 2012). Pertinently, FOXO1 is a key regulator of hESC decidualisation (Kajihara et al., 2013a). FOXO1 is upregulated during hESC decidualisation (Grinius et al., 2006) and has also been shown to be enhanced by DHT in decidualised hESCs (Kajihara et al., 2012). Thus, it may be speculated that ultimate modulation of FOXO1 by androgen and metformin may explain the enhancement of hESC decidualisation reported herein but requires further study.

DHT did not alter the expression of putative androgen-regulated genes during exposure to the low dose of metformin (100μM) in decidualising cells. In hindsight the higher concentration of metformin (1000μM) should also have been tested as, in the absence of DHT, 1000μM metformin was able to alter decidualisation-associated gene expression (steroid enzyme CYP19A1 and putative androgen-regulated and decidualisation-regulated genes RGS2, SIK1, SNCAIP and AXIN2). This higher dose of metformin has been shown to modulate gene expression of PI3K, glucose transporter GLUT4 and the insulin receptor in a very different decidualised-hESC model that consisted of hESCs treated with 10nM oestradiol, 1µM P₄, 100ng/ml insulin and 1µM DHT (a concentration higher than that used in the current studies or reported in the circulation) (Ferreira et al., 2014a, Ferreira et al., 2014b). However, the role of DHT in these studies was not specifically addressed. In addition, review of the literature indicated that a blood concentration of metformin of 1000μM may be higher than that found in patients after therapeutic doses (Robert et al., 2003) and thus may not reflect actual or even tolerable physiological concentrations.

Similar studies on the interaction between androgen and metformin were not extended to the expression of androgen-regulated genes in undifferentiated hESCs due to lack of time.
7.3 Limitations of the studies

The use of bioinformatics for the analysis of microarray data is limited by our current knowledge. In particular, the information regarding androgen action in the literature is biased towards other tissues, i.e. it is mainly composed of studies carried out in male cell types (prostate, prostate cancer, gonadal development). Although there is some androgen action data originating from female cell types (ovarian follicular development) there is very little data regarding androgen action in the endometrium and specifically in hESCs. Thus the current bioinformatics findings may, accordingly, be biased.

Another limitation of the work is the lack of functional and mechanistic analyses to confirm experimentally the impact of androgens on cellular pathways predicted by bioinformatics (due to lack of time). For example, the predicted androgen regulation of cellular proliferation and cell cycle progression processes require confirmation by specific pathway focussed/functional qPCR arrays (gene expression) or by appropriate functional assays in undifferentiated hESCs. There is also a lack of functional studies to correlate with the gene expression findings from the cell models (due to lack of time). For example, missing mechanistic studies would be helpful to dissect (and confirm) the interaction of androgen action and metformin action in decidualised hESCs.

Statistical analysis of some of the *in vitro* cell study data was limited due to difficulties in carrying out such experiments (e.g. availability of cells/tissues from women within defined clinical criteria, cell culture conditions), lack of time and subsequent reduced data collection that underpowered the results. In some instances, increasing the data collection would improve confidence in some findings and in other instances, enable statistical significant changes (in gene expression) to be reached.

The use of DHT alone in the current studies is crucial to understand the the role of androgen/AR signalling in hESCs but this approach is reductionist when we consider that in normal and abnormal physiologies (*e.g.* in PCOS) androgens are not present alone. For example, the balance between oestrogens and androgens was not
considered and thus it could be argued that the present findings were obtained in conditions very different to physiological conditions. In future, the inclusion of oestradiol, for example, would go towards building a more complete hESC cell model. Similarly, the studies were carried out in hESC monocultures where cell-to-cell interactions were absent and not considered (in particular stromal-epithelial interactions). To include and study the contributions of such interactions, co-culture systems should be employed.

7.4 Implications for women’s health
Cloke and Christian (2012) have speculated that androgen balance, following the Goldilocks principle, is required for endometrial reproductive function and that altered androgen levels (excess or deficiency) lead to functional dysregulation that affects fertility. In particular, androgen/AR signalling is reported to enhance hESC decidualisation markers and morphological characteristics (in vitro) (Cloke et al., 2008, Kajihara et al., 2013), the timely progression of which is required for successful embryo implantation, trophoblast invasion and pregnancy (Gellersen and Brosens, 2014).

Dysregulated decidualisation has been associated with higher rates of implantation failure and miscarriage (fertility reduction) in women with PCOS (Palomba et al., 2012), who also commonly exhibit hyperandrogenism (O’Reilly et al., 2014). The management of PCOS with metformin is reported to reduce the risk of pregnancy loss (Lautatzis et al., 2013, Zheng et al., 2013, Feng et al., 2015) but it is unclear if metformin affects decidualisation directly. The current very preliminary findings in hESCs suggest that normalised androgen signalling may help to explain the local endometrial effects of metformin that speculatively, may contribute to improve fertility and pregnancy outcomes by increasing decidualisation in metformin treated patients with PCOS. Further specific interactions between androgen but also oestrogen signalling (at normal or higher concentrations) and metformin action in endometrial cells remain to be elucidated and their implications for womens health remain speculative. In women, the study of the effects of androgens or metformin upon the endometrium is difficult to do in isolation due to the complexity of
interacting factors in the pathophysiology of PCOS. For example, while hyperandrogenism may directly impact the endometrium, it also inhibits ovarian function (ovulation and progesterone production) that, in the end, critically affects endometrial function. Similarly, the action of metformin improves ovarian function (Sung et al., 2014) affecting the endometrium indirectly that may overlap with any direct effects of metformin upon endometrial function. Therefore, studies using in vitro cell models such as the decidualised hESC model or animal models are still required.

If the predicted androgen regulation of cellular proliferation and cell cycle progression processes are confirmed then androgens may be important in maintaining a balance between proliferation and differentiation in the endometrium and it may be speculated that androgens may be exploited to treat disorders where there is excessive proliferation in hESCs such as that reported in endometriosis (Aghajanova et al., 2011). Pelvic endometriotic lesions predominantly exhibit AR positive immunoreactivity (Carneiro et al., 2008). Together these findings may help explain why the synthetic steroid Danazol which has some weak androgenic properties can be a beneficial therapeutic for women with pain and endometriosis (systematic review by Brown and Farquhar, 2014). Thus, the use of Danazol or other AR selective agonists may be helpful in the management of such disorders. Local intrauterine delivery systems could be used to deliver smaller effective doses and minimize the unwanted androgenic side effects such as hirsutism.

Excessive proliferation is also a hallmark of cancer and most endometrial cancers are of epithelial cell origin (Rose, 1996) and exhibit elevated AR expression (ref). Whilst the role of AR in these pathologies remains speculative, the studies herein showed that the RGS2, SIK1 and Synphilin-1 proteins may also be expressed in endometrial epithelial cells. Further study of the expression of these genes/proteins in epithelial cells including their regulation by androgens and expression in endometrial cancers may subsequently help to further characterise endometrial cancer and inform therapeutic interventions. Especially as the mRNA and/or protein expression of all three genes has been altered in association with cell proliferation and/or progression in other cancers (Cao et al., 2006, Cheng et al., 2009, Hurst et al., 2009, Jiang et al.,
The potential role of androgens in endometrial cancer risk was highlighted in a recent review by our colleagues (Gibson et al., 2014).

Changes in the expression of inflammatory genes in hESCs, possibly driven by excess androgens and altered androgen/oestrogen balance (among other factors such as insulin), in patients with PCOS may alter normal cell-to-cell interactions regulated by oestrogen and progesterone that instead favour the development of endometrial hyperplasia (Piltonen et al., 2013) or contribute to endometrial infertility in PCOS (Piltonen et al., 2015). In this way, it is speculated that dysregulated expression of androgen-regulated genes in hESCs may contribute indirectly to the early etiology of some endometrial cancers or may affect immune function in early pregnancy (altered chemokine profile) (Salamonsen et al., 2007). However, functional confirmation of such speculation is still necessary before the proposal of any therapeutic strategies based on targeting androgen/AR signalling can be made. Additional considerations include interactions with oestrogen/progesterone signalling and the mode of therapeutic delivery (to limit unintended effects upon ovarian function).

### 7.5 Future studies

A series of further studies designed to specifically unravel the regulation of proliferation and differentiation by androgens may be proposed (some have already been introduced above). On the one hand, future studies may aim to validate the regulation of other specific microarray-derived putative androgen-regulated genes by DHT (informed by bioinformatics analyses). Confirmation of AR-DNA specific binding and expression regulation by AR could also be carried out to confirm AR-mediated effects (Bolton et al., 2007). Additionally, to elucidate androgen regulation of particular cell processes predicted by bioinformatics analyses, a combination of specific qPCR gene arrays and subsequent pathway inhibitor studies may be used to delineate the effects of androgen signalling upon specific cell pathways. Functional assays could be used to confirm androgen regulation of predicted cell processes. Such assays, in particular proliferation assays (not yet reported in undifferentiated hESCs) are still required to quantify the effects of androgens upon undifferentiated...
hESCs proliferation (with/without AR inhibitors) or in undifferentiated hESCs with AR-knocked-down as previously shown in decidualised hESCs (Cloke et al., 2008). The use of oestradiol should also be considered in these experiments as oestradiol may facilitate the detection of changes in proliferation in these assays due to hESCs exhibiting a low proliferative rate compared to epithelial cells even in the oestrogen-dominant proliferative phase endometrium (Mertens et al., 2002). In addition, the inclusion of oestradiol also better simulates physiological conditions.

The impact that androgen signalling has upon stromal-epithelial interactions can also be investigated in co-culture systems (Chen et al., 2013b) and murine cell specific AR knock-out models when available. The current available murine models could be exploited to study androgen action in the endometrium in vivo and in the context of other steroid hormones. For example, the human menstrual cycle may be modelled with oestrogen and progesterone (Cousins et al., 2014) in ovariectomised normal and female AR KO mice with the addition of DHT. Although there are anatomical and molecular differences compared to humans, the role of androgens could be examined during each of the simulated proliferative, secretory and menstrual phases with a focus upon quantification of proliferation and characterisation of cell cycle progression markers and differentiation markers. The balance of oestrogens and androgens speculated to be required for successful endometrial reproduction function could also be facilitated by using animal models that enable the manipulation and control of confounding factors.

Other future studies may include the study of the interactions between androgen/AR signalling and other steroids/receptor signalling. For example, some of the putative androgen-regulated genes were separately regulated in decidualising hESCs. Upon the demise of the corpus luteum and progesterone withdrawal, it is not known if androgen regulation of putative androgen-regulated genes plays a role at this time, during menses or during transition into the proliferative phase. This should be investigated not only in endometrial stromal cells but also in the epithelial cells as upregulation of AR immunoexpression has been associated with progesterone withdrawal in these cells (Marshall et al., 2011). Equally, the gene/protein expression
of putative androgen-regulated genes should be extended to endometrial tissues from women with PCOS, endometriosis or endometrial cancer.

Finally, the regulation of AR expression and activity regulation in the endometrial stromal cell is complex and is far from being elucidated. The possible role of one of the androgen-regulated genes, RGS2, could be investigated as RGS2 is suggested to be a regulator of AR localisation (Cao et al., 2006, Rimler et al., 2007). This should be evaluated in endometrial stromal cells using knock-down and fluorescent cell imaging techniques.

7.6 Conclusion

Findings presented in this thesis complement and extend studies on the regulation of human endometrial biology. Specifically, bioinformatic analysis of microarray data derived from a DHT-treated hESC experiment predicted that androgens acting within endometrial stromal cells play a more important role in endometrial function than previously recognised by regulating key processes including proliferation and differentiation. Novel putative androgen-regulated genes with roles associated with these cell processes were identified in undifferentiated human endometrial stromal cells and endometrial tissues. Although there were difficulties in obtaining significant changes in gene expression, the regulation of some of the candidate genes by androgens was demonstrated. Functional studies are still required to confirm the role of androgens upon, for example, hESC proliferation in undifferentiated hESCs.

Interestingly, most of the candidate genes were also found to be regulated in association with decidualisation in endometrial tissues and some of the identified novel androgen-regulated genes (in undifferentiated hESCs) were regulated in hESCs by in vitro decidualisation. Androgens may also modulate the effects of drugs in the endometrium as exemplified by the ability of DHT to sensitize decidualising hESCs to the action of metformin with an impact upon decidualisation. Mechanistic studies are still required to elucidate the interaction of androgens and metformin in hESCs.

While novel androgen-regulated genes were identified in hESCs, firm conclusions cannot be made from the data regarding the regulation of cellular processes by
androgens in hESCs. Nonetheless, collectively the novel data represent a spur for the study of androgen action both in the proliferative and secretory phase endometrium bringing to light new knowledge (Cloke et al., 2008, Marshall et al., 2011, and reviewed by Cloke and Christian, 2012), new hypotheses and avenues of investigation.
References


References


References


References


References


References


References


References


References


References


References


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interdomain communication with the NH(2)-terminal domain. J Biol Chem, 274, 37219-25.


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undergoing assisted reproductive technology procedures. *Int J Gynaecol Obstet*, 131, 111-6.
References


References


References


References


References


References


programming window and development of male reproductive organs. *Int J Androl*, 33, 279-87.


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References


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References


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References


References


References


References


References


References


# Appendix A: Full gene names

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Full gene name (and other aliases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AXIN2</td>
<td>NM_004655</td>
<td>axin 2</td>
</tr>
<tr>
<td>CDC25A</td>
<td>NM_001789</td>
<td>cell division cycle 25A</td>
</tr>
<tr>
<td>ELK4</td>
<td>NM_001973</td>
<td>ETS-domain protein (SRF accessory protein 1)</td>
</tr>
<tr>
<td>FOXL2</td>
<td>NM_023067</td>
<td>forkhead box L2 (BPES)</td>
</tr>
<tr>
<td>GADD45G</td>
<td>NM_006705</td>
<td>growth arrest and DNA-damage-inducible, gamma (DDIT2)</td>
</tr>
<tr>
<td>ID3</td>
<td>NM_002167</td>
<td>inhibitor of DNA binding 3, dominant negative helix-loop-helix protein (HEIR-1)</td>
</tr>
<tr>
<td>JMY</td>
<td>NM_152405</td>
<td>junction-mediating and regulatory protein, p53 cofactor</td>
</tr>
<tr>
<td>LKB1</td>
<td>NM_000455</td>
<td>serine/threonine kinase 11 (STK11)</td>
</tr>
<tr>
<td>MAP3K8</td>
<td>NM_005204</td>
<td>mitogen-activated protein kinase kinase kinase 8 (TPL2)</td>
</tr>
<tr>
<td>MGC16121</td>
<td>NR_024607</td>
<td>MIR503 host gene (long non-coding RNA, non-protein coding)</td>
</tr>
<tr>
<td>NR4A2</td>
<td>NM_006186</td>
<td>nuclear receptor subfamily 4, group A, member 2 (NURR1)</td>
</tr>
<tr>
<td>PRLR</td>
<td>NM_000949</td>
<td>prolactin receptor</td>
</tr>
<tr>
<td>RGS2</td>
<td>NM_002923</td>
<td>regulator of G-protein signalling 2, 24KDa</td>
</tr>
<tr>
<td>SIK1</td>
<td>NM_173354</td>
<td>salt-inducible kinase 1 (SNF1LK)</td>
</tr>
<tr>
<td>SLC6A6</td>
<td>NM_003043</td>
<td>solute carrier family 6 (neurotransmitter transporter, taurine), member 6 (TAUT)</td>
</tr>
<tr>
<td>SNCAIP</td>
<td>NM_005460</td>
<td>synuclein, alpha interacting protein (protein: Synphilin-1)</td>
</tr>
</tbody>
</table>

### Decidualisation markers

<table>
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<tr>
<th>Gene</th>
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<th>Description</th>
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<tbody>
<tr>
<td>IGFBP1</td>
<td>Nm_000596</td>
<td>insulin-like growth factor binding protein 1</td>
</tr>
<tr>
<td>PRL</td>
<td>Nm_000948</td>
<td>prolactin</td>
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</table>

### Steroid receptors

<table>
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<td>AR</td>
<td>Nm_000044</td>
<td>androgen receptor</td>
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<td>ESR1 (ERα)</td>
<td>Nm_000125</td>
<td>estrogen receptor 1</td>
</tr>
<tr>
<td>PR</td>
<td>Nm_000926</td>
<td>progesterone receptor (PGR)</td>
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</table>

### Steroidogenesis

<table>
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<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP19A1</td>
<td>Nm_031226</td>
<td>cytochrome P450, family 19, subfamily A, polypeptide 1 (aromatase)</td>
</tr>
</tbody>
</table>
Appendix B: ‘Data not shown’ from Chapter 3

The lists of microarray-derived putative androgen-regulated genes in hESCs at 2 and 8 hours (DHT)

This appendix lists the microarray-derived putative androgen-regulated genes in hESCs (at 2 and 8 hours) provided by the FDMC (Tables AB1 and AB2). The Fold change threshold was 1.2 (P<0.01). The expression fold change is the fold change of the control value relative to DHT-treated value therefore: a negative fold-change value denotes the genes that are upregulated by DHT (compared to vehicle); a positive fold-change value denotes the genes that are downregulated by DHT (compared to vehicle). The gene identities are those used by Illumina (section 3.2.5).

Table B.1 The microarray putative DHT-regulated genes in hESCs at 2 hours.

<table>
<thead>
<tr>
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<th>Gene</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_014725.2</td>
<td>STARDB8</td>
<td>-1.252</td>
<td>9.59E-05</td>
</tr>
<tr>
<td>NM_004571.3</td>
<td>PKNOX1</td>
<td>-1.222</td>
<td>0.00101</td>
</tr>
<tr>
<td>NM_144675.1</td>
<td>GSG1L</td>
<td>-1.301</td>
<td>0.000293</td>
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<td>XM_928338.1</td>
<td>LOC653598</td>
<td>-1.261</td>
<td>0.000481</td>
</tr>
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<td>NM_015481.1</td>
<td>ZNF385A</td>
<td>-1.253</td>
<td>0.00846</td>
</tr>
<tr>
<td>NR_003083.2</td>
<td>SLC6A10P</td>
<td>-1.242</td>
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<td>XM_943313.2</td>
<td>LOC648405</td>
<td>-1.206</td>
<td>0.001784</td>
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<td>NM_002697.2</td>
<td>POU2F1</td>
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<td>NM_001013722.1</td>
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<td>NM_018449.2</td>
<td>UBAP2</td>
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<td>0.00214</td>
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<td>NM_001080447.1</td>
<td>TTL8</td>
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<td>NM_000487.3</td>
<td>ARSA</td>
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<td>0.005436</td>
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<td>NM_012262.2</td>
<td>HS2ST1</td>
<td>1.2</td>
<td>0.009693</td>
</tr>
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<td>NM_016472.3</td>
<td>C14ORF129</td>
<td>1.2</td>
<td>0.009439</td>
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<td>NM_198402.2</td>
<td>PTPNB</td>
<td>1.245</td>
<td>0.007265</td>
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<td>NM_018639.3</td>
<td>WSB2</td>
<td>1.309</td>
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<td>NM_020332.4</td>
<td>RTN4</td>
<td>1.238</td>
<td>0.006403</td>
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<td>NM_182765.2</td>
<td>HECTD2</td>
<td>1.208</td>
<td>0.005223</td>
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<td>BX113955</td>
<td>HS.7415</td>
<td>1.2</td>
<td>0.00477</td>
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<td>NM_024006.4</td>
<td>VKORC1</td>
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<td>XM_938297.1</td>
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<td>NM_018981.1</td>
<td>DNAJC10</td>
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<td>NM_004779.4</td>
<td>CNOT8</td>
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<td>NM_006227.2</td>
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<td>BE041597</td>
<td>HS.544911</td>
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</table>
Table B.2  The microarray putative DHT-regulated genes in hESCs at 8 hours.
The genes examined using qRTPCR are highlighted in bold.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene</th>
<th>Fold change</th>
<th>P Value</th>
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<tbody>
<tr>
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<td>-1.706</td>
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<td>LRP5L</td>
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<td>NM_001079670.1</td>
<td>CAB39L</td>
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<td>NM_152553.2</td>
<td>RNF217</td>
<td>-1.538</td>
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