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**Studies on the Androgen Receptor and Steroid  
Metabolising Enzymes in the Human  
Endometrium**

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**Studies Conducted  
in the  
Centre for Reproductive Biology  
The University of Edinburgh**

**MD  
The University of Glasgow  
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## Abstract

Activation of sex steroid receptors is a key event in the regulation of uterine function. The expression of sex steroid receptors such as Oestrogen and Progesterone have been well characterised in normal human endometrial tissue. Expression of the androgen receptor (AR) has not been well characterised. The characterisation of AR in human endometrium is important to determine the role of androgens in physiological uterine events such as implantation and menstruation.

The term "Intracrinology" refers to the ability of a peripheral target tissue to synthesise sex steroids. The expression pattern of metabolising enzymes determines a tissues steroidogenic potential. Key sex steroid metabolising enzymes include the 17 $\beta$ -hydroxysteroid dehydrogenases (17 $\beta$ -HSD), 5 $\alpha$ -reductases (5 $\alpha$ R), 3 $\beta$ -hydroxysteroid dehydrogenases (3 $\beta$ -HSD) and Aromatase.

The objectives of this thesis were thus to determine in human endometrium:

1. The spatial and temporal expression of androgen receptor mRNA and protein in endometrium across the menstrual cycle.
2. The expression of critical androgen metabolising enzymes across the menstrual cycle - 17 $\beta$ -HSD type 2 mRNA and protein; 17 $\beta$ -HSD type 5 mRNA; 3 $\beta$ -HSD type 1 and 2 mRNA; and 5 $\alpha$ R types 1 and 2 mRNA.
3. The spatial and temporal localisation, in endometrium exposed to high dose intra-uterine levonorgestrel, of AR mRNA and protein; 17 $\beta$ -HSD2 mRNA and protein; 17 $\beta$ -HSD5 mRNA; 3 $\beta$ -HSD1 and 2 mRNA; and 5 $\alpha$ R1 and 2 mRNA.

Endometrial biopsies from both normal endometrium and endometrium exposed to high dose intra-uterine levonorgestrel were subjected to optimised immunohistochemical protocols to determine the spatial and temporal immunolocalisation of the androgen receptor and 17 $\beta$ -HSD2. In-situ hybridisation (ISH) techniques were employed to localise AR mRNA in full thickness endometrial biopsies. Taqman real time RT-PCR examined the temporal variation in mRNA expression for AR; 17 $\beta$ -HSD2 and 5; 3 $\beta$ -HSD1 and 2; and 5 $\alpha$ R1 and 2 in normal endometrium and endometrium exposed to high dose intra-uterine levonorgestrel.

The results determined that AR is expressed in the endometrial stromal compartment with down regulation of AR protein and mRNA in the late secretory

phase. This localisation was confirmed with ISH data. Endometrium exposed to high dose intra-uterine levonorgestrel exhibits a significant decrease in stromal AR protein immunoreactivity when compared with proliferative endometrium.

Temporal variations in expression of steroid metabolising enzymes were studied. Significantly elevated levels of  $17\beta$ -HSD2 are expressed in the glandular compartment of mid secretory phase endometrium. Endometrium exposed to high dose levonorgestrel exhibited high levels of endometrial  $17\beta$ -HSD2 protein in the first month after insertion of a LNG IUS, associated with high levels of  $17\beta$ -HSD2 mRNA expression in endometrial tissue at the three month time point. Thus, increased levels of  $17\beta$ -HSD2 would indicate the potential for lowered intracellular oestradiol levels at this time (since this enzyme converts oestradiol to the less potent oestrogen, oestrone). Thereafter, levels of  $17\beta$ -HSD2 protein and mRNA significantly decline.  $17\beta$ -HSD5 mRNA is also significantly increased in the mid secretory phase.

The peri-menstrual period is associated with significant elevations in mRNA levels for both  $3\beta$ -HSD enzymes and  $5\alpha$ R 2.  $5\alpha$ R 1 is significantly elevated in the mid-cycle phases. In endometrium exposed to high dose intrauterine levonorgestrel, an increased expression of  $3\beta$ -HSD and a reduced expression of  $5\alpha$ R 2 are noted.

In conclusion, AR had been localised to the endometrial stromal compartment with a significant down regulation noted in the late secretory phase. The expression pattern of metabolising enzymes identified in this study is consistent with the secretory phase human endometrium possessing significantly greater steroidogenic potential than the proliferative phase. Furthermore, the available literature suggests that secretory endometrium is a significant source of androgen production. Data has been published that implicates androgens as having an important role in physiological events such as implantation. However, the precise role and regulation of androgens, the androgen receptor and the metabolising enzymes in human endometrium requires further study.

Novel data is also described here regarding the effect of high local doses of progestogen on local tissue androgen receptor and sex steroid metabolising enzymes. Such data suggests a potential role for progestogens in influencing the uterine environment that may lead to novel interventions for the problematic breakthrough bleeding suffered by progestogen only contraceptive users.

## **Declaration**

Except where due acknowledgement is made by reference, the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Studies investigating the expression of Androgen Receptor (AR) mRNA expression in human endometrium using in-situ hybridisation were conducted by Dr Ov Slayden. This aspect of the study was performed at the Oregon National Primate Research Centre (ONPRC).

Dr Kevin Anthony Burton

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## Abbreviations

Analysis of Variance	(ANOVA)
Androgen Receptor	(AR)
Aromatase	(P450arom)
Avidin-Biotin Peroxidase	(ABC)
BLAST	(Basic Local Alignment Search Tool)
Breakthrough Bleeding	(BTB)
Cyclooxygenase 2	(COX2)
Complementary DNA	(cDNA)
Diaminobenzidine tetra hydrochloride	(DAB)
Dhydroepiandrosterone	(DHEA)
Dehydroepiandrosterone-sulphate	(DHEAS)
Dihydrotestosterone	(DHT)
Deoxyribonucleic Acid	(DNA)
Extracellular Matrix	(ECM)
Follicle Stimulating Hormone	(FSH)
Glucocorticoid receptor	(GR)
Hormone Response Elements	(HRE)
In-situ Hybridisation	(ISH)
Immunohistochemistry	(IHC)
Immunoglobulin	(IgG)
Levonorgestrel Releasing Intra-Uterine System	(LNG-IUS)
Luteinising Hormone	(LH)
Matrix Metalloproteinases	(MMP)
Messenger RNA	(mRNA)
Mifepristone	(RU486)
Oestrogen Receptor	(ER)
Oestrogen Receptor alpha	(ER $\alpha$ )
Oestrogen Receptor beta	(ER $\beta$ )
Phosphate-buffered saline	(PBS)
Polycystic ovarian syndrome	(PCOS)
Polymerase Chain Reaction	(PCR)
Progesterone receptor	(PR)

Progesterone receptor A	(PR-A)
Progesterone receptor B	(PR-B)
Real Time Quantitative Reverse Transcription - PCR	(Q-RT PCR)
Ribonucleic Acid	(RNA)
Standard Error of the Mean	(SEM)
Tris buffered saline	(TBS)
Uterine NK cells (CD 56 <sup>Bright</sup> CD 16 <sup>-</sup> )	(uNK)
Vascular endothelial growth factor	(VEGF)
3 $\beta$ -Hydroxysteroid Dehydrogenases	(3 $\beta$ -HSD)
5 $\alpha$ -Reductases	(5 $\alpha$ R)
17 $\beta$ -Hydroxysteroid Dehydrogenases	(17 $\beta$ -HSD)
20 $\alpha$ -Hydroxysteroid Dehydrogenase	(20 $\alpha$ -HSD)

# Chapter 1

## Literature Review

## 1.1 General Introduction

The endometrium is a dynamic target organ, the development and function of which is regulated by the action of sex steroids such as oestrogens and progesterone. The other major group of sex steroids are the androgens, and their contribution to endometrial function constitutes an under researched aspect of endometrial biology. This thesis summarises the published data regarding the role of androgens and the androgen receptor in human endometrium and highlights under researched areas pertaining to androgen receptor expression and regulation in the human endometrium. Furthermore, new data are presented that will contribute to our understanding of the role of androgens in endometrial physiology.

Menstruation signals the end of one ovarian cycle and the initiation of a new cycle. Oestradiol levels rise during the proliferative phase of the cycle and are responsible for stimulating endometrial growth and proliferation. The combined activity of oestradiol and progesterone in the secretory phase then prepares the endometrium for possible blastocyst implantation. Following the release of an oocyte at ovulation, the secretory phase of the cycle commences and there is concurrent luteinisation of the theca cells to produce the corpus luteum. The corpus luteum is then responsible for the production and secretion of progesterone producing the elevated levels associated with the secretory phase. The secretory endometrium then undergoes functional and morphological differentiation under the influence of progesterone, preparing for blastocyst implantation. However, should implantation fail to occur then luteolysis of the corpus luteum results in falling progesterone levels, initiating a cascade of events that results in shedding of the superficial endometrium, menstruation, and thereafter a new cycle begins.

The physiological mechanisms controlling endometrial growth, development and menstruation are areas of ongoing research where the precise mechanisms remain elusive. Only through establishing the normal physiological events can the mechanisms of endometrial dysfunction be better understood. Endometrial dysfunction encompasses a range of clinical problems including subfertility, menorrhagia, and benign or malignant neoplastic changes. Through understanding the normal physiology of endometrial development, we may then be able to target and manipulate normal physiological events to achieve therapeutic interventions for

endometrial dysfunction. For example, progesterone only contraceptive users may benefit from an intervention to prevent the troublesome problem of breakthrough bleeding.

Sex steroids (ligands) play a critical role in regulating physiological endometrial function. The response elicited will be determined by which ligand binds to which sex steroid receptor. Therefore, factors controlling ligand and receptor availability are important in determining the response of target organs. Ligand and receptor availability may be regulated in endocrine, paracrine and autocrine mechanisms but more recently research has highlighted the important role that intracrine regulation has to play through influencing ligand availability.

Intracrinology is a term that was first used by Labrie et al in 1988 (Labrie 1988). In essence it refers to the ability of peripheral target tissues to activate or inactivate steroid ligands in the same cell that the steroid action is exerted, without release and dilution of the active hormones in the extracellular space or general circulation (Labrie 1991). Thus, the rate of formation of each sex steroid ligand depends upon the activity of the specific sex steroid metabolising enzymes in each cell of each tissue (Labrie 1991; Stewart et al. 1992). The key enzymes responsible for androgen, oestrogen and progesterone interconversion include the  $17\beta$ -hydroxysteroid dehydrogenases,  $3\beta$ -hydroxysteroid dehydrogenases,  $5\alpha$ -reductases, and Aromatase. The expression of the different isoforms for each enzyme family has been under researched in part due to the recent determination of human isoforms that were previously unknown.

The temporal and spatial distribution of the oestrogen (ER) and progesterone receptor (PR) in human endometrium has been well characterised. In contrast, a clear characterisation has not yet been established for the androgen receptor (AR). When the temporal and spatial distribution of AR has been established, it may then be possible to hypothesise as to its role in human endometrium.

The literature review that follows will expand on the published data, which have been summarised in this short introduction, to illustrate the important role that androgens may play in endometrial physiology and where research may be directed.

## 1.2 Steroidogenesis

The uterus and in particular the endometrium are, as previously stated, steroid responsive tissues. The classical endocrine organs responsible for steroid production in the female are the adrenal gland and the ovary. In particular, the production and secretion of sex steroids, such as oestradiol and progesterone, is a major function of the ovary.

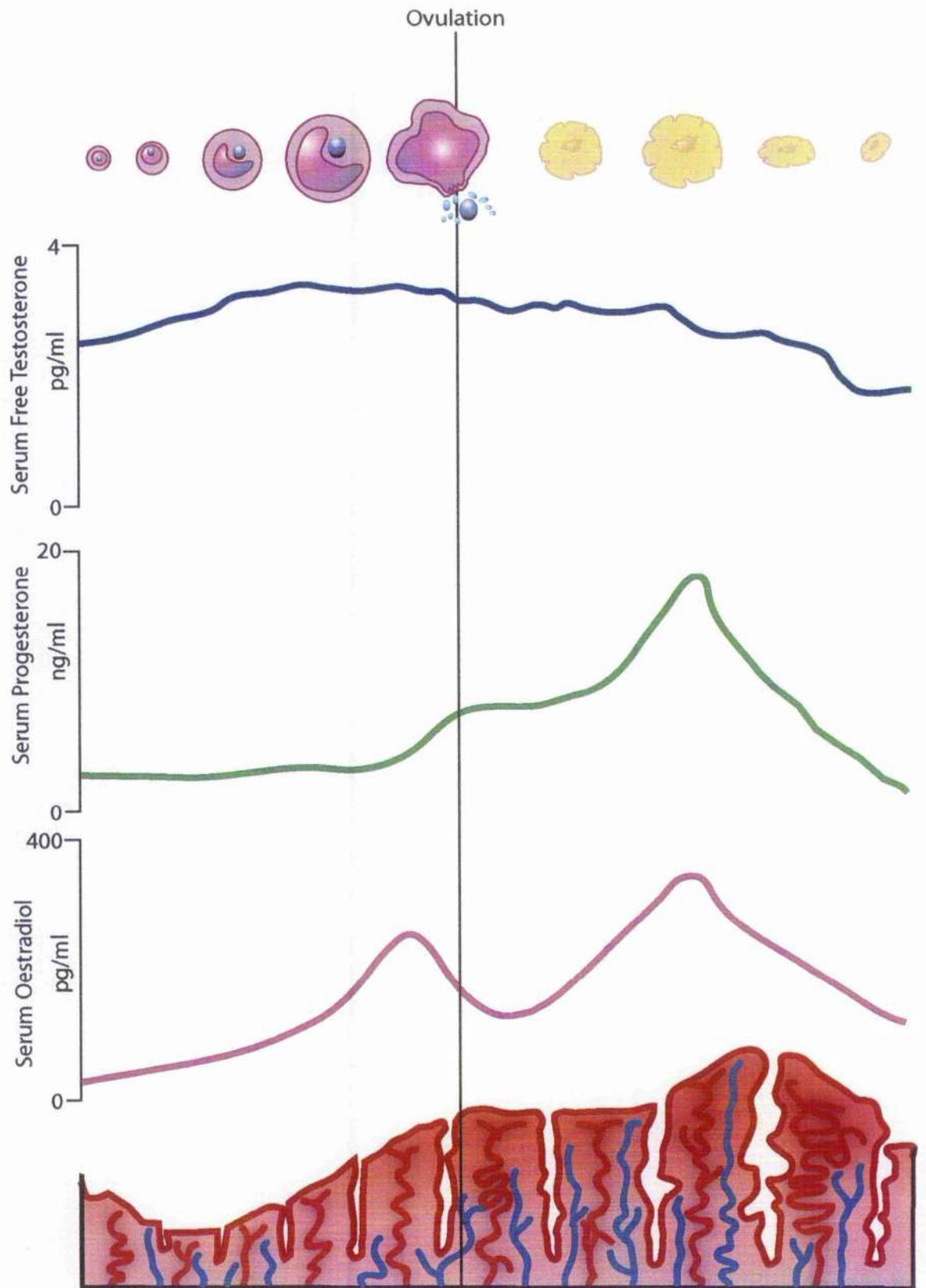
The hormone secreting component of the ovary is the theca cell-granulosa cell unit. Following a brief overview each component shall be described in turn illustrating the production of oestrogen and progesterone through the cycle. The temporal secretion of sex steroids in the female circulation is illustrated in Figure 1.1.

The primordial follicles of the ovary are single oocytes surrounded by a layer of granulosa cells that are in turn surrounded by stromal cells of the ovary that will become the theca interna and externa. The ovary has its highest complement of oocytes in-utero where at a gestation of 5 months the number of oocytes is approximately 7 million. By birth, this number has declined to 1 million and by the menopause to less than 1000. Mathematical models based upon studies examining the follicle content of human ovaries at a range of maternal ages have shown this decline to be bimodal with a constant rate of decline from birth followed by a rapid phase of decline after the age of 37.5 years (Faddy et al. 1992).

Following puberty, the arcuate nucleus of the hypothalamus secretes gonadotrophin releasing hormone (GnRH) in a pulsatile manner. This is then transported via and secreted from the hypophyseal portal venous plexus connecting the hypothalamus to the anterior pituitary gland. In the anterior pituitary GnRH stimulates secretion of Follicle Stimulating Hormone (FSH) and Luteinising Hormone (LH). Elevated FSH recruits follicles from the ovary with a dominant follicle being selected and the remaining follicles becoming atretic. This dominant follicle will then become the major source of oestradiol synthesis. Figure 1.3 illustrates the two cell two gonadotrophin (Short 1962; Lieberman 1996) mechanism of oestradiol synthesis.

Figure 1.1

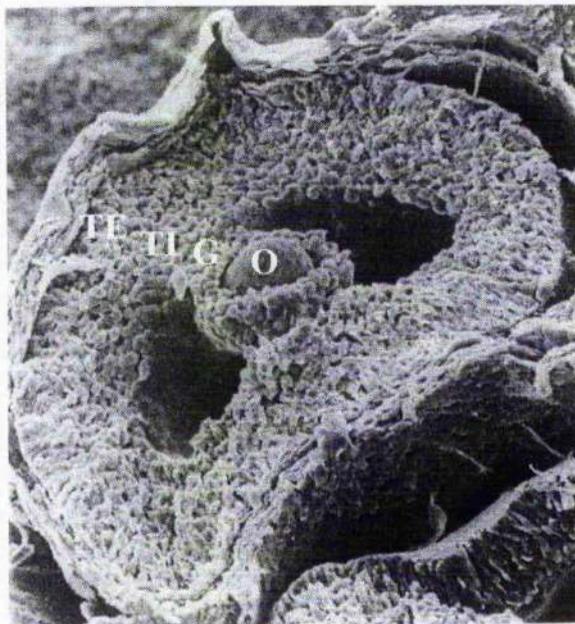
Temporal variation in serum oestradiol, progesterone and testosterone levels graphically depicted in relation to the menstrual cycle and ovarian follicle/corpus luteum development. There is no significant change in serum free testosterone through the menstrual cycle (Massafra et al. 2003).



Under the influence of LH, theca cells metabolise cholesterol into androstenedione. The granulosa cells then metabolise the androstenedione produced by the theca cells into oestradiol under the control of FSH. Thus, both cell types and both gonadotrophins are crucial to oestrogen synthesis (McNatty et al. 1980). Figure 1.2 shows an electron microscope image of a graffian follicle with all the components of the follicle labeled.

Following ovulation, luteinisation of the theca-granulosa layers occurs commencing production and secretion of progesterone. This ovarian region is now termed the corpus luteum and is the principal source of progesterone production in the secretory phase of the menstrual cycle.

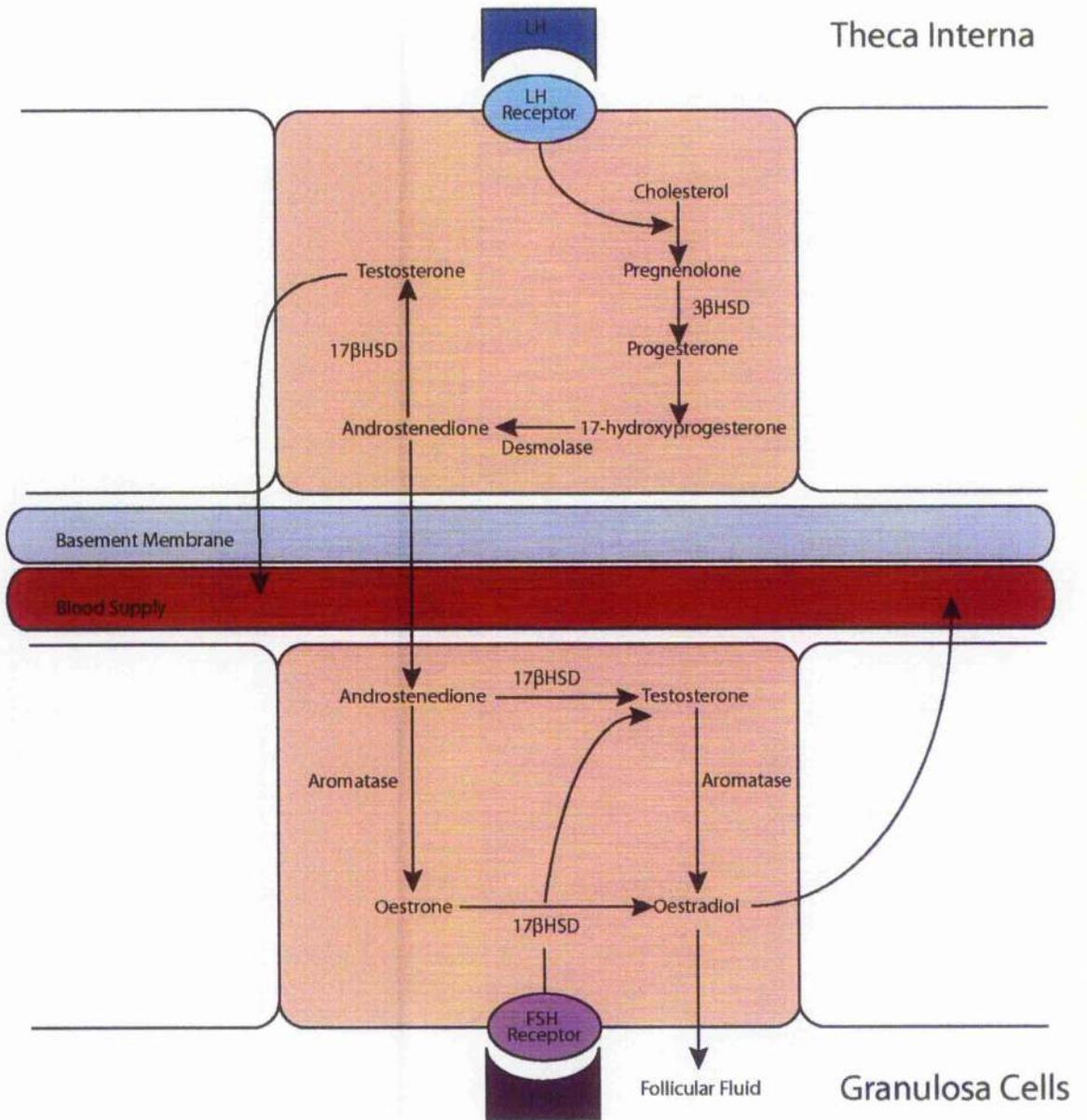
Serum androgens are abundant in the systemic circulation as the adrenal is the major producer of androgens in the form of dehydroepiandrosterone (DHEA). In addition to being an androgen, DHEA is also an important substrate for the production of oestradiol, progesterone and testosterone.



**Figure 1.2** An electron microscopy image of a graffian follicle with the oocyte **O**, Granulosa cells **G**, Theca Interna **TI**, and Theca Externa **TE** indicated on the image.

**Figure 1.3**

Two-cell two-gonadotrophin theory. Steroid synthesis pathways shown in respective cells with key enzymes also indicated. Androstenedione produced by the theca cells is then converted to oestradiol in the granulosa cells. 3 $\beta$ -HSD – 3 $\beta$  hydroxysteroid dehydrogenase, 17 $\beta$ -HSD – 17 $\beta$  hydroxysteroid dehydrogenase



### 1.2.1 Theca cell Androgen Synthesis

The classic pathway of sex steroid biosynthesis in the ovary ( $C_{27}$  cholesterol  $\rightarrow$   $C_{21}$  progesterone  $\rightarrow$   $C_{19}$  androgen  $\rightarrow$   $C_{18}$  oestrogen) commences with either cholesterol that has been synthesised *de novo* or obtained from the peripheral circulation. Cholesterol is then metabolised to pregnenolone by the enzyme cholesterol side-chain cleavage (P450<sub>scc</sub>) (Sasano et al. 1989). 17-hydroxylase/ $C_{17-20}$ -lyase (P450<sub>c17</sub>) is then responsible within the theca cells for the metabolism of pregnenolone to DHEA (Sasano et al. 1989). DHEA is then metabolised to androstenedione by 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD). 3 $\beta$ -HSD can also metabolise progesterone to androstenedione by the sequential actions of P450<sub>c17</sub> and 3 $\beta$ -HSD (Hillier et al. 1997).

Diffusion of androstenedione then occurs from the theca interna to the granulosa cells surrounding the oocyte. Theca cells do not express aromatase and are therefore unable to synthesise oestrogens *de novo* from androgenic precursors such as androstenedione.

### 1.2.2 Granulosa Cell Oestrogen Synthesis

Following diffusion of androstenedione, the granulosa cell is responsible for the production of oestrogens. While the granulosa cells do not express the enzymes necessary to produce androgens from cholesterol, they do express; members of the 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) family (Tremblay et al. 1989; Qin et al. 2000), 3 $\beta$ -HSD (Sasano et al. 1990), as well as aromatase. Aromatase in particular is almost exclusively expressed in granulosa cells (Sasano et al. 1989). Hillier et al. demonstrated that 99.9% of aromatase activity was in this cell layer (Hillier et al. 1981). The association of such enzymes in the granulosa cell allows the interconversion of androgens and oestrogens with the main function being production of oestradiol with FSH playing a key role in production.

In the granulosa cell, androstenedione may be metabolized to oestradiol by either of two pathways. Firstly, it may be aromatised to oestrone then metabolised to oestradiol by the action of 17 $\beta$ -HSD 1 or alternatively androstenedione may be metabolised to testosterone by 17 $\beta$ -HSD 1 and then aromatised to oestradiol by aromatase (Figure 1.4). The oestradiol produced may then enter the fluid of the antral follicle or be secreted into the bloodstream.

### 1.2.3 Corpus Luteum

Following the selection of a dominant pre-ovulatory follicle, it is favoured developmentally through the activation of a positive feedback loop. The oestrogen produced by the follicle stimulates granulosa cell proliferation, in addition to augmenting cellular responsiveness to gonadotrophins, thereby generating additional oestrogen formation (McNatty 1981). The dominant follicle contributes 95% of the serum oestradiol concentrations illustrating its ability to produce oestradiol (Baird et al. 1974). Oestradiol augments pituitary LH release, although the mechanism is unknown (Shoham et al. 1996).

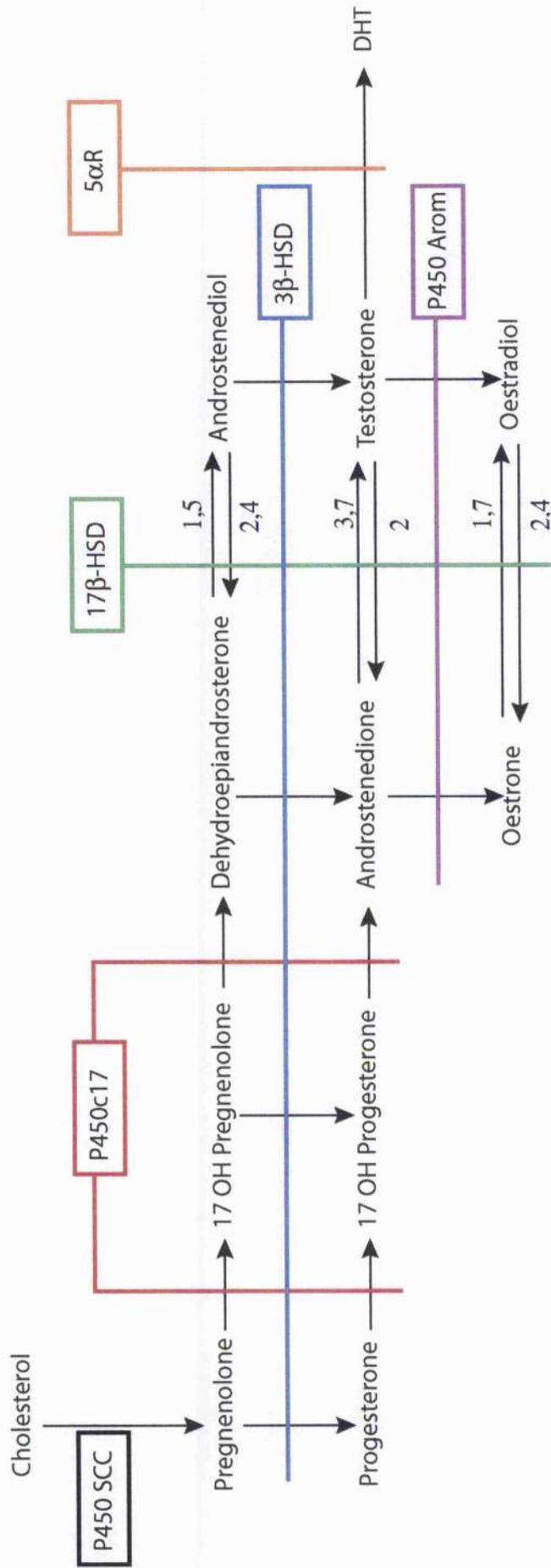
Ovulation is triggered by the mid-cycle LH surge, which is itself triggered by the sustained high circulatory level of oestradiol produced by the pre-ovulatory follicle. The LH surge initiates resumption of meiosis in the oocyte, luteinisation of the granulosa cells, and synthesis of prostaglandins (Shoham et al. 1996).

Following ovulation the granulosa and theca cells undergo angiogenic and mitogenic stimulation (McCracken et al. 1999). The luteal cells accumulate lipoid vacuoles rich in cholesterol, producing the characteristic yellow appearance. Cholesterol acts as a substrate for steroidogenesis with progesterone being the principal sex steroid produced. Progesterone secretion peaks about 8 days following the LH surge inducing secretory changes in the endometrium, in anticipation of possible blastocyst implantation. To maintain the corpus luteum and its attendant steroidogenesis, it requires Human Chorionic Gonadotrophin (HCG) secreted from a developing placenta. When no blastocyst implantation occurs, the corpus luteum undergoes luteolysis.

Luteolysis and the resultant decline in serum progesterone, results in a cascade of events leading to endometrial breakdown and menstruation. The role of progesterone in menstruation shall be discussed in Chapter 1.8.1.

Figure 1.4

Pathways of ovarian steroid hormone synthesis. Enzymes: Cholesterol side-chain cleavage (P450<sub>scc</sub>); 17-hydroxylase/C<sub>17-20</sub>-lyase (P450<sub>c17</sub>); 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) with numbers representing the isoform that catalyses the adjacent reaction; 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD); aromatase (P450<sub>arom</sub>), 5  $\alpha$  reductase (5 $\alpha$ R), Dihydrotestosterone (DHT). (Zeleznik and Hillier, 1996).



#### 1.2.4 Adrenal Androgen Steroidogenesis

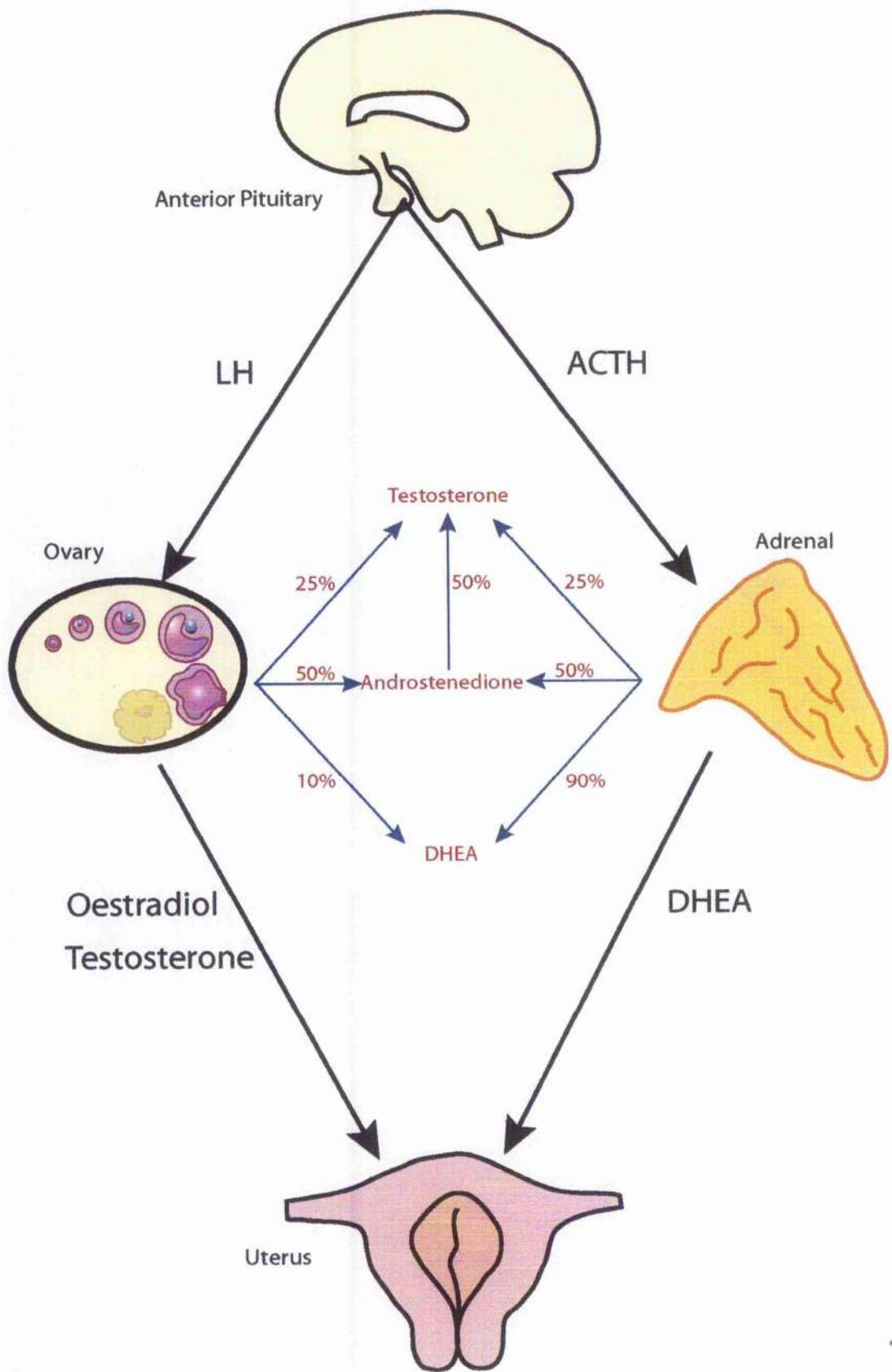
The adrenal gland is divided into the medulla and cortex with the cortex divided into a further 3 zones; the outer zona glomerulosa, the zona fasciculata and the inner reticularis. Adrenal androgens are thought to arise from the reticularis zone and it contains the enzymatic profile that is favourable for DHEA and DHEAS production (McKenna et al. 1997).

The onset of adrenal androgen production is termed adrenarche, occurring in children from the age of 6 – 8. 60 – 75% of circulating androgens in women arise directly or indirectly from the adrenal glands and 25 – 40% from the ovaries (McKenna et al. 1997). Androgens include testosterone, dihydrotestosterone, androstenedione, dehydroepiandrosterone (DHEA), and DHEA-sulphate (DHEAS). With regards to serum testosterone the adrenal gland is responsible for 25%, the ovary for 25% and the remaining 50% from peripheral conversion. The adrenal gland is the dominant source of testosterone precursors. The mechanism whereby adrenal androgen secretion is controlled is not yet known but it is thought to be related in part to adrenocorticotrophin (ACTH) secretion (McKenna et al. 1991). Figure 1.5

Cortisol secretion from the adrenal gland is known to be controlled by ACTH from the pituitary gland. During childhood, cortisol and ACTH levels are closely correlated but adrenal androgens bear no such close correlation (Cutler et al. 1979). However, exogenous ACTH results in elevated cortisol and adrenal androgen production. ACTH is known to be under very sensitive negative feedback control from cortisol, but no such mechanism is thought to exist for adrenal androgens. It would therefore appear that ACTH plays a role in the regulation of adrenal androgen secretion but other factors are thought to be involved. These factors include prolactin, growth hormone, insulin, sex steroids and a cortical androgen stimulating hormone has been proposed (McKenna et al. 1997).

Figure 1.5.

Higher control of ovarian and adrenal androgen production via luteinising hormone (LH) and adrenocorticotrophin (ACTH). The predominant androgen produced by each tissue is indicated, with the uterus shown as an example target organ. The ovarian and adrenal contribution to serum androgen secretion is also shown with red figures inset between the illustrations.



### **1.3 Sex Steroid Hormones Mechanism of Action**

Having established in broad terms the sites of sex steroid hormone synthesis and the temporal variation of the endocrine levels of hormone through the menstrual cycle it remains to describe their mechanism of action in target tissues. The steroid ligands classically bind to nuclear receptors, initiating a genomic response. In essence the nuclear receptors are ligand-inducible transcription factors whose primary function is to mediate the transcriptional response in target cells to hormones such as the sex steroids (androgens, oestrogens and progestins), adrenal steroids (glucocorticoids, mineralocorticoids), vitamin D<sub>3</sub>, thyroid hormones and retinoid hormones, in addition to a variety of other metabolic ligands. These protein receptors constitute the Nuclear Receptor Superfamily (Evans 1988; Soontjens et al. 1996; McKenna et al. 1999). More recently a further, Non-Genomic, mechanism of action for sex steroids has been recognised. Non-Genomic responses are in contrast to genomic responses, rapid and their response is not inhibited by agents that inhibit transcription or translation, e.g. actinomycin-D or cycloheximide.

#### **1.3.1 Genomic Mechanism of Action**

Phylogenetic studies in the mid to late 80's determined that the members of the family were evolutionarily related (Amero et al. 1992; Laudet et al. 1992). They are subdivided into 3 subclasses: Type I, Type II and Orphan receptors. Type I or "Classical" receptors includes the AR, PR, ER, Glucocorticoid receptor (GR), and Mineralocorticoid receptor. Type II receptors encompass the thyroid receptor, retinoic acid receptors, and vitamin D<sub>3</sub> receptors. Orphan receptors are a relatively new class of receptors. Cloning studies have identified a large number of previously unknown genes that have sequence homology to the steroid hormone receptor superfamily, however the ligands for these proteins (Orphan Receptors) are not known and are currently being characterised (Soontjens et al. 1996; McKenna et al. 1999).

The receptors share a common domain structure with the regions of the receptor being subdivided into regions A – F. The A/B domain contains a transactivation function that allows the activation of target genes and presumably determines target gene specificity. The C region is the DNA binding domain and is

responsible for DNA site recognition and dimerisation. The variable hinge region, D domain, allows the protein to change shape or alter conformation. The complex ligand binding domain, E region, also possesses a number of functions including Ligand binding, HSP binding, dimerisation, nuclear localisation and transactivation (Soontjens et al. 1996; Podczaski et al. 2001). No specific function has been assigned to the F domain. An illustration of the domain structure is shown in Figure 1.6

Figure 1.6

Domain Structure of the "Classic" Nuclear Receptor.



Activation Function-1  
(AF-1)



DNA Binding  
(DBD)



P-box  
(half-site specificity)



Dimerisation



Nuclear Localisation Signal  
(NLS)



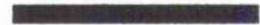
C-terminal extension  
(Flanking DNA binding specificity)



Ligand Binding



Ligand Dependant Activation



Activation Function-2  
(AF-2)



Repression



Type I receptors in the absence of ligand binding are bound to heat shock proteins (HSP) and in this state are unable to effect changes in cellular transcription. However, once ligand binds to the receptor the HSP dissociate, the receptor-ligand complex translocates to the nucleus, and the activated complex is now able to undergo dimerisation with other steroid receptors. The dimers formed are then able to bind to specific regions of DNA termed hormone response elements (HRE). Once bound to the HRE the receptor can alter transcription of genes aided by the recruitment of co-regulators with resultant activation or suppression of function (McKenna et al. 1999). Type II receptors are able to bind to the HRE of DNA in the absence of ligand. If a type II receptor binds to DNA in the absence of ligand then it may repress the function of that gene, "silencing". The receptor must still undergo dimerisation and unlike type I receptors, that usually form homo-dimers, the type II receptors may form hetero-dimers with other type II receptors and this may modulate the amplitude of the transcriptional response to ligand binding (McKenna et al. 1999).

If a gene is activated, RNA polymerase transcribes the information from the gene into messenger RNA (mRNA). Initially the transcribed product contains the intron and exon information but prior to transport from the nucleus the introns are spliced out joining the exons in a continuous sequence. This molecule then transfers the information decoded from the gene into the cytoplasmic compartment of the cell. The information is then decoded (translated) by the ribosomes of the rough endoplasmic reticulum to produce the protein appropriate to the gene. Finally, post translational modifications may occur prior to the protein producing its function within the cell.

Gene activation can also be modulated by coregulators which may be coactivators or corepressors. Coregulators are, by definition, rate limiting for nuclear receptor activation and repression, but do not significantly alter basal transcription (McKenna et al. 1999). This field is vastly complex and a comprehensive overview is out with the scope of this thesis. However, in general terms the initiation of transcription by steroid hormones requires the assembly of a complex of general transcription factors. Ultimately, it is by influencing the rate of assembly of such complexes that nuclear receptors, in association with their coregulators, achieve transcriptional regulation. The precise physiological role of nuclear receptor coregulators has yet to be determined. McKenna et al. provide a comprehensive review of this field (McKenna et al. 1999). Figure 1.7 illustrates the genomic mechanism of action.

### 1.3.2 Non-Genomic Mechanism of Action

Unlike classical genomic steroid responses, non-genomic steroid responses are rapid, within minutes, and are not sensitive to inhibitors of translation and transcription. The ability of steroids to initiate second messenger signaling cascades, including Phospholipase C; Phosphoinositide turnover; intracellular pH; intracellular free Calcium concentration; and Protein Kinase C, have been described for all classes of steroids (Falkenstein et al. 2000).

The precise mechanism of non-genomic steroid action is currently not known. Several possibilities have been suggested and if non-genomic progesterone action is used as an example, the suggestions are:

1. High concentrations of progesterone may insert into the plasma membrane of the cell and affect membrane fluidity that will elicit an intracellular response.
2. Direct progesterone activity with a subset of classic intracellular receptors bound to the cell membrane.
3. Progesterone interaction with a partner ligand via a non-progesterone receptor.
4. Direct progesterone action via a non-classical cell membrane bound receptor.

Recent data from Harding et al. have described the presence of membrane progestin receptors in the membrane fraction from endometrial fibroblasts (Harding et al. 2003).

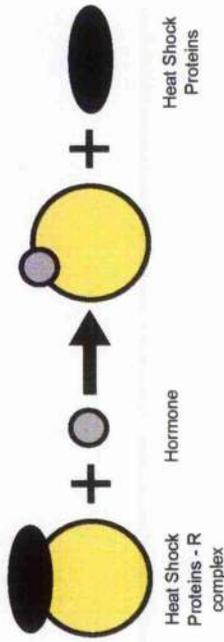
Rapid effects of androgens on cells such as human granulosa-lutein cells, prostate cancer cells, adult rat brain and human breast cancer cells have been described (Falkenstein et al. 2000). The data indicated the response was not initiated via a classical genomic response, as androgen receptor antagonists did not prevent a response. Furthermore, in some of the studies, the use of a plasma membrane impermeable testosterone conjugate elicited a response, suggesting action via cell surface receptors (Falkenstein et al. 2000).

Figure 1.7.

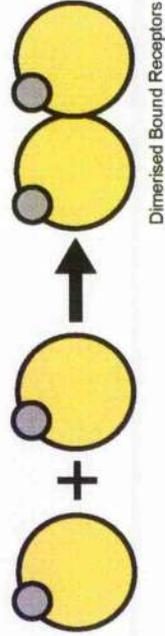
Diagram illustrating the mechanism of action for steroid hormones. Receptor binding dissociates heat shock proteins enabling receptor dimerisation with subsequent binding to the HRE – hormone response element. A transcriptional complex is then formed that produces mRNA.

# Model of Steroid Hormone Action

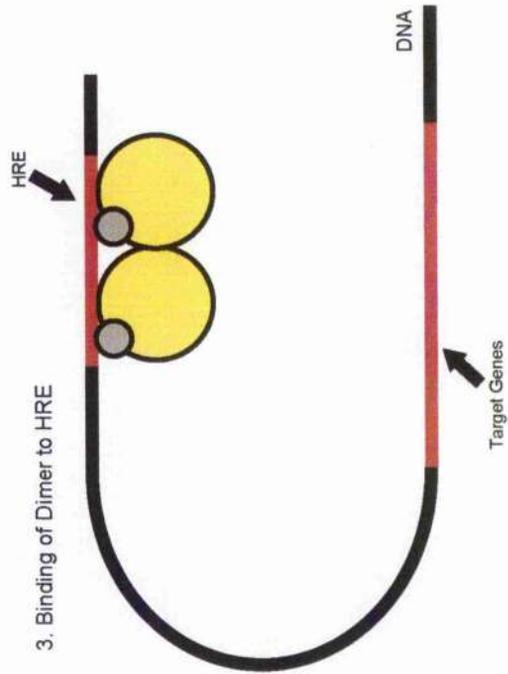
1. Binding of Hormone to its Receptor (R)



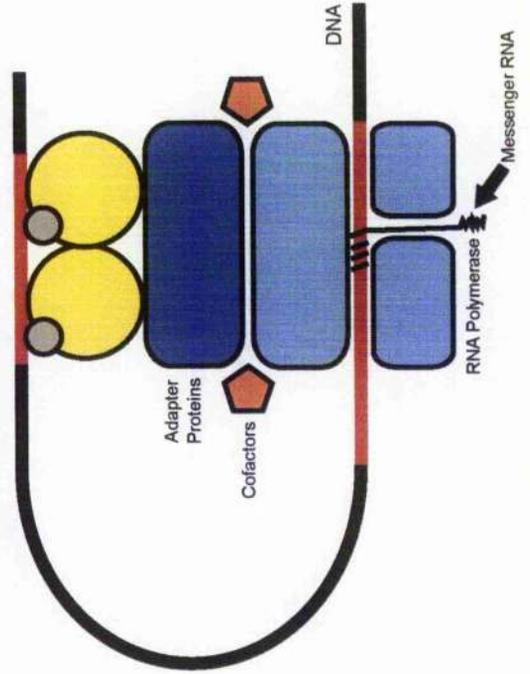
2. Dimerisation of Bound Receptors



3. Binding of Dimer to HRE



4. Formation of Transcriptional Machinery and mRNA from Target Genes



## 1.4 Intracrinology

Humans have a sophisticated, stratified regulatory system for the control of steroidogenesis and steroid hormone activity. The stratification of control includes endocrine, paracrine, autocrine and most recently described intracrine regulation. Classical endocrine systems secrete steroid hormones from endocrine glands into the systemic circulation whereby they are transported to target cells in peripheral tissues. Paracrine activity refers to the influence of hormone secreted into the extracellular space upon adjacent/proximal cells. Autocrine activity is the influence of hormone secreted into the extracellular space upon the cell from which it was secreted. Intracrine activity is described as the influence exerted by hormones within the same cell where synthesis occurred without release into the extracellular space. Figure 1.8

The term "Intracrinology" was first described by Labrie et al. (Labrie 1991). The importance of this concept is that peripheral target tissues have autonomous control over their sex steroid formation and metabolism according to the requirements of the target tissue (Labrie 1991). Figure 1.4 details the metabolic pathways in the production of sex steroids and the enzymes responsible for each step. In the female, oestradiol and progesterone have long been considered the biologically active sex steroids with a lesser contribution from the androgen, testosterone. The main source of all is ovarian steroidogenesis under the control of FSH and LH secretion. They are not however the most abundant sex steroids in the systemic circulation, dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEA-S) are both synthesised in large quantities by the adrenal gland under the control of ACTH.

The secretion of DHEA and DHEA-S in the adrenal gland first commences with adrenarche, which occurs in the region of 6-8 years of age. From this point forward elevated levels of both are maintained through adulthood. In comparative terms the levels of DHEA-S is 1000 -10 000 higher than those of Oestradiol in the adult female (Labrie et al. 2000). Such elevated levels of this inactive steroid precursor provides a large reservoir of substrate that may later be converted into alternative biologically active sex steroids such as oestradiol, progesterone and testosterone within peripheral target tissues. The importance of intracrinology was first realised in a clinical context in the treatment of prostate cancer.

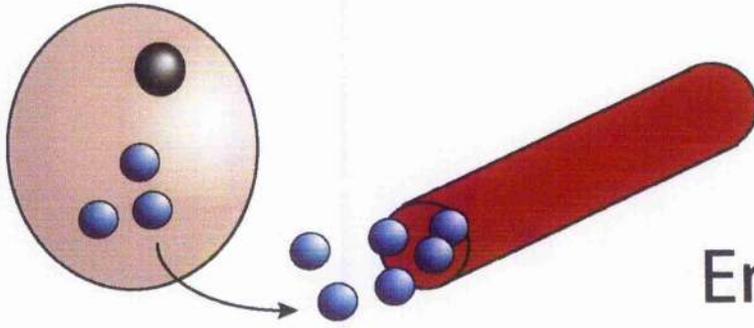
Prostate cancer is an endocrine responsive cancer with prognosis related in part to serum and tissue levels of androgens. Endocrine modulation is an important element in the treatment of prostate cancer where surgical or medical castration (GnRH agonists) were shown to reduce serum testosterone levels by 90-95%, however tissue levels of dihydrotestosterone (DHT) were only 50% of normal. As the main pathway of DHT production is the 5 alpha reduction of testosterone this suggested another source of androgens - the adrenal - persisted following castration that could be 5 alpha reduced to DHT in the prostate (Labrie et al. 1996). This important discovery has now altered the treatment of prostate cancer with therapy now aimed at blocking the effect of both adrenal and testicular androgens - combined androgen blockade. This combined androgen blockade in essence inhibits both the androgen receptor and the enzyme 5 $\alpha$  reductase which are both expressed in the prostate. Prospective randomized control trials have now shown that this approach improves survival in patients with prostate cancer (PCTCG 2000).

The role of peripheral intracrine sex steroid formation is also seen in postmenopausal females affected by breast cancer, a condition that is also endocrine responsive. In the postmenopausal female, oestradiol production is via extra-ovarian aromatisation of oestrone. Inhibition of oestradiol action has been targeted by blockade of aromatisation with aromatase inhibitors (Buzdar et al. 1996) and the oestrogen receptor with selective oestrogen receptor modulators (SERM'S) (Cummings et al. 1999). Both interventions were shown to improve outcomes in patients diagnosed with breast cancer.

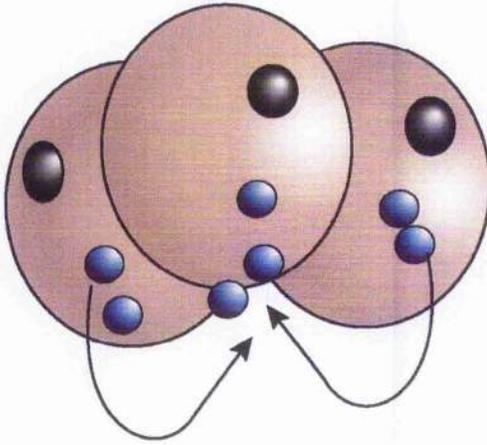
The target cell responses to steroid hormones are dependent on the target cell concentration of steroid and their binding affinity to their respective receptors. Through ligand binding, gene transcription is activated to express steroid hormone actions. Apart from receptor concentration, another key regulatory factor in steroid action is the intracellular steroid concentration. The enzymes that play a key role in regulating intracrine sex steroid metabolism include the isoenzymes of 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD), 5 $\alpha$ -reductase (5 $\alpha$ R), 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) and Aromatase.

Figure 1.8

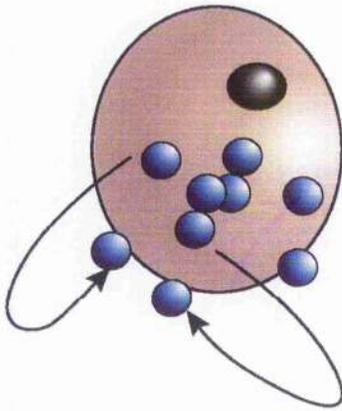
Illustration of endocrine, paracrine, autocrine and intracrine secretion. Classically, endocrine activity includes the hormones secreted in specialized glands, called endocrine glands, for release into the general circulation and transport to distant target cells. Alternatively, hormones released from one cell can influence neighboring cells (paracrine activity) or can exert a positive or negative action on the cell of origin (autocrine activity). Intracrine activity describes the formation of active hormones that exert their action in the same cells where synthesis took place without release into the pericellular compartment.



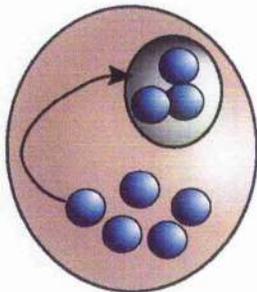
Endocrine



Paracrine



Autocrine



Intracrine

### 1.4.1 17 beta hydroxysteroid dehydrogenases

The 17 $\beta$ -hydroxysteroid dehydrogenases (17 $\beta$ -HSD) are enzymes involved in the formation of activation and inactivation of sex steroids, including testosterone (T), oestradiol (E2), 5-androstenediol (5-diol) and dihydrotestosterone (DHT). They catalyse the last and key step in the formation of all oestrogens and androgens, the reduction of the 17-keto group to the 17 $\beta$ -hydroxyl on the steroid nucleus, increasing the affinity of the steroids to their cognate receptors (Peltoketo et al. 1996).

There are currently eight known isoforms in the 17  $\beta$ -HSD family with seven human isoforms having been identified (Luu-The 2001). Each member of the 17  $\beta$ -HSD family is a separate gene product from a different chromosome making it a multi-gene family. The enzymes play an important role in the control of active sex steroids with a wide distribution in tissues throughout the body. As well as being localised to classical steroidogenic tissues such as the human placenta (Luu-The et al. 1990), ovary (Luu-The et al. 1990), and testis (Inano et al. 1986; Geissler et al. 1994), the 17  $\beta$ -HSDs are found in peripheral intracrine tissues (Martel et al. 1992), such as adipose tissue (Bleau et al. 1974), endometrium (Casey et al. 1994), ilcum (Inano et al. 1986), liver (Breuer et al. 1966), and vaginal mucosa (Weinstein et al. 1968).

Despite catalysing similar reactions often with the same substrates, the various 17  $\beta$ -HSDs only share in the region of 20% homology in their gene sequence (Peltoketo et al. 1999; Luu-The 2001). In keeping with other dehydrogenases the 17  $\beta$ -HSDs were thought to facilitate reversible interconversion between substrate and product. However, transfection studies without exogenous cofactors to drive reactions have shown that within an intracellular environment the direction of reaction is unidirectional and is likely to depend upon the intracellular ratio of NAD<sup>+</sup> to NADPH (Luu-The et al. 1995; Luu-The 2001). It is noteworthy that all the enzymes catalyzing the reduction reaction prefer a phosphorylated cofactor (NADPH) while the enzymes that catalyze the oxidative reaction prefer NAD (Luu-The 2001). The enzymes, except 17  $\beta$ -HSD 5, are all members of the short-chain alcohol dehydrogenase/reductase family (SDR). 17  $\beta$ -HSD 5 is a member of the aldoketoreductase (AKR) protein family (Deyashiki et al. 1995). The seven human isoforms are 17  $\beta$ HSD types 1, 2, 3, 4, 5, 7 and 8. The substrate preference, function and tissue distribution of the enzymes are detailed in Table 1.1.

Type/Protein Family	Subcellular Localisation	Tissue Distribution
1/SDR	Cytosolic	Ovary Placenta Breast
2/SDR	Microsomal	Placenta, Liver Gastrointestinal tract, Kidney Uterus, Breast, Prostate
3/SDR	Microsomal	Testis
4/SDR	Peroxisomal	Widely distributed
5/AKR	Cytosolic	Liver, Kidney Testis, Prostate Adrenal, Bone
6/SDR (Rat)	Membrane bound	Prostate, liver
7/SDR (Rat and mouse)	Membrane bound	Ovary Placenta Mammary gland
8/SDR	Unknown	Liver, Pancreas, Kidney Skeletal Muscle, Liver Ovary, Testis, Spleen

**Table 1.1** List of the cloned 17 HSD/KSR enzymes (Peltoketo et al., 1999).

#### 1.4.1.1 17 $\beta$ -HSD 1

The molecular structure of a human type1 17 $\beta$ -HSD cDNA and its corresponding gene encodes a predicted protein of 327 amino acids with the gene consisting of 6 exons and 5 introns within a genomic DNA fragment of 3.2 kb. 17 $\beta$ -HSD 1 enzyme is a protein that catalyzes predominantly the conversion of oestrone to oestradiol using NAD (H) or NADP (H) as cofactor (Labrie et al. 1997; Labrie et al. 2000).

The type1 17 $\beta$ -HSD enzyme plays an active role in the regulation of oestradiol production in the ovary. It is exclusively expressed in granulosa cells being at low levels in antral follicles, up regulated during follicular maturation, and highest in Graffian follicles. These data suggest that type1 17 $\beta$ -HSD is the principal 17-HSD isoenzyme involved in ovarian oestradiol production. Similarly, to P450 aromatase, 17 $\beta$ -HSD 1 expression is regulated by gonadotrophins, and the effects of gonadotrophins are modulated by oestrogens, androgens and growth factors. Moreover, 17 $\beta$ -HSD 1 is also expressed in human corpus luteal, as well as in human granulosa luteal cells and is involved in the production of significant amounts of oestrogens (Poutanen et al. 1995; Zhang et al. 1996).

In addition to the ovary, 17 $\beta$ -HSD 1 participates in oestradiol biosynthesis in the human placenta (Fournet-Dulguerov et al. 1987). Northern blotting experiments show that 17 $\beta$ -HSD 1 is highly expressed in the placenta and the enzyme has been immunolocalised to the syncytiotrophoblast cells of the human placenta (Labrie et al. 1997; Takeyama et al. 1998; Bonenfant et al. 2000). Two mRNA species have been cloned for 17 $\beta$ -HSD 1, a major RNA species of about 1.3 kb and a minor RNA species of 2.3 kb being detected. The 1.3 kb and 2.3 kb mRNA species are also detected in other tissues. The 1.3 kb mRNA is very abundant in placental and ovarian tissues. It has been suggested that the 1.3 kb mRNA is present only in tissues producing oestrogens, whereas the 2.3 kb mRNA has been found in steroid-forming tissues and some target tissues of steroid hormone action, such as prostate and breast tissue, as well as in breast and endometrium cancer cell lines (Vihko et al. 1991; Poutanen et al. 1995).

Until recently there has been a lack of clarity in the literature regarding the expression of 17 $\beta$ -HSD 1 and 2. It appears from reviewing the literature that, before cloning of a separate enzyme for 17 $\beta$ -HSD 1 and 2, 17 $\beta$ -Oestradiol Dehydrogenase

was generally referred to as the enzyme responsible for the conversion oestrone to oestradiol, in keeping with 17 $\beta$ -HSD 1 activity as is now understood (Luu The et al. 1989; Vihko et al. 1991). However, a series of papers was published by Maentausta et al. that referred to 17 $\beta$ -Oestradiol Dehydrogenase as the enzyme responsible for the inactivation of oestradiol to oestrone, in keeping with 17 $\beta$ -HSD 2 activity as it is now understood (Maentausta et al. 1990; Maentausta et al. 1991; Maentausta et al. 1993). Maentausta et al. described the progesterone up regulation of the enzyme, 17 $\beta$ -HSD 2, and subsequent papers misquoted this reference as meaning up regulation of the enzyme responsible for catalysing the conversion of oestrone to oestradiol (Miettinen et al. 1996; Peltoketo et al. 1996; Mustonen et al. 1998).

In summary, type1 17 $\beta$ -HSD is involved in the endocrine and intracrine production of oestradiol in females, in classical steroidogenic cells and in some peripheral, oestrogen target tissues, respectively. This suggests a central role for the enzyme in regulating the circulating oestradiol concentration, as well as its local production in oestrogen target cells.

#### 1.4.1.2 17 $\beta$ -HSD2

The type2 17 $\beta$ -HSD cDNA encodes a predicted protein of 387 amino acids with a molecular mass of 42,782 Da (Wu et al. 1993). The gene is comprised of seven exons, which give rise to the 387-amino acid type IIA 17 $\beta$ -HSD and a predicted 291-residual type IIB 17 $\beta$ -HSD proteins through alternative splicing. Cloning of the human type 2 17 $\beta$ -HSD gene confirmed that type IIA and IIB 17 $\beta$ -HSD transcripts originate from a single gene. Sequencing of the exons and of the adjoining intron sequences revealed that the 17 $\beta$ -HSD IIA protein is encoded by exon 1-3 and 6-7, whereas type IIB protein is encoded by exon 1-7 inclusively (Labrie et al. 1995). Such detail is important for the design of molecular probes to allow distinction between the active and inactive gene products.

The type IIA 17 $\beta$ -HSD, but not type IIB, has been shown to catalyse the conversion of oestradiol into oestrone, testosterone into androstenedione and androstenediol into DHEA. It also demonstrates 20 $\alpha$ -hydroxysteroid dehydrogenase activity, i.e. the conversion of 20 $\alpha$ -dihydroprogesterone to the more active progestin, progesterone (Wu et al. 1993). The function of the type IIB 17 $\beta$ -HSD protein is

unknown but it is hypothesized that the type IIB protein might regulate type IIA 17 $\beta$ -HSD activity by forming heterodimers with type IIA 17 $\beta$ -HSD (Labrie et al. 1995). Future reference to 17 $\beta$ -HSD 2 in this text will refer to type IIA 17 $\beta$ -HSD.

The enzyme is a member of the short-chain alcohol dehydrogenase super family, sharing in the region of 20% sequence identity with the enzyme encoded by the 17 $\beta$ -HSD1 gene, typical of the 17 $\beta$ -HSD multigene family. The type2 17 $\beta$ -HSD isoenzyme utilizes NAD as a cofactor and preferentially catalyzes the oxidative reaction (Andersson et al. 1997; Labrie et al. 2000).

17 $\beta$ -HSD 2 is principally expressed in the placenta (Casey et al. 1994; Moghrabi et al. 1997; Takeyama et al. 1998; Bonenfant et al. 2000), endometrium (Maentausta et al. 1990), and liver (Wu et al. 1993) with lower levels in the prostate (Wu et al. 1993), kidney, colon and pancreas (Casey et al. 1994; Andersson 1995). Virtually undetectable levels are found in testis, ovary, skeletal muscle, brain, heart, spleen and thymus (Casey et al. 1994; Andersson 1995).

In human placenta, both immunohistochemistry and northern blot analysis have shown that 17 $\beta$ -HSD 2 is expressed in the endothelial cells of capillaries and sinusoids in the chorionic villi (Moghrabi et al. 1997; Takeyama et al. 1998; Bonenfant et al. 2000). Gurpide et al demonstrated that 90% of the oestradiol and oestriol produced by human placental trophoblast enters the maternal circulation (Gurpide et al. 1966) and it is generally agreed that both oestradiol and oestriol, but not oestrone are secreted directly via the intervillous space to the maternal circulation (Moghrabi et al. 1997). Investigators have also established that oestrone is the major oestrogen product in foetal umbilical veins (Walsh et al. 1981; Gurpide et al. 1982). Following trophoblast synthesis of oestradiol, the oestrogen must first cross the intervillous space prior to traversing the fetal capillary wall. The localisation of 17 $\beta$ -HSD 2 in the endothelial cells lining this space will therefore be in a position to metabolise oestradiol to oestrone. One proposed physiological role of 17 $\beta$ -HSD 2 may therefore be the protection of the fetus from excess placental and maternally derived bioactive oestrogens and androgens that are produced by the syncytiotrophoblast cells (Moghrabi et al. 1997). Such a catabolic role would also be consistent with the expression of 17 $\beta$ -HSD 2 in the liver

Human endometrium also expresses 17 $\beta$ -HSD 2. Temporal variation in the expression of 17 $\beta$ -HSD 2 was first shown by Gurbide and colleagues. They demonstrated oestradiol conversion to oestrone in human secretory endometrium that was up-regulated by the action of progestins both in vivo and in vitro (Tseng et al. 1975; Gurbide et al. 1976; Gurbide et al. 1977; Tseng et al. 1977; Tseng et al. 1979; Gurbide et al. 1981; Holinka et al. 1981). Further, the activity was localised to secretory glandular epithelium (Scublinsky et al. 1976; Satyaswaroop et al. 1982). Following on from these early reports of enzyme activity, the protein has been immunolocalised using a polyclonal antibody. Highest levels were noted in the mid-secretory endometrium and the progesterone regulation was also demonstrated in vivo (Maentausta et al. 1990; Maentausta et al. 1991; Maentausta et al. 1992; Maentausta et al. 1993). Higher levels of the 17 $\beta$ -HSD 2 mRNA were also demonstrated in the mid- to late-secretory phase endometrium in contrast to relatively low levels in proliferative phase endometrium (Casey et al. 1994; Mustonen et al. 1998).

The precise role of 17 $\beta$ -HSD 2 in human endometrium is not known. It is hypothesized that the effect of increasing oxidative activity during the luteal phase of the cycle is to not only down-regulate oestrogen action, but to also up-regulate progesterone action in the endometrium during this phase of the cycle in keeping with the enzyme's ability to catalyse the formation of progesterone (Wu et al. 1993; Mustonen et al. 1998). The immunolocalisation of 17 $\beta$ -HSD 2 with a monoclonal antibody is now possible following the characterisation of a novel monoclonal antibody (Moghrabi et al. 1997). This should enable further characterisation of the enzyme in a spatial and temporal context within the endometrium. The role of 17 $\beta$ -HSD 2 in androgen and androgen receptor metabolism within human endometrium also remains to be determined.

#### 1.4.1.3 17 $\beta$ -HSD 3

17 $\beta$ -HSD 3 is expressed predominantly in the testis (Geissler et al. 1994). It has also been detected in the brain (Stoffel-Wagner et al. 1999) and in both tissues it catalyses the conversion of androstenedione to testosterone. In addition it can also catalyse DHEA to androstenediol and oestrone to oestradiol (Geissler et al. 1994). 17 $\beta$ -HSD

3 cDNA was cloned in 1994 also belongs to the short chain alcohol dehydrogenase super family (Geissler et al. 1994; Andersson 1995).

Testosterone is synthesized by the Leydig cells of the testis and is the main hormone of the male gonad. Testosterone is required for the development of internal male reproductive structures (epididymis, vas deferens, seminal vesicles and ejaculatory ducts) derived from the Wolffian ducts while its  $5\alpha$  reduced form, dihydrotestosterone, induces formation of the male external genitalia (penis and scrotum) and prostate gland (Andersson et al. 1997; Twosten et al. 2000). A failure in testosterone biosynthesis or a failure in conversion of testosterone to  $5\alpha$  dihydrotestosterone can lead to insufficient differentiation of the male external genitalia, and hence to defective virilisation. This may result in genotypic males with female phenotypes, male pseudohermaphroditism.

Molecular defects in the  $17\beta$ -HSD 3 enzyme are among the genetic defects that cause this syndrome (Andersson et al. 1997; Twosten et al. 2000). The defect was first described by Saez et al. (Saez et al. 1971; Saez et al. 1972). It is inherited in an autosomal recessive pattern producing a characteristic phenotype of 46 XY with testis, Wolffian duct-derived male internal genitalia, but an absence of prostate gland, and with female external genitalia (Andersson et al. 1997).

Attempts were made to localise  $17\beta$ -HSD 3 in human endometrium but northern blotting failed to detect this isoform of the enzyme in human endometrium or endometriotic tissue (Zeitoun et al. 1998).

#### **1.4.1.4 $17\beta$ -HSD4**

Human  $17\beta$ -HSD 4 was cloned by Adamski et al in 1995 (Adamski et al. 1995) and it catalyses the conversion of oestradiol to oestrone and androstenediol to DHEA. Human mRNA encoding type 4  $17\beta$ -HSD is expressed in virtually all human tissues examined. The highest mRNA level of human  $17\beta$ -HSD 4 was observed in liver, followed by heart, prostate and testis. Moderate expression occurred in lung, skeletal muscle, kidney, pancreas, thymus, ovary, intestine, and term placenta. Weak signals were observed in brain, spleen, colon and lymphocytes (Adamski et al. 1995). It's expression has now been demonstrated in endometrial epithelial cell cultures (Husen et al. 2000). It has not however been demonstrated in intact human endometrium.

17 $\beta$ -HSD 2 also catalyses the same reaction but there is only 16% amino acid identity between the two enzymes (Labrie et al. 1997). It is likely to play a role in inactivation of oestrogens in peripheral tissues, although its activity is low and its physiological importance remains to be established in human (Labrie et al. 2000).

#### 1.4.1.5 17 $\beta$ -HSD 5

17 $\beta$ -HSD 5 differs from the other 17-hydroxysteroid dehydrogenases in that it belongs to the aldo-keto reductase family. Identification of the enzyme was confusing as it has also been recognised as dihydrodiol dehydrogenase and 3 $\alpha$ -hydroxysteroid dehydrogenase type 2 (3 $\alpha$ -HSD 2) which is also known as AKR1C3. 3 $\alpha$ -HSD 2 was originally cloned by Khanna et al. from human liver (Khanna et al. 1995). They localised the enzyme mRNA using RT-PCR to liver, kidney, placenta, brain and testis (Khanna et al. 1995). Subsequently it has been demonstrated in human prostate by northern blotting and in this same study northern blotting also identified the mRNA in leukocytes, colon, small intestine, ovary, thymus, spleen, pancreas, skeletal muscle, and heart (Lin et al. 1997). The enzyme was also known to possess reductive 17 $\beta$ -HSD activity but the 3 $\alpha$ -HSD 2 activity was thought to be the predominant activity of the enzyme. However, Dufort et al. established that human 3 $\alpha$ -HSD 2 was not the predominant activity and a higher percentage conversion of androstenedione to testosterone and progesterone to 20 $\alpha$ -hydroxyprogesterone occurred when a stably expressed enzyme was studied. It was felt from this and prior studies that homogenization protocols had destroyed much of the 17 $\beta$ -HSD activity of the enzyme, suggesting that it was highly labile (Dufort et al. 1999). It is therefore now recognised as 17 $\beta$ -HSD 5.

The 17 $\beta$ -HSD 5 activity of the enzyme catalyses androstenedione to testosterone (Penning 1997; Dufort et al. 1999). The 20 $\alpha$ -hydroxysteroid dehydrogenase activity will inactivate progesterone to 20 $\alpha$ -hydroxyprogesterone (Dufort et al. 1999). The 3 $\alpha$ -HSD 2 activity will catalyse the inactivation of DHT to androstenediol (Penning et al. 2000).

Active androgens in the male are formed mainly within the testis under the control of 17 $\beta$ -HSD3, an enzyme that in humans is functional mainly within the male (Geissler et al. 1994). The enzyme may be subject to mutations with the affected male individuals suffering male pseudohermaphroditism, to date no females with

similar mutations have been shown to suffer an equivalent condition (Mendonca et al. 1999). As the ovary is the main source of serum androgens in the female, studies were carried out to localise 17 $\beta$ -HSD3 in the ovary but it was not found (Zhang et al. 1996). However, 17 $\beta$ -HSD3 shares the same substrate specificities as 17 $\beta$ -HSD5, catalysing the conversion of androstenedione to testosterone (Penning 1997), and 17 $\beta$ -HSD5 has been localised in the ovary (Luu-The et al. 2001). It would therefore appear that 17 $\beta$ -HSD5 is the hydroxysteroid dehydrogenase responsible for androgen production in the female. However, in the male 17 $\beta$ -HSD5 is also present in peripheral target tissues such as the prostate. In this tissue its role is thought to be a combination of androgen formation and progesterone inactivation via its 20 $\alpha$ -hydroxysteroid dehydrogenase (20 $\alpha$ -HSD) activity, protecting the prostate from the action of progesterone (Dufort et al. 1999).

In the female 17 $\beta$ -HSD5 has now been localised to the ovary, breast and uterus (Pelletier et al. 1999). Uterine 17 $\beta$ -HSD5 was localised to glandular and surface epithelium; however the stage of the cycle in which it was detected was not stated (Pelletier et al. 1999). As endometrial AR localisation is thought to be predominantly stromal, it may be that local 17 $\beta$ -HSD5 androgen production produces a paracrine effect in the stroma. Alternatively, its role may relate to its 20 $\alpha$ -HSD activity, inactivating progesterone. To elucidate its role, more details regarding the immunolocalisation of 17 $\beta$ -HSD5 in endometrium through the menstrual cycle would be required.

#### **1.4.1.6 17 $\beta$ -HSD 6**

17 $\beta$ -HSD 6 selectively catalyzes the oxidation of 3 $\alpha$ -androstane-2,3-diol to androsterone. Additionally it may catalyse DHT to androstane-2,3-dione and testosterone to androstenedione but at an approximately 50 to 100 fold lower rate. This enzyme belongs to the retinol dehydrogenase family. Northern blotting analysis shows high expression of 17 $\beta$ -HSD 6 in rat liver and prostate. The human counterpart has not yet been described, and a human clone that possesses the characteristics of rat 17 $\beta$ -HSD 6 has not been found (Labric et al. 2000; Luu-The 2001)

#### **1.4.1.7 17 $\beta$ -HSD 7**

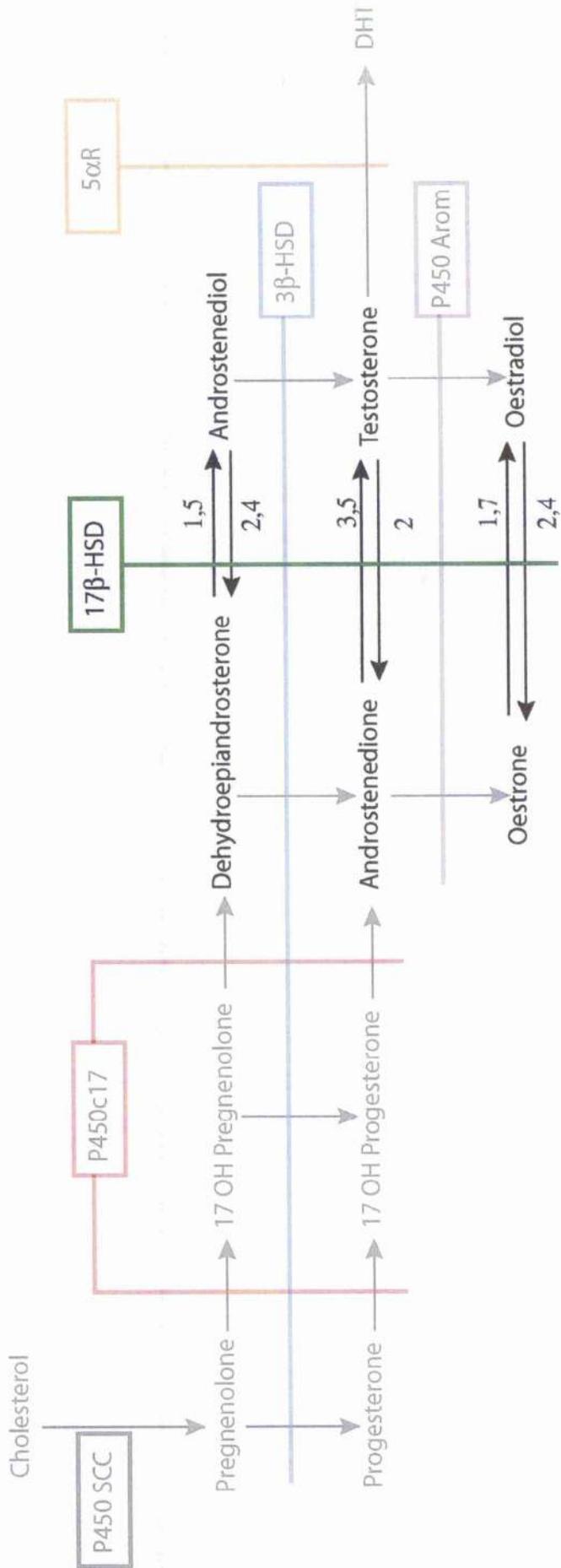
17 $\beta$ -HSD 7 was first cloned in a rat corpus luteum cDNA library and was identified as prolactin receptor associated protein (Duan et al. 1997). It efficiently and selectively catalyzes oestrone to oestradiol (Nokelainen et al. 1998). A human type7 17 $\beta$ -HSD has been cloned and using RT-PCR the enzyme was detected in the ovary, breast, prostate, placenta, liver and brain (Krazeisen et al. 1999). As present, the endometrium has not been examined.

#### **1.4.1.8 17 $\beta$ -HSD 8**

17 $\beta$ -HSD 8 catalyses oestradiol to oestrone most efficiently and to a lesser extent the oxidation of androgens. The conversion of testosterone to 4-dione is about 25% of that oestradiol into oestrone. It was cloned in humans by Kikuti et al (Kikuti et al. 1997) and determined to have 17 $\beta$ -HSD activity by (Fomitcheva et al. 1998). This enzyme is expressed abundantly in the kidney, liver, gonads and spleen. In the ovary, type8 17 $\beta$ -HSD is present in cumulus cells but not in granulosa or luteal cells, unlike type1 and type7 17 $\beta$ -HSD, respectively (Peltoketo et al. 1999; Labrie et al. 2000).

Figure 1.9.

Summary of the principal pathways involving members of the 17 $\beta$ -hydroxysteroid dehydrogenase family. While steroids can be interconverted by members of the 17 $\beta$ -hydroxysteroid dehydrogenase family, each reaction is unidirectional for specific isoforms with some isoforms catalysing more than one specific reaction. The highlighted pathways are those corresponding to 17 $\beta$ -hydroxysteroid dehydrogenase.



### 1.4.2 3 beta hydroxysteroid dehydrogenase

3 $\beta$ -hydroxysteroid dehydrogenase/ $\Delta$ 5- $\Delta$ 4 isomerase (3 $\beta$ -HSD) isoenzymes are essential for the biosynthesis of all classes of hormonal steroids, namely progesterone, glucocorticoids, mineralocorticoids, androgens and oestrogens (Lachance et al. 1990; Labrie et al. 1992). Additionally they catalyse the formation and/or degradation of 5 $\alpha$ -androstanes and 5 $\alpha$ -pregnanes such as 5 $\alpha$ -dihydrotestosterone and 5 $\alpha$ -dihydroprogesterone respectively (Mason et al. 1993; Mason et al. 1997). Two isoenzymes are present in humans with the type 2 gene coding for the 3 $\beta$ -HSD isoenzyme predominantly expressed in the adrenal gland, ovary and testis, whereas expression of the type 1 gene appears to account for the 3 $\beta$ -HSD activity found in peripheral target tissues (Simard et al. 1996; Mason et al. 1997). Type 1 encodes a protein of 372 amino acids and was first sequenced by Luu-The et al. (Luu-The et al. 1990) with type 2 encoding a protein of 371 amino acids being sequenced by Rheume et al. (Rheume et al. 1991). The two isoenzymes are highly homologous sharing 93.5% identity of amino acids (Simard et al. 1996). The type 1 displays a higher substrate affinity than the type 2 (Rheume et al. 1991) that may facilitate steroid formation from relatively low concentrations of substrate usually present in peripheral target tissues (Simard et al. 1996).

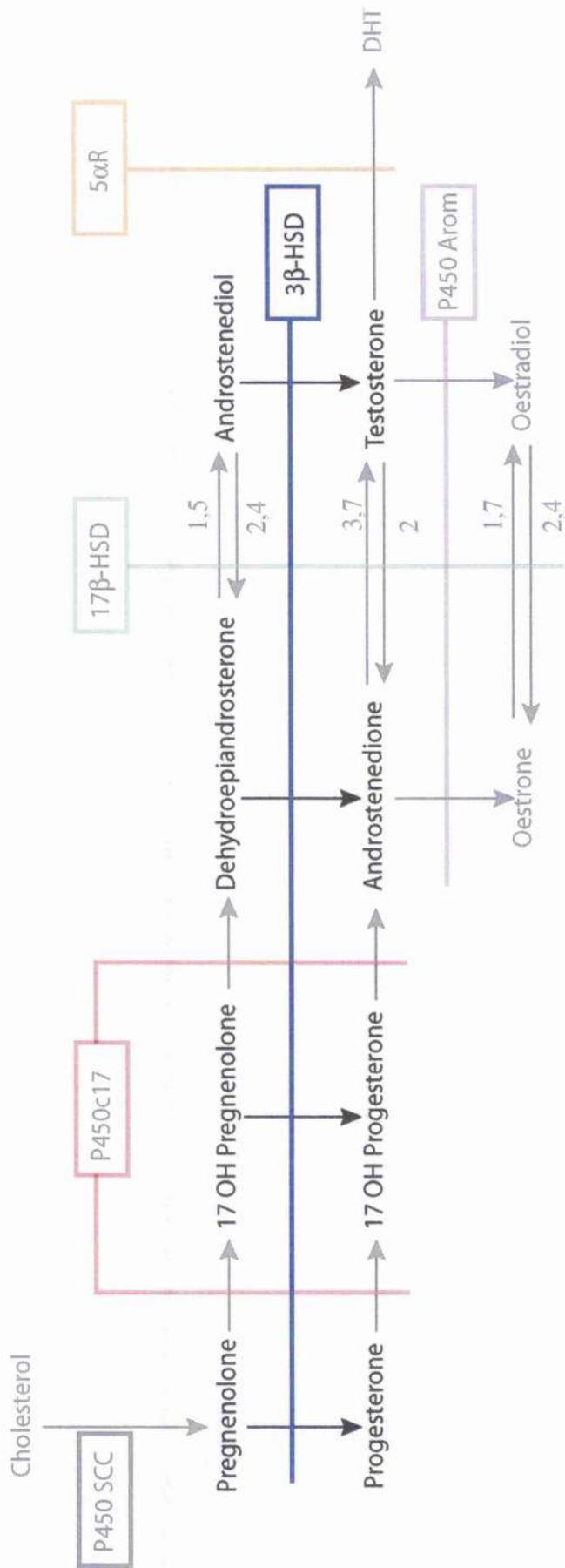
As a clinical entity this enzyme is important as it is a cause of congenital adrenal hyperplasia (CAH). CAH is the commonest cause of ambiguous genitalia and adrenal insufficiency in newborns (Simard et al. 1996). 3 $\beta$ -HSD deficiency is included with 21-hydroxylase and 11 $\beta$ -hydroxylase deficiencies as specific enzyme defects that impair cortisol biosynthesis that in turn increase ACTH secretion from the anterior pituitary as cortisol negative feedback is absent. This produces a compensatory hypertrophy of the adrenal cortex. 3 $\beta$ -HSD deficiency affects both the adrenal and gonads with consequent impairment of sex steroid biosynthesis. A range of phenotypes are noted with 3 $\beta$ -HSD deficiency suggesting a range of complete to partial inhibition of activity. As such 3 $\beta$ -HSD deficiency may present as late onset CAH at the time of expected puberty (Rosenfield et al. 1980).

The role of  $3\beta$ -HSD expressed in the human ovary is to produce C19 steroids in the thecal cells that can be aromatised to oestrogens in the granulosa cells of the ovary. The enzyme is also expressed in the luteal cells of the ovary following ovulation where its role is extended to the production of both progesterone and oestrogens (Doody et al. 1990; Sasano et al. 1990). Towards menstruation expression of  $3\beta$ -HSD in the ovary declines presumed due to a loss of response to LH (Benyo et al. 1993) as it has been demonstrated that the primate corpus luteum requires LH for continued  $3\beta$ -HSD expression (Ravindranath et al. 1992).

In the placenta  $3\beta$ -HSD is expressed principally within the syncytiotrophoblast from as early as 6 weeks gestation (Riley et al. 1992). At this point the source of progesterone is still the corpus luteum. However, with advancing gestation there is an increase in the expression of  $3\beta$ -HSD that corresponds to the increase in plasma progesterone during this time (Riley et al. 1992). This suggests that the placenta synthesises progesterone with  $3\beta$ -HSD expression being a key factor in the steroidogenesis.

$3\beta$ -HSD activity has also been demonstrated in human (Tang et al. 1993) and rhesus macaque endometrium (Martel et al. 1994). Tang et al. suggested that expression may be progesterone regulated following an increase in enzyme expression following culture of endometrium with medroxyprogesterone acetate. A recent publication by Rhee et al. have now immunolocalised expression of the enzyme in the endometrial glandular epithelium with higher expression in the secretory phase than the proliferative phase (Rhee et al. 2003). The role that  $3\beta$ -HSD may play in endometrial function is not yet known.

Figure 1.10  
Summary of reactions catalysed by 3 $\beta$ -hydroxysteroid dehydrogenase. The highlighted area of the pathway indicates those reactions catalysed by 3 $\beta$ -hydroxysteroid dehydrogenase.



### 1.4.3 5 alpha reductase

5 $\alpha$ -reductase (5 $\alpha$ R) is multifunctional and catalyses the reduction of many steroid substrates (Wilson 1975) such as testosterone to the more potent dihydrotestosterone and progesterone to the inactive 5 $\alpha$ -pregnan-3,20-dione (Fisher et al. 1978). Two isoenzymes have now been cloned and characterised (Russell et al. 1994) and are designated 5 $\alpha$ R 1 and 5 $\alpha$ R 2 according to the order in which they were cloned. They play an essential role in the development of the male phenotype where there are differential roles for testosterone and the more potent androgen of dihydrotestosterone (DHT). DHT possesses an affinity for the AR four times higher than that of testosterone (Kovacs et al. 1984; Grino et al. 1990; Poletti et al. 1998). Moreover, the activated AR is also stabilized by DHT and DHT exhibits a fivefold slower dissociation rate from the hormone binding domain of the receptor than its precursor testosterone (Kovacs et al. 1984; Grino et al. 1990). Consequently, DHT activates transcription of androgen-dependent genes at concentrations significantly lower than testosterone. This ability would enable androgens to contribute to a local intracrine environment through formation of DHT in target tissues. To date little is known regarding the expression of 5 $\alpha$ R in human females and what role it may play.

The 5alpha-reductase type 1 gene (SRD5A1) encodes an isoenzyme with an alkaline pH optimum (Jenkins et al. 1991; Jenkins et al. 1992) whereas the 5alpha-reductase type 2 gene (SRD5A2) encodes an isoenzyme with an acidic pH optimum (Andersson et al. 1991; Thigpen et al. 1992). The two 5alpha-reductase isoenzymes can also be distinguished by their 4-azasteroid inhibitor, finasteride, pharmacokinetics as 5 $\alpha$ R 2 is inhibited by this compound (Liang et al. 1984; Vermeulen et al. 1989). Tissue distribution of the isoenzymes has been described with 5 $\alpha$ R 1 localised to the hair follicles, sebaceous glands and liver predominantly and 5 $\alpha$ R 2 localised to genital skin, seminal vesicles, epididymis, liver as well as being the only isoenzymes detected in the prostate (Thigpen et al. 1993). The use of finasteride as a 5 $\alpha$ R 2 inhibitor has significantly improved the outcome for patients with prostatic carcinoma, an androgen responsive carcinoma, whereby in combination with anti-androgens it will provide suppression of intra-prostatic DHT formation to inhibit androgen stimulation of the carcinoma (Prostate Cancer Trialist Collaborative Group 2000. 2000).

In physiological terms, androgens play an important role in the expression of the male phenotype with Testosterone and DHT having specific roles. Testosterone is required for differentiation of the Wolffian ducts, spermatogenesis and it provides feedback control for the secretion of Luteinising hormone. DHT is required for differentiation of the urogenital sinus and external genitalia as well as playing a role in sexual maturation at puberty (Wilson et al. 1993). The role of DHT in differentiation of the external genitalia is another area where 5 $\alpha$ R 2 has a direct clinical relevance. 5 $\alpha$ R gene mutations result in the failure of 5 $\alpha$ R 2 enzyme expression, contributing as one cause of the androgen insensitivity syndrome or male pseudohermaphroditism as the condition is alternatively called (Imperato-McGinley et al. 1974; Wilson et al. 1974; Brinkmann et al. 1996). The external genitalia require the more potent action of dihydrotestosterone (DHT) for the development of the male external genital phenotype during organogenesis and development whereas the internal genitalia, Wolffian ducts, are able to utilise testosterone for adequate differentiation. Within the families identified with 5 $\alpha$ -reductase gene mutations a group of females homozygous for the defect have been studied. In this small group the females were noted to have delayed menses and a higher than expected twin pregnancy rate (Katz et al. 1995). The authors suggest the delay in menarche implies a regulatory role for 5 $\alpha$ R 2 in the initiation of puberty at the hypothalamic/pituitary and/or gonadal level and in view of the twin pregnancy rate there may be a role for 5 $\alpha$ R 2 or DHT in ovarian follicular maturation (Katz et al. 1995). As yet no defect in 5 $\alpha$ R 1 gene expression has been published but murine studies suggest this enzyme has an important contribution for fertility and parturition (Mahendroo et al. 1999).

On the basis of tissue distribution and biochemical properties 5 $\alpha$ R 1 is thought to mainly act as a catabolic agent whereas 5 $\alpha$ R 2 is essentially an anabolic agent (Andersson et al. 1990; Normington et al. 1992; Wilson et al. 1993). No naturally occurring mutations have been described in the 5 $\alpha$ R 1 gene (Mahendroo et al. 1999). Therefore, to explore the physiological role of 5 $\alpha$ R 1 Mahendroo et al. generated mice with a null mutation in the 5 $\alpha$ R 1 gene. Male mice with this defect appeared to virilise normally and appeared to suffer no adverse consequences from the loss of this gene. It is thought the male compensates by utilising its 5 $\alpha$ R 2 activity. In humans and mice with 5 $\alpha$ R 2 gene defects the males fail to virilise properly but the female expresses a normal phenotype with no apparent consequences (Mahendroo et al. 1999).

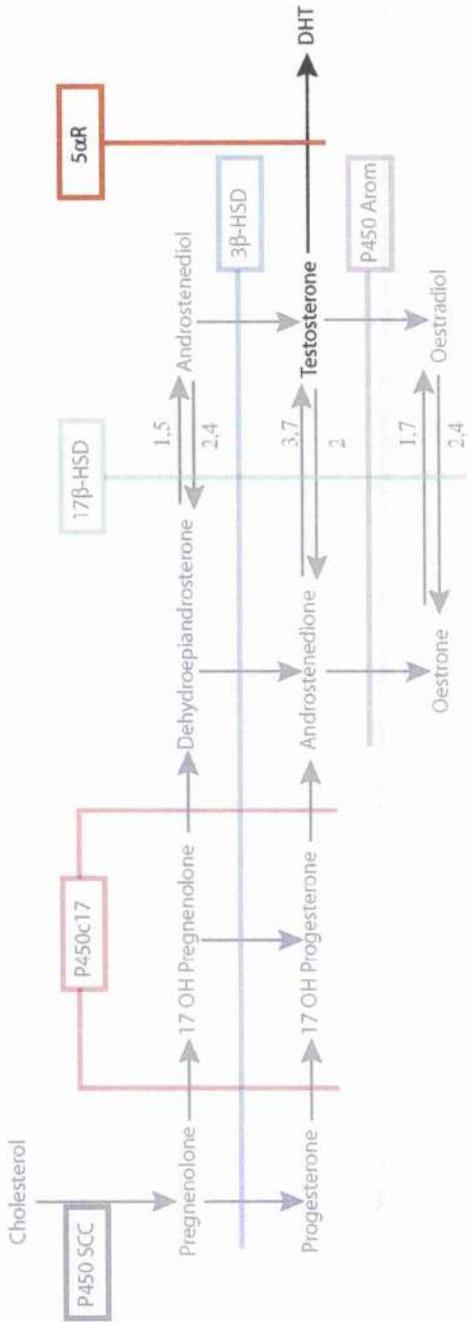
Mahendroo et al. in their review show that mice affected by the null mutation in 5 $\alpha$ R 1 suffer adverse reproductive outcomes and hypothesise that 5 $\alpha$ R 1 is the important isoenzyme in females whereas 5 $\alpha$ R 2 is the important isoenzyme for males (Mahendroo et al. 1999).

An adverse reproductive outcome has been noted in the 5 $\alpha$ R 1 knockout mouse. 70% of 5 $\alpha$ R 1 knockout mice died in utero (Mahendroo et al. 1997). Fertilisation, implantation and early fetal development were normal but at the time of 5 $\alpha$ R 1 gene induction in the decidua an increased rate of intrauterine death occurred. At this time an increase in the serum oestradiol levels was also noted, correlating with a surge in placental androgens at this gestation (Mahendroo et al. 1997). Mahendroo et al. hypothesise that this increase in androgenic substrate, that may have been metabolised by 5 $\alpha$ R1, would now alternatively be metabolised by aromatase leading to the increase in serum oestradiol at this time resulting in an intrauterine environment that was toxic to the fetuses (Mahendroo et al. 1997). In support of this hypothesis an oestrogen receptor antagonist and an aromatase inhibitor were able to prevent the intrauterine deaths (Mahendroo et al. 1997).

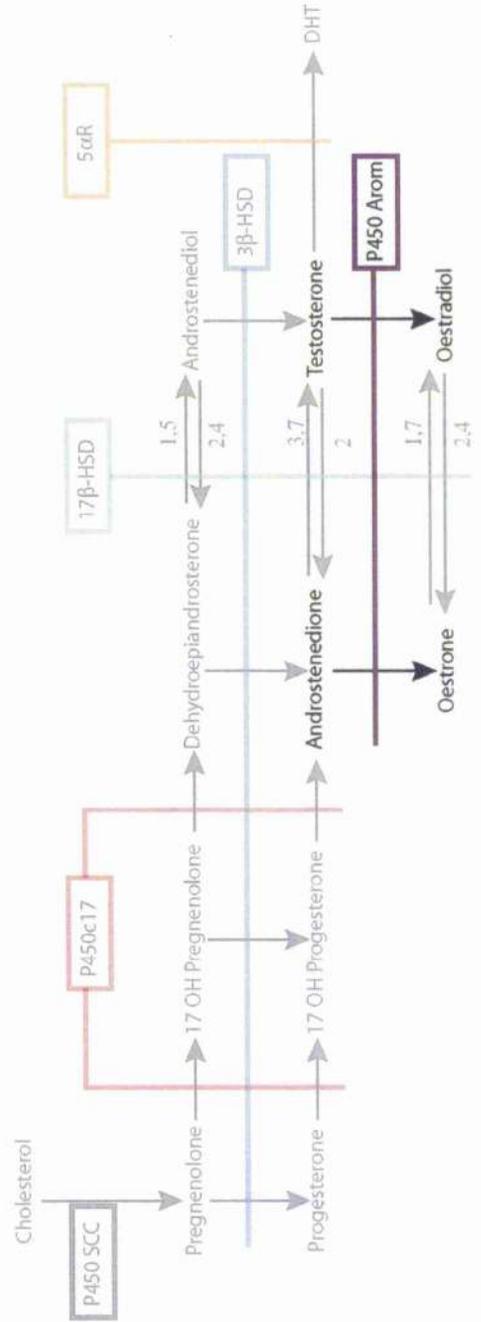
While not directly exploring the role of 5 $\alpha$ -reductase in the endometrium these studies have indicated that 5 $\alpha$ -reductase may have an important role to play in female reproductive biology. The only study to examine 5 $\alpha$ R expression in female reproductive tissues was published very recently by Ito et al (Ito et al. 2002). They described the immunolocalisation of 5 $\alpha$ R1 and 5 $\alpha$ R2 in the glandular epithelium of the endometrium with no temporal variation. Their sample numbers were small which may have precluded the demonstration of significant differences. In addition to normal endometrium, they examined 44 cases of endometrial carcinoma and determined that 5 $\alpha$ R was expressed in 80% of specimens. This would appear to confirm that 5 $\alpha$ R is present in normal cycling endometrium but the paper does not provide answers as to the role of 5 $\alpha$ R in normal or malignant endometrium.

**Figure 1.10**  
Summary of reactions catalysed by 3 $\beta$ -hydroxysteroid dehydrogenase. The highlighted area of the pathway indicates those reactions catalysed by 3 $\beta$ -hydroxysteroid dehydrogenase.

### 5-alpha Reductase Pathway



### Aromatase Pathway



#### 1.4.4 Aromatase

The primary source of oestrogen biosynthesis in the non pregnant pre-menopausal female is the ovary. Aromatase is the enzyme complex that catalyses the synthesis of oestrogens from androgens, and therefore it has unique potential to influence the physiological balance between the sex steroid hormones (Conley et al. 2001). The balance between androgen and oestrogen production is not only essential for normal sexual development and reproduction, but also for normal physiological well being. Abnormalities in female aromatase expression have been associated with disease processes such as breast cancer, endometrial cancer, and endometriosis.

Aromatase is the product of the CYP19 gene (Means et al. 1989; Harada et al. 1990) which is a member of the cytochrome P450 superfamily of genes (Simpson et al. 1997). Regulation of aromatase (P450arom) expression in humans is controlled by tissue specific promoters and alternative splicing techniques (Simpson et al. 1997; Simpson et al. 2002). Consistent with its fundamental biological importance, P450arom is highly conserved especially among mammals (Conley et al. 2001). Androstenedione and testosterone are the commonest and physiologically perhaps most important substrates for aromatase, being converted to oestrone and oestradiol respectively. However, in pregnancy 16 hydroxy-androstenedione arising from hepatic hydroxylation of foetal adrenal dehydroepiandrosterone sulphate (DHEAS), is an important substrate for placental oestriol synthesis (Siteri 1982).

Extra-gonadal oestrogen biosynthesis has been identified in mesenchymal cells of the adipose tissue and skin (Simpson et al. 1997), osteoblasts (Bruch et al. 1992), aortic smooth muscle cells (Sasano et al. 1999) as well as a number of sites in the brain (Naftolin et al. 1975). In postmenopausal women these extra-gonadal sites become the main contributors to serum oestrogen (Simpson et al. 1997). In support of the observation that post menopausal oestrogen levels are primarily the product of extra-gonadal oestrogen biosynthesis and adipose tissue in particular, Bulun et al. demonstrated increasing P450arom mRNA expression in adipose tissue with advancing age (Bulun et al. 1994). In pre-menopausal women the role of extra-gonadal aromatase will primarily be the provision of local oestrogen biosynthesis. This will allow high tissue concentrations that will likely exert a significant local influence in keeping with the principles of intracrinology (Labrie 1991).

Aberrant P450arom expression in tissues that do not normally have high concentrations of the enzyme is associated with many pathological conditions in humans. These include breast cancer (Bulun et al. 1997; Sasano et al. 1998), endometrial cancer (Bulun et al. 1997; Sasano et al. 1998), and endometriosis (Noble et al. 1996; Bulun et al. 1997; Kitawaki et al. 1997). Understanding what turns 'on' the expression of aromatase in disease or what causes a 'switch' in promoter usage is currently an area of intense investigation (Conley et al. 2001). The power of local oestrogen biosynthesis is illustrated in the case of postmenopausal breast cancer. It has been determined that the concentration of oestradiol present in breast tumours of postmenopausal women is at least 20-fold greater than that present in the plasma (Pasqualini et al. 1996). Overall, it is thought that about half of the oestrogen is produced locally within the tumour or surrounding tissue (Miller 1999). With aromatase inhibitor therapy, intra-tumoural concentrations of oestradiol and oestrone drop precipitously, together with a corresponding loss of intra-tumoural aromatase activity, consistent with this activity being within the tumour and the surrounding breast adipose tissue and being responsible for these high tissue concentrations (de Jong et al. 1997).

Initial studies investigating aromatase activity in human endometrium suggested that it may indeed possess such activity (Tseng et al. 1982; Neulen et al. 1987). However, the presence or absence of the enzyme in endometrium was still thought to be controversial with Bulun et al. unable to demonstrate aromatase activity nor mRNA using sensitive RT-PCR in normal endometrium, decidua or endometrial stromal cell culture (Bulun et al. 1993). The latter findings by Bulun et al. have been subsequently substantiated by other studies suggesting that aromatase is not expressed in normal human endometrium but is expressed in endometrium associated with pathological conditions such as endometrial cancer and endometriosis (Bulun et al. 1994; Noble et al. 1996; Kitawaki et al. 1997).

The expression of aromatase in pathological conditions, benign and malignant, affecting the endometrium is an important finding that may have important implications for the management of endometriosis and endometrial cancers with aromatase inhibitors (Sasano et al. 1999; Berstein et al. 2002).

## 1.5 The human uterus and endometrium

The uterus is a fibro-muscular target organ that is essential for human reproduction. Embryologically it is derived from the müllerian duct and it is composed of a smooth muscle outer layer lined by endometrium and is grossly divided in three anatomical portions; the body of the uterus, the isthmic portion and the cervix.

The body of the uterus constitutes the thickest layer with the largest proportion being smooth muscle. The smooth muscle cells are steroid responsive, undergoing hyperplastic and hypertrophic changes at key points in a women's reproductive life. Pre-puberty the body of the uterus is in equal proportion to the cervix and following puberty, under the influence of ovarian oestrogens, the body of the uterus becomes twice as large as the cervix. Holm et al. have demonstrated pubertal maturation of the uterus using ultrasound. They determined that uterine and ovarian volume increased before the onset of clinical puberty and continues several years after the menarche (Holm et al. 1995).

A significant change in uterine size occurs in pregnancy where the cells undergo extensive hyperplasia, hypertrophy, and remodelling under the influence of placental steroidogenesis; allowing the growing foetus to develop within the uterus.

The body of the uterus tapers towards its lower pole where the isthmic portion is in essence a transition zone that in late pregnancy forms the lower uterine segment. Through this zone the composition of the tissue gradually changes to become increasingly fibrous in nature as it becomes the cervix.

The endometrium lines the body of the uterus and is a dynamic steroid responsive tissue that must undergo rapid regeneration and remodelling each menstrual cycle to prepare for possible conceptus implantation. Human endometrium can be identified as three distinct layers: the superficial zona compacta, the intermediate zona spongiosa and a basal layer. The term functional layer refers to the compacta and spongiosa layers and it responds to the temporal variations in serum sex steroid levels as secreted by the ovary. Oestradiol initially drives proliferative change prior to progesterone mediating secretory phase differentiation. Following luteolysis and withdrawal of progesterone, menstruation occurs, shedding the functional layer (Cameron et al. 1996). The basal layer is in contact with the myometrium and undergoes little cyclic change throughout the menstrual cycle and is not sloughed during menstruation but provides the platform for regeneration of the functional layer following menstruation.

## **1.6 Endometrial Morphology during the Menstrual Cycle**

The menstrual cycle is divided into a pre-ovulatory proliferative phase and a post-ovulatory secretory phase that precedes menstruation. The proliferative and secretory stages are further divided into early-, mid-, and late- categories classically based upon the seminal work by Noyes et al. A number of researchers had produced papers detailing histological changes in endometrium through the menstrual cycle in both monkeys and human. Noyes et al., recognizing the need for a quantitative interpretation, reviewed papers produced regarding endometrial morphology in addition to their depth of experience to produce a guide for the dating of endometrial biopsies that is still referred to this day. The following is a brief description of the characteristics for each stage of the cycle (Noyes et al. 1950; Buckley et al. 1989).

### **1.6.1 Proliferative Phase**

#### **Early proliferative phase (day 4–day 7).**

Identified by a regenerating surface epithelium, especially between the mouths of the glands. Most of the straight, short, narrow glands are of proliferative type, as shown by epithelial mitosis. Some still show cuboidal, ragged, inactive epithelium in keeping with involution. The stroma is compact with stellate or spindle-shaped cells with scanty cytoplasm and anastomosing processes making the nuclei appear relatively large.

#### **Mid-proliferative phase (Day 8-Day 10)**

This phase is recognizable by columnar surface epithelium, longer, curving glands, and a variable amount of stromal oedema, which tends to regress. Mitosis are numerous in the stroma.

#### **Late-proliferative phase (Day 11-Day 14)**

Characterised by an undulant surface with tortuous glands and pseudostratification of epithelium. There is a moderately dense, actively growing stroma.

### **1.6.2 Secretory Phase**

#### **Early Secretory Phase (Post Ovulation Day (P.O.D) 1-Day 4)**

Subnuclear glycogen-rich vacuolation of the glandular epithelium becomes prominent in at least 50% of cells, lasting for up to 6 days post ovulation (Buckley et al. 1989). The nuclei lose the pseudostratified configuration with an accompanying increase in the diameter and tortuosity of the glands. Mitoses cease to be seen.

#### **Mid Secretory Phase (post ovulation Day 5-Day 9)**

Acidophilic intra-luminal secretions reach a peak. Stromal oedema is characteristically marked in the mid-secretory phase, peaking about day 8-9. The stroma at this phase looks like small, dense, nuclei with only filamentous cytoplasm. The spiral arterioles, previously somewhat difficult to distinguish in the oedematous stroma, become much more prominent peaking at day 9-10. These findings constitute the earliest visible pre-decidual change.

#### **Late Secretory Phase (post ovulation Day 10-Menses)**

Eosinophilic pre-decidual cells may be identified around the arterioles and stromal proliferation recurs, as evidenced by mitosis. The differentiation of pre-decidea is accompanied by a sharp increase in lymphocytic infiltration. Also accompanying the pre-decidual change there is stromal regression and loss of endometrial height (Buckley et al. 1989). In the absence of pregnancy, gland secretion diminishes to a variable extent, and involution of gland epithelium occurs. The glands are dilated and tortuous, and the epithelium is thrown into tufts causing the characteristic saw-toothed effect; but the previously tall columnar epithelium is now low, the nuclei are shrunken, and the cytoplasmic edges are ragged and indistinct.

### **1.6.3 Menstrual Phase**

The menstrual cycle commences with the first day of blood flow. The upper two thirds of the endometrium, the superficial layer, are shed. Endometrium in the early menstrual phase exhibits focal areas of sub-epithelial necrosis with subsequent glandular collapse and necrosis. As the menses cease a typical shallow dense endometrium remains that is composed of the basal layer and residual deeper functional layer. From the third day of the cycle regeneration commences in glands and stromal elements (Buckley et al. 1989).

## **1.7 Endometrial Sex Steroid Receptor Expression.**

The important role of oestrogen and progesterone in endometrial growth, differentiation and function has been long recognised. Their spatial and temporal distribution within the endometrium has been well characterised as researchers have attempted to unravel the field of endometrial biology. Other members of the classical nuclear receptor super family such as AR and GR have not been so intensely researched and hitherto their spatial and temporal localisation have not been well characterised in normal endometrium. In recent years it has also become apparent that even the well characterised ER and PR distribution will require further refining as subtypes and variants of the receptors are recognised and their localisation determined using monoclonal antibodies.

### **1.7.1 Oestrogen and Progesterone Receptor Expression**

The development of specific monoclonal antibodies to ER and PR in addition to improved antigen retrieval techniques has advanced immunohistochemical localisation. Immunohistochemistry has therefore been employed to determine receptor distribution in human endometrium. The ER and PR are immunolocalised to the nuclear compartments of cells with the description of their spatial localisation in broad terms divided between the superficial layer and basal layer of the endometrium. The layers are further categorised into stromal, glandular, endothelial, and perivascular regions. The following summary of receptor localisation will keep within this convention and will also cover the factors thought to regulate sex steroid receptor expression.

There are two isoforms of human PR known, PR-A and PR-B, which arise from the same gene (Clarke et al. 1990). The A isoform 779 amino acids (aa) in length is a truncated version of the B isoform that is 933 aa in length, the truncation of 164 aa being from the N terminal end of the protein (Tung et al. 1993). There are also two distinct subtypes of ER that arise from two different genes: ER $\alpha$  (Kuiper et al. 1996) and ER $\beta$  (Enmark et al. 1997). ER $\beta$  was originally cloned from rat prostate (Kuiper et al. 1996) and later in the human (Mosselman et al. 1996), with the classical ER now designated ER $\alpha$ .

Temporal variation in the expression of the ER and PR was initially detailed through studies by Lessey et al. comparing monoclonal antibody immunolocalisation with ligand binding studies (Lessey et al. 1988). These data revealed an increase in ER concentrations throughout the proliferative phase peaking in the early secretory phase of the cycle; thereafter the ER concentrations decline. A parallel but delayed increase in the PR was also described with a decline in the secretory phase of the cycle. Further immunohistochemical studies have demonstrated the nuclear localisation of the ER and PR in the endometrial compartments. In the stromal and glandular compartments, ER expression was maximal in the late proliferative phase. In the secretory phase, ER expression declined in the glandular and stromal compartments, with the ER decrease in the glandular compartment being more gradual. The PR also exhibits marked immunoreactivity in the nuclei of glands and stroma during the proliferative phase. In the secretory phase this immunoreactivity declines in the glandular compartment with a modest persistence of stromal immunoreactivity (Lessey et al. 1988; Snijders et al. 1992; Critchley et al. 1993).

With the development of two anti-progesterone receptor antibodies, a specific polyclonal raised against PR-B and the other a commercially available monoclonal raised against PR subtype A+B, it has been possible to refine the cellular localization of PR subtype A and B in the normal endometrium throughout the menstrual cycle. As PR-A is a shorter splice variant it was not possible to raise an antibody specific to it alone, all immunohistochemical analysis of PR subtype A has further to been determined by subtractive inference (Wang et al. 1998). Using subtractive inference it was determined that the isoform of PR that mainly persists in the secretory phase stroma is PR-A (Wang et al. 1998). In addition, both PR-A and -B are present in a perivascular location in the secretory phase, but not in the proliferative phase. Their expression supports a role of progesterone action either directly or indirectly influencing growth, development and function of the spiral arterioles in this phase of the menstrual cycle (Wang et al. 1998; Brosens et al. 1999).

It is also now possible to localise ER $\alpha$  and ER $\beta$  using monoclonal antibodies. ER $\alpha$  and ER $\beta$  are both immunolocalised in the nuclei of the glandular epithelial and stromal cells of functional and basal endometrium in the proliferative phase. The expression of ER $\alpha$  declines in the glandular and stromal of the functional layer while expression of ER $\beta$  declines in the glandular cell nuclei, but not stroma, during the

secretory phase. Levels of expression of ER $\alpha$  and ER $\beta$  in all cellular compartments remain unchanged in the basal layer (Critchley et al. 2001).

Interestingly, unlike ER $\alpha$ , ER $\beta$  was present in the nuclei of the endothelial cells of most blood vessels, including spiral arteries, capillaries, and veins. The intensity of immunoreactivity of ER $\beta$  in the endothelial cells shows little change throughout the proliferative and secretory phases. These data supported the hypothesis that oestrogen action for regulation of the vasculature and angiogenesis is mediated via binding to ER $\beta$  (Critchley et al. 2001).

### **1.7.2 Androgen Receptor Expression**

Data available describing the immunolocalisation of AR has been conflicting reflecting the variety of antibodies and immunohistochemical techniques employed. The presence of an androgen receptor in human endometrium was initially shown with ligand binding studies (Muechler 1987; Fujimoto et al. 1994). However, subsequent immunolocalisation studies using the mouse monoclonal antibody (F39.4) localised AR in a variety of human tissues, but no receptors were localised to the uterus (Ruizeveld de winter et al. 1991). Horie et al., using rat anti-human monoclonal antibody, then reported the presence of the receptor in the ovary throughout the menstrual cycle (Horie et al. 1992). Using the same monoclonal antibody, this group found the androgen receptor to be present in endometrial glands and stroma throughout the menstrual cycle. The endometrial immunoreactivity was mainly in the functional layer with less in the basal layer (Horie et al. 1992). Kimura et al. and Takeda et al. also found immunoreactivity in endometrial stroma and glands but Takeda et al. less so in the glands (Takeda et al. 1990; Kimura et al. 1993). Further contradiction arose when Chadha et al. also used the mouse monoclonal antibody, F39.4, to compare the endometrium of transsexual women on testosterone with the endometrium of pre-menopausal women to look for evidence of AR up-regulation. This study indicated absent or focal areas of AR immunoreactivity in the endometrium of pre-menopausal women compared with more intense and diffuse immunoreactivity in the testosterone treated patients. In both groups the glandular epithelium was negative for AR (Chada et al. 1994). Recently Mertens et al. has localised the receptor to stromal cells with the most profound immunoreactivity in the basal layer and only minimal immunoreactivity in the

glandular epithelium. This study also found no immunoreactivity in the late secretory phase indicating there may be temporal variation in expression within the menstrual cycle (Mertens et al. 1996). Mertens et al followed this study with further work in 2001 confirming their previous findings (Mertens et al. 2001).

Most studies confirm the presence of AR in the uterus. The description of a stromal distribution of AR is not consistent but the more recent studies suggest that the most abundant immunoreactivity is within the stromal areas of the basal layer. The glandular epithelium appears to stain only very weakly for AR. As stated earlier the differences between these studies may be due to variations in immunohistochemical protocols and the different antibodies used.

Unlike the functional variants for ER and PR, AR has not yet been shown to have functional variant isoforms. Wilson et al. identified A (87k) and B (110-112K) isoforms of the androgen receptor. Both isoforms were distributed widely in the body with the longer AR-B isoform being the more common. The isoform pattern is similar to the progesterone receptor, which also has two isoforms derived from the same gene producing a longer B isoform, and a shorter N terminal truncated A isoform. The presence of two isoforms suggests that each may effect a different function depending upon the tissue or cell where it is sited (Wilson et al. 1996). However, the possibility that the AR-A isoform might be a proteolysed product of AR could not be ruled out. As no follow-up studies were reported about the N-terminally truncated isoform of AR, the concept of AR isoforms is not widely accepted (Lee et al. 2003).

With improvements in immunohistochemical techniques it would be hoped that a greater consistency in the localisation of AR may emerge. Further work is necessary to characterise the spatial and temporal variation in expression of the AR in human endometrium through the menstrual cycle in addition to confirming that protein expression is substantiated by evidence of AR mRNA production.

### **1.7.3 Regulation of Endometrial Sex Steroid Receptors**

#### **1.7.3.1 Oestrogen and Progesterone Receptor Regulation**

The PR is controlled by both oestrogen and its own ligand progesterone. In the oestrogen dominated proliferative phase PR is up regulated secondary to the oestradiol up regulation of PR mRNA via an oestrogen response element that results in increased PR protein synthesis (Clarke et al. 1990). In the progesterone dominated secretory phase it's own ligand, progesterone, initiates down regulation at both the transcriptional, via the same oestrogen response element (Savouret et al. 1991), and post transcriptional levels (Chauchereau et al. 1992).

In keeping with the regulation of PR, ER is controlled by its own ligand, oestrogen, and progesterone. In the proliferative phase, oestradiol interacts with the oestrogen response element up regulating the production of ER mRNA with resultant increase in receptor levels. In the secretory phase, progesterone initiates down regulation of ER at the ER mRNA level via interaction with its hormone response element resulting in reduced ER (Clarke et al. 1990).

#### **1.7.3.2 Androgen Receptor Regulation**

While the functional significance of the androgen-AR receptor pathway in the male reproduction system is well studied, that in the female has been little understood due to the lack of a proper model system. The generation of an AR knockout (ARKO) female mouse model has long been hampered, because the AR gene is located on the X chromosome (Lee et al. 2003). However, recently ARKO female mice have been successfully generated (Yeh et al. 2002). The study demonstrated that homozygous ARKO female mice produced a smaller number of pups per litter with an average of four, whereas the counterpart wild-type female mice produced an average of eight pups per litter. This result indicates that AR may play a role in female fertility and/or ovulation (Yeh et al. 2002). Further study indicated that homozygous ARKO female mice suffer from defective folliculogenesis due to poor follicular maturation and defective implantation due to subnormal uterine development (Lee et al. 2003).

In human endometrium data regarding the regulation of the AR is, similar to its immunolocalisation, conflicting and lacking in clarity. Research has been carried out to determine possible mechanisms regulating the androgen receptor expression

with many studies being ligand binding studies and only a few immunohistochemical studies. No quantitative methods of determining mRNA have been employed.

Fujimoto et al. studied women given an intramuscular injection of oestradiol dipropionate (ED) with or without testosterone cypionate (TC) 1 week prior to hysterectomy. ED alone significantly increased AR mRNA and testosterone binding sites but when administered with TC the testosterone binding sites were significantly reduced and the AR mRNA level showed a non significant increase. Suggesting that while TC significantly reduced testosterone binding sites it had no significant effect on AR mRNA levels(Fujimoto et al. 1995).

Iwai et al experimenting on endometrial stromal cell cultures found that progesterone and testosterone independently were able to decrease ER mRNA, PR mRNA, and AR mRNA levels in a dose dependant manner (Iwai et al. 1995). It is difficult to compare the Fujimoto et al. results with those of Iwai et al. as the patients in Fujimoto's study were part of an in-vivo study and the patients received ED as well as TC. However, the Iwai et al. study did show a decrease in AR mRNA levels occurred after 9 days in culture. This finding may explain the difference with Fujimoto's results as these patients were operated upon only seven days after their testosterone injections.

Studies regarding AR regulation in endometrium have been carried out in Rhesus monkeys. In 1999 Adesanya-Famuyiwa et al. showed a non-significant increase in AR mRNA with oestradiol treatment alone but significant increases were seen in stromal, glandular and myometrial compartments when oestradiol was given in combination with progesterone or testosterone (Adesanya-Famuyiwa et al. 1999).

More recent immunohistochemical data is available from Mertens et al. and Chadha et al. Mertens et al., while not examining regulation directly, described temporal variation in expression of the AR with a down regulation of the receptor in the late secretory phase (Mertens et al. 1996; Mertens et al. 2001). As the reduction in receptor expression occurs during the progesterone dominated secretory phase, it may be suggested that the down regulation is secondary to a progesterone regulated mechanism. Chada et al. were able to obtain the uteri of patients receiving testosterone supplementation as a component of treatment to become a transsexual. In this situation where testosterone was administered in a continuous regimen the

patients exhibited more intense AR immunoreactivity compared with controls suggesting possible androgen mediated up-regulation (Chada et al. 1994).

In summary, a consistent increase in AR mRNA is seen when estrogen is given with progesterone or testosterone. When oestradiol is given alone a significant increase may occur in AR mRNA in addition to increasing testosterone binding sites. When progesterone is administered alone, a decrease in AR mRNA is noted. However, when testosterone is administered alone a decrease or increase in the AR has been observed. In some aspects it would appear to parallel the pattern of progesterone receptor regulation. PR is up regulated by oestradiol and is down regulated by its own ligand, progesterone. There is yet no clarity in this field with further work required to establish the mechanism of AR regulation.

## 1.8 Menstruation

Menstruation is a physiological event involving shedding of the superficial layer of the endometrium producing cyclical uterine bleeding. Normal menstruation occurs with the fall in progesterone levels secondary to luteal regression. The mechanisms regulating menstruation continue to be the subject of intense research despite the gross physiological events being known for over sixty years.

Classic studies unlikely to be reproduced were conducted by Markee in the rhesus monkey. Endometrial explants were transplanted into the anterior chamber of the eye allowing direct visualisation of stromal and vascular events following progesterone withdrawal. The events noted include stromal shrinkage, increased coiling of spiral arterioles and vascular stasis. Subsequently there was a period of vasodilatation and perivascular bleeding followed 24 hrs later by intense vasoconstriction, tissue necrosis and menstruation (Markee 1940).

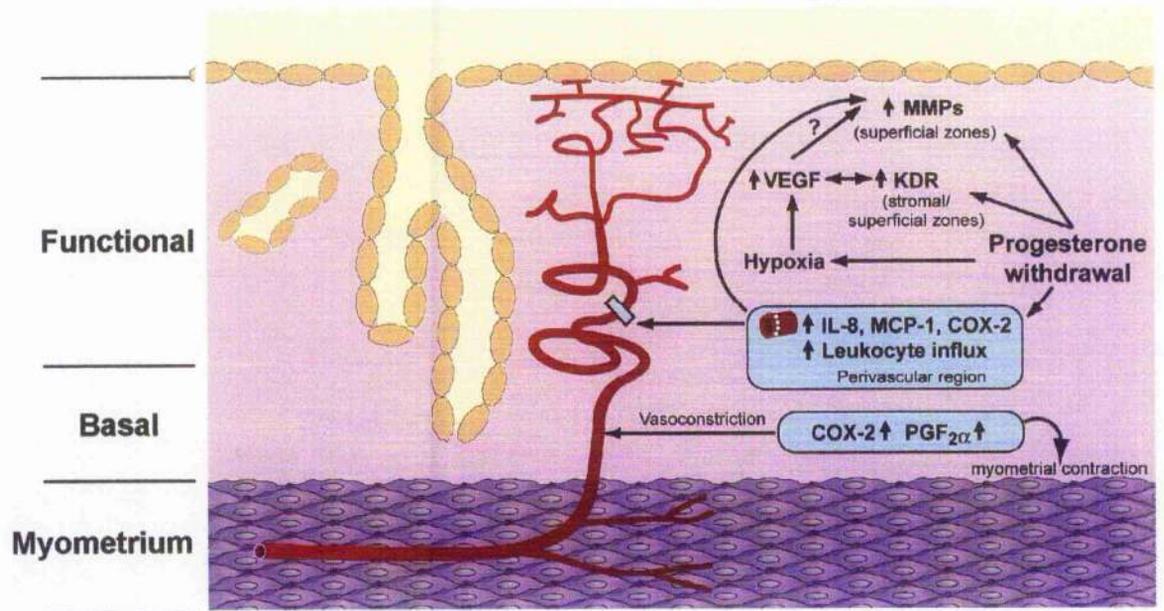
The sequence of events resulting in menstruation is initiated if the oocyte released at ovulation is not fertilised. The failure of HCG secretion in the absence of a conceptus leaves no drive for the corpus luteum to continue steroidogenesis, resulting in luteal regression and a fall in serum progesterone. Following progesterone withdrawal in the secretory phase there is an increase in the stromal density of a unique population of leukocytes named uterine NK cells (CD 56<sup>Bright</sup> CD 16<sup>+</sup>) associated with an increase in local cytokine production (Loke et al. 1995; King 2000) and MMP expression. The association of leukocyte invasion, cytokine release and MMP expression has led to menstruation being described as an inflammatory event (Finn 1986; Kelly 1994; Critchley et al. 2001).

It is hypothesised by Kelly et al. (Kelly et al. 2001) that menstruation is a biphasic event with an initial progesterone dependant phase followed by a progesterone independent phase. In the initial phase up-regulation of inflammatory mediators such as cytokines, prostaglandins and vasoconstriction are potentially reversible progesterone dependant mechanisms. The subsequent phase is then inevitable with the changes at this point occurring independently of progesterone withdrawal and likely secondary to endometrial hypoxia. Figure 1.12 provides an illustrative overview of the induction of menstruation.

Figure 1.12

Illustration summarising the effects of progesterone withdrawal and hypoxia on the mechanisms that may be involved with induction of menstruation. Progesterone withdrawal results in an up-regulation of inflammatory mediators, production of MMPs, a leukocyte influx and expression of stromal KDR in the upper endometrial zones. There is coincident hypoxia and an up-regulation of VEGF. VEGF binds to its type 2 receptor, KDR and there is a paracrine/autocrine action on the up-regulation of MMP production in the same endometrial upper zone stromal cells. Menstrual sloughing takes place from the functional layers of the endometrium (Critchley et al. 2001).

## Menstrual Induction Hypothesis



### 1.8.1 Progesterone dependant mechanisms in menstruation

In the initial phase, the withdrawal of progesterone modulates factors that will encourage leukocyte recruitment and activation in the endometrium in addition to factors that induce changes in vascular permeability, such as prostaglandins.

Leukocyte influx during the secretory phase consists of uterine NK cells, neutrophils, mast cells, macrophages and other haematopoietic cells. It is likely that this influx will represent a combination of in-situ proliferation and migration from the peripheral circulation (Salamonsen 2000). Regulation of leukocyte proliferation and migration is not yet clear. Factors that have been examined include sex steroids, cytokines and prostaglandins.

In a hormonally driven target organ such as the endometrium it would be anticipated that sex steroids would play a key role in events such as leukocyte migration. However, ER $\alpha$  and PR have not been immunolocalised in the major perimenstrual leukocyte, the uterine NK cell (King et al. 1996; Stewart et al. 1998), suggesting these sex steroid receptors have no direct role. The PR is however prominent in a perivascular location (Perrot-Aplanat et al. 1988; Perrot-Aplanat et al. 1994; Wang et al. 1998) with an absence of endothelial cell immunoreactivity (Kohnen et al. 2000) suggesting that indirect mechanisms for progesterone action may exist within the perivascular region. A role for AR, the other major sex steroid, has not as yet been examined.

The cells that compose the perivascular region are heterogeneous (Roberts et al. 1992) with the main type appearing to myofibroblasts, they express smooth muscle  $\alpha$ -actin and the fibroblast marker vimentin (Kohnen et al. 2000), a quality shared by decidual cells. The perivascular region is known to be a rich source of inflammatory mediators such as prostaglandins and cytokines. Key inflammatory mediators such as Cyclooxygenase 2 (COX-2), Interleukin-8 (IL-8) and Monocyte Chemotactic Peptide-1 (MCP-1) have all been localised to the perivascular region with significant up regulation in the late secretory phase (Jones et al. 1997). Prostaglandins are also up regulated in the secretory phase of the menstrual cycle (Smith et al. 1981) with PGE in particular expressed in a perivascular location (Cheng et al. 1993). Progesterone stimulation of the perivascular region may be responsible for the prostaglandin and cytokine production.

Evidence for progesterone regulation of these inflammatory mediators is available. All are up regulated in the late secretory phase, a point when progesterone levels are in decline, coincident with leukocyte accumulation in the endometrium (Jones et al. 1997). In vivo administration of the anti-progestogen RU486 (Mifepristone) when decidualised endometrium is present results in significantly increased expression of PGE in the perivascular region of the decidua (Cheng et al. 1993). Similarly, up regulation of IL-8 is also noted in endometrium (Critchley et al. 1999) and decidua (Critchley et al. 1996) following administration of mifepristone.

The up-regulated cytokines and prostaglandins are believed to act synergistically in the proposed mechanisms of menstruation (Kelly et al. 2002). IL-8 is chemotactic for both neutrophils and NK cells. It is produced by monocytes and fibroblasts and in endometrium it has been localised to the perivascular region (Critchley et al. 1994; Jones et al. 1997; Milne et al. 1999). MCP-1 is also produced by monocytes, fibroblasts and additionally by epithelial cells. It has been localised to the perivascular region in human endometrium (Jones et al. 1997; Critchley et al. 1999) and is chemotactic for both monocytes and NK cells. An in-vivo experimental model of progesterone withdrawal demonstrated up-regulation of IL-8 and COX-2 mRNA and protein 48 hours following progesterone withdrawal (Critchley et al. 1999).

The peri-menstrual influx of haematopoietic cells releases more cytokines providing further signals for leukocyte migration. The leukocytes release enzymes that play direct and indirect roles in menstruation. Secretory products of leukocytes such as elastase digest elastin or certain collagen types (Salamonsen et al. 2000). Indirect mechanisms involve the activation of Matrix Metalloproteinases (MMP). MMP's are enzymes that can degrade the extracellular matrix (ECM) and basement membranes of tissues but are not usually synthesised in normal connective tissue (Birkedal-Hansen et al. 1993). However, they have been reported as key enzymes in the breakdown of the endometrial ECM with a focal distribution in the peri-menstrual phase (Salamonsen et al. 1999). The focal MMP expression suggested local regulation and due to the spatial and temporal distribution of leukocytes within the endometrium they must be considered as a possible mechanism of regulation. Leukocytes may directly release MMP's and indirectly via paracrine interactions

they can induce endometrial cells to produce MMP or activate MMP's secreted by local cells (Salamonsen et al. 2000).

In-vitro experiments have shown sex steroids to be regulators of MMP expression with up-regulation of MMP's following progesterone withdrawal. The action of progesterone upon the oestrogen primed endometrium inhibits MMP-1, a collagenase, preventing degradation of the ECM but upon its withdrawal the up regulation of MMP-1 allows ECM degradation which is thought to be a key element of menstruation (Lockwood et al. 1998). Evidence also exists for progesterone regulation of MMP-2, a gelatinase, in-vitro data showing up-regulation following withdrawal of progesterone (Irwin et al. 1996).

Other inflammatory mediators that are regulated by progesterone include the prostaglandins (Abel et al. 1980). Elevated levels of which are found in the endometrium (Pickles 1957; Downie et al. 1974). Indeed prostaglandin synthetase inhibitors remain a component of the management strategies for the treatment of menorrhagia (Cameron et al. 1990; Fraser 1992) and their role in menstruation is reviewed by Baird et al (Baird et al. 1996). Prostaglandin synthetase has an isoform, Cyclooxygenase 2 (COX2), which is expressed in human endometrium. The enzyme is inducible by a variety of factors and inhibited by progesterone and glucocorticoids (Baird et al. 1996). Highest levels of COX2 have been shown in the menstrual phase (Jones et al. 1997) in keeping with the secretory phase decline in serum progesterone levels. Further evidence of its progesterone regulation is shown data from Hapangama et al. where administration of mifepristone in the mid luteal phase produced a significant increase in the expression of COX2 (Hapangama et al. 2002). In addition, this study also showed that a significant decline in the luteal phase expression of Prostaglandin dehydrogenase (PGDH) following administration of mifepristone (Hapangama et al. 2002). PGDH is responsible for the inactivation of prostaglandins and its down regulation will therefore result in elevated levels of prostaglandins. COX2 products that appear to be of particular importance are PGE-2 and PGF-2 $\alpha$ . PGE-2 is a vasodilator that acts synergistically with bradykinin to increase oedema and pain (Williams 1979). PGF-2 $\alpha$  is a vasoconstrictor that stimulates myometrial and other smooth muscle contractility; and intrauterine administration induces menstruation (Lundstrom 1977; Martin Jr et al. 1978).

A number of key events in the peri-menstrual phase are under the influence of progesterone. Progesterone withdrawal, as first described by Markee, induces vasoconstriction of the spiral arterioles and up-regulates an inflammatory response. The prostaglandins up-regulated in the inflammatory response may also be the mechanism of vasoconstriction. This however has yet to be determined with other candidates having been proposed, such as endothelins (O'Reilly et al. 1992) and angiotensin II (Ahmed et al. 1995). Of particular interest the perivascular stromal cells are reported to be intensely immunoreactive for angiotensin II, a region that also expresses the progesterone receptor. Slayden et al. also provide data to support this hypothesis. In their paper if progesterone is "added back" within 36 hours of progesterone withdrawal, menstruation can be prevented. If progesterone is added back more than 36 hours following progesterone withdrawal then menstruation is inevitable (Slayden et al. 1999). It is therefore after this period that progesterone independent mechanisms are important in the menstrual process.

### **1.8.2 Progesterone Independent mechanisms in menstruation**

Progesterone dependent events include mechanisms that produce hypoxic conditions within the endometrium. In the premenstrual phase, constriction of the spiral arteries after progesterone withdrawal induces a local endometrial hypoxic injury. In this phase it has been shown that prior to the onset of bleeding there is degeneration in the tissue architecture supporting the decidualised endometrial cells and the endothelium of the endometrial blood vessels (Roberts et al. 1992). The degradation of the extracellular matrix (ECM), as previously noted, is likely to involve the MMP's. A close association between menstruation and MMP activity has been shown (Salamonsen et al. 1996; Hulboy et al. 1997; Rudolph-Owen et al. 1997). The greatest MMP activity was noted in the pre-menstrual and menstrual phases with activity confined to the functional layer of the endometrium which is shed during menstruation. Progesterone has a role to play in the control of MMP activity but P withdrawal is a generalised endocrine action and MMP up regulation in endometrium is focal (Salamonsen et al. 1996). It has been hypothesised that the hypoxia produced by spiral artery constriction, which is greatest in the superficial functional layer, may be the event that initiates the cascade culminating in MMP up-regulation and ECM degradation.

Hypoxia also stimulates local mediators including cytokines and Vascular endothelial growth factor (VEGF). VEGF is present in human endometrium through the menstrual cycle (Shifren et al. 1996) with up-regulation noted in the menstrual phase (Charnock-Jones et al. 1993; Sharkey et al. 2000). In addition to being up-regulated under hypoxic conditions (Popovici et al. 1999; Sharkey et al. 2000) VEGF has also been shown to up-regulate MMPs (Unemori et al. 1992; Wang et al. 1998). The close spatial (functional layer) and temporal association (premenstrual phase) of VEGF and MMP's was described by Nayak et al. (Nayak et al. 2000).

Nayak et al described the up-regulation of the VEGF receptor type2 (KDR) in the stromal cells of the functional layer of the endometrium following progesterone withdrawal. KDR is normally found only in endothelial cells but in this case was significantly up-regulated in stromal cells of the upper endometrial zones. Hypoxia is not thought to directly induce such effects (Gerber et al. 1997). It was therefore hypothesised that the up-regulation of KDR may be as a consequence of hypoxia induced VEGF up-regulation (Sharkey et al. 2000) indirectly up-regulating KDR. Therefore, as VEGF can affect MMP expression the up-regulation of KDR is a possible mechanism whereby VEGF can produce such a ligand binding effect.

Hypoxic induction of the VEGF-MMP pathway contributes to other proposed factors where expression is influenced by the premenstrual phase. These other factors include tumour necrosis factor (TNF)- $\alpha$  (Tabibzadeh 1996), prostaglandins (Baird et al. 1996) and tissue plasminogen activator (Schatz et al. 1995). The role of, and interplay between, these mechanisms with regards to menstruation remain to be resolved.

Progesterone does appear to have an important role to play as its withdrawal following luteal regression initiates a cascade of factors that are implicated in the mechanism of menstruation. Hitherto a role for the androgens has not been suggested or investigated. Chapter 1.7.2 describes the expression of the AR in human endometrium and there is evidence to suggest that it may be temporally expressed with a down regulation in the late secretory phase. If the reduced availability of AR is confirmed in the late secretory phase, androgens will have a diminished ability to influence endometrial function which may be of significance with regards to menstruation.

## **1.9 Decidualisation**

In humans, the ability of the endometrium to undergo the morphological changes that result in decidualisation is a key event to allow blastocyst implantation. Despite decidualisation being an important intrauterine event, little is known regarding the exact mechanisms involved (King 2000).

The precise role of decidualisation in allowing blastocyst implantation and successful placentation is unknown. A possible role may be to control the extent of maternal spiral arteriole invasion by trophoblast cells (Osteen 1998; Pijnenborg 1998). The importance of such a control mechanism is exhibited in patients where trophoblast invasion exceeds the limit of the decidua and becomes morbidly adherent to the myometrium (Placenta Accreta) or invades through the entire myometrial wall of the uterus (Placenta Percreta). Such clinical problems are potentially life threatening due to hemorrhage and are more common when implantation occurs at sites where there may be defective decidualisation such as the uterine cornu or at the site of a caesarean section scar. Decidual trophoblast interaction may therefore alter matrix metalloproteinase (MMP) expression, cytokine production, surface amounts of integrin and major histocompatibility complex (MHC) molecules (Loke et al. 1995). While the mechanisms that initiate decidualisation are not yet understood, the key role of progesterone acting upon oestrogen primed tissue is well recognized (Maslar 1988). Key morphological and functional changes occur in endometrial stromal cells, glandular epithelium, spiral arterioles and endometrial leukocyte population.

### **1.9.1 Stromal Cells**

Stromal cells enlarge mainly due to an increase in cytoplasm that stains pale pink with haematoxylin and eosin. The stromal cells produce prolactin (Jones et al. 1998), insulin-like growth factor binding protein 1 (IGFBP-1) (Pekonen et al. 1992; Rutanen et al. 1997), and tissue factor among other substances. The exact role that this change in function plays is not yet known. An additional functional change that is also not fully understood is the formation and deposition of extracellular matrix proteins, such as fibronectin, in a peri-cellular manner. A proposed function is that this provides a framework for trophoblast cells to manoeuvre towards spiral

arterioles and myometrium. Progesterone does however appear to play a critical role in such functional changes (Lokc et al. 1995).

### **1.9.2 Glandular Epithelium**

The glands in decidualised endometrium, in contrast to their late secretory saw tooth appearance, continue to produce glandular secretions especially in the lower third of the endometrium where stromal cells are not so evident (Decidua Spongiosa). The upper two thirds of the decidua (Decidua Compacta) stops secretion and the glands become attenuated (King 2000).

### **1.9.3 Spiral arterioles**

The spiral arterioles, in keeping with other endometrial components, undergo dynamic changes through the menstrual cycle. From thin walled vessels in the proliferative phase they obtain actin rich thicker walls as the menstrual cycle advances also increasing in tortuosity (Kam et al. 1999). While the arterioles may become more prominent, there is no angiogenesis on the same level as visualised in the placentation of other species. Instead, human placentation involves the destruction of existing arterial walls and subsequent dilation of the vascular space.

### **1.9.4 Endometrial Leukocytes**

Endometrial leukocyte populations vary throughout the menstrual cycle. However, standard morphological stains such as haemotoxylin and eosin will not reveal leukocytes, therefore Immunohistochemical localisation with leukocyte markers is necessary (King et al. 1998).

In the human endometrium, spatial differences in leukocyte populations are noted. The basal layer contains lymphoid aggregates populated by mainly T cells with occasional B cells. The functional layer contains a scattering of uterine Natural Killer (uNK) cells, T cells and B cells. Of this group the macrophages show an increase pre-menstrually and significant increase at the implantation site. The uNK cells are present in low numbers during the proliferative phase but during the secretory phase they increase significantly in number until the pre-menstrual phase, in the absence of pregnancy (King et al. 1989).

In decidua, uNK cells remain in significantly increased numbers if pregnancy occurs and in particular in the basal decidua where trophoblast cells are also greatest in number. The uNK cells appear to be a distinct cell type different from those NK cells found in the systemic circulation. Other terms for this unique cell type include large granular lymphocytes (LGL) (King et al. 1991), endometrial granulocytes (Hamperl et al. 1958), K cells (Spornitz 1992), endometrial granulated lymphocytes (Bulmer et al. 1991), or decidual granulated lymphocytes (Deniz et al. 1996). To encompass all terms this cell type is now called the uterine NK cell and this cell is the predominant leukocyte at the time of decidualisation (King 2000). The uNK cells are distinguished from systemic NK cells by their surface cluster determination (CD) markers. Uterine NK cells express high levels of the universal NK cell marker CD 56 and do not express CD 16 (CD 56<sup>Bright</sup> CD 16<sup>-</sup>) whereas systemic NK cells have low concentrations and do express CD 16 (CD 56<sup>Dim</sup> CD16<sup>+</sup>) (King 2000).

High levels of uNK cells at the time of decidualisation suggest that they may have an important role to play in the process. uNK cells increase in number during the early secretory phase, particularly around both arteries and glands. The proportion of uNK cells in the stromal compartment reaches 30-40% by the late secretory phase. Despite their rise and persistence in decidua occurring at the time of progesterone dominance, in addition to studies showing that progesterone can increase CD 56 cell numbers (Inoue et al. 1996), it would not appear to be an event directly regulated by progesterone. uNK cells have not been shown to express classic genomic sex steroid receptors (King et al. 1996; Stewart et al. 1998). Recently, Henderson et al. have described the expression of ER $\beta$  and Glucocorticoid receptor in uNK cells (Henderson et al. 2003). However, it may be that progesterone has an important paracrine influence in surrounding stromal cells, which may in turn influence uNK cell function, as the stromal cells express PR at this time.

The role that uNK cells play in the decidualisation process is unknown. Their importance may be inferred from studies in patients with recurrent miscarriage (Lachapelle et al. 1996) or failed implantation with in vitro fertilisation therapy (Fukui et al. 1999). In both groups, mid secretory endometrium revealed altered leukocyte profiles when compared with normal. The endometrium had fewer CD 56<sup>Bright</sup> CD 16<sup>-</sup> and more CD 56<sup>Dim</sup> CD16<sup>+</sup> cells. This suggests that the altered profile produces a hostile implantation environment, leading to fetal loss. Further data to

support a role for CD56 cells in decidualisation are available from murine studies where similar cell types are noted. Mice lacking these cell types showed a failure of decidualisation and a lack of spiral arteriole development (Ashkar et al. 2001).

The increase in uNK cells in the progesterone dominated secretory phase in addition to their persistence in decidualised endometrium would tend to suggest they are likely to play an important role but the precise nature of their regulation and the role played in endometrial physiology is yet to be determined.

## 1.10 Implantation

Blastocyst implantation is the initiating event in a 9-month long complex, highly coordinated and controlled maternal-embryonic interaction. Among the initial events important for implantation is ovulation allowing release of an oocyte and formation of the corpus luteum that will drive the progesterone dominated secretory phase. As discussed in Chapter 1.9 the endometrium undergoes morphological and functional differentiation in the presence of progesterone that is termed pre-decidual and following conception decidual formation is stimulated to facilitate implantation.

To establish implantation of the blastocyst there must be initial apposition to the maternal epithelium, intrusion of trophoblast, and then invasion of the endometrial compartments (Lessey 2000). The embryo enters the uterine cavity 72-96 hrs after fertilisation. Hatching of the embryo by day 5 allows release of the zona pellucida exposing receptors, cell adhesion molecules (CAMs), MMPs and extracellular matrix components (ECM) that are likely to be involved in the next stage of implantation (Lessey 2000).

The initial step of blastocyst attachment is thought to involve the extraction of fluid from the uterine lumen by specialized endometrial apical protrusions called pinopods. Pinopods are thought to be progesterone-dependent cellular organelles appearing between days 19 and 21 of the cycle and could therefore be markers of the implantation window. The maximum life span of these pinopods is only 48 hours. As pinopods extract fluid from the uterine lumen, the blastocyst is drawn towards the uterine epithelium. This process is termed blastocyst apposition (Sunder et al. 2000).

In the next stage, adhesion of the blastocyst, trophoblastic cells establish close contact with the apical surface of the uterine epithelium. Adhesion molecules are thought to be important in modulating adhesion with MUC-1 being the most extensively studied to date. MUC-1 has been localised to human endometrium, is an oestrogen responsive cell membrane glycoprotein that appears to have anti-adhesive properties and is expressed at the luminal endometrial surface in the mid-secretory phase (Mcseguir et al. 1998). This observation suggests the existence of a barrier to the implanting embryo and the need for a locally acting mechanism for the removal of inhibition. It is possible that, as in rabbits, the local down-regulation of maternal MUC-1 is in response to a paracrine signal from the embryo. Murine studies have

shown that high levels of MUC-1 create a non-receptive endometrium but this role has not yet been demonstrated in humans (Carson et al. 1998; Meseguer et al. 1998). The role of MUC-1, its hormonal regulation and its properties as an anti-adhesion molecule are controversial in humans but animal studies suggest that it may be an important factor for successful adhesion (Simon et al. 2000).

Other adhesion molecules that appear to play a role include Trophinin (Aplin 2000; Sunder et al. 2000) and integrins (Sunder et al. 2000), both of which are expressed in the implantation window of endometrium. In particular, the integrins may have a role in integrating trophoblast with decidua, embryo attachment and initial invasion of the embryo into the extracellular matrix. Some of the integrins facilitate the expression of MMP-like collagenases, gelatinases and stromelysins that play a major role in trophoblast invasion (Sunder et al. 2000).

The next stage of implantation is invasion of the trophoblast into the uterine epithelium. Uterine NK cells interact with invading trophoblast to generate leukemia inhibitory factor (LIF). LIF is thought to induce urokinase plasminogen activator (uPA) and gelatinase enzymes that play a role in trophoblast invasion. NK cells may also have an important role to play through their interactions with HLA-G and HLA-C class antigens, antigens expressed by the invading trophoblast. This response may in part explain the successful interactions between tissues of disparate immunogenic sources, the embryo and the endometrium, in implantation (Loke et al. 2000; Sunder et al. 2000).

While other factors have been suggested to play an important role in blastocyst implantation, they are too expansive for inclusion in this thesis. However, specific data pertaining to a potentially important role for androgens exists.

Most recently murine studies carried out in androgen receptor knockout mice revealed impaired fertility compared to wild type mice, possibly related to subnormal uterine development (Lee et al. 2003). In human females with elevated serum androgens low levels of glycodeilin-A (previously known as placental protein 14 – PP14) have been shown in uterine flushings (Okon et al. 1998). Another factor important for endometrial receptivity that has been negatively correlated with androgens is HIOXA-10 (Cermik et al. 2003).

Glycodeilin-A is a major protein secreted by the secretory endometrium and gestational decidua that can be found in uterine flushings (Li et al. 1993). Its role in

implantation is thought to be mediated through inhibition of the maternal immune system. Specifically it inhibits uterine NK cell cytotoxicity (Okamoto et al. 1991) and diminishes the IL-2 enhanced cytotoxicity of the NK cells (Seppala et al. 2001). Secretory phase levels of glycodelin-A in the plasma and uterine flushings of women who have recurrent miscarriages are lower than those of normal fertile women (Dalton et al. 1995; Tulppala et al. 1995).

Endometrial development and receptivity requires transcriptional regulation by HOX genes (Cermik et al. 2003). Specifically, HOXA-10 is essential for the development of the uterus during organogenesis (Block et al. 2000) and it is also essential for allowing uterine receptivity to implantation (Taylor 2000). Female mice deficient in HOXA-10 are viable but have infertility caused by uterine defects, unable to support implantation (Satokata et al. 1995). In women, HOXA-10 expression varies during the menstrual cycle, with a dramatic rise in the mid-luteal phase (Taylor et al. 1998). This rise corresponds to the time of implantation and remains elevated throughout the rest of the luteal phase. Regulation of maternal HOXA-10 expression is essential to blastocyst implantation and reproductive success. HOXA-10 is up regulated by oestrogen and progesterone in endometrium (Taylor et al. 1998). However, recent work by Cermik et al. has shown that testosterone will diminish endometrial expression of HOXA-10 (Cermik et al. 2003). Such data would be consistent with elevated androgens, associated with PCOS and recurrent miscarriage, producing an endometrial environment unreceptive to implantation, resulting in impaired fertility.

### 1.11 Effects of Levonorgestrel on the Endometrium

Levonorgestrel is a potent progestogen which also has a binding affinity for the androgen receptor (Kloosterboer et al. 1988). The Levonorgestrel Releasing Intra-Uterine System (LNG-IUS) was initially developed as a contraceptive with the Pearl index (a measure of pregnancy rate) varying from 0.0-0.2 per 100 women-years with its 5-year contraceptive duration (Luukkainen et al. 1990). Additionally the LNG-IUS will significantly reduce menstrual blood loss (Andersson et al. 1990), menstrual pain and is associated with a low rate of infection (Luukkainen et al. 1990). The contraceptive effect of the LNG-IUS, the reduction in menstrual blood loss, and amenorrhea in some cases, are thought to be due to local effects on the endometrium (Nilsson et al. 1984). Ovarian function appears to be unaffected with studies confirming ongoing ovulation with LNG-IUS treatment (Nilsson et al. 1984; Xiao et al. 1995).

The LNG-IUS has a T-shaped body (32 x 32 mm) made of polyethylene. An elastomer sleeve is mounted around its vertical part. This sleeve consists of a 1 to 1 mixture of polydimethylsiloxane and 52 mg of levonorgestrel. The sleeve is covered with a drug release controlling membrane of medical grade polydimethylsiloxane. This system releases levonorgestrel over an extended time of up to 5 years at a practically constant rate. The initial release rate of levonorgestrel is 20 µg per 24 h. At the end of 5 years, the release rate is still above 10 µg per 24 h. The LNG-IUS was first introduced in Finland in 1990 (Lahteenmaki et al. 2000).

The histological appearances after an insertion time of 1 month are uniform suppression throughout the thickness of the functional layer of the endometrium. The histological picture is independent upon the distance from the intrauterine device. Typical findings are a 1 to 3 mm endometrium with a swollen stroma and enlarged cells consistent with a pseudo-decidual appearance. The endometrial glands are scarce, extremely narrow, and atrophic and lined by inactive, flattened cuboidal epithelium with no signs of mitotic activity or irregularity. No difference could be seen between specimens obtained during the luteal phase and those obtained during the follicular phase or those obtained from subjects not ovulating during treatment (Nilsson et al. 1978; Silverberg et al. 1986; Critchley et al. 1998). The changes in

endometrial morphology return to normal within 1-3 months following removal of the LNG-IUS (Silverberg et al. 1986).

The endometrial tissue concentrations of LNG are much higher with the LNG-IUS, far exceeding the tissue levels found with high systemic doses of LNG. Indeed endometrial LNG tissue levels are 1,000 times greater than serum concentrations (Nilsson et al. 1982). Therefore, the endogenous, systemic progesterone effect on the endometrium would be masked, despite circulating progesterone concentrations remaining in the normal follicular-luteal phase range (Critchley et al. 1998). Additionally, there is no reduction in oestrogen concentrations because women using an LNG-IUS as a rule have normal ovulatory cycles.

Sub-dermal LNG administered as a long term contraceptive exhibits an increase in immunoreactivity of the stromal progesterone receptor when compared with control endometrium at all stages across the menstrual cycle (Critchley et al. 1993). Endometrial treatment with the LNG-IUS providing continuous high local LNG levels, down regulates oestrogen and progesterone receptors in both glands and stroma with suppression being maintained until the 12 month time point (Critchley et al. 1998). Similar findings were also published by Zhu et al where again after 12 months treatment with the LNG-IUS a down regulation of oestrogen and progesterone receptors in both glands and stroma was noted (Zhu et al. 1999). However, progesterone receptor A levels appear to increase in the stroma by the 12 month time point (Critchley et al. 1998). This finding for the progesterone receptor subtype expression in LNG pseudo-decidualised endometrium is consistent with the observations in first trimester decidua (Wang et al. 1998). The lack of endometrial sensitivity to oestradiol during intra-uterine exposure to LNG is likely reflected in the down regulation of endometrial ER. However, the presence of LNG-IUS also disrupts oestradiol mediated proliferation of the endometrium by the stimulation of Insulin-like growth factor (IGF) binding protein production in endometrium. This prevents IGF-1 from performing its normal mediation of oestradiol activity to induce endometrial proliferation (Pekonen et al. 1992; Rutanen 2000).

Other endometrial factors that have been shown to be affected by intra-uterine LNG-IUS administration treatment are prolactin receptors, granulocyte-macrophage colony stimulating factor (GM-CSF) and uterine NK cells. There are

significant increases in prolactin receptors and granulocyte-macrophage colony stimulating factor (GM-CSF) immunoreactivity in the glandular epithelial and pseudo-decidual stromal cells. GM-CSF is an activating growth factor for granulocytes and monocytes whereas prolactin is an immune regulation agent and may play a role in leukocyte function. As a result, there is a significant increase in the endometrial leukocyte population, the majority of which are CD56+ uterine NK cells (Critchley et al. 1998).

LNG is known to bind to both the androgen receptor and the progesterone receptor (Kloosterboer et al. 1988). It does not possess any binding affinity for the oestrogen receptor. It can be metabolised by the same enzymes responsible for sex steroid metabolism and this can influence the binding affinity of LNG. Lemus et al. examined tissue metabolism of LNG in rats and demonstrated that following 5 $\alpha$  reduction it will have a greater binding affinity for androgen receptor (AR) but diminished binding for progesterone receptor (PR) (Lemus et al. 1992).

Users of a LNG-IUS often report breakthrough bleeding (BTB). Indeed this complaint is the commonest reason for discontinuation of the method. The manner in which it affects mechanisms involved with vascular control such as VEGF, tissue factor, vessel density and vessel size has been the subject of much research. These findings will be discussed at greater length in Chapter 1.12.

The precise role of altered sex steroid receptor expression in the formation of a pseudo-decidualised endometrium, contraception, reduced menstrual blood loss and the clinical problem of breakthrough bleeding associated with the LNG-IUS remain to be resolved. As yet data relating to the expression of the androgen receptor and the enzymes responsible for the metabolism of sex steroid ligands in LNG-IUS treated endometrium have not been published.

## 1.12 Endometrial Breakthrough Bleeding

All progesterone only contraceptives are associated with the clinical problem of breakthrough bleeding (BTB) (Critchley et al. 1998). BTB is spotting between periods that may become prolonged. It is most likely to occur in the first 6 months following insertion of the LNG-IUS (Lahteenmaki et al. 1998). BTB is commonest in the early months following insertion and improves with duration of use. In a study by Zalel et al. the incidence at 2 months was 44%, subsequently falling to 8% by 6 months (Zalel et al. 2003). Over this same time the cessation of menstrual bleeding increased from 5% at 2 months to 66% by 6 months.

The LNG-IUS is increasingly being used as a contraceptive and as a method of treating menstrual dysfunction. Patient acceptability is high but many patients will discontinue therapy due to the adverse effect of breakthrough bleeding (BTB) (Anderson 1992; Findlay 1996). The aetiology of BTB is unknown at present but is likely to involve angiogenic factors, matrix metalloproteinase activation, and altered haemostatic factors that result in increased endometrial vessel fragility (Hickey et al. 2000).

The presence of unscheduled breakthrough bleeding and reduction in menstrual blood loss suggests that progestogens might affect the endometrial vascular system directly (Roopa et al. 2003). A broad range of molecules identified as angiogenic growth factors are present in the endometrium. These polypeptides, which have been implicated in angiogenesis, include vascular endothelial growth factor (VEGF) (Chamock-Jones et al. 1993), epidermal growth factor (Nelson et al. 1991), basic fibroblast growth factor (Ferriani et al. 1993) and transforming growth factor-beta (Roberts et al. 1986). These angiogenic factors promote endothelial cell proliferation, migration and tube formation, and also facilitate the changes in extracellular matrix needed to permit new vessel formation. VEGF has also been shown to be produced by endometrial stromal cells in culture following stimulation by oestradiol and progesterone (Perrot-Appianat et al. 2000). Further, Roopa et al. have demonstrated that treatment with LNG-IUS increases VEGF expression at 3 and 6 months following insertion (Roopa et al. 2003). VEGF stimulates plasminogen activators which could be involved in the proteolytic degradation of the basement membrane of blood vessels, thereby leading to increased

vascular fragility (Roopa et al. 2003). Superficial vascular dilatation is seen in the endometrium exposed to low dose progestogens (Hickey et al. 1998) and similar findings have also been described in endometrium exposed to high dose LNG following treatment with the LNG-IUS (McGavigan et al. 2003). VEGF is also known to stimulate the potent vasodilator, nitric oxide from endothelial cells which would be expected to promote vasodilatation (Papapetropoulos et al. 1997).

Additional molecular factors, implicated in BTB, reported in the endometrium of levonorgestrel users includes activation of matrix metalloproteinases 1, 3 and 9 to a level consistent with that seen in menstrual phase endometrium (Galant et al. 2000; Rodriguez-Manzanaque et al. 2000; Vincent et al. 2000). This may explain the features of focal stromal breakdown and collagen lysis noted in endometrium from users of Norplant, sub-dermal LNG delivery (Galant et al. 2000). Other factors reported that relate to vessel fragility include a decrease in endometrial perivascular alpha smooth muscle actin in Norplant users (Rogers et al. 2000) and decreased endothelial cell proliferation (Goodger et al. 1994; Rodriguez-Manzanaque et al. 2000; Hague et al. 2002). Haemostatic factors may also play an important role as a decrease in tissue factor protein and mRNA has been reported in levonorgestrel exposed endometrium (Runic et al. 1997). The aforementioned factors all suggest a disruption in the mechanisms that control vessel growth and haemostasis.

While the knowledge base regarding local mechanisms that may be involved with BTB (experienced by progesterone only contraceptive users) is ever expanding, the precise mechanisms are not yet fully understood. The data thus far would support an important role for the factors summarised above that are important for angiogenesis and the maintenance of vascular integrity. The role of sex steroids and their respective receptors and the influence they exert on the above local factors requires further research. In particular, despite LNG demonstrating a binding affinity for AR, the role of androgens and androgen metabolising enzymes in LNG-IUS treated endometrium has to date not been elucidated.

## Chapter 2

# Hypothesis and Aims of Thesis

## 2 Hypothesis and Aims of Thesis

Sex Steroids and their cognate receptors play a critical role in the regulation of normal endometrial physiology. Well studied regulators of endometrial function are oestradiol and progesterone, acting via their respective receptors. The foregoing literature review highlights an under researched area of endometrial physiology, namely the role of androgens and the androgen receptor (AR) in endometrial physiology.

To date there have been inconsistent data reporting the spatial and temporal localisation of AR messenger RNA (AR mRNA) and AR protein in human endometrium throughout the menstrual cycle. Therefore, the role of androgens in physiological processes such as implantation and menstruation is currently unknown. Furthermore, the role that androgens play in endometrial dysfunction has received little attention. Androgens have been implicated in defective implantation in the hyper-androgenic polycystic ovarian syndrome.

The availability of sex steroids to bind to sex steroid receptors, and in particular the AR, will also determine the predominant sex steroid regulated physiological events at any particular point in the menstrual cycle. The key determinants of sex steroid availability in endometrium are metabolising enzymes that include the  $17\beta$ -hydroxysteroid dehydrogenases,  $3\beta$ -hydroxysteroid dehydrogenases,  $5\alpha$ -reductases, and Aromatase. The expression of the aforementioned enzymes will allow a target tissue/cell autonomous control over its sex steroid requirements.

Furthermore, progestogenic agents are commonly employed in gynaecology and they possess androgenic activity. Knowledge of the effect of progestogens, such as levonorgestrel, on endometrial biology will contribute to our understanding of the role of androgens in endometrial biology and their mode of action.

Thus the based upon the available evidence to date the following hypotheses are proposed:

1. Normal endometrial function requires a stable ratio of intracellular androgens and oestrogens that favours lower intracellular oestrogen concentrations at specific times in the menstrual cycle, for example, during the secretory phase. Low intracellular oestrogen concentrations are maintained by the sex steroid

metabolising enzymes: 17 $\beta$ -hydroxysteroid dehydrogenases, 3 $\beta$ -hydroxysteroid dehydrogenases, and the 5 $\alpha$ -reductases.

2. Raised local concentrations of androgens are deleterious for normal endometrial function. Hence, aberrant menstrual bleeding patterns and defective implantation may be a consequence of abnormalities in endometrial AR expression and/or expression of 17 $\beta$ -hydroxysteroid dehydrogenases, 3 $\beta$ -hydroxysteroid dehydrogenases, and the 5 $\alpha$ -reductases.
3. Exposure of human endometrium to high doses of intra-uterine levonorgestrel influences the expression patterns of AR and/or the sex steroid metabolising enzymes. Altered expression of such factors may be relevant to the problematic bleeding patterns seen during treatment with the levonorgestrel releasing intrauterine system

The aims of this thesis were thus to establish in human endometrium:

1. The spatial and temporal expression of androgen receptor mRNA and protein in endometrium across the menstrual cycle.
2. The expression of critical androgen metabolising enzymes across the menstrual cycle - 17 $\beta$ -hydroxysteroid dehydrogenase type 2 and 5; 3 $\beta$ -hydroxysteroid dehydrogenase type 1 and 2; and 5 $\alpha$ -reductase type 1 and 2.
3. The spatial and temporal localisation, in endometrium exposed to high dose intra-uterine levonorgestrel, of AR mRNA and protein; 17 $\beta$ -hydroxysteroid dehydrogenase type 2 mRNA and protein; 17 $\beta$ -hydroxysteroid dehydrogenase type 5 mRNA; 3 $\beta$ -hydroxysteroid dehydrogenase type 1 and 2 mRNA; and 5 $\alpha$ -reductase type 1 and 2 mRNA.

# Chapter 3

## General Methods

### 3.1 Human Endometrial Tissue Collection.

Human endometrial tissue samples were obtained from patients attending the Royal Infirmary of Edinburgh Gynaecology department. Institutional ethical approval was obtained and all women provided written informed consent.

#### 3.1.1 Endometrial Specimens

The first group consisted of fertile women (n=53) with regular menstrual cycles lasting between 25 and 35 days who had not been using hormonal preparations for the preceding 3 months. Samples were obtained at the time of hysterectomy for benign indications. Subjects with significant uterine pathology (for example, fibroids) were excluded. Endometrial tissue was collected from women during the proliferative phase (n=29), early secretory (n=6), mid secretory (n=8) and late secretory phase (n=10) of the menstrual cycle.

The following specimens were collected immediately following removal of the uterus:

1. A *full thickness* biopsy of the endometrium (including adjacent endometrial-myometrial junction) was obtained and fixed in 4% paraformaldehyde.
2. A separate endometrial sample was snap frozen in liquid nitrogen at collection and subsequently stored at -80°C.
3. In addition, venous blood samples were collected at the time of tissue collection for determination of circulating oestradiol and progesterone concentrations.

The full thickness endometrial biopsy extended from the uterine lumen continuously through to the myometrial layer, therefore including the superficial and basal endometrium in addition to the adjacent myometrium (Figure 3.1). Following fixation in paraformaldehyde at 4°C the specimen was embedded in paraffin blocks. Histological dating according to the criteria of Noyes et al (Noyes et al. 1950) was performed to confirm the stage of the menstrual cycle was consistent with the patients reported last menstrual period. Venous blood samples were centrifuged at 3000 rpm for 7 min to separate serum allowing oestradiol and progesterone concentrations to be determined by radioimmunoassay (RIA) again to confirm levels were consistent with the designated cycle stage. Figure 3.2

The second group consisted of fertile women (n=30) with regular menstrual cycles lasting between 25 and 35 days that had not been using hormonal preparations for the preceding 3 months. Samples were obtained at the time of laparoscopic sterilisation using an endometrial aspirating curette (Pipelle de cornier, Laboratoire CCD, Paris, France). Subjects with significant uterine pathology (for example, fibroids) were excluded. Endometrial tissue was collected from women during the early proliferative phase (n= 5), mid proliferative phase (n= 5), late proliferative phase (n= 5), early secretory (n=5), mid secretory (n=5) and late secretory phase (n=5) of the menstrual cycle. At collection, samples were divided and collected in 4% paraformaldehyde and snap frozen in liquid nitrogen and stored at -80°C. Paraformaldehyde fixed specimens were embedded in paraffin blocks. Histological dating according to the criteria of Noyes et al (Noyes et al. 1950) was performed to confirm the stage of the menstrual cycle was consistent with the patients reported last menstrual period. Serum oestradiol and progesterone levels were consistent with the designated cycle stage. Figure 3.3

### **3.1.2 Endometrial Specimens from Women Using a Levonorgestrel Releasing Intra-Uterine System**

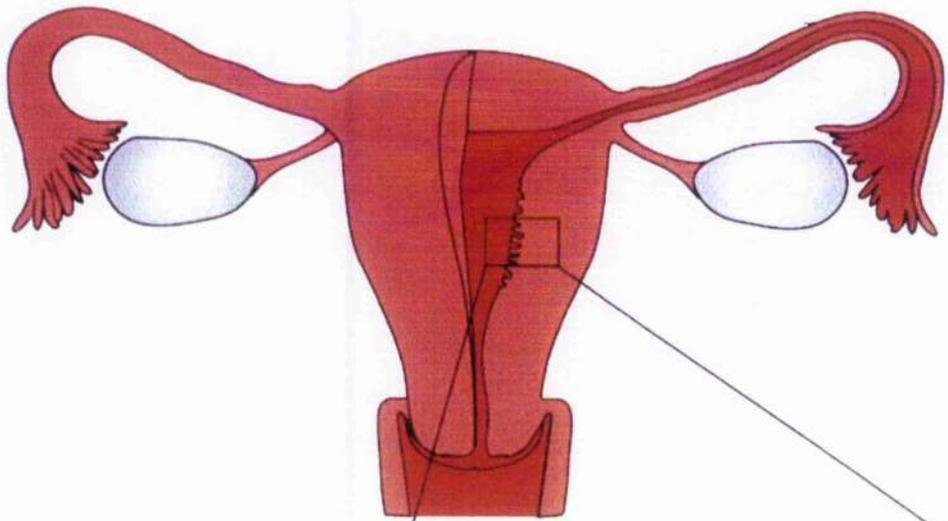
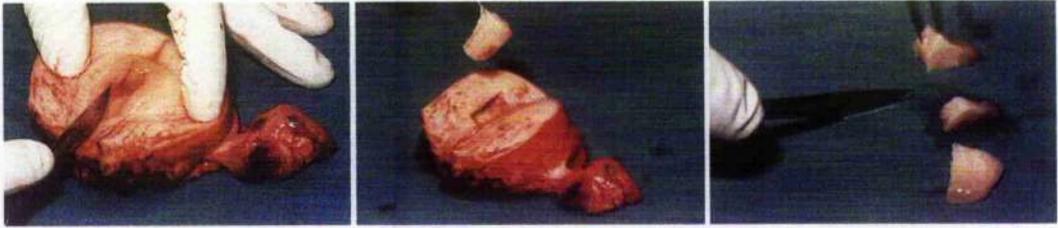
This patient group consisted of fertile women (n=10) with regular menstrual cycles lasting between 25 and 35 days and who had not been using hormonal preparations for the preceding 6 months. The patients were to have a LNG-IUS inserted as a method of long-term hormonal contraception. All patients underwent a pre-insertion endometrial biopsy immediately after which a LNG-IUS (Mirena, Schering HC, UK) was inserted as an outpatient procedure. The stage of the cycle prior to insertion of the LNG-IUS was determined by reference to histological dating by the criteria of Noyes et al. (Noyes et al. 1950) and reported date of last menstrual period. Further endometrial samples were collected at 1 (n=2), 3 (n=5), 6 (n=5) and 12 months (n=5) following insertion of the LNG-IUS. Specimens were divided into portions that were either fixed in 10% neutral buffered formalin at 4°C then embedded in paraffin for immunohistochemical analysis or snap frozen in liquid nitrogen at collection and subsequently stored at -80°C. An additional cohort of women using a LNG-IUS also consented to endometrial biopsies being performed. These patients were attending a

menstrual problems clinic with regular periods but suffering from heavy menstrual blood loss and were managed with a LNG-IUS. Endometrial samples were collected from patients attending the clinic with an LNG-IUS in-situ for 3 months, 6 months, or greater than 1 year. Endometrial samples were frozen immediately upon collection in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

Figure 3.1

Images illustrate the bisection of the uterus enabling identification of the uterine cavity. A biopsy incorporating the endometrium and a portion of the myometrium is then dissected free. Sample is then divided into separate portions for subsequent processing as detailed in 3.1.1.

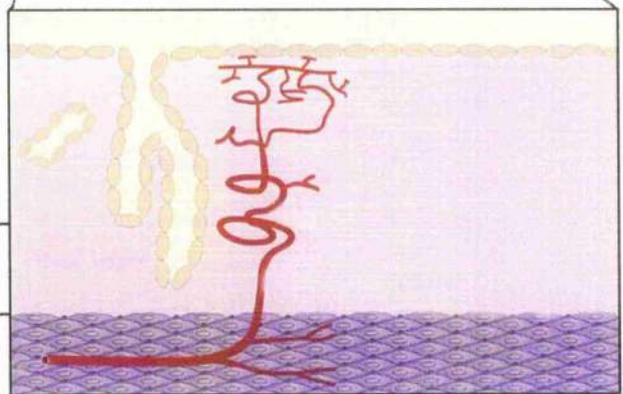
The diagram illustrates the layer architecture for the full thickness endometrial biopsy with the functional layer adjacent to the uterine lumen and the basal layer adjacent to the myometrial layer.



Functional Layer

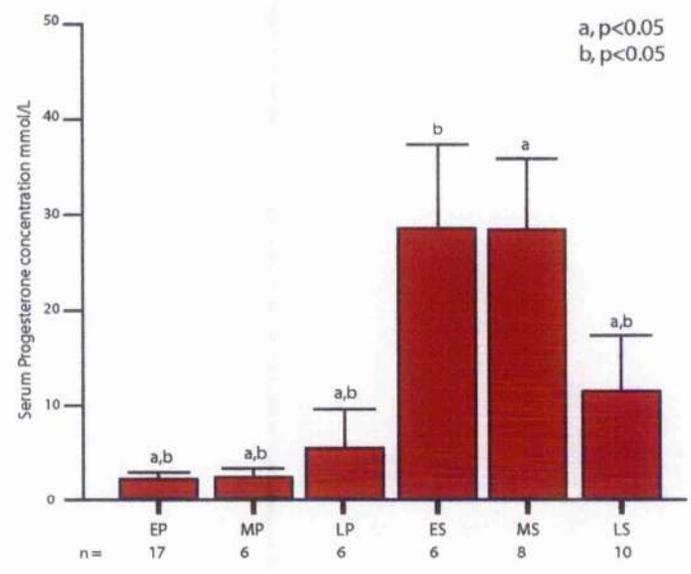
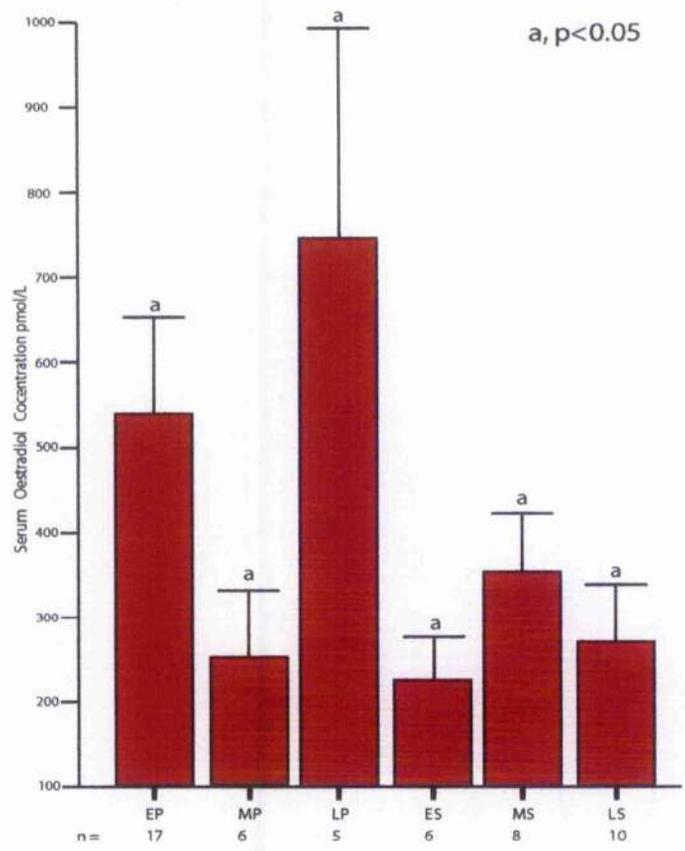
Basal Layer

Myometrium



**Figure 3.2**

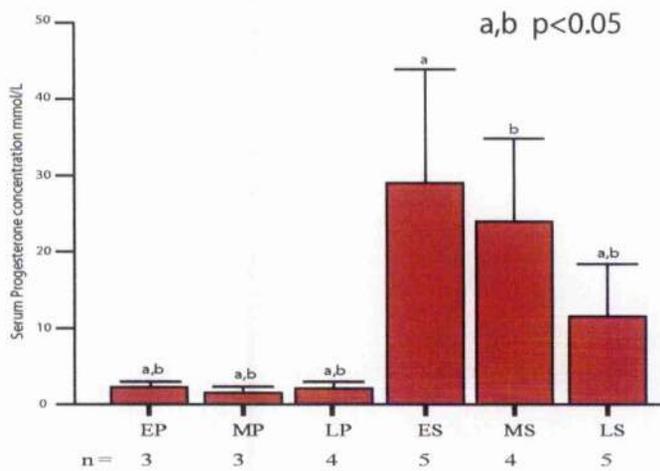
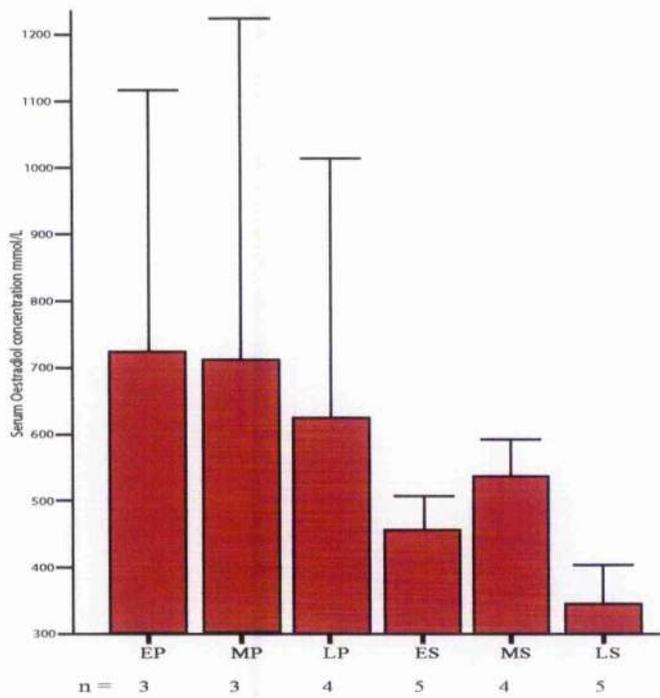
Bar charts illustrating serum oestradiol levels and serum progesterone levels from full thickness endometrial specimens used for immunohistochemical analysis. The table indicates mean serum values for oestradiol and progesterone for each cycle stage, standard error of the mean is shown in brackets. A significant decline in serum progesterone is noted in the late secretory phase of the menstrual cycle.



	Mean Serum E2 ( $\pm$ SEM) pmol/L	Mean Serum P4 ( $\pm$ SEM) mmol/L
<b>Proliferative Phase</b>		
Early	533.4 ( $\pm$ 97.5)	2.8 ( $\pm$ 0.41)
Mid	252 ( $\pm$ 66.9)	2.8 ( $\pm$ 0.42)
Late	737.2 ( $\pm$ 255)	5.5 ( $\pm$ 3.9)
<b>Secretory Phase</b>		
Early	232.7 ( $\pm$ 38.9)	28.5 ( $\pm$ 7.8)
Mid	352 ( $\pm$ 44.6)	28.8 ( $\pm$ 7.3)
Late	294 ( $\pm$ 63.0)	12.0 ( $\pm$ 5.2)

**Figure 3.3**

Bar charts illustrating serum oestradiol levels and serum progesterone levels from endometrial specimens used for real time RT-PCR analysis. The table indicates mean serum values for oestradiol and progesterone for each cycle stage, standard error of the mean is shown in brackets. A significant decline in serum progesterone is noted in the late secretory phase of the menstrual cycle.



	Mean Serum E2 ( $\pm$ SEM) pmol/L	Mean Serum P4 ( $\pm$ SEM) mmol/L
<b>Proliferative Phase</b>		
Early	721 ( $\pm$ 337)	2.7 ( $\pm$ 0.44)
Mid	713 ( $\pm$ 512)	1.5 ( $\pm$ 0.27)
Late	624 ( $\pm$ 191)	2.3 ( $\pm$ 0.33)
<b>Secretory Phase</b>		
Early	452 ( $\pm$ 13)	15.9 ( $\pm$ 7.1)
Mid	524 ( $\pm$ 54)	23.9 ( $\pm$ 1.1)
Late	342 ( $\pm$ 73)	10.7 ( $\pm$ 3.0)

## **3.2 Immunohistochemistry.**

Immunohistochemistry (IHC) was performed for localisation of the Androgen Receptor (AR), 17 $\beta$ -hydroxysteroid dehydrogenase (HSD) type 2 enzyme, and Aromatase enzyme. No commercially available antibodies were available to determine the immunolocalisation of other 17 $\beta$ -HSD iso-enzymes or the 5 alpha reductase iso-enzymes. All protocols were optimised to determine the best conditions for maximal immunostaining. The protocols are summarised in Table 3.1.

### **3.2.1 General Immunohistochemistry Protocol.**

Paraffin block specimens were cut to 5 micron width sections and mounted on superfrost glass slides (BDH, Merck House, Poole, Dorset, England). Slides were placed in histoclear for 10 minutes, then rehydrated through graded alcohols, 100% ethanol (2 min), 95% ethanol (2min), 70% ethanol (2min), and then into distilled water for 10 minutes. Thereafter the slides were washed in phosphate buffered saline (PBS, Sigma-Aldrich Ltd. Poole, Dorset, England) for 10 minutes.

The next step in the protocol involved an antigen retrieval step. Formaldehyde fixation primarily involves its reacting with the basic amino acid lysine to form cross-linked 'methylene bridges'. This means that there is relatively low permeability to macromolecules and that the structures of intracytoplasmic proteins are not significantly altered. This generally allows good cytoplasmic preservation and immunolocalisation with a minimum of antigen masking. However, some epitopes are difficult to expose in formalin-fixed paraffin-embedded tissues. Therefore, an antigen retrieval step such as pressure cooking, microwaving, or proteolytic digestion is used to unmask the antigenic site hidden by cross-linked proteins. When pressure cooking antigen retrieval is required, sections are heated in boiling 0.01M Na Citrate (pH 6.0) for 5 minutes, then left to cool for 20 minutes. Thereafter the sections are washed in 0.01M PBS for 10 minutes before the next step of immunohistochemical procedure.

To reduce background staining, further steps may also be included. Endogenous peroxidase activity is quenched by immersion in 3% hydrogen peroxide solution (Merck, Poole, UK) for 10 minutes followed by a 10 minute wash in PBS. Next endogenous biotin and avidin activity is blocked with a 15 minute incubation period with each in turn. A 5 minute PBS wash follows each incubation. Finally, to

reduce non-specific background binding the slides are incubated with a non immune serum block diluted in buffer that is from the same species as the biotinylated secondary antibody.

Next, a standard Avidin-Biotin Peroxidase immunohistochemical protocol was followed (Figure 3.4). The ABC complex method utilized the high affinity of avidin for biotin. Avidin has four binding sites for biotin (dissociation constant  $10^{-19}$  M). The sequence of reagent application is primary antibody (e.g. mouse), biotinylated secondary antibody (biotin is covalently linked to the antibody, e.g. Biotinylated horse anti-mouse) specific for the primary antibody and diluted in serum as a second concurrent non immune block. Then preformed avidin-biotin enzyme complex is added. This preformed avidin-biotin complex has open sites on avidin to allow binding to the biotin on the secondary antibody. The peroxidase substrate Diaminobenzidine tetra hydrochloride (DAB) is employed as the chromagen. The peroxidase enzyme causes oxidation of DAB, which results in a stable enzyme-substrate complex and a brown precipitate allowing visualization of the stain (Figure 3.4). Sections were counterstained with Harris' haemotoxylin, dehydrated in ascending grades of alcohol and mounted from xylene in Pertex.

To improve the detection of antigens the ABC-Elite system can be employed. ABC-Elite is the most sensitive peroxidase-based detection system, providing about 5-10 times more sensitivity than the regular ABC in immunohistochemical staining. When the avidin and biotinylated peroxidase in the ABC-Elite kits are combined, a smaller, very uniform and highly active complex is formed which allows a greater number of enzyme complexes to diffuse through a tissue section and bind to a biotinylated target.

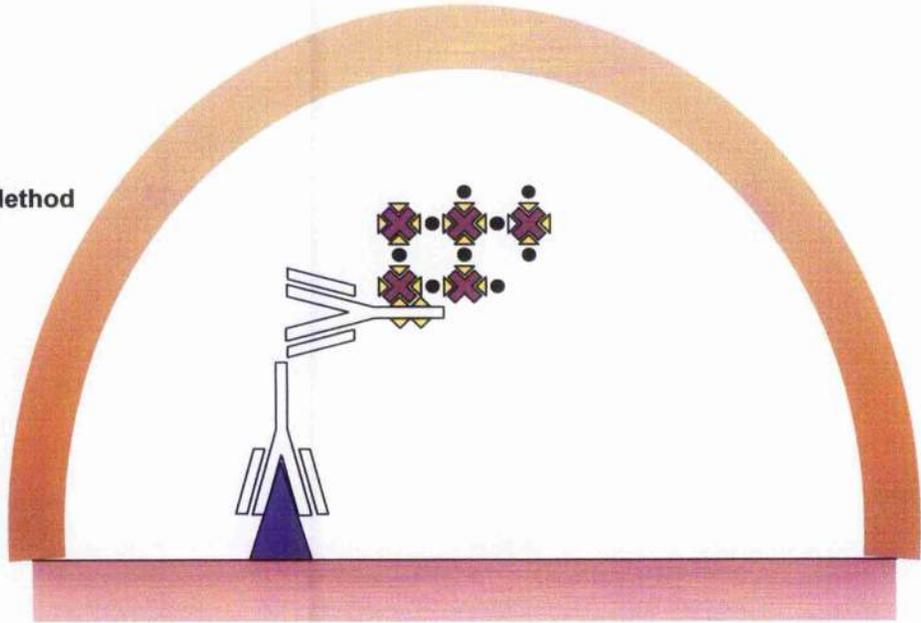
	Primary Antibody	Antigen Retrieval Step	Dilution	Incubation
Androgen Receptor	$\alpha$ F 39.4	Pressure Cooking	1:480	Overnight 4 <sup>0</sup> C
17 $\beta$ -HSD 2	Mab C2-12	Pressure Cooking	1:20	Overnight 4 <sup>0</sup> C
Aromatase	In House	Not Required	1:50	Overnight 4 <sup>0</sup> C

Table 3.1 Summary of Immunohistochemical Protocols.

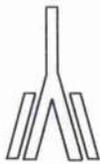
**Figure 3.4**

Diagrammatic representation of the ABC immunohistochemical method as referred to in 3.2.1. Sequential addition of primary antibody, biotinylated secondary antibody and Diaminobenzidine allows visualisation of antigen detection by the primary antibody.

ABC Method



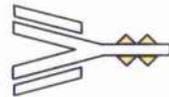
Key



Primary  
Antibody



Tissue  
Antigen



Biotinylated  
Antibody

▲ Biotin

● Peroxidase

⊗ Avidin



Avidin-Biotin  
Peroxidase  
Complex



DAB  
Reaction

### **3.2.2 Androgen Receptor (AR) Immunohistochemistry.**

5 micron paraffin sections were de-waxed and re-hydrated as in the general protocol. Following the washing steps detailed in the general protocol an antigen retrieval step was included. This step involved heating the sections in a pressure cooker containing 0.01M sodium citrate at pH 6 for 5 minutes, then cooling the sections in the mixture for 20 minutes. The blocking steps included were the same as detailed in the general protocol with normal horse serum as the 20 minute non immune blocking step in a humidified chamber. The antibody raised against the AR was a commercially available monoclonal antibody called  $\alpha$ F39.4 (IgG<sub>1</sub>; Biogenex Laboratories, Inc., San Ramon, CA, USA). Tissue sections were incubated overnight at 4°C in the humidified chamber with 50  $\mu$ l of  $\alpha$ F39.4 diluted 1:480 in PBS/Bovine Serum Albumin (BSA) - employed as an additional method to minimise background staining. The primary antibody was substituted with equimolar concentration of mouse immunoglobulin (IgG) in negative control sections. After the primary antibody incubation, sections were washed between each stage for 10 minutes with PBS/Tween buffer solution. Sections were incubated with horse anti-mouse biotinylated secondary antibody and then with ABC-Elite detection system (Vectastain, Vector Laboratories, Inc., Peterborough, UK), both for 60 minutes at room temperature. DAB (DAKO Liquid, DAKO Corporation, California, USA) was then added to the sections allowing visualization of the stain. Sections were counterstained with Harris' haematoxylin, dehydrated in ascending grades of alcohol and mounted from xylene in Pertex.

### **3.2.3 17 Beta-Hydroxysteroid Dehydrogenase Type 2 (17 $\beta$ -HSD 2) Immunohistochemistry.**

This protocol was identical to that for androgen receptor immunolocalisation. The primary antibody, Mab C2-12, detected 17 $\beta$ -HSD 2. This is a monoclonal antibody that was a kind gift from Stefan Andersson (University of Texas, Dallas, USA). Details regarding antibody production and specificity have been previously published (Moghrabi et al. 1997). It was diluted 1:20 in normal horse serum and applied overnight at 4°C in a humidified chamber. Negative control sections were incubated under the same conditions with equimolar mouse IgG.

### **3.2.4 Aromatase Immunohistochemistry.**

This protocol differed from previous protocols as Tris buffered saline (TBS) was used for washes throughout and no antigen retrieval step was required. The anti Aromatase antibody was a mouse monoclonal antibody and details regarding its production and specificity have been previously published (Turner et al. 2002). The antibody was a kind gift from Dr P. Saunders (Human Reproductive Sciences Unit, University of Edinburgh, UK), a co-author for the paper describing the new antibody. The first non immune block was performed with normal rabbit serum diluted in TBS/BSA (Diagnostics Scotland, Edinburgh, Midlothian, UK) for 30 minutes at room temperature. Primary antibody incubation was at a 1:50 dilution overnight at 4 °C. Rabbit anti-mouse biotinylated secondary antibody was employed diluted with normal rabbit serum as above and incubated at room temperature for 60 minutes. The ABC complex used was ABC-HRP (Vectastain, Vector Laboratories, Inc., Peterborough, UK) and this incubation step was also at room temperature for 45 minutes.

### **3.2.5 Immunohistochemical Analysis and Statistics.**

An objective quantitative scoring system was employed for the analysis of AR immunoreactivity in normal human endometrium. A semi-quantitative scoring system was employed for the analysis of all other IHC experiments

#### **3.2.5.1 Quantitative image analysis**

Quantitative image analysis involved capturing images at x20 magnification via a Zeiss microscope that was in turn connected to an Apple McIntosh G3 computer. Images were captured using a real time digital video camera connected to the Zeiss microscope. Analysis was performed on the Apple McIntosh G3 using image analysis software - openlab 3.0 (Improvision inc., Lexington, MA.). 12 random images were captured from the functional and basal layers of each tissue section. After 12 fields were captured the running mean stabilised, ensuring inter-image variance is <5% (Lowrey et al. 1995).

To allow separate analysis of the stroma and epithelial glands, the glands were interactively removed from each of the 12 images for each tissue section. This allowed later, separate analysis of the epithelium. Using hue, saturation and intensity

values to determine optimal colour discrimination, the automated software generated a mask that calculated the total number of DAB positive brown cells and subsequently the total number of negative blue cells in each of the stromal and glandular epithelial compartments. Calculation of the percentage of chromagen positive cells in each compartment could then be performed. Knowing the value for each of the 12 random images per section allowed a mean percentage of positive cells for each compartment of each section to be calculated.

A one way analysis of variance (ANOVA) with least significant difference (LSD) analysis was used to evaluate statistical differences in compartments through the menstrual cycle. Results with a p value <0.05 were considered to be significant differences.

#### **3.2.5.2 Semi-quantitative analysis**

Semi-quantitative analysis was employed for assessment of intensity and localisation of immunoreactivity in entire tissue sections. Scoring was performed by two independent observers blinded to the stage of the menstrual cycle. A score of 0 = absent immunoreactivity; 1 = faint immunoreactivity; 2 = strong immunoreactivity; 3 = very strong immunoreactivity. This scoring system was previously validated in a study that compared semi-quantitative and quantitative scoring systems as described above (Wang et al. 1998). In this study a high correlation, with a regression coefficient of 0.963 was found between the two methods. This provides a high level of confidence in the semi-quantitative scoring system and takes much less time to perform than quantitative analysis.

Due to the discontinuous nature of the semi-quantitative data the non parametric Kruskal-Wallis test was performed in addition to Dunns multiple post test to evaluate statistical differences between sections. Results with a p value <0.05 were considered to be significant differences.

### **3.3 Real Time Quantitative Reverse Transcription – PCR (Q-RT PCR).**

#### **3.3.1 RNA Extraction**

Tissue was collected as specified above whereupon it was immediately frozen in liquid nitrogen at collection and stored at  $-80^{\circ}\text{C}$ . Endometrium was easily homogenised and the extraction method is detailed below. All tissue handling was conducted to minimise the possibility of RNase contamination. All instruments and disposables used in the procedure below were autoclaved or were certified RNase free and used sterile.

Trizol reagent (Invitrogen Life technologies Ltd, Paisley, UK) was used for RNA isolation from endometrial tissues. This reagent is a monophasic solution of phenol and guanidine isothiocyanate. During sample homogenization, Trizol reagent maintains the integrity of the RNA while disrupting cells and dissolving cell components. Addition of chloroform followed by centrifugation separates the solution into an aqueous phase and an organic phase. RNA remains exclusively in the aqueous phase. After transfer of the aqueous phase, the RNA is recovered by precipitation with isopropyl alcohol.

Tissue samples were homogenized in 1 ml of Trizol reagent per 50-100 mg of tissue using a hand-held homogenizer. Then each homogenized sample was placed in a phase-lock gel heavy tube and incubated for 5 minutes at 15 to  $30^{\circ}\text{C}$  to permit the complete dissociation of nucleoprotein complex. After adding 0.2 ml of chloroform per 1 ml of Trizol reagent, sample tubes were capped and mixed by inversion for 15 seconds, and incubated at room temperature for 3 minutes. Then the samples were centrifuged at no more than 13,000 rpm for 15 minutes at  $4^{\circ}\text{C}$  for separation of the mixture into two portions, the aqueous RNA containing phase above and the DNA/protein content was contained below the gel in the phenol red layer.

The aqueous phase, the top portion, was transferred to 2ml eppendorf tube. Then 0.5 ml of 100% isopropyl alcohol per 1 ml of Trizol reagent used for the initial homogenization was added to allow precipitation the RNA from the aqueous phase. After that, samples were incubated at room temperature for 10 minutes and centrifuged at no more that 13,000 rpm for 10 minutes at  $4^{\circ}\text{C}$ . The resulting supernatant was removed leaving a pellet containing RNA precipitate. The RNA

pellet was washed once with 75% ethanol by adding at least 1 ml of 75% ethanol per 1 ml of Trizol reagent used for the initial homogenization, mixing the samples by vortexing. Samples were then centrifuged at 7,500 rpm for 10 minutes at 4<sup>0</sup>C. At the end of the procedure, the RNA pellet was briefly air dried, and then resuspended in RNA storage solution (Ambion, Austin, Texas, USA) and stored at -80<sup>0</sup>C until required for Taqman PCR analysis.

### **3.3.2 RNA Quantitation**

To quantify the concentration of RNA, optical density measurements were performed using the Genequant machine. This machine automated the calculation of the concentration of RNA. A 1:10 dilution of RNA was made by dilution of 2  $\mu$ l of RNA in 8  $\mu$ l of nuclease free H<sub>2</sub>O. A ratio of the optical density at a wavelength of 260nm to a wavelength of 280nm (260:280) was calculated. If the ratio was less than 1.6 the sample was considered not to have sufficient purity for use in the studies. The concentration was then recorded.

### **3.3.3 Reverse Transcription**

The reverse transcription polymerase chain reaction (RT-PCR) is a sensitive method for detection of low-abundance mRNA. RT-PCR assays are the most common method for comparing mRNA levels in different sample populations (Orlando et al. 1998). As RNA cannot serve as a template for PCR, the first step in an RT-PCR assay is the reverse transcription of the RNA template into cDNA, followed by its exponential amplification in a PCR reaction. An advantage of the RT step being carried out separately from the PCR step is that the generated pool of cDNA can be stored for later use.

Taqman primers and probes were designed to span an intron where possible as this should prevent amplification of contaminant genomic DNA. Where Taqman primers and probes did not span an intron samples were DNase treated prior to reverse transcription to prevent the risk of genomic DNA contamination. The initial Taqman primers and probe to detect androgen receptor spanned an intron and samples were not DNase treated. All subsequent reverse transcriptions were performed with DNase pre-treatment as the Taqman primers and probes did not span

an intron with each design due to specific criteria that had to be followed in Taqman primer probe design.

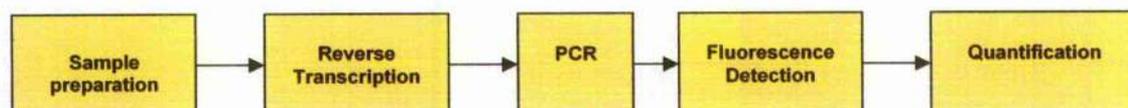
DNase pre-treatment of RNA stock solutions was carried out as follows. 1 µg of each RNA was added to individual mixtures containing 1 µl of DNase buffer and 1 µl of DNase with the remaining volume being made up to 10 µl with nuclease free water. The mixture was incubated at room temperature for 15 minutes at which point 1 µl of 25 mM EDTA was added to stop the digestion reaction. Then the mixture was incubated on a PCR block for 5 minutes at 99°C to denature the DNase. The mixture was then cooled on ice and samples given a pulse centrifuge to collect any sample condensed on the eppendorf wall. Samples could then be stored at -80°C until required for cDNA preparation.

The following is the protocol for reverse transcription. The RT-PCR reaction was performed in a 10 µl volume of reaction solution. 2 µl of DNase treated mixture containing 200 ng of RNA or RNA stock was diluted in nuclease free H<sub>2</sub>O to prepare the concentration of 100 ng/µl from which 200 ng (2 µl) of each sample RNA, including a positive control, were added into 8 µl of master mix solution. Master mix solution contained 10x Taqman buffer, MgCl<sub>2</sub> (5.5 mmol/l), deoxyNTP's, random hexamers (2.5 µmol/l), multiscribe reverse transcriptase (1.25 IU/µl), RNase inhibitor (0.40 IU/µl), and nuclease free water (Perkin-Elmer, Applied Biosystems, Warrington, UK). Each tube was mixed by vortexing, pulse centrifuged and overlaid with mineral oil. PCR reactions were carried on a Hybaid Omn-E thermal cycler (Hybaid, Ashford, U.K.). Reverse transcription reaction was conducted at 25°C for 60 minutes, 48°C for 45 minutes, and 95°C for 5 minutes for 1 cycle. Thereafter samples were stored at -20°C.

#### **3.3.4 Real Time Quantitative RT-PCR**

The Taqman assay (Perkin-Elmer–Applied Biosystems, Foster City, CA, USA) is a real time RT-PCR assay. It requires the use of the ABI Prism 7700 - a combined thermal cycler/detector (Perkin-Elmer–Applied Biosystems, Foster City, CA, USA) which utilises sequential laser stimulation of its 96 reaction wells to detect fluorescence between 500 nm and 660 nm. Detected fluorescence is then analysed to

quantify the amount of amplicon of interest in the samples being analysed. A summary of this process is shown below.



Following reverse transcription the assay requires the annealing of specific oligonucleotide sequences to the cDNA under examination to be successful. Two specific oligonucleotide primers define the endpoints of the amplicon providing the first level of specificity with additional specificity from the use of an oligonucleotide probe that hybridises to the amplicon during the annealing/extension phase of the PCR. To avoid amplification of contaminant genomic DNA the primers should be designed to span an intron. This is sometimes not possible and in such cases the RNA to be quantified should be DNase treated with RNase free DNase to minimise the potential for genomic contamination of the cDNA.

The assay utilises the 5'-3' nuclease activity of the DNA polymerase, Taq polymerase, to hydrolyse a hybridisation probe that is bound to its amplicon. The probe used for Taqman assay is a 5' labelled FAM (6-carboxyfluorescence; a fluorophore) 3' labelled TAMRA (6-carboxytetramethylrhodamine; a quencher) FRET (Fluorescence Resonance Energy Transfer) probe. In FRET probes, the fluorophore donor transfers its energy to the quencher suppressing fluorescence due to its close proximity. If no amplicon complementary to the probe is amplified during the PCR, the probe remains unbound and its fluorescence cannot be emitted for reporting. If the correct amplicon has been amplified, the probe can hybridise to that amplicon after the denaturation step. It remains hybridised while the polymerase extends the primers until it reaches the hybridised probe, when it displaces its 5' end to hold it in a forked structure. The enzyme continues to move from the now free end to the bifurcation of the duplex, where cleavage takes place. This separates the FAM reporter and TAMRA quencher dyes meaning the quencher can no longer suppress the reporter. Hence, the fluorescence is increased allowing measurement of the amount of PCR product (Bustin 2000).

Figure 3.5

Diagrammatic representation of Taqman real time RT-PCR process.

#### Polymerisation

During PCR, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence.

#### Strand Displacement

As polymerization of the strand continues, the probe fragments are displaced from the target sequence.

#### Cleavage

During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, resulting in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Taq DNA Polymerase cleaves the probe only if it hybridises to the target.

#### Polymerisation Complete

The 3'-end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, any nonspecific amplification is not detected.



Reporter

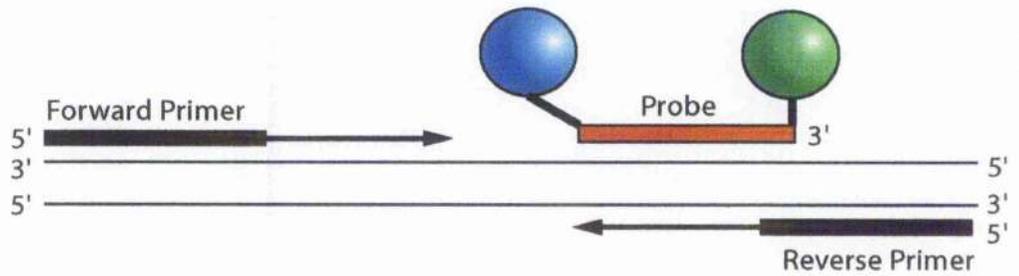


Signal

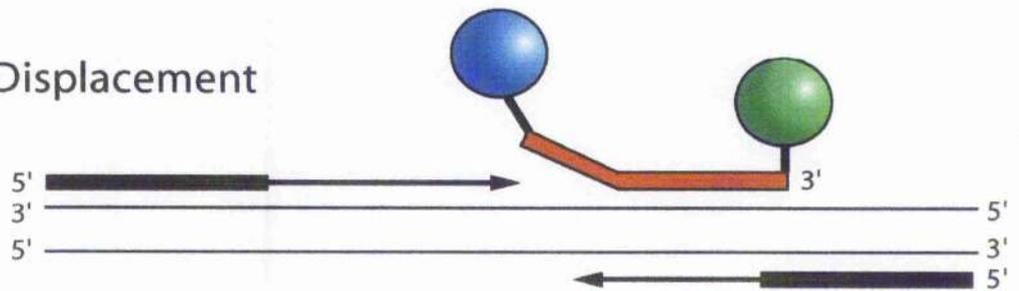


Quencher

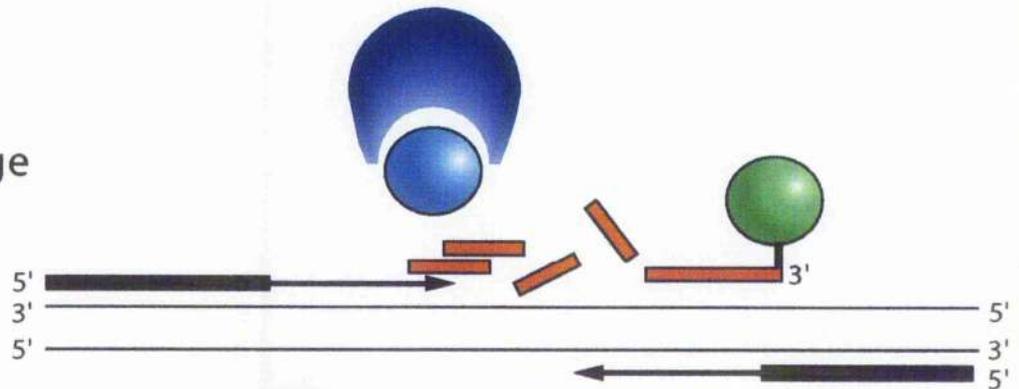
### Polymerisation



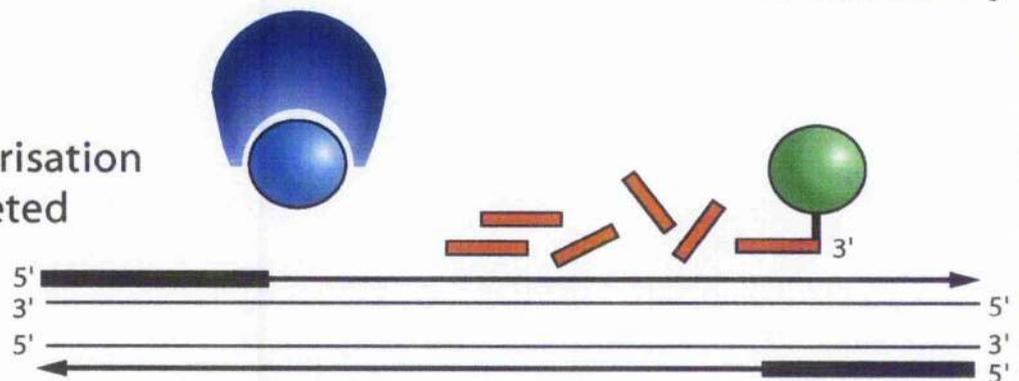
### Strand Displacement



### Cleavage



### Polymerisation Completed



### 3.3.4.1 Primer/Probe Design and Validation

The primers and probes for use in the Taqman system need to be designed to very exacting specifications. The Taqman system incorporates its own design program, Primer Express, to facilitate adherence to the design specifications for the Taqman oligonucleotides that will in turn ensure successful hybridisation with the template cDNA and efficient amplification. BLAST (Basic Local Alignment Search Tool) searches were also conducted to determine the presence of sequences in the scientific databases that are similar to those amplified by each set of primers.

In the experiments, a multiplexing strategy was utilised to allow relative quantitation of cDNA between samples. Multiplexing is the use of more than one primer pair in the same tube. In relative quantitation, one primer pair amplifies the target and the other amplifies an endogenous reference in the same tube. The details of multiplexing shall be explained later in this Chapter.

An endogenous reference is employed as there may be difference in the amount of starting material between samples. The accepted method for minimising these errors and correcting for sample-to-sample variation is to amplify, simultaneously with the target, a cellular RNA that serves as an internal reference against which other RNA values can be normalised (Karge et al. 1998). The ideal internal standard should be expressed at a constant level among different tissues, at all stages of development, and should be unaffected by any experimental treatment. Three references are currently used;  $\beta$ -Actin, GAPDH, and ribosomal RNA (rRNA). The endogenous reference used in these experiments was rRNA. It was used as it constitutes 85-90% of cellular RNA, their levels are unlikely to vary under conditions that may affect expression of mRNA (Barbu et al. 1989), and it has also been shown to be more reliable than the other two normalisation genes in a comparison study of human malignant cell lines (Zhong et al. 1999).

VIC-labeled ribosomal 18s RNA probe and primers were used for normalisation in this research project (Perkin-Elmer, Applied Biosystems, Warrington, UK). VIC is an alternative fluorescent probe that is detected at a different wavelength from FAM hence allowing discrimination between the two different labeled probes. To prevent consumption of reagents by the abundant 18s within tissue samples, the concentration of 18s primers and probes were limited to

the same degree in all samples in accordance with the suggested protocol by applied biosystems.

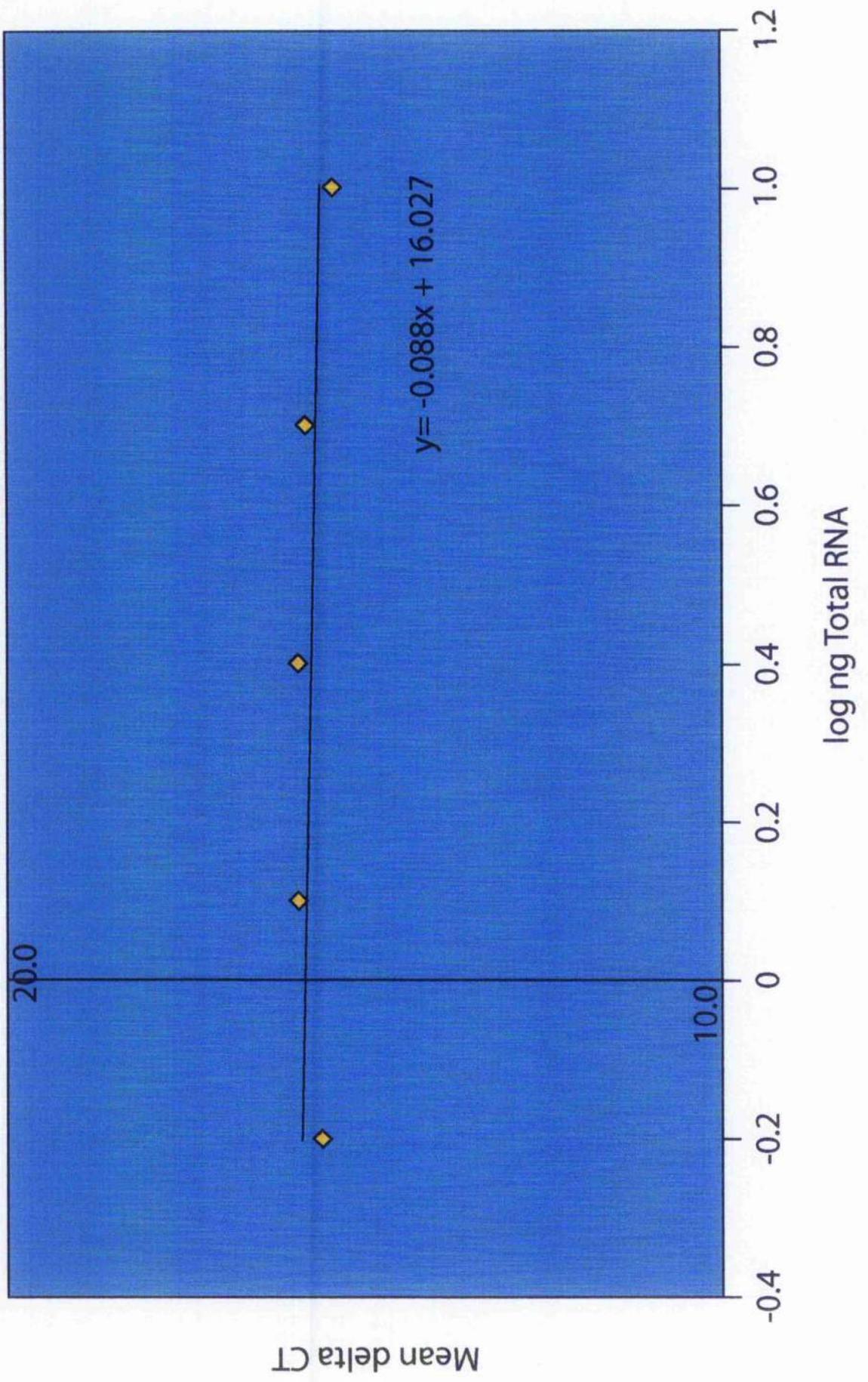
Prior to using the primers and probe for quantitation, the linearity of the response was determined by serial dilutions of both measuring primer probe amplification against a standard pool of RNA (cDNA) that expresses the target gene. The dilutions were up to 1/64. The log of the ng total RNA was plotted against the  $\Delta C_t$ . The  $C_t$  is the cycle number at which the PCR signal crosses a designated threshold; and the  $\Delta C_t$  is the difference between the  $C_t$  values for the specific amplicon and 18s. The gradient of the best fit line through these points should be  $< 0.1$ . An example is shown in Figure.3.6.

Figure.3.6.

Validation of 17  $\beta$ -HSD 5 primers and probe.

Representative plot for Taqman primer and probe validation. Validation was performed for each set of primers and probes used in this thesis. The mean Delta Ct is represented on the y-axis and the log concentration of total RNA is represented on the x-axis. The equation for the gradient of the slope is shown and the gradient is  $< 0.1$ . This figure is representative of the validation experiments.

Table 3.2 details the sequence of the primers and probes used in this study with the BLAST E value also indicated.



**Table 3.2**

**Details of Taqman primers and probes with sequences, accession numbers, and the Blast E value for the Taqman probe. The lower the E value then the more significant the match between the probe and the sequence it is designed to detect.**

	Accession Number	Forward Primer (5' - 3')	Tagman Probe (5' FAM - TAMRA 3')	Reverse Primer (5' - 3')	Blast E Value
Androgen Receptor	A39248	GTACCCGTGGCGGCATGGF	AGCAGAGTCCCTATCCCAAGTCCCA	CCCATTTCGCTTTTGACACA	5.00E-05
17 $\beta$ -HSD 2	L11708	TGTCAGCAGCATGGGAGGA	CCCCAATGGAAAGGCTGGCATCTT	GGTCACAGCCCGCTTTTGAT	2.00E-04
17 $\beta$ -HSD 5	NM003739	ATTGCTAGATTTCTGCAAGTCGAA	TCCGAGAGCACTATAGGCCAACCCAGAACAA	CCCATCGTTTGTCTCGTTGA	3.00E-07
3 $\beta$ -HSD 1	M35493	GCTCCATACCACACAGCAA	CCCGTTAGCCGCCAGTACAGCCCTT	TGGGTGGTAAGCCACAAAGTG	2.00E-04
3 $\beta$ -HSD 2	NM001198	GCAIGGGGTATTGTTAGGAAATG	CATCAAACTCCACCCACCTGGCTTCAT	TGCCCTGTTCGCCITCTG	4.00E-06
5 $\alpha$ Reductase 1	AF052126	TCCTCCTGGCCATGTCCT	TCCACTACGGGCATCGGTGCTTAAATTT	TCCTCCTGGCATCAGAAAACG	4.00E-06
5 $\alpha$ Reductase 2	M74047	GGGCCTCTTCTCCGTACATTAC	TCCACAGGACATTTGTGTACTCACTGCICA	GCCTTGAAGGACTCCCAITCCC	1.00E-07

#### **3.3.4.2 Taqman Protocol**

Real-Time relative quantitative RT-PCR was used to measure the levels of Androgen Receptor, 17  $\beta$ -HSD 2, 17 $\beta$ -HSD 5, 5 alpha-reductase type 1, 5 alpha reductase type 2, and 3  $\beta$ -HSD types 1 and 2 in the cDNA samples. A reaction mixture was made containing Stratagene Taqman buffer, MgCl<sub>2</sub> (5.5mmol/l), Sure Start Taq DNA polymerase (0.025 IU/ $\mu$ l; Stratagene, Amsterdam, Netherlands), dATP (200  $\mu$ mol/l), dCTP (200  $\mu$ mol/l), dGTP (200  $\mu$ mol/l), dUTP (200  $\mu$ mol/l), specific target amplicon forward and reverse primers (300nmol/l; Biosource, Nivelles, Belgium), and specific probe (200nmol/l; Biosource, Nivelles, Belgium). Additionally specific primers and probe for ribosomal 18S were also added at 50nmol/l (Perkin-Elmer; Applied biosystems, Warrington, UK).

The mixture was then aliquoted into separate tubes for each cDNA sample. An amount of 2.5  $\mu$ l/replicate of 1: 2.5 dilution cDNA was added to each tube. After mixing, 23 $\mu$ l of each sample were added to the wells on a PCR reaction plate with each sample being added and analysed in triplicate. Wells were sealed with optical caps and the PCR reaction run on the ABI prism 7700 using standard conditions.

To assess if genomic DNA contamination was present, two negative controls were added to the plate in triplicate. A no template (containing water) control and no reverse transcriptase (containing RNA) control.

#### **3.3.5 Taqman Analysis and Statistics**

PCR data was analysed using the formula  $2^{-\Delta\Delta CT}$  (as described above the Ct is the cycle number at which the PCR signal crosses a designated threshold). The  $\Delta CT$  is the difference between the Ct value at the threshold for the target amplicon and the internal normalisation control, 18S.  $\Delta\Delta CT$  is the difference between the  $\Delta CT$  and the internal reference control, which all samples are compared to thus providing a relative value to the reference. As each cDNA sample was analysed in triplicate the mean  $\Delta CT$  was used for each.

Statistical analysis was conducted using SPSS 11.0 for Windows. One-way analysis of variance (ANOVA) with Least Significant Difference (LSD) post testing used to evaluate whether there were significant differences in mean target mRNA expression between samples with error bars signifying the standard error of the mean. Results with a p value of <0.05 were considered statically significant.

## Chapter 4

# Androgen Receptor Expression in Human Endometrium

## 4.1 Introduction

The role of the androgen receptor (AR) in endometrial biology has been subject to limited study to date. Immunolocalisation of the AR in human endometrium has failed to reveal a consensus regarding its expression and no studies have exhibited its expression at the messenger RNA levels in human endometrium. However, studies do agree that the AR is expressed in human endometrium.

The regulation of its expression provides conflicting data. Oestrogen stimulation of endometrium appears to produce an up regulation of androgen receptor (Fujimoto et al. 1995; Adesanya-Famuyiwa et al. 1999; Apparao et al. 2002) while progestogens produce a down regulation (Iwai et al. 1995; Apparao et al. 2002). Further research is therefore required to elucidate such mechanisms and provide clarity in this area. A more detailed overview of the published literature regarding androgen receptor expression and regulation can be found in Chapter 1.7.2.

Sex steroids play an important role in the regulation of endometrial function. To play such a role there must be a mechanism for their action and this may be direct or indirect. In the case of androgens and endometrium, the presence of the AR would be vital to the direct action of androgen receptor ligands such as testosterone and dihydrotestosterone. Indirect mechanisms such as enzymatic conversion to a more or less biologically active form or even to an alternate sex steroid represent indirect mechanisms for androgen action. Indirect mechanisms may occur as paracrine, autocrine or intracrine processes. Intracrine mechanisms have been established in other tissue systems and appear to be important areas for enzymatic action. An intracrine mechanism allows each target cell the autonomous control over its own sex steroid requirements. While the biological role of androgens and AR remains to be determined, data have been published that examine their possible role in endometrial function.

A fundamental role of the endometrium is the provision of a receptive endometrium for conceptus implantation, allowing progression to a viable pregnancy. Upon establishment of a viable pregnancy, the endometrium will continue to play a role in its maintenance under the influence of sex steroids. If a conceptus is not established then menstruation will occur. Elevated serum androgen levels are associated with recurrent miscarriage (Okon et al. 1998) and raised

androgen levels are a feature of polycystic ovarian syndrome (PCOS), a condition associated with subfertility. The role that androgens may play in the pathogenesis of subfertility or miscarriage is not yet known but its effect on factors associated with poor endometrial receptivity have been studied.

Further clinical evidence of the effect that androgens have upon endometrial function is available by observing the actions that pharmacological agents used in gynaecology have upon the endometrium. Many pharmacological agents used in gynaecology such as, progestogens and danazol, are known to bind to the AR (Punnonen et al. 1982; Hoppen et al. 1987; Kumar et al. 2000).

In-vitro cell cultures where progestogens and androgens have been added were shown to inhibit endometrial proliferation (Neulen et al. 1987). Additional studies have also revealed that androgenic progestogens can induce endometrial atrophy in both eutopic endometrium (Floyd 1980; Jeppsson et al. 1984; Rose et al. 1988; Fedele et al. 1995) and the ectopic endometrium of endometriosis (Chimbira et al. 1980). Paradoxically, it is also known that treatment with the anti-progestogens (such as mifepristone, RU486) will also induce endometrial atrophy (Batista et al. 1992; Cameron et al. 1996).

Atrophic endometrial changes were also reported by Miller et al. who studied an interesting group of 32 young preoperative transsexual females receiving long term androgen therapy. The histopathology of this group of patients revealed endometrial atrophy (Miller et al. 1986). Chada et al. also investigated testosterone treated female transsexual patients, specifically looking at AR expression, and found AR expression to be increased but failed to comment upon the finding of endometrial atrophy (Chada et al. 1994).

Another pharmacological agent that induces atrophy is danazol, also an androgenic drug used in the management of menorrhagia, endometriosis and endometrial hyperplasia (Sedati et al. 1992). As it is an effective agent in inducing endometrial atrophy, it is also now employed in the pre-operative thinning of the endometrium prior to endometrial ablation treatment for menorrhagia. It has been shown to be as effective as gonadotrophin down regulation with gonadotrophin releasing hormone agonists (Sowter et al. 2000). A similar ability to induce endometrial atrophy is seen with the use of the commonly used androgenic progestogens, norethisterone and levonorgestrel, both of which are used in the

management of menorrhagia and endometriosis due to their ability to induce endometrial atrophy.

Medroxy-Progesterone Acetate (MPA), another androgenic progestogen, has been utilised in the management and palliation of endocrine responsive endometrial carcinoma (Quinn 1999; Thigpen et al. 1999). Their use has resulted in improved progression free survival.

While AR has been shown to be expressed in endometrial carcinoma (Prodi et al. 1980; Ito et al. 2002) it is not known if androgens play a direct role by binding to the AR. Data could also support indirect androgen effects following aromatisation to oestrogens. Aromatase has been shown to be expressed in endometrial adenocarcinoma (Tseng et al. 1984; Yamamoto et al. 1993; Sasano et al. 1996) with abundant precursor for conversion being available within the serum and the post menopausal ovary (Jongen et al. 2002). In addition, high levels of androgens have been detected in the utero-ovarian vein and the ovary itself has been shown to have an increased capacity for the production of androgens with significantly higher levels associated with endometrial adenocarcinoma (Bremond et al. 1982; Nagamani et al. 1986; Nagamani et al. 1992).

In summary, there is no clear consensus regarding the expression or regulation of the androgen receptor in human endometrium. The observation that expression is decreased in the late secretory phase at the time of progesterone dominance may suggest a role for progesterone in the regulation of endometrial AR expression. Additional observations from research into clinical conditions such as polycystic ovarian syndrome suggest that there may be important direct and indirect roles for androgens. This is reinforced by observations from studies that have employed pharmacological agents that have a binding affinity for the androgen receptor.

In this Chapter, I aim to add clarity to the expression of the androgen receptor at the messenger RNA and protein levels. I will also explore the possible mechanisms for endogenous regulation of AR by studying the effect that anti-progestogens have on endometrial AR expression when administered in the secretory phase.

## 4.2 Material and Methods

Material and Methods have been described in Chapter 3. Details that follow summarise pertinent material and methods for this area of the study.

### 4.2.1 Tissue Collection

The patient group for AR immunohistochemistry consisted of fertile women (n= 32) with regular menstrual cycles lasting between 25 and 35 days who had not been using hormonal preparations for the preceding 3 months. Endometrial tissue included samples collected from women during the proliferative phase (n=15), early secretory (n=5), mid secretory (n=5) and late secretory phase (n=7) of the menstrual cycle. In this group all samples were full thickness biopsies of the uterus. The stage of the menstrual cycle was consistent with the patients reported last menstrual period and was confirmed by histological dating, according to the criteria of Noyes *et al* (Noyes *et al.* 1950). Furthermore, serum samples collected at the time of endometrial tissue collection for determination of circulating oestradiol and progesterone concentrations by RIA were consistent with the designated cycle stage and a significant decline in progesterone concentrations was apparent between biopsies taken in the mid and late secretory phase of the cycle, see chapter 3.1.

The patient group for AR real-time quantitative RT-PCR similarly included women with regular menstrual cycles lasting between 25 and 35 days who had not been using hormonal preparations for the preceding 3 months. Samples were dated according to their last menstrual period and the criteria of Noyes *et al* (Noyes *et al.* 1950). Endometrial samples from all stages of the menstrual cycle samples were divided into proliferative (n=12) and secretory (n=15) phases with further classification of samples as early-(n=4), mid-(n=3), and late-proliferative (n=5) and early-(n=5), mid-(n=5), and late-secretory (n=5) phases.

Some samples were also collected in Tissue Tek II OCT (Van Waters and Rogers International, South Plainfield, NJ). The samples collected in Tissue Tek were immersed in a 10% sucrose PBS solution for 15 minutes then microwave stabilised prior to freezing in Tissue Tek solution with liquid nitrogen to  $-70^{\circ}\text{C}$  in keeping with the protocol devised by Slayden *et al.* (Slayden *et al.* 1995). Samples were stored at  $-80^{\circ}\text{C}$  and when required were cryo-sectioned to 10 micron thickness and shipped on dry ice to the Oregon National Primate Research Centre (ONPRC)

for in-situ hybridisation studies of AR. (performed by collaborators Professor R.M. Brenner and Dr Ov.D. Slayden)

Archived paraffin embedded sections from two additional patient groups were also available for analysis from previous studies performed within Professor Critchleys' department (Cameron et al. 1996; Cameron et al. 1997).

The first of these two groups consisted of six healthy women with regular menstrual cycles (25–35 days). The effects of daily low-dose RU 486 on endometrial maturation and proliferation have already been described in this group of women (Cameron et al. 1996). No subject had used hormonal contraception in the preceding 3 months. The subjects were studied in a control and a treatment cycle. Two placebo capsules were taken daily from the onset of menses of the first cycle (control). These were then replaced with identical capsules of RU 486 (two 1 mg capsules), commencing on the first day of menses of the second (treatment) cycle and daily for 30 days. Endometrial biopsies were collected with a Pipelle endometrial sampling device (Prodimed, Neuilly-en-Thelle, France) in the control cycle, 7–11 days after the plasma LH surge and on the corresponding day of treatment with RU486 (days 19–28). In the previous report on samples obtained from these women (Cameron et al. 1996) it was noted that RU 486 treatment suppressed mitotic activity in the endometrial glands and elevated ER and progesterin receptor levels. The AR was not studied. Paraffin embedded tissue from these studies were available for the current work.

The second group consisted of an additional 10 healthy women with regular menses (25–35 days), all of whom had participated in a study evaluating the effects of postovulatory administration of anti-progestogens on the endometrium (Cameron et al. 1997). Again, no subject had used hormonal contraception in the preceding 3 months. Archival endometrial biopsies from this group of women were available for evaluation of AR localisation. Each subject had been studied over a control cycle and a treatment cycle. In the treatment cycle, the woman received 200 mg oral RU 486 on the second day after the onset of the LH surge in urine (LH + 2). Endometrial biopsies were collected with a pipelle endometrial sampler, either 4 or 6 days after the LH surge in the control cycle and on the corresponding day of the treatment cycle, that is, 2 or 4 days after oral administration of RU486.

#### **4.2.2 Androgen Receptor Immunohistochemistry.**

5 micron paraffin sections were de-waxed and re-hydrated as in the general protocol (see Chapter 3.2.1). Following the washing steps detailed in the general protocol a pressure cooker 0.01M sodium citrate antigen retrieval step was included. The blocking steps included normal horse serum as the 20 minute non-immune blocking step in a humidified chamber. The antibody raised against the AR was  $\alpha$ F39.4 (IgG<sub>1</sub>; Biogenex Laboratories, Inc., San Ramon, CA, USA). Tissue sections were incubated overnight at 4 °C in the humidified chamber with 50  $\mu$ l of  $\alpha$ F39.4 diluted 1:480 in PBS/Bovine Serum Albumin (BSA). The primary antibody was substituted with equimolar concentration of mouse immunoglobulin (IgG) in negative control sections. After the primary antibody incubation, sections were washed between each stage for 10 minutes with PBS/Tween buffer solution. Sections were incubated with horse anti-mouse biotinylated secondary antibody made in normal horse serum to incorporate a second blocking step. The final incubation was performed using the ABC-Elite detection system (Vectastain, Vector Laboratories, Inc., Peterborough, UK). DAB (DAKO Liquid, DAKO Corporation, California, USA) was then added to the sections allowing visualisation of the stain. Sections were counterstained with Harris' haematoxylin, dehydrated in ascending grades of alcohol and mounted from xylene in Pertex. (See Chapter 3.2.2)

#### **4.2.3 Androgen Receptor In-Situ Hybridisation.**

AR in-situ hybridisation was generously performed by Dr Ov Slayden from the Oregon National Primate Research Centre whilst collaborating on research into expression of AR protein and messenger RNA in human and macaque endometrium. The following is a summary of the material and method for the in-situ hybridisation (ISH) performed by Dr Slayden in human and macaque endometrium (Slayden et al. 2001).

ISH of 10 micron frozen sections of human endometrium was conducted with a riboprobe labeled with [35S]UTP (NEN Life Science Products) derived from a rhesus monkey-specific 330-bp AR complementary DNA (cDNA) (GenBank accession number AF092930). The nucleotide sequence of this AR cDNA has 99% homology with the corresponding AR human sequence (Abdelgadir et al. 1999). Frozen sections of endometrium were mounted on SuperFrost Plus slides (Fisher

Scientific) and fixed in 4% paraformaldehyde in PBS for 20 min at 4°C. The tissue sections were rinsed in 2x SSC (standard saline citrate), then acetylated with 0.25% acetic anhydride in 0.1 mol/L tri-ethanolamine (pH 8.0) for 10 min. Slides were then rinsed in 2x SSC, dehydrated through an ascending series of alcohols, and air-dried. At this point at least one slide per tissue group was treated with ribonuclease A [RNase A; 20 mg/mL, 0.5 mol/L NaCl, 0.01 mol/L Tris, and 1 mmol/L ethylenediamine tetraacetate (EDTA), pH 8.0] as a negative control. The slides were prehybridized for 1 h at 42°C in 10 mmol/L dithiothreitol, 0.3 mol/L NaCl, 20 mmol/L Tris (pH 8.0), 5 mmol/L EDTA, 1× Denhardt's solution, 10% dextran sulfate, and 50% formamide. Sections were then incubated at 55°C overnight in the same solution containing the appropriate concentrations of the sense and antisense probes (5 million cpm/mL). The most appropriate concentrations of labeled probes were empirically determined by serial dilution trials. RNase-treated sections were incubated with the antisense probe. After treatment with RNase A-containing buffer (20 mg/mL; 0.5 mol/L NaCl, 0.01 mol/L Tris, and 1 mmol/L EDTA, pH 8.0) at 37°C for 30 min to inactivate nonhybridized probe, the slides were rinsed in a descending series of SSC (2, 1, and 0.5 x) and then incubated in 0.1 x SSC at 65°C (high stringency) for 30 min. Sections were dehydrated in an ascending series of alcohol dilutions, vacuum-dried, coated with NTB2 autoradio-graphic emulsion (Eastman Kodak Co., Rochester, NY), stored at 4°C for 2 weeks, developed in D-19 (Eastman Kodak Co.), lightly counterstained with haematoxylin, dehydrated in an ascending series of alcohol dilutions, cleared with xylene, and coverslipped with Permount (Fisher Scientific). Sense- and RNase-treated controls had no specific signals.

Silver grains were counted over stroma and glandular epithelium in sections hybridized with radiolabeled probe. The counts were made with Optimas (Media Cybernetics, Silver Spring, MD) on images captured at x 250 original magnification. In RNase-treated slides, regardless of hormone treatment or state, grains over the tissues were identical to those over the glass slide. Therefore, a region of the slide away from the section was counted as background, and these counts were subtracted from each epithelial and stromal field counted. The abundance of silver grains in the field was expressed as the number of silver grains per nucleus (stromal cell and glandular cell, respectively). These counts were then expressed as a percentage of the maximum signal in all the sections analyzed.

Statistical analysis was performed using one way analysis of variance with Fishers' protected least significant difference post test (SPSS 11.0). A p value <0.05 was considered significant.

#### **4.2.4 Temporal expression of androgen receptor messenger RNA in endometrium during the menstrual cycle.**

Real-Time quantitative RT-PCR was used to measure the levels of Androgen Receptor in menstrual cycle cDNA samples. Frozen samples of endometrium stored at -80°C were first homogenised, and then total RNA was extracted using the commercially available product Trizol (Invitrogen Life technologies Ltd, Paisley, UK). RNA extraction, quantitation and reverse transcription methods have been detailed in Chapter 3. (See Chapters 3.3.1; 3.3.2; 3.3.3)

Menstrual cycle samples were divided into proliferative (n=12) and secretory (n=15) phases with further classification of samples as early-(n=4), mid-(n=3), and late-proliferative (n=5) and early-(n=5), mid-(n=5), and late-secretory (n=5) phases.

AR primers and probe were designed using the applied biosystems primer express program. A reaction mixture was made containing Stratagene Taqman buffer, MgCl<sub>2</sub> (5.5mmol/l), Sure Start Taq DNA polymerase (0.025 IU/μl; Stratagene, Amsterdam, Netherlands), dATP (200 μmol/l), dCTP (200 μmol/l), dGTP (200 μmol/l), dUTP (200 μmol/l), AR forward and reverse primers (300nmol/l; Biosource, Nivelles, Belgium), and AR probe (200nmol/l; Biosource, Nivelles, Belgium). Additionally specific primers and probe for ribosomal 18S were also added at 50nmol/l (Perkin-Elmer; Applied biosystems, Warrington, UK).

The mixture was then aliquoted into separate tubes for each cDNA sample. An amount of 2.5 μl/replicate of 1: 2.5 dilution cDNA was added to each tube. After mixing, 23μl of each sample were added to the wells on a PCR reaction plate with each sample being added and analysed in triplicate. Wells were sealed with optical caps and the PCR reaction run on the ABI prism 7700 using standard conditions.

Statistical analysis was performed using one way analysis of variance with Fishers' protected least significant difference post test (SPSS 11.0). A p value <0.05 was considered significant.

## 4.3 Results

### 4.3.1 Immunohistochemical localisation of AR in human endometrium.

Menstrual cycle samples were divided into proliferative phase (n=15), early secretory phase (n=5), mid secretory phase (n=5), and late secretory phase (n=7). Immunolocalisation of the AR revealed a predominantly endometrial stromal distribution with no (or barely detectable) staining in the glands (Figure. 4.1).

To facilitate objective image analysis the sections were digitalised and treated as described previously in Chapter 3.2.5. This provided a mean percentage positive result for the expression of AR in the stromal and glandular compartments of the endometrium. Spatial variation in expression was determined by separate analysis of the functional and basal layers of the endometrium. Temporal analysis was assessed by comparison of the functional and basal layers across the menstrual cycle stages.

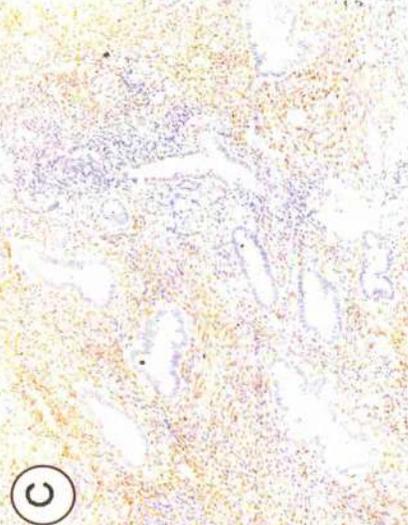
The spatial distribution of the AR was similar in the functional and basal layers throughout the menstrual cycle with stromal immunoreactivity being the predominant feature. Significant temporal variation in the expression of the AR was noted.

The abundance of AR-positive cells was similar in the functional and basal stroma throughout the entire proliferative, early secretory and mid secretory phases of the cycle, but there was a clear decrease in immunostaining in the late secretory phase. This decrease was particularly marked in the functional layer but was noted in both layers. Quantitation of the frequency of AR-positive cells showed that the decrease in the late secretory phase was highly significant (Figure. 4.2).

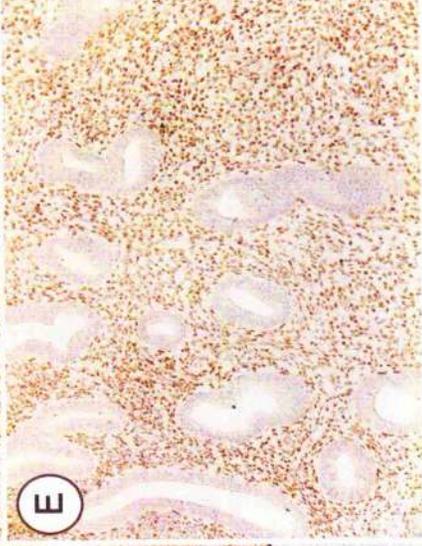
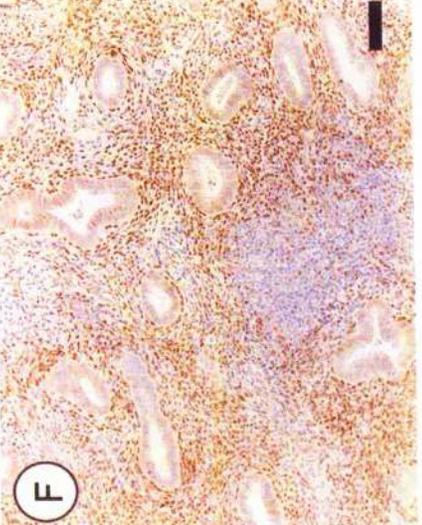
Figure 4.1

Immunohistochemical localisation of AR in human endometrium. Images A, B and C represent the functional layer AR immunoreactivity. Images D, E and F represent the basal layer AR immunoreactivity. All images were taken at x20 magnification. AR immunoreactivity in the proliferative (A and D), early secretory (B and E), and late secretory phases (C and F) was evident only in the stroma of with minimal glandular immunoreactivity being noted. The number of positively immunoreactive cells was lowest in the late secretory phase, significantly less in both layers compared to any other menstrual cycle stage. A negative control from the basal layer proliferative phase is inset in image D. Scale Bar represents 50  $\mu$ m.

Late Secretory



Early Secretory



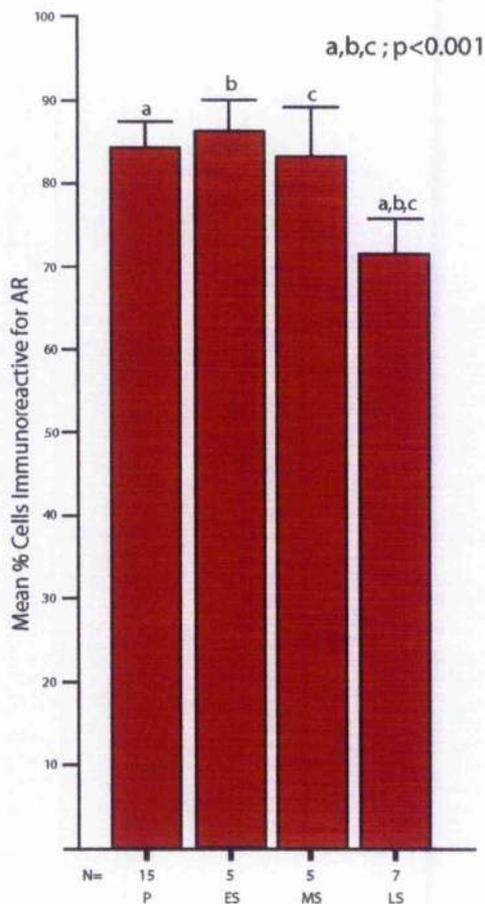
Functional

Basal

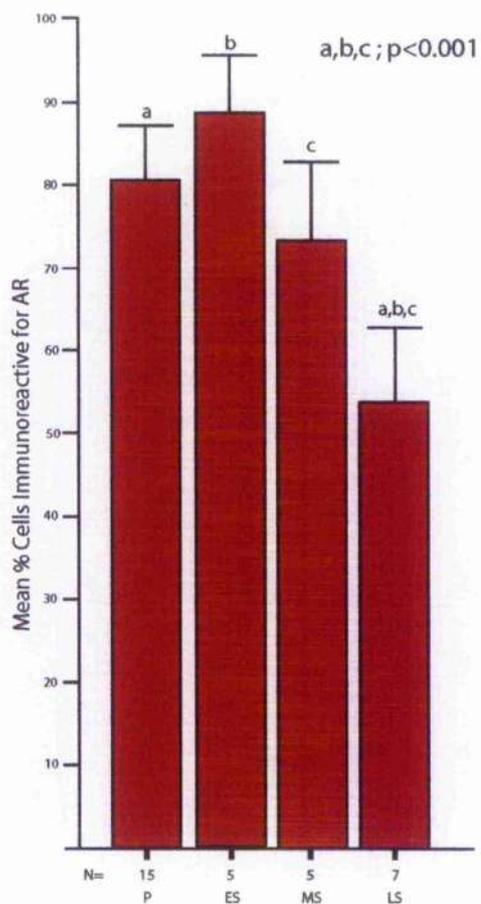
Figure 4.2

Quantification of AR positive cells in human endometrial Stroma. Data are expressed as mean ( $\pm$ SEM) percentage of cells staining positive for AR during the menstrual cycle (P, Proliferative; ES, early secretory; MS, mid secretory; LS, late secretory). The percentage of AR immunoreactive stromal cells was significantly lower ( $P < 0.05$ ) during the late secretory phase compared with other phases of the cycle in both the functional and basal layers.

# Stromal AR Immunoreactivity



Functional Layer



Basal Layer

#### **4.3.2 In-Situ hybridisation of AR mRNA in human endometrium.**

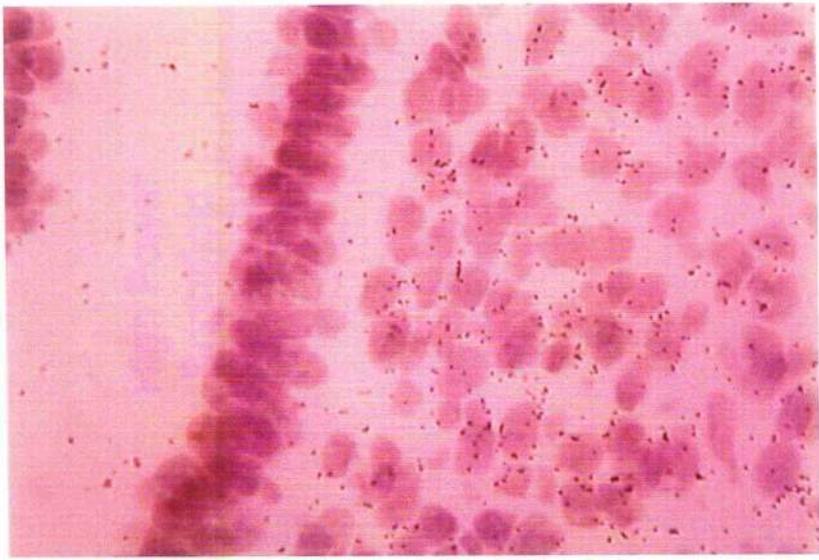
To assess if human endometrial AR mRNA expression was consistent with AR protein expression, in-situ hybridisation (ISH) was performed in addition to quantitative RT-PCR. ISH of AR mRNA in human endometrium aimed to determine whether the same cells that expressed the AR protein also expressed AR mRNA and ascertain if a similar decline in AR mRNA is noted in the late secretory phase.

A representative sample from the early proliferative phase is depicted at low magnification with bright field illumination (Figure 4.3), dark field illumination (Figure 4.3) and at high magnification bright field (Figure 4.3). These images are consistent with the immunohistochemistry (IHC) data and show that AR mRNA was predominantly localised in the endometrial stroma and not the glandular epithelium. As with IHC, ISH of late secretory phase endometrium showed a decline in the intensity of the stromal cell signal in the late secretory phase (Slayden et al. 2001).

Figure 4.3

In situ hybridisation (ISH) of AR in early proliferative phase endometrium. A comparison of low (x 2.5) magnification bright field and dark field optics shows that the AR mRNA signal is in the stroma. A higher magnification (x 50) of the ISH clearly shows that the glands are negative.

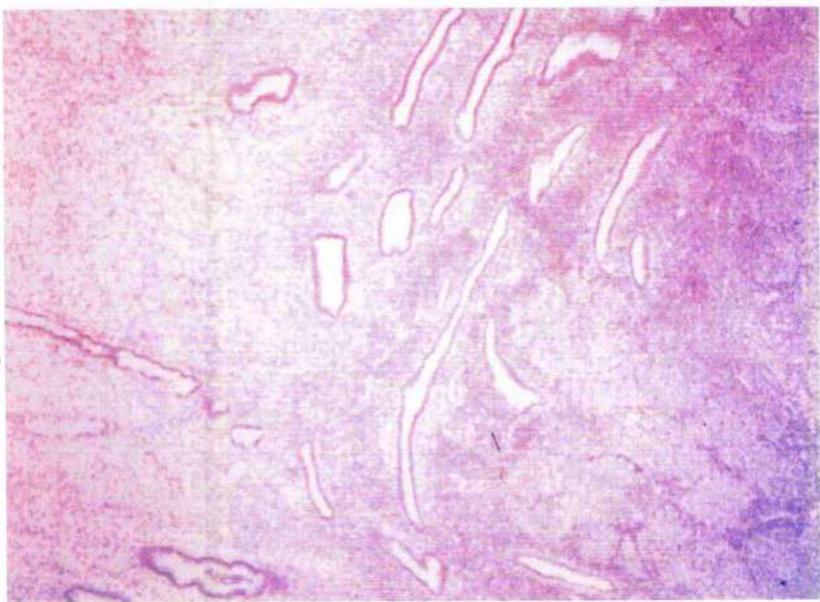
High Power  
Brightfield



Low Power  
Darkfield



Low Power  
Brightfield



### **4.3.3 Temporal expression of AR mRNA with quantitative RT-PCR.**

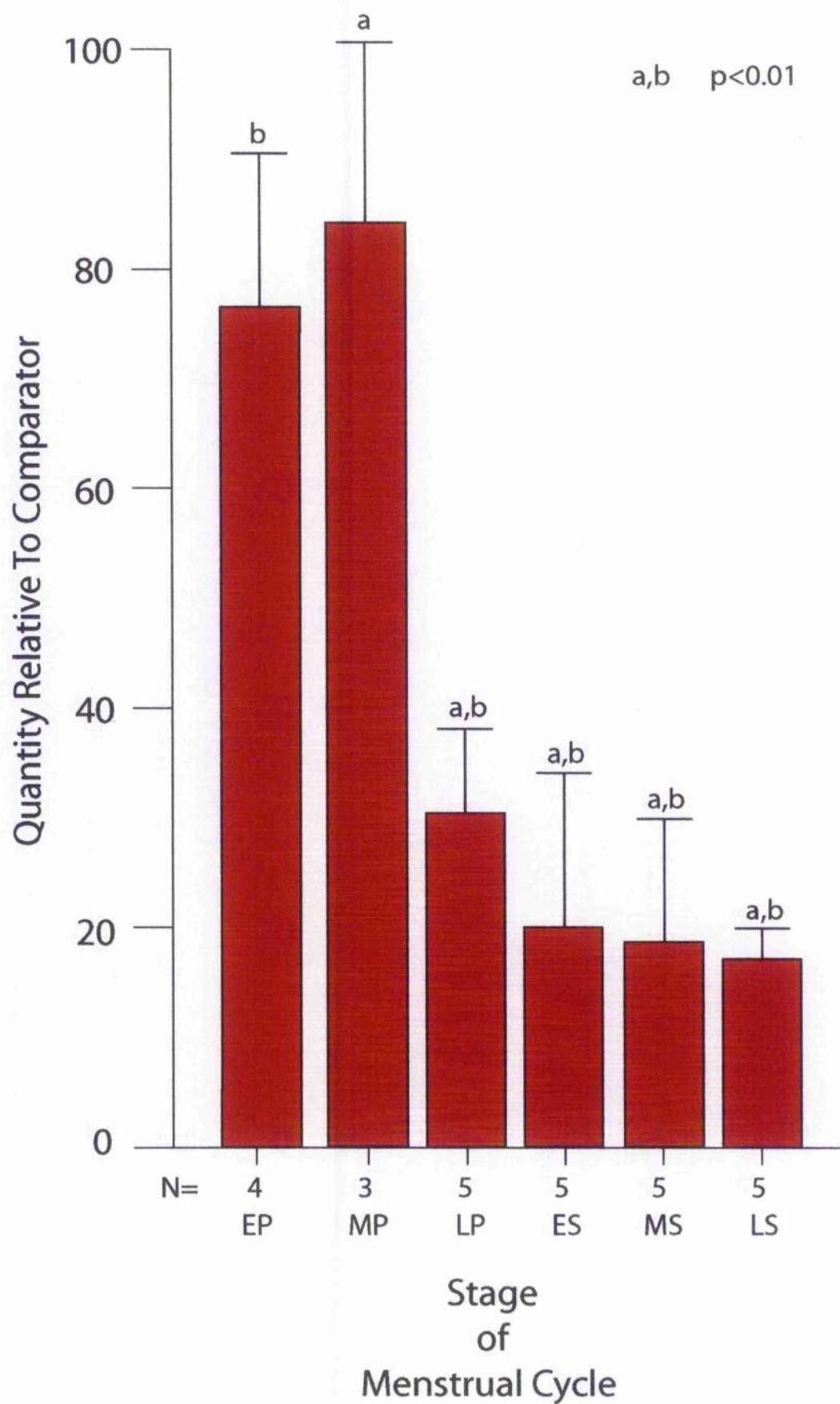
The menstrual cycle samples were divided into proliferative (n=12) and secretory (n=15) phases with further classification of samples as early-(n=4), mid-(n=3), and late-proliferative (n=5) and early-(n=5), mid-(n=5), and late-secretory (n=5) phases.

Significantly higher levels of AR mRNA were observed in the early and mid proliferative phase endometrial tissue samples compared to the remainder of the menstrual cycle stages. From the mid proliferative phase of the menstrual cycle there is a progressive decline in the relative expression of AR mRNA (Figure 4.4).

The reduced expression of AR protein occurs in the late secretory phase human endometrium and this is supported from the findings of ISH and quantitative RT-PCR.

Figure 4.4

Quantitative evaluation of AR mRNA in endometrium across the stages of the menstrual cycle. All endometrial tissue samples were compared to an internal control (comparator) obtained during the early proliferative stage of the menstrual cycle. AR mRNA levels were high in the early and mid proliferative samples but fell significantly in samples taken from the late proliferative and early to late secretory samples ( $p < 0.01$ ). EP-early proliferative, MP-mid proliferative, LP-late proliferative, ES-early secretory, MS-mid secretory, LS-late secretory.



#### **4.3.4 AR Localisation in Anti-Progestogen Treated Endometrium**

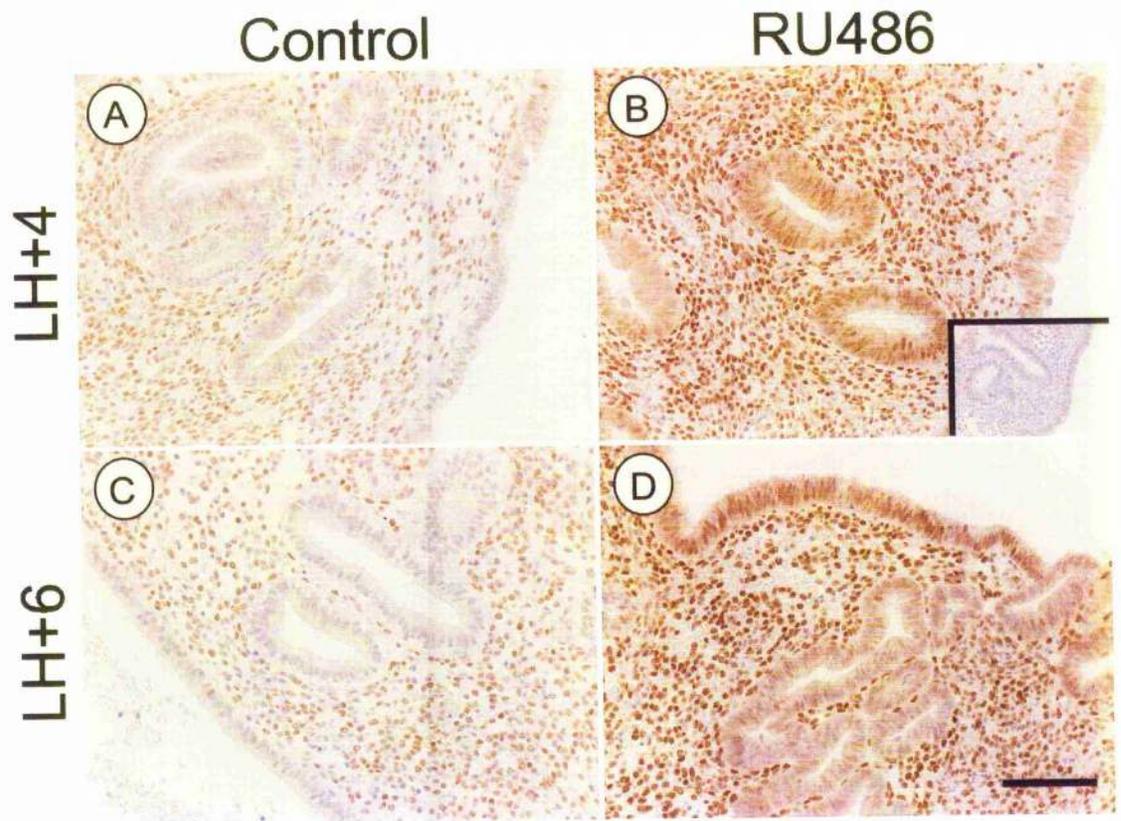
The immunohistochemical assessment of endometrial samples from six control patients and six women treated with 2 mg/ day RU 486 for 30 days for AR immunoreactivity was performed as described in Chapter 3.2.

In the control specimens there was essentially no immunoreactivity in the glandular or surface epithelium, but there was distinct immunostaining of the stroma in keeping with the findings detailed previously (Figure 4.5). After 30 days of RU 486, there were distinct and notable increases in the AR immunostaining of the glands and surface epithelium plus some enhancement of stromal AR immunostaining.

Furthermore, we evaluated the IHC preparations from five placebo controls and five patients treated with a single dose of 200 mg RU 486 on day LH + 2 and sampled on days LH + 4 and LH + 6. The controls, who were sampled on days LH + 4 and LH + 6, had essentially no AR staining in the glands or surface epithelium, but had definite stromal AR immunostaining (Figure 4.5). Figure 4.5 is representative of the controls from both the 2-mg and the 200-mg studies. On both days LH + 4 and LH + 6 after the 200 mg RU 486 dose there were clear increases in glandular and surface epithelial immunostaining and some increase in stromal AR immunostaining (Figure 4.5).

Figure 4.5

Immunohistochemical localisation of AR in anti-progestogen treated endometrium. The findings shown in the photomicrographs are representative of the 2mg and 200mg studies. Images A and C represent the control endometrial samples taken on days LH+4 and LH+6 respectively. Images B and D represent the treatment groups on days LH+4 and LH+6 respectively following administration of 200mg of RU486 on day LH+2 (Image D inset is negative control). AR is demonstrated in the stromal cells of both the treatment and control groups. When compared to the control groups, treatment with RU486 increases AR immunoreactivity in the endometrial glandular and surface epithelial cells. Scale bar represents 50  $\mu\text{m}$ .



#### 4.4 Discussion

The assessment of the biological role of androgens in endometrial function has been hindered by a lack of consistent findings from published data. To generate a hypothesis for their role it was necessary to undertake descriptive studies to provide a reliable platform for further research. This study determined that AR is expressed in the endometrial stromal compartment with down regulation of AR protein and messenger RNA in the progesterone dominated late secretory phase. Furthermore, treatment of endometrium with the anti-progestogen RU486 revealed up-regulation of AR in the stromal, glandular and surface epithelial compartments. These latter findings suggest a role for progesterone in the regulation of AR.

The experiments performed on human endometrium were carried out in collaboration with colleagues at the Oregon National Primate Research Centre. Our collaborators studied nonhuman primates, Rhesus Macaques, to investigate the role of androgens in macaque endometrium (Slayden et al. 2001). The antibody utilised in both human and nonhuman primate tissues was the monoclonal antibody F39.4 and the in situ hybridisation protocols employed were identical. In keeping with the present results from human endometrium, Macaque endometrial stromal cells were the predominant cell population expressing AR. In addition, the studies on the macaque endometrium suggested a role for progesterone in the regulation of endometrial AR expression. Using three independent technical approaches (binding assays, Immunohistochemistry, and In-situ hybridisation) it was demonstrated in ovariectomised macaques that expression of AR protein and message was minimal and were increased significantly following 28 days of oestradiol treatment. Addition of progesterone for the last 14 days inhibited this increase. This finding in the macaque is analogous to human endometrial tissue.

Human endometrial immunohistochemical sections were analysed using objective image analysis to determine the expression of AR protein in stromal cells. High levels of AR protein in the endometrium biopsied from the oestrogen dominated proliferative phase were demonstrated. In-situ hybridisation confirmed mRNA localisation to the stromal compartment. Quantitative real time RT-PCR revealed temporal down regulation of mRNA expression in the secretory phase. AR protein was also significantly down regulated in the progesterone dominated

secretory phase. Overall these current findings are most similar to the most recently published studies by Mertens et al. (Mertens et al. 2001) for human endometrium and Adesanya et al. (Adesanya-Famuyiwa et al. 1999) for rhesus Macaques.

In human endometrium, the use of full thickness endometrial biopsies maintained architectural integrity allowing a spatial and temporal description of the AR in all cellular compartments. The findings were consistent between human and nonhuman primate specimens unlike previously published studies on the androgen receptor, which lacked a consistency in their observations. The in-situ hybridisation study also provided novel data in human endometrium, as no studies have previously localised AR mRNA in human endometrium. The use of full thickness cryosections again allowed accurate spatial description of AR mRNA expression. The use of Taqman probes permitted the relative quantitation of AR mRNA expression to determine variances in AR mRNA expression through the menstrual cycle, another novel finding in this study.

The nonhuman primate studies were able to create animal models that exposed the endometrium to oestradiol alone then added in progesterone and/or RU486 for subsequent cycles to examine the effect of the single or combined sex steroid effect upon nonhuman primate endometrial AR expression. Such experiments were not feasible in human patients. The animal models simulated the human in-vivo situation studied. However, both human and nonhuman endometrium displayed consistent findings, namely: down regulation of AR in a progesterone dominated environment and up-regulation of AR following treatment with an antiprogestogen (RU486). These data suggest a role for progesterone in the regulation of endometrial AR expression. Indeed it has already been demonstrated in nonhuman primate endometrium that antiprogestogen treatment will up-regulate other sex steroid receptors such as oestrogen (ER) and progesterone receptor (PR) (Neulen et al. 1990; Slayden et al. 1994; Slayden et al. 1998). The expression of ER and PR are under dual control of oestrogen and progesterone and it would appear that a similar mechanism might exist for the regulation of AR.

The pattern of AR expression in human endometrium would suggest that any action of androgens would be predominantly in the proliferative and peri-ovulatory phases of the menstrual cycle. Endometrial tissue sections exhibit no substantial immunoreactivity in the glandular, endothelial or perivascular areas, as stromal

expression of AR predominates. This suggests that any genomic action of androgens would have to be mediated via the endometrial stromal cells. While the endogenous action of androgens remains to be elucidated, a common androgenic action appears to be endometrial atrophy. The data presented suggests a role for progesterone in the regulation of AR and allows a hypothesis to be formulated that may explain the mechanism of androgen/antiprogestogen endometrial atrophy.

Antiprogestogen treatment up-regulates AR, ER and PR in human endometrium (Cameron et al. 1996; Slayden et al. 2001). The underlying mechanism for this action may paradoxically be related to an enhanced action of oestradiol (E2) at the molecular level. Animal studies on mice and rabbits indicate that unoccupied PR (in the presence of E2 and absence of P) can down-modulate the effects of E2. Lydon et al. studied the action of E2 on the uterus and oviducts of wild type and PR knockout mice (Lydon et al. 1995). E2 produced hyperplasia in the uterus and oviducts of the PR knockout mice but in the wild type mice a far less dramatic effect was noted. This suggests that unoccupied PR can have a "braking" effect on E2 action. A similar role for unoccupied PR has also been described in rabbit endometrium (Chwalisz et al. 1991) and brain (Chappell et al. 2000). Anti-progesterone treatment effectively occupies PR and removes it from play; therefore, any braking effect of PR on E2 action would effectively be removed. Accordingly, the action of E2 at the molecular level would be enhanced resulting in elevated levels of ER, PR and AR. As such, the increased expression of AR may play a role in the mechanism of endometrial atrophy.

Increased endometrial AR could lead to increased binding of endometrial androgens, which may antagonise the effect of oestrogens on endometrial growth. Testosterone and danazol have been shown to inhibit endometrial cell proliferation in tissue cultures that contained phenol red in sufficient concentrations to provide an oestrogenic stimulus (Rose et al. 1988). The authors suggest that the anti-proliferative effects of danazol may involve a direct anti-oestrogenic effect. A further example of the ability of danazol to inhibit endometrial proliferation is demonstrated in patients suffering from endometrial hyperplasia who were treated with danazol. The changes of hyperplasia were reversed in 97% of patients (Bulletti et al. 1987). More recently longitudinal evaluation of the endometrium of patients on long term

danazol with light and electron microscopy has again shown its ability to induce endometrial atrophy (Fedele et al. 1995).

Androstenedione has also been shown to inhibit human endometrial cell growth and secretory activity *in vitro* (Tuckerman et al. 2000), and high plasma androgen levels have been associated with adverse reproductive outcome, including recurrent miscarriage (Tulppala et al. 1993; Okon et al. 1998; Bussen et al. 1999; Cermik et al. 2003). Okon et al showed that hyperandrogenism correlated highly with the increased endometrial production of glycodefin, a protein correlated with endometrial dysfunction. Their explanation presumed that this was due to androgen antagonism of the ability of oestrogen to prime the endometrium during the proliferative phase. Brenner et al., (personal communication from our collaboration) have preliminary data from studies on ovariectomised macaques to support the anti-oestrogenic effect of androgens in the endometrium. Systemic treatment with dihydrotestosterone blocked the stimulatory effect of E2 on ER and PR levels, oviductal ciliogenesis and endometrial mitosis (Hirst et al. 1992). The above findings give credibility to the possibility that elevated AR may mediate the anti-oestrogenic effect of androgens in the endometrium during antiprogesterone treatment.

A paradoxical observation to this hypothesis is that the risk of endometrial hyperplasia and invasive endometrial adenocarcinoma are increased in patients affected by PCOS. PCOS is a complicated metabolic disorder that results in elevated serum androgen, oestrogen and insulin levels. If, as suggested above, androgens exhibit anti-oestrogenic activity in the endometrium and PR is unoccupied then there should theoretically be mechanisms *in-vivo* to prevent endometrial proliferation. *In-vivo* this does not appear to be the case as proliferation and hyperplasia still occur. However, the endometrial endocrine phenotype is different from that of patients without PCOS. The elevated insulin levels associated with PCOS have been shown to induce aromatase activity in stroma and glands of endometrium whereas normally endometrium has no such capacity for intracrine synthesis of oestrogens (Randolph et al. 1987). Therefore, in PCOS the elevated free androgens may be converted to active oestrogen metabolites in both the endometrium and peripheral aromatase containing tissues, further driving endometrial proliferation. Oestrogenic stimulation drives cell proliferation and by the increase in the number of cell divisions the risk of random DNA replication errors (activation of oncogenes, inactivation of tumour-

suppression genes) increases. Depending on the kind of genetic damage a malignant phenotype may arise with progressive growth under the influence of oestrogens (Jongen et al. 2002). Thus, oestrogens act as tumour promoters but not as carcinogens. The action of oestrogen would then be augmented by the ability of endometrial cancers to produce aromatase within the tumour (Bulun et al. 1994; Watanabe et al. 1995). The above provides a mechanism for the indirect participation of androgens in endometrial hyperplasia/adenocarcinoma.

An interrelation may exist between ovarian morphology, local androgen production and hyperplastic and malignant diseases of the endometrium. It has been shown that the testosterone levels in the utero-ovarian vein blood samples from women with endometrial carcinoma were significantly elevated as compared with the utero-ovarian vein levels from women without endometrial carcinoma, without significant increases in the peripheral levels of these androgens (Bremond et al. 1982). In PCOS, ovarian stromal hyperplasia with increased ovarian androgen production is noted and rather than supplying precursor for further metabolism this may directly stimulate the AR in endometrium to produce malignant change (Hackenberg et al. 1996).

The paradoxical association of AR with endometrial atrophy and endometrial hyperplasia/adenocarcinoma remains to be resolved with further study. Indeed the biological role of AR in endometrial function requires attention from future research.

In conclusion, the data presented here provide a consistent description of the temporal and spatial expression of AR in human endometrium at the protein and mRNA levels. Evidence is also provided to support a role for progesterone in the regulation of endometrial AR expression. Additionally a mechanism for the anti-progesterone induced endometrial atrophy with reference to progesterone regulation of AR is also discussed.

## Chapter 5

# Levonorgestrel Regulation of Androgen Receptor (AR) and 17 $\beta$ hydroxysteroid dehydrogenase type 2 (17 $\beta$ -HSD2) Expression in Human Endometrium.

## 5.1 Introduction

Levonorgestrel (LNG) is a potent progestogen, which has a binding affinity for the androgen receptor, and is a common constituent of contraceptive preparations. The levonorgestrel-releasing intrauterine system (LNG IUS; Schering HC, West Sussex, UK) releases 20 micrograms LNG locally to the endometrium over 24 hours (Luukkainen et al. 1990). Patient acceptability is high but many patients will discontinue therapy due to the adverse effect of breakthrough bleeding (BTB) (Anderson 1992; Findlay 1996). The mechanism responsible for BTB remains poorly understood but is likely to involve angiogenic factors, matrix metalloproteinase activation, and altered haemostatic factors that result in increased endometrial vessel fragility (Hickey et al. 2000). Such a pathogenesis may be directly related to the high dose of local LNG. Alternatively, indirect actions of LNG may be responsible, such as its influence upon endometrial sex steroid receptor expression, or ligand availability in the endometrium.

Sex steroid ligands play a pivotal role in the control of uterine function by binding to their respective receptors (See Chapters 1.7 and 4.1). Briefly, AR has been spatially localised to human endometrial stroma with up regulation in the oestrogen-dominated proliferative phase followed by down regulation in the late secretory phase (Mertens et al. 2001; Slayden et al. 2001). ER and PR are also up-regulated in both glandular and stromal tissue in the proliferative phase. However, in the secretory phase a down-regulation of ER is noted in both glands and stroma. PR is also down regulated in the glands but expression persists in stromal tissues (Garcia et al. 1988; Lessey et al. 1988; Snijders et al. 1992; Critchley et al. 1993). The progesterone receptor subtype that persists in the stroma is predominantly the PR<sub>A</sub> isoform (Wang et al. 1998; Brosens et al. 1999).

LNG, delivered locally by the LNG IUS, is known to down regulate ER and PR expression in the human endometrium (Critchley et al. 1998). This alteration in sex steroid receptor expression may affect endometrial cytokine release which in turn may contribute to the pathogenesis of breakthrough bleeding (BTB) (Jones et al. 2000). The cellular and molecular expression of the androgen receptor in endometrium exposed to high dose intra-uterine levonorgestrel has not been

previously reported and AR may play an important role in endometrial physiology (Slayden et al. 2001).

In reproductive tissues, the local actions of sex steroids are modulated by hydroxysteroid dehydrogenases (HSDs). The various dehydrogenases are multi-gene families. The human  $17\beta$ -HSD family has six known members, each being a separate gene from a different chromosome with distinct properties in terms of substrate and redox direction. The type 2 isoform ( $17\beta$ -HSD2) has a major role in the inactivation of oestradiol to oestrone.  $17\beta$ -HSD2 is also the enzyme responsible for converting androgens to less potent forms while also activating progesterone. The  $17\beta$ -HSD2 isoform is expressed in endometrial glandular epithelium, and is up regulated by progesterone (Macentausta et al. 1993). Its activity decreases when progesterone concentrations decline premenstrually or following antiprogestin administration (Maentausta et al. 1993; Mustonen et al. 1998). LNG is a potent androgen that binds avidly to the AR (Kloosterboer et al. 1988). The availability of other androgenic ligands to bind to the AR may be influenced by the availability of the enzyme,  $17\beta$ -HSD2.

In this Chapter, the expression of both AR and  $17\beta$ -HSD2 in a longitudinal study of endometrial samples taken from naturally cycling women and from women with a LNG IUS over a twelve month time frame has been studied. The results provide new data on the expression patterns of these two molecular components that may be relevant to the bleeding patterns reported with intrauterine delivery of levonorgestrel.

## 5.2 Material and Methods

Material and Methods have been described previously in Chapter 3. Details that follow summarise pertinent material and methods for this area of the project. Human endometrial samples were obtained from three different patient groups.

### 5.2.1 Tissue Collection

The first group consisted of fertile women ( $n=24$ ) with regular menstrual cycles lasting between 25 and 35 days who had not been using hormonal preparations for the preceding 3 months. The tissue samples were a subset of the larger set described in 3.1.1 and 3.1.2. Endometrial tissue included samples collected from women during the proliferative phase ( $n=6$ ), early secretory ( $n=6$ ), mid secretory ( $n=6$ ) and late secretory phase ( $n=6$ ) of the menstrual cycle. In this group all tissue samples were full thickness endometrial biopsies. The stage of the menstrual cycle was consistent with the patients reported last menstrual period and was confirmed by histological dating, according to the criteria of Noyes *et al* (Noyes et al. 1950). Furthermore, serum samples collected at the time of endometrial tissue collection for determination of circulating oestradiol and progesterone concentrations by RIA were consistent with the designated cycle stage. A significant decline in progesterone concentrations was apparent between biopsies taken in the mid and late secretory phase of the cycle see chapter 3.1.

The second group consisted of fertile women ( $n=12$ ) with regular menstrual cycles lasting between 25 and 35 days that had not been using hormonal preparations for the preceding 6 months. These same samples were also a subset of the larger set of tissue samples detailed in Chapters 3.1.2. The patients were scheduled to have a LNG IUS inserted for long-term hormonal contraception. All patients underwent a pre-insertion endometrial biopsy in either the proliferative ( $n=5$ ) or secretory phase ( $n=5$ ) of the cycle immediately following which a LNG IUS (Mirena, Schering HC, UK) was inserted as an outpatient procedure. The stage of the cycle prior to insertion of the LNG IUS was determined by reference to serum sex steroid concentrations, stated date of last menstrual period and histological dating by the criteria of Noyes et al. (Noyes et al. 1950). Further endometrial samples were collected 1 ( $n=2$ ), 3 ( $n=5$ ), 6 ( $n=5$ ) and 12 months ( $n=5$ ) following insertion of the LNG IUS.

The third patient group included women using a LNG IUS for management of regular but heavy menstrual loss. Endometrial samples were collected from patients with a LNG IUS in-situ for 3 months, 6 months, or greater than 1 year. Endometrial tissue samples were frozen immediately upon collection in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

### **5.2.2 RNA extraction and real time quantitative RT-PCR**

Frozen samples of endometrium stored at  $-80^{\circ}\text{C}$  were first homogenised, and then total RNA was extracted using the commercially available product Trizol (Invitrogen Life technologies Ltd, Paisley, UK). The methods of extraction, quantification, cDNA preparation and quantitative RT-PCR are the same as detailed in Chapters 3.3.1; 3.3.2; and 3.3.3.

Samples of total RNA to be used for quantification of AR mRNA required pre-treatment with RNase free DNase (Invitrogen Life technologies Ltd, Paisley, UK) as this primer probe preparation did not span an intron. Samples of RNA to be used for quantification of  $17\beta$ -HSD2 mRNA did not require pre-treatment with RNase free DNase as this primer probe preparation spanned an intron.

Real-time quantitative PCR was performed in an ABI 7700 Sequence Detection System (Perkin-Elmer, CA, USA). Oligonucleotide forward and reverse primers and oligonucleotide Taqman probes for AR and  $17\beta$ -HSD2 were designed, with the use of Primer Express version 1.0 (Perkin-Elmer Applied Biosystems, Cheshire, UK), from sequences entered in the GenBank database. The sequences for the androgen receptor and  $17\beta$ -HSD2 primers and probe are shown in table 3.2.

Numeric data from the real time quantitative RT-PCR were analysed by one way ANOVA, followed by Fisher's protected least significant difference test.

### 5.2.3 AR and 17 $\beta$ -HSD2 Immunohistochemistry

As detailed in Chapters 3.2.2 and 3.2.3 the specificity and properties of the antibodies for the immunolocalisation of the AR (F39.4; (Slayden *et al.* 2001) and 17 $\beta$ -HSD2 (Mab C2-12; (Moghrabi *et al.* 1997) have been previously described.

Paraffin sections (5 $\mu$ m) were dewaxed and rehydrated through a series of alcohols then washed in distilled water and 0.01M phosphate-buffered saline (PBS, Sigma-Aldrich Ltd. Poole, Dorset, England) The slides were then subjected to antigen retrieval by pressure-cooking in 0.01mol/L sodium citrate at pH 6 for 5 minutes. After cooling for 20 minutes the slides were washed in PBS. Endogenous peroxidase activity was quenched with immersion in 3% hydrogen peroxide (Merck, Poole, UK) in distilled water for 10 minutes at room temperature. Non specific binding of the antibodies was blocked by incubating the sections for 20-30 minutes at room temperature in non immune horse serum (Vectastain, Vector Laboratories, Inc., Peterborough, UK).

For localisation of the AR, sections were incubated with the mouse monoclonal antibody F39.4 (IgG<sub>1</sub>; Biogenex Laboratories, Inc., San Ramon, CA, USA) overnight at 4°C at a 1:480 dilution in PBS/BSA gelatin or similarly with a control mouse IgG<sub>1</sub> antibody at a 1:600 dilution. For localisation of 17 $\beta$ -HSD2, sections were incubated with the mouse monoclonal Mab C2-12 overnight at 4°C at a 1:20 dilution in normal horse serum or similarly with a control mouse IgG antibody at a 1:400 dilution. Following a wash in PBS with Tween 20, the slides were incubated in biotinylated horse anti-mouse secondary antibody (Vectastain, Vector Laboratories, Inc., Peterborough, UK.) in normal horse serum for 60 minutes at room temperature. They were then reacted with the avidin-biotin peroxidase complex (Vectastain Elite) for 60 minutes at room temperature and visualised with the substrate and chromagen 3, 3'-diaminobenzidine (DAKO Liquid, DAKO Corporation, California, USA).

The immunostaining intensity of epitopes in all tissue sections was assessed in a semi-quantitative manner on a four point scale: 0 = no immunostaining, 1 = mild immunostaining, 2 = moderate immunostaining and 3 = intense immunostaining. All tissue sections were scored blind by two observers. We had previously validated this scoring system in a subset of tissue sections in which immunoreactivity was

measured with a computerised image analysis system, and a strong correlation between quantitative data derived from image analysis and subjective scores by a trained observer was obtained (Wang et al. 1998). Semi-quantitative scoring results were analysed by a non-parametric method, the Kruskal Wallis test, followed by Dunn's multiple post test.

## 5.3 Results

### 5.3.1 Temporal expression of endometrial AR messenger RNA following high dose intrauterine delivery of levonorgestrel.

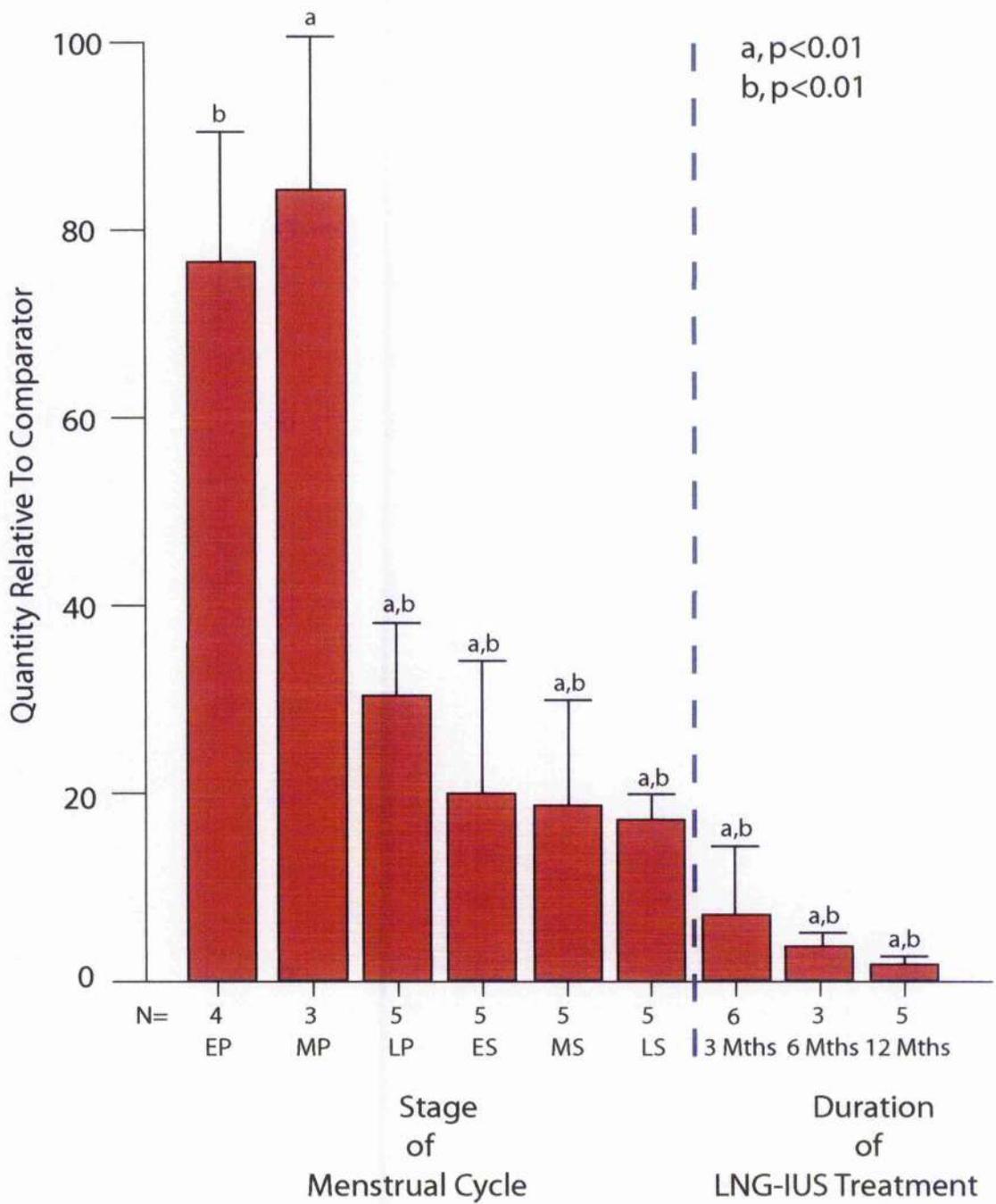
The menstrual cycle samples analysed were the same as those in the previous Chapter and accordingly the findings are also presented in Chapter 4.2.4 showing significantly higher levels of AR mRNA in the early and mid proliferative phase endometrial tissue samples compared to the remainder of the menstrual cycle stages.

Endometrial tissue samples from women using a LNG-IUS for 3 months, 6 months or more than 12 months were studied. Samples treated for greater than 12 months were analysed together as maximal reduction in blood loss occurs by 12 months (Andersson et al. 1994), and the period of breakthrough bleeding is confined usually to the first 3-6 months after initiation of treatment. To allow comparison with the menstrual cycle, samples were statistically compared to an early proliferative phase sample as a control.

Comparison revealed a significantly greater production of AR mRNA in the proliferative phase when compared to any duration of treatment with the LNG IUS ( $p < 0.01$ ) (Figure 5.1). No significant differences were noted between the secretory phase and the LNG IUS exposed endometrium.

Figure 5.1

Quantitative evaluation of AR mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of LNG. All endometrial tissue samples were compared to an internal control (comparator) obtained during the early proliferative stage of the menstrual cycle. AR mRNA levels were high in the early and mid proliferative samples but fell significantly in samples taken from the late proliferative and early to late secretory samples ( $p < 0.01$ ). Levels of AR mRNA were again low in the LNG-IUS treated samples when compared to the proliferative stage of the cycle ( $p < 0.01$ ). EP-early proliferative, MP- mid proliferative, LP-late proliferative, ES-early secretory, MS-mid secretory, LS-late secretory.



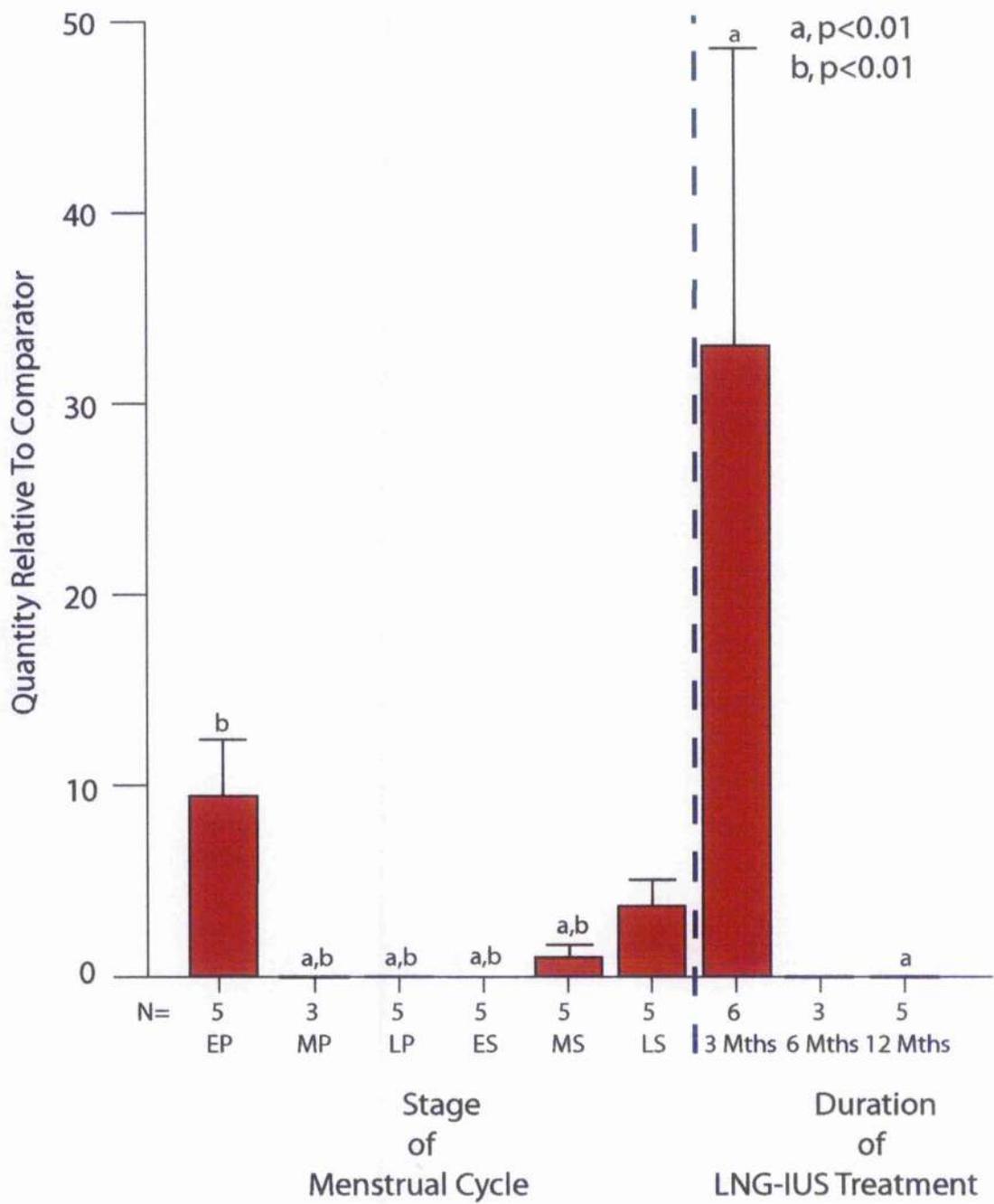
### **5.3.2 Temporal expression of 17 $\beta$ -HSD2 messenger RNA in endometrium through the menstrual cycle and following high dose intrauterine delivery of levonorgestrel.**

The same samples analysed for AR expression were studied for the expression of 17 $\beta$ -HSD2 mRNA. The highest levels of 17 $\beta$ -HSD2 mRNA expression was noted in the early proliferative phase, with the levels significantly greater than the other stages of the menstrual cycle apart from the late secretory phase ( $p < 0.008$ ) (Figure 5.2).

17 $\beta$ -HSD2 mRNA was also detected in endometrium exposed to high dose intrauterine LNG. Samples were again analysed after 3, 6 or >12 months in vivo treatment with an LNG IUS. Relatively higher levels of 17 $\beta$ -HSD2 mRNA were detected following 3 months treatment with LNG IUS. This was significantly greater than levels noted during the menstrual cycle stages and the other LNG IUS treatment groups ( $p < 0.01$ ; Figure 5.2).

Figure 5.2

Quantitative evaluation of  $17\beta$ -HSD2 mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of LNG. All endometrial tissue samples were compared to an internal control (comparator) obtained during the early proliferative stage of the cycle.  $17\beta$ -HSD2 mRNA levels were significantly higher in the early proliferative phase of the menstrual cycle when compared to the mid-late proliferative and the early-mid secretory phase samples ( $p < 0.008$ ). In LNG-IUS treated samples relatively higher levels of  $17\beta$ -HSD2 mRNA were detected following 3 months. This was significantly greater than levels noted during the menstrual cycle and the other LNG IUS treated samples ( $p < 0.01$ ). EP-early proliferative, MP- mid proliferative, LP-late proliferative, ES-early secretory, MS-mid secretory, LS-late secretory.



### **5.3.3 AR Immunostaining In Endometrium Exposed To High Dose Intra-Uterine Levonorgestrel.**

Presence of LNG IUS resulted in a typically pseudo-decidualised stroma with atrophic glands and these histological features were observed in all post insertion endometrial biopsies, as previously shown by our laboratory (Critchley et al. 1998). Prior to insertion all proliferative phase endometrial tissue samples displayed strongly positive nuclear immunoreactivity for AR in the endometrial stroma with minimal glandular nuclear immunoreactivity. As we previously noted AR immunoreactivity in secretory phase biopsies was localised to stromal nuclei and was clearly decreased compared to the proliferative phase (Figure 5.3).

Intra-uterine delivery of levonorgestrel via a LNG IUS was associated with minimal AR immunoreactivity in the stromal compartment with a significantly less immunoreactivity by 12 months treatment with a LNG IUS (Figures 5.3; 5.4).

Figure 5.3.

Immunohistochemical localisation of AR in human endometrium prior to and following treatment with LNG IUS at x 40 magnification. (A) Proliferative phase -high level of stromal immunoreactivity. (B) Secretory endometrium - decreased stromal immunostaining. (C) Negative-no immunoreactivity. Endometrial biopsies at 3 months (D); 6 months (E) and 12 months (F) following insertion of LNG IUS. Note persistent down-regulation of stromal immunoreactivity. Scale bar 50  $\mu$ m.

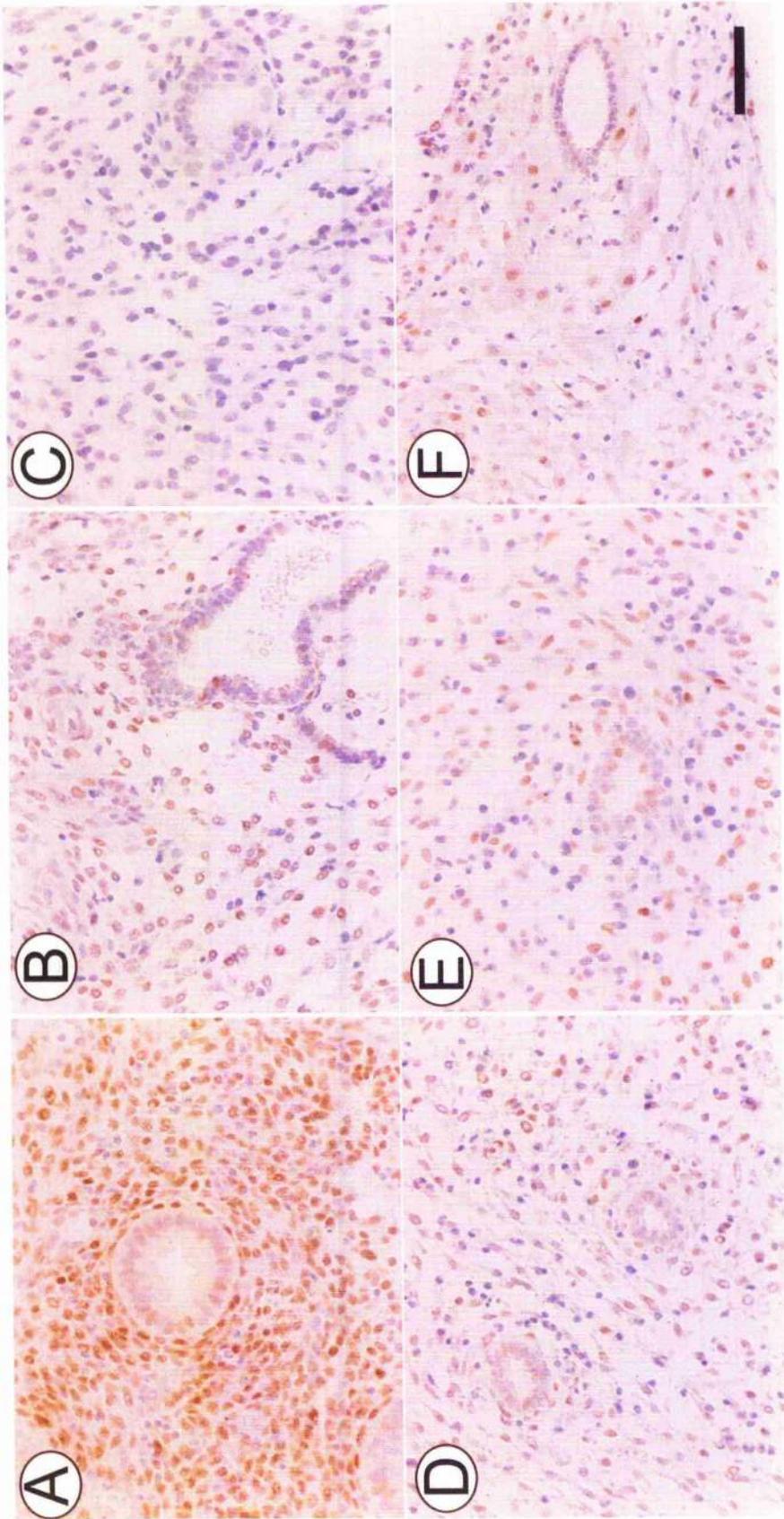
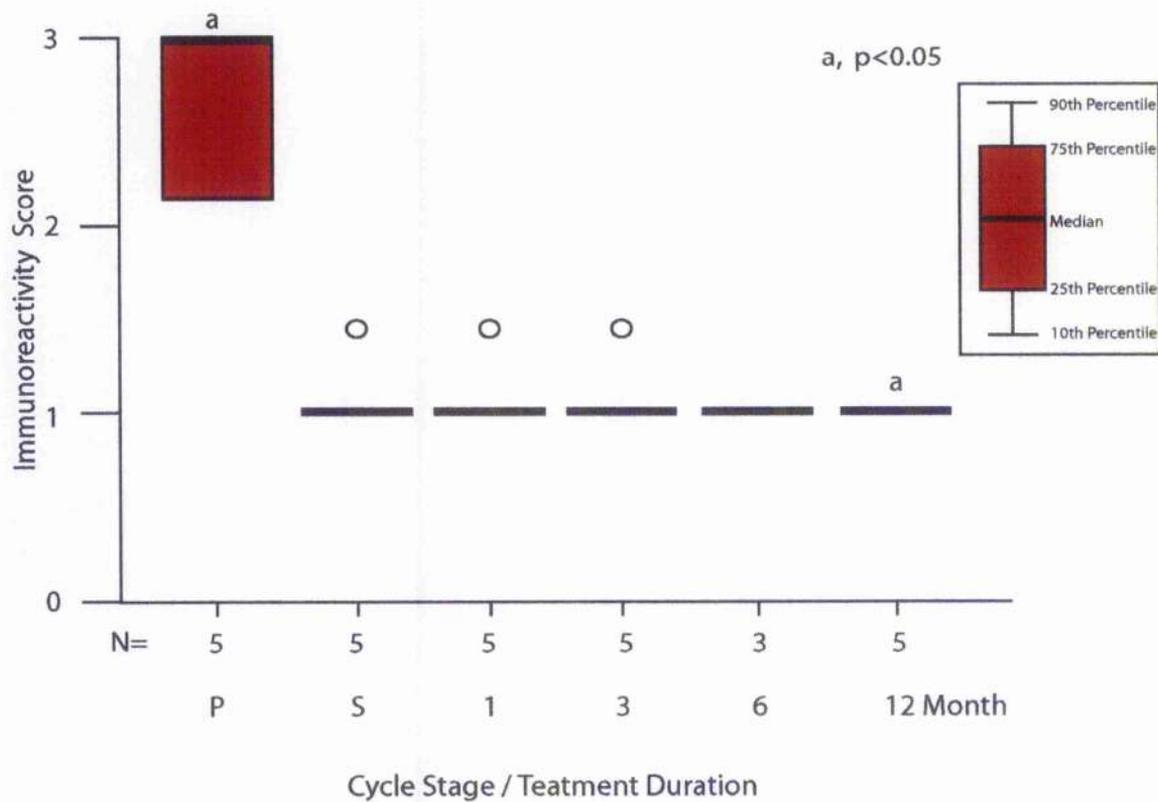


Figure 5.4.

AR immunoreactivity scores in endometrial stromal compartments throughout the menstrual cycle and following high dose intrauterine LNG exposure (1-12 months). Box and whisker plots; box represents the 25<sup>th</sup> and 75<sup>th</sup> percentiles and the heavy bar represents the median. Symbols (O) represents outliers. P = proliferative, S = secretory.



### **5.3.4 17 $\beta$ -HSD2 immunostaining Endometrium Exposed To High Dose Intra-Uterine Levonorgestrel.**

In full thickness endometrial biopsies 17 $\beta$ -HSD2 was immunolocalised to the cytoplasm of glandular epithelium with no stromal immunoreactivity observed (Figure 5.5). Maximal immunoreactivity was noted in the secretory phase during which both superficial and basal layers displayed strong immunoreactivity. Basal layer immunoreactivity was first evident in the early secretory phase. Immunostaining in both the early and mid secretory phase biopsies was significantly greater than in the proliferative phase ( $p < 0.05$ ). Superficial layer immunoreactivity for 17 $\beta$ -HSD2 was also first evident in the early secretory phase with the mid secretory phase biopsies displaying significantly greater levels of immunostaining than the proliferative phase ( $p < 0.01$ ). In the late secretory phase following the mid secretory peak there was persistent superficial and basal immunoreactivity.

One month after insertion of the LNG IUS there was still detectable 17 $\beta$ -HSD2 immunostaining, and the staining intensity was greater than that normally found during the proliferative phase. Thereafter, at three months after insertion there was a significant decline in 17 $\beta$ -HSD2 expression compared to the level of 17 $\beta$ -HSD2 immunoreactivity in the secretory phase ( $p < 0.01$ ; Figures 5.5; 5.6). This low level of immunoreactivity was maintained in the 12 month biopsy specimens. The low degree of immunostaining for 17 $\beta$ -HSD2 between 3 and 12 months post insertion was not significantly different from that seen during the proliferative phase (Figure 5.5)

Figure 5.5

Immunohistochemical localisation of 17 $\beta$ -HSD2 in human endometrium prior to and following high dose intrauterine LNG exposure. (x 20 magnification). (A) Proliferative endometrium – no immunoreactivity in epithelial cells. (B) Secretory endometrium – immunoreactivity localised to glandular epithelium and absent stromal immunoreactivity. Inset is negative control. (C) Biopsy performed 1 month after LNG IUS insertion – definite, though reduced glandular immunoreactivity. Endometrium 3 months (D); 6 months (E) and 12 months (F) after exposure to high dose intrauterine LNG. Negligible/absent immunostaining observed at these time points. Scale bar 50  $\mu$ m.

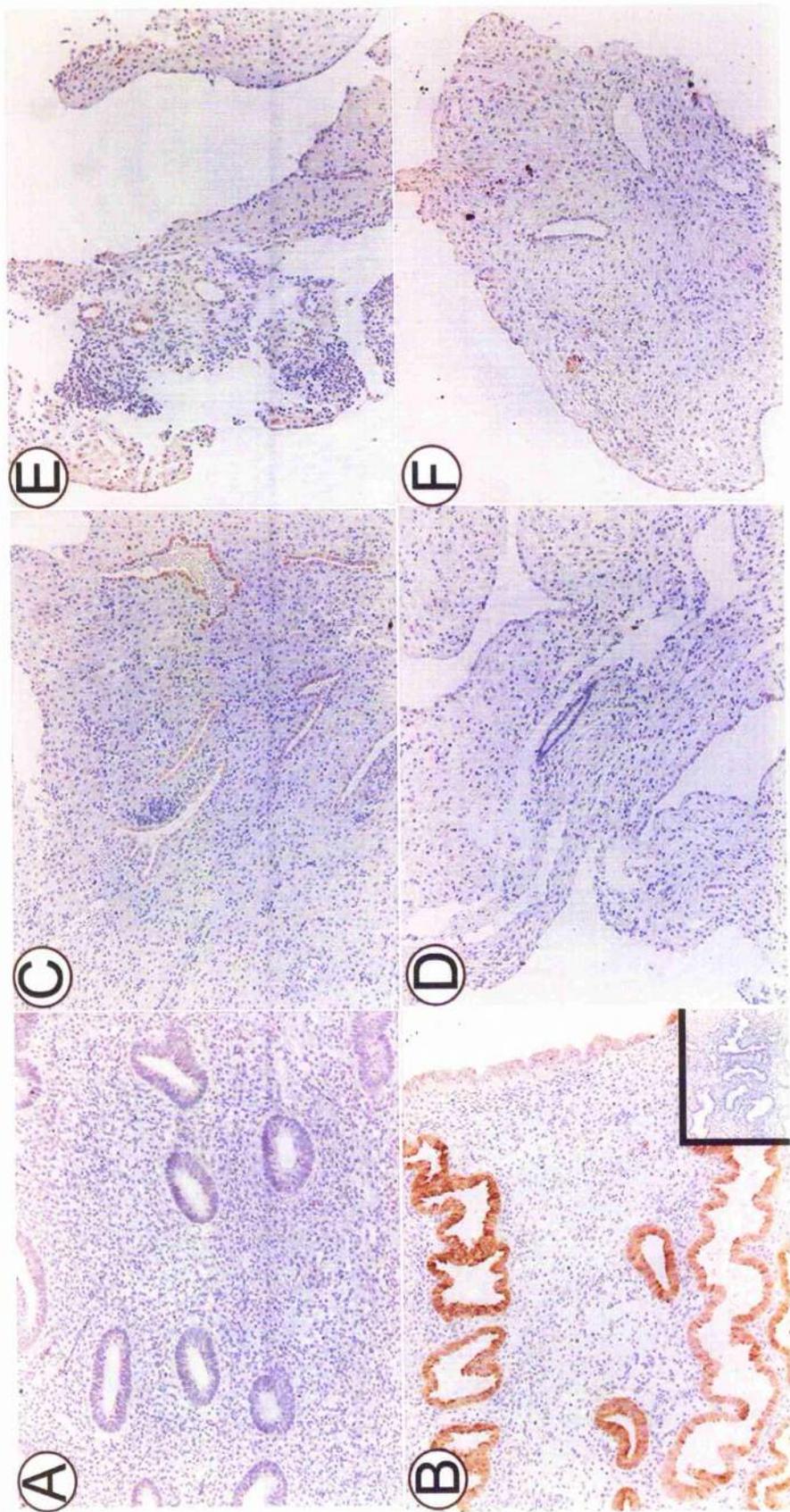
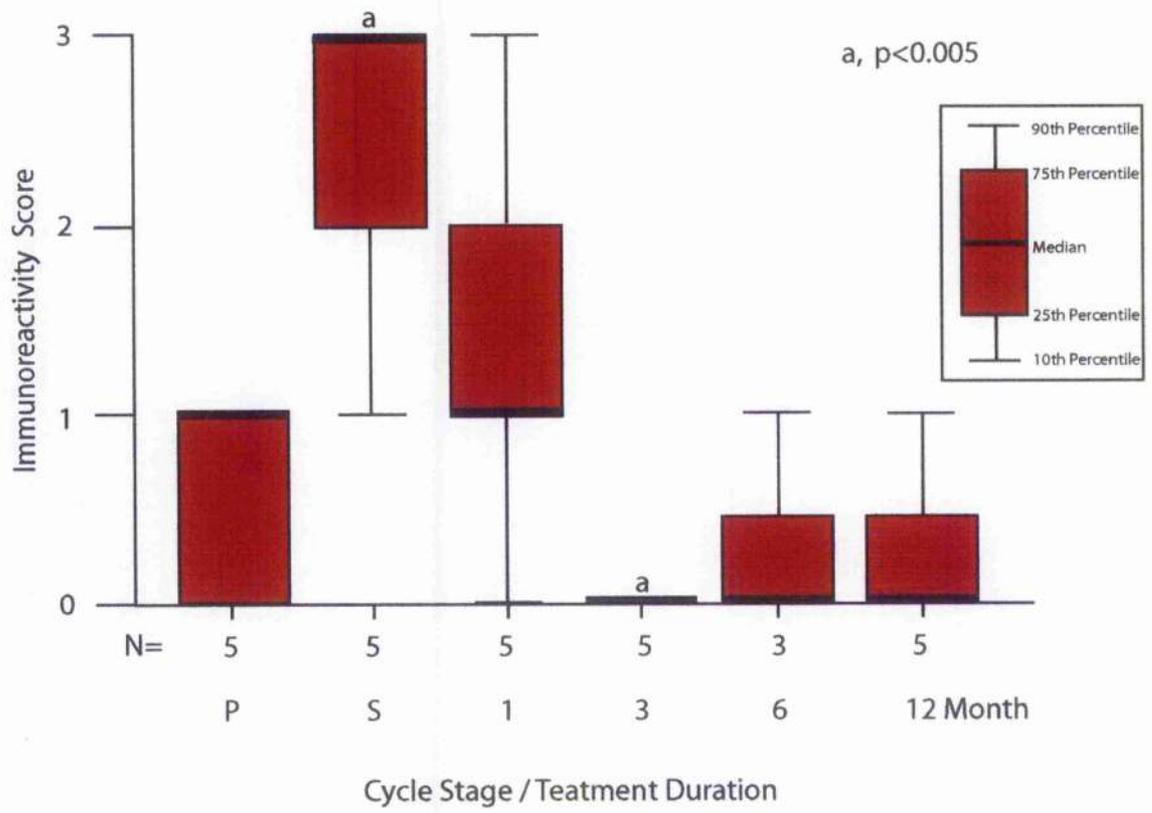


Figure 5.6.

$17\beta$ HSD2 immunoreactivity scores in endometrial glandular compartments of superficial endometrium across the menstrual cycle and following LNG IUS exposure (1-12 months). Box and whisker plots; boxes represent the 25<sup>th</sup> and 75<sup>th</sup> percentiles and the heavy bar represents the median. The whiskers represent the 10<sup>th</sup> and 90<sup>th</sup> percentile. P = proliferative, S = secretory.



## 5.4 Discussion

The mechanism of problematic and unscheduled breakthrough bleeding (BTB) experienced by users of long-term progestin only contraception remains poorly understood. Consequently there are no reliable therapeutic intervention strategies available to overcome this problem and improve patient satisfaction. The intrauterine exposure of the endometrium to high doses of LNG results in marked endometrial atrophy, inactive glands and a heavily decidualised endometrium. There is a marked down-regulation of sex steroid receptors (PR, ER and AR) and there are higher local concentrations of prostaglandins in the initial months of LNG IUS exposure (Critchley et al. 1998; Jones et al. 2000). Another consistent feature in the endometrium of long-term progestin only contraceptive users are the abnormalities reported in the structure of endometrial microvessels (Hickey et al. 2000). The endometrium of long term progestin users have an associated increase in blood vessel fragility that may contribute to the pathogenesis of endometrial breakthrough bleeding. Also, in normal endometrium, progesterone receptors are absent in the endothelium but prominent in the perivascular cells (Koji et al. 1994; Critchley et al. 2001). LNG is reported to induce changes in vessel fragility that may be mediated through perivascular cells rather than the endothelium (Roberts et al. 1992).

The present data demonstrate that endometrium exposed to high dose intrauterine delivery of levonorgestrel exhibits a significant decrease in stromal AR protein immunoreactivity when compared with control proliferative endometrium. This significant down regulation is in keeping with levels exhibited in the secretory phase and is maintained for at least 12 months following insertion of the LNG IUS. In addition there is a down regulation of AR messenger RNA in LNG IUS exposed endometrium which, in this case, is closely reflected by the pattern of expression of the AR protein.

AR has been shown to be predominantly expressed in the human endometrial stroma with minimal glandular immunoreactivity and down regulated in the late secretory phase of the menstrual cycle (Mertens et al. 1996; Mertens et al. 2001; Slayden et al. 2001). The mechanism of AR up regulation in the normal cycle is thought to be mediated by the action of oestradiol and its down regulation mediated by the action of progesterone as shown by the data in Chapter 4 (Slayden et al.

2001). Chapter 4 also provides evidence that administration of mifepristone resulted in an up-regulation of AR in the stromal and glandular nuclei. The suggestion from these data was that PR provided a tonic inhibitory control over the ability of ER to up regulate AR, but when this PR inhibition was removed by the action of an antiprogestogen (RU486, mifepristone) there was an up regulation of AR by the action of oestradiol acting through ER. Notably, mifepristone treatment, which suppresses bleeding and increases amenorrhea, also increases endometrial AR (Brown et al. 2002). By inference, the lowered levels of AR in LNG treated endometrium may be linked to the sporadic, breakthrough bleeding typically seen in such patients.

Levonorgestrel binds to both the AR and PR (Kloosterboer et al. 1988; Lemus et al. 1992). The role of androgens in the regulation of the AR protein and mRNA is not yet known, with some studies suggesting an up regulation in response to androgens and others suggesting a down regulation (Chada et al. 1994; Fujimoto et al. 1994; Iwai et al. 1995; Apparao et al. 2002). In situ hybridisation studies in the primate uterus suggested that testosterone can synergize with oestradiol to up-regulate AR (Adesanya-Famuyiwa et al. 1999). Levonorgestrel has similar progestogenic and androgenic activity shown by its relative binding affinity for the PR when compared with AR (Kloosterboer et al. 1988). It is therefore unclear as to the mechanism of AR down regulation in levonorgestrel exposed endometrium, but it could be a consequence of both progestogenic and androgenic action.

The data presented have identified high levels of endometrial 17 $\beta$ -HSD2 protein in the first month after insertion of the LNG IUS associated with high levels of 17 $\beta$ -HSD2 mRNA expression in endometrial tissue at the three month time point. Since 17 $\beta$ -HSD2 converts oestradiol to the less potent oestrogen, oestrone, the consequence is that the endometrial glands are exposed to more oestrone than oestradiol during the first three months after LNG IUS insertion. Any oestradiol dependent products of the glands that may have paracrine actions throughout the endometrium would thus be suppressed or perturbed at this time. It is during the first months after LNG IUS insertion that the complaint of breakthrough and unscheduled bleeding is most frequent. We therefore propose that the unscheduled breakthrough bleeding experienced by women at this time may be due in part to an intracellular "oestrogen deficiency" that either directly or indirectly leads to vascular fragility.

17 $\beta$ -HSD2 protein expression thereafter is significantly reduced following three months treatment, coincident with the onset in improvement in menstrual bleeding patterns. However, the decline in 17 $\beta$ -HSD2 mRNA expression appears to be staggered, as these mRNA levels do not become undetectable until 6 months of LNG IUS treatment.

In human endometrium, steroidal regulation of receptor action is dependent upon ligand availability. The 17 $\beta$  hydroxysteroid dehydrogenase family is a large family, so far comprising 8 members of which 6 human isoforms have been characterized. Its primary role is the inactivation of oestradiol to oestrone and testosterone to androstenedione but in addition it also converts the inactive 20 $\alpha$ dihydroprogesterone to active progesterone (Peltoketo et al. 1999; Labrie et al. 2000). The observations in the present study are consistent with those of previous publications showing an up regulation of 17 $\beta$ -HSD2 in the progesterone dominated secretory phase (Casey et al. 1994). With intrauterine delivery of levonorgestrel, we found an increase in the expression of 17 $\beta$ -HSD2 protein when compared with the proliferative phase of the menstrual cycle followed by a down regulation of the 17 $\beta$ -HSD2 protein in glandular epithelium after 3 months treatment that was maintained up to the 12 month time point. A significant up regulation of the 17 $\beta$ -HSD2 mRNA in the initial 3 months was also noted followed by a down regulation in patients treated for longer than 6 months. This is consistent with the down regulation of the PR receptor over this same time frame (Critchley et al. 1998).

Of interest, the decline in 17 $\beta$ -HSD2 mRNA expression appears to be staggered from that of the protein with a decrease in protein expression being noted prior to mRNA decline. This may be due to enhanced stability of 17 $\beta$ -HSD2 mRNA possibly resulting in a persistence of the mRNA for long periods without translation into protein. It has been shown that mRNA can be stable for days at a time and this may be regulated in some cases by sex steroids (Day et al. 1998; Staton et al. 2000). Analysis of 17 $\beta$ -HSD2 mRNA through the menstrual cycle also shows a lack of correlation with protein expression with higher levels of RNA noted following increased protein expression with highest levels of mRNA noted in the early proliferative phase when no protein expression is noted. A similar pattern of 17 $\beta$ -HSD2 mRNA expression is seen in the data reported by Casey *et al.* (1994); Mustonen *et al.*, (1998) and Kitawaki *et al.*, (2000). Northern blot analysis revealed

17 $\beta$ -HSD2 mRNA in the early proliferative phase (Casey et al. 1994) and Kitawaki *et al* found comparable levels of 17 $\beta$ -HSD2 mRNA in proliferative and secretory phase tissue specimens (Kitawaki et al. 2000). Quantitative analysis of 17 $\beta$ -HSD2 mRNA in human endometrium reported by Mustonen *et al.*, also revealed a lack of correlation with protein levels, higher levels of mRNA expression occurring in the late secretory phase following the serum progesterone peak and previously noted mid secretory increase in protein expression (Mustonen et al. 1998).

Molecular factors reported in endometrium of levonorgestrel users includes activation of matrix metalloproteinase's 1, 3 and 9 to a level consistent with that seen in menstrual phase endometrium (Galant et al. 2000; Rodriguez-Manzanique et al. 2000; Vincent et al. 2000) in addition to decreased tissue inhibitors of metalloproteinases (TIMPs). This may explain the features of focal stromal breakdown and collagen lysis noted in endometrium from users of Norplant, subdermal LNG delivery (Galant et al. 2000). Other factors reported that relate to vessel fragility include a decrease in endometrial perivascular alpha smooth muscle actin in Norplant users (Rogers et al. 2000) and decreased endothelial cell proliferation (Goodger et al. 1994; Rodriguez-Manzanique et al. 2000; Hague et al. 2002). Haemostatic factors may also play an important role as a decrease in tissue factor protein and mRNA has been reported in levonorgestrel exposed endometrium (Runic et al. 1997). The aforementioned factors all suggest a disruption in the mechanisms that control vessel growth and haemostasis.

In summary, exposure of the endometrium to high dose intrauterine LNG results in decidualisation and atrophy. Breakthrough bleeding problems with this method of long-term progestin delivery are maximal during the first few months following insertion of an LNG IUS. The data presented demonstrated that during this period after LNG IUS insertion, the steroid metabolising enzyme 17 $\beta$ -HSD2 is elevated and steroid receptors, including AR, are suppressed. As a consequence, intracellular levels of the less potent oestrogen, oestrone are raised and the more potent oestrogen, oestradiol lowered. Problematic breakthrough bleeding usually improves after 3-6 months of LNG IUS usage. At this time, these studies have shown that levels of 17 $\beta$ -HSD2 protein are low and thus intracellular concentrations of oestradiol would be raised and oestrone lowered. The levels of both AR mRNA and protein remain suppressed. There remains no doubt that the mechanisms underlying

the breakthrough bleeding associated with use of LNG IUS and indeed other long-term progestin only methods of contraception are complex. Hopefully, data such as presented here will contribute further to our understanding of local progestin action and how this relates to BTB in human endometrium.

## Chapter 6

# Endometrial Intracrinology: Studies of Normal Endometrium and Endometrium Exposed to Intra-Uterine Levonorgestrel

## 6.1 Introduction

Humans possess a sophisticated endocrine system where the bioavailability of active sex steroids depends upon not only on the concentrations in the peripheral circulation, but to a large extent sex steroid synthesis within peripheral target tissue cells (Labrie et al. 2000). This process is termed "intracrinology" and it essentially allows peripheral target tissues to have control over sex steroid formation and metabolism according to the requirements of the target tissue (Labrie 1991). The ligand that is available to bind to the sex steroid receptors in a given target tissue will depend upon the enzymes present in the tissue.

Ligand formation is dependent upon a precursor substrate and the adrenal gland is such a source. Circulating dehydroepiandrosterone (DHEA) and dehydroepiandrosterone-sulphate (DHEA-S) provide the high levels of substrate required for conversion into potent androgens and oestrogens in peripheral tissues. The role of adrenal DHEA in androgen formation was discovered in patients being treated for prostatic carcinoma. As this is an androgen-responsive cancer, treatment involved medical (GnRH agonists) or surgical castration to reduce serum testosterone (T) levels by 90-95% (Labrie et al. 2000). However, it was noted that when compared to normal prostate tissue, prostate tissue removed from castrated patients still contained 50% dihydrotestosterone (DHT). This suggested another source of adrenal androgen formation (Labrie et al. 1996). This observation led to the introduction of combined androgen and 5 $\alpha$ -reductase blockade that was the first intervention shown to prolong life in prostate cancer (Prostate cancer trialist collaborative group 2000).

A number of enzymes are involved in the metabolism of sex steroids as discussed in the literature review (see Chapter 1.4). The enzymes studied here are important in the formation and metabolism of androgens. They include 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) types 1 and 2; 17 $\beta$ -hydroxysteroid dehydrogenase (17 $\beta$ -HSD) types 1, 2, and 5; 5 $\alpha$ -reductase (5AR) types 1 and 2; and aromatase.

Knowledge of peripheral target tissue enzyme expression is important as it has already been shown, as with prostate cancer, that therapeutic interventions can be

developed to manipulate local endocrine environments. Such knowledge may progress the fields of menstrual disorders, contraception and endocrine related cancers.

Further examples of clinical interventions to manipulate enzyme function in target tissues are seen in breast cancer and endometriosis. In breast cancer, aromatase inhibitors may be used to prevent oestrogen formation, while selective oestrogen receptor modulators are used to inhibit binding of active oestrogens. Aromatase inhibitors are now also being explored for their use in benign conditions such as endometriosis (Bulun et al. 2000). While endometriotic tissue is histologically akin to endometrium, it has been shown to have a different intracrine system (Bulun et al. 1994; Noble et al. 1996; Noble et al. 1997), containing aromatase and  $17\beta$ -HSD1 whilst lacking expression of  $17\beta$ -HSD2. This combination favours production of oestradiol driving the persistence of endometriosis that produces pain and pelvic scarring in patients.

Peripheral sex steroid metabolism is complex with physiological and pathological variations in enzyme expression and tissue specific isoforms utilising various steroid substrates. Within endometrium further complexity is added as, under the control of the menstrual cycle, endometrium regenerates in a 28 day cycle. This has the potential to lead to temporal and spatial variations in endometrial receptor and enzyme expression. Little is known regarding endometrial enzyme expression and what has been published to date is summarised below.

$3\beta$ -HSD has two highly homologous isoforms, 1 and 2, that are important in the formation of sex steroids from precursors as seen in figure 1.4 (Lachance et al. 1991; Labrie et al. 1992). The tissue expression of the isoforms varies, type 1 being present mainly in the placenta, liver, kidney and skin (Mason et al. 1997). Type 2 is expressed principally in the gonads and adrenals (Mason et al. 1997). The function of type 2 is consistent with its tissue of expression namely steroidogenesis. However the exact function of type 1 is less certain but it appears to be responsible for the formation and/or degradation of  $5\alpha$ -androstanes and  $5\alpha$ -pregnanes such as  $5\alpha$ -dihydrotestosterone and  $5\alpha$ -dihydroprogesterone respectively (Mason et al. 1997).  $3\beta$ -HSD activity has been demonstrated in the endometrium of human (Tang et al. 1993) and rhesus macaque endometrium (Martel et al. 1994). Tang et al suggested that expression might even be progesterone regulated as an increase in enzyme

expression was demonstrated following culture of endometrium with medroxyprogesterone acetate. The most recent paper by Rhee et al has demonstrated expression of the enzyme in the endometrial glandular epithelium with higher expression in the secretory phase than the proliferative phase (Rhee et al. 2003). The paper also demonstrated the expression of the enzyme in human endometrium at the molecular level using RT-PCR. They did not report the temporal or spatial variation in expression of the enzyme at either the molecular or the protein level.

To form active androgens and oestrogens the steroid product of 3 $\beta$ -HSD will require the presence of 17 $\beta$ -hydroxysteroid dehydrogenase(s). This is a multigene family discussed previously in Chapter 5. There is little homology (usually in the region of 20%) between members of this family (Labrie et al. 1997). The enzymes in this family are unidirectional within intact cells either reducing sex steroids to active forms or oxidising to inactive forms. The expression of 17 $\beta$ -HSD2 in secretory phase endometrial glandular epithelium at the protein and messenger RNA level has been discussed in Chapter 5. 17 $\beta$ -HSD2 is responsible for the inactivation of oestrogens and androgens as well as the 20 $\alpha$ -dehydrogenation of inactive 20 $\alpha$ -dihydroprogesterone to active progesterone. 17 $\beta$ -HSD1 is responsible for the conversion of oestrone to the active oestradiol (Bartlett et al. 1991). The publications to date have failed to establish occurrence of 17 $\beta$ -HSD1 enzyme or its messenger RNA in endometrium.

Active androgens in the male are formed mainly within the testis under the control of 17 $\beta$ -HSD3, an enzyme that in humans is functional mainly within the male (Geissler et al. 1994). The enzyme may be subject to mutations, with the affected male individuals suffering male pseudohermaphroditism. To date no females with similar mutations have been shown to suffer an equivalent condition (Mendonca et al. 1999). As the ovary is the main source of serum androgens in the female, studies were carried out to localise 17 $\beta$ -HSD3 in the ovary but it was not found (Zhang et al. 1996). However, 17 $\beta$ -HSD3 shares the same substrate specificities as 17 $\beta$ -HSD5, catalysing the conversion of androstenedione to testosterone (Penning 1997), and 17 $\beta$ -HSD5 has been localised in the ovary (Luu-The et al. 2001). It would therefore appear that 17 $\beta$ -HSD5 is the hydroxysteroid dehydrogenase responsible for androgen production in the female. However, in the male, 17 $\beta$ -HSD5 is also present in peripheral target tissues such as the prostate. In this tissue its role is thought to be a

combination of androgen formation and progesterone inactivation, via its 20 $\alpha$ -hydroxysteroid dehydrogenase (20 $\alpha$ -HSD) activity, protecting the prostate from the action of progesterone (Dufort et al. 1999). In the female 17 $\beta$ -HSD5 has now been localised to the ovary, breast and uterus (Pelletier et al. 1999). Uterine 17 $\beta$ -HSD5 was localised to the glandular and surface epithelium, however, the stage of the cycle in which it was detected was not stated (Pelletier et al. 1999). As androgen receptors are expressed largely in the endometrial stroma it may be that local androgen production via 17 $\beta$ -HSD5 produces a paracrine effect in the stroma. Another possible role may relate to its 20 $\alpha$ -HSD activity, inactivating progesterone. To elucidate its role, more details regarding the immunolocalisation of 17 $\beta$ -HSD5 in endometrium through the menstrual cycle would be required.

Endometrial production of androgens may however require further metabolism to the more biologically active androgen dihydrotestosterone or even to an alternate sex steroid such as oestradiol before eliciting a response. The enzymes responsible for testosterone conversion to oestradiol and dihydrotestosterone are aromatase and 5 $\alpha$ -reductase respectively.

Aromatase is not expressed in disease-free human endometrium (Bulun et al. 1993) but has been shown to be expressed in benign and malignant pathological conditions involving the uterus such as endometrial cancer (Bulun et al. 1994; Sasano et al. 1999), uterine leiomyoma (Bulun et al. 1994), and endometriosis (Noble et al. 1996; Kitawaki et al. 1997). Due to the expression of aromatase in these pathological conditions, the clinical use of aromatase inhibitors has been the subject of research, in particular their role in endometriosis and endometrial cancer. Endometrial cancer has been shown to be responsive to aromatase inhibitor treatment. This was demonstrated in short term in-vivo studies where treatment with Letrozole, an aromatase inhibitor, for two weeks prior to theatre produced some positive clinical changes (Berstein et al. 2002). Berstein et al. now propose longer term randomized trials to assess the effectiveness of aromatase in endometrial cancer (Berstein et al. 2002). Endometriosis has also been successfully treated with aromatase inhibitors (Takayama et al. 1998). The modulation of aromatase activity in ectopic and eutopic endometrium of endometriosis may even be crucial to its treatment. A recent study indicated that commonly used treatments such as danazol or GnRH analogues will reduce aromatase expression and therefore oestradiol

production (Ishihara et al. 2003). Therefore, while aromatase inhibitors may be an attractive therapeutic option in a condition that is difficult to treat, large scale randomized trials will be required to assess the effectiveness and adverse effect profile of aromatase inhibitors (Bulun et al. 2000).

5 $\alpha$ -reductase is present as two isoenzymes, types 1 and 2 (Russell et al. 1994), and is responsible for the activation of androgens and the inactivation of progesterone (Fisher et al. 1978). The presence of 5 $\alpha$ -reductase in human endometrium was first raised by Rose et al. where endometrium cultured with testosterone was shown to produce significant quantities of 5 $\alpha$ -dihydrotestosterone (Rose et al. 1978). In general, type 1 is a catabolic agent favouring progesterone (Andersson et al. 1990) as a substrate and is highly expressed in the liver (Mahendroo et al. 1999). The finding that males deficient in type 2 are still able to convert exogenous testosterone to dihydrotestosterone would suggest that the type 1 enzyme does have at least some type 2 like activity. Type 2 is an anabolic enzyme expressed mainly in androgenic target tissues and favours testosterone as a substrate (Normington et al. 1992).

In the male 5 $\alpha$ -reductase type 2 deficiency will result in male pseudohermaphroditism where a genotypic male has female external genitalia and male internal genitalia (Wilson et al. 1993). Females have been identified with 5 $\alpha$ -reductase type 2 deficiency in families where male members are also affected by this condition (Wilson et al. 1993; Katz et al. 1995). Delayed menarche was demonstrated in this group but no other clinical symptoms or signs were discovered that would suggest 5 $\alpha$ -reductase type 2 played an essential role in female endocrine physiology. Neither has a role for 5 $\alpha$ -reductase type 1 been elucidated in human females. However, female knockout mouse models suggest an important reproductive role for 5 $\alpha$ -reductase type 1.

In the female mouse, 5 $\alpha$ -reductase type 1 is localised to the uterus, placenta and cervix. When absent, the mice are less likely to deliver their young at term and have smaller litter sizes (Mahendroo et al. 1997). Initial fertilisation and implantation occurs but at the time of the expected androgen surge from the placenta fetal demise appears to occur with a 2-4 fold elevation in serum oestradiol levels noted (Mahendroo et al. 1997). It would therefore seem that aromatisation of androgens to oestrogen occurs leading in some way to fetal demise. This suggests an important

catabolic role for 5 $\alpha$ -reductase type 1. In the fetuses that do survive 70% fail to labour and steroid levels are normal apart from low levels of a 5 $\alpha$  reduced metabolite, 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol. If exogenous 5 $\alpha$ -reduced metabolites are administered then no parturition defect occurs (Mahendroo et al. 1996). Finally it would also appear that a failure of cervical ripening is also noted in type 1 deficient mice suggesting that the presence of 5 $\alpha$ -reductase type 1 is also important for cervical ripening (Mahendroo et al. 1999). It is suggested by Mahendroo et al. that the failure of progesterone catabolism leads to this failure in cervical ripening.

While detailed mouse models exist, there are to date few studies examining the localisation and regulation of the 5 $\alpha$ -reductase isoenzymes in human uterine tissues to determine if a similar role for 5 $\alpha$ -reductase exists in human females. The only study to examine 5 $\alpha$ -reductase expression in female reproductive tissues was published recently by Ito et al. (Ito et al. 2002). They described the immunolocalisation of 5 $\alpha$ -reductase type 1 and 5 $\alpha$ -reductase type 2 in the glandular epithelium of the endometrium with no temporal variation. Their sample numbers were small which may have precluded significant differences. In addition to normal endometrium, they examined 44 cases of endometrial carcinoma and determined that 5 $\alpha$ -reductase was expressed in 80% of specimens. This would appear to confirm that 5 $\alpha$ -reductase is present in normal cycling endometrium but the paper does not provide answers as to the role of 5 $\alpha$ R in normal or malignant endometrium.

There are thus a number of enzymes that are important in the formation and degradation of sex steroids in peripheral tissues. The degree of expression of enzymes and the availability of substrate will therefore determine the ligand available to exert a specific function. This knowledge will allow the refinement of pharmacological agents to target a specific tissue or condition, such as the use of aromatase inhibitors to treat endometriosis. As discussed in Chapter 5 a consequence of LNG IUS treatment is the alteration in 17 $\beta$ -HSD2 expression in the endometrium leading to intracellular levels oestrone being raised and intracellular levels of oestradiol lowered. We hypothesise that this alteration in the intracrine environment may be important in the pathogenesis of breakthrough bleeding (BTB). Further examination of the enzymes that compose the intracrine environment in normal cycling endometrium and endometrium exposed to intrauterine delivery of levonorgestrel will aid to complement our current knowledge and understanding of

the processes that may contribute to BTB. Such knowledge may direct future research to be focused upon manipulation of enzyme expression as a mechanism for preventing BTB.

This Chapter therefore explores the expression of the above enzymes in human endometrium across the normal menstrual cycle and in patients where the endometrium has been exposed to high dose local levonorgestrel via the LNG-IUS. Where antibodies are available, the protein is examined but otherwise real time quantitative RT-PCR is used to assess temporal variation in enzyme mRNA expression.

## 6.2 Materials and Methods

Material and Methods have been described previously in Chapter 3. Details that follow summarise pertinent material and methods for this area of the research. Human endometrial samples were obtained from two different patient groups.

### 6.2.1 Tissue collection

The samples that were to be analysed for temporal expression of mRNA consisted of endometrium from fertile women ( $n=30$ ) with regular menstrual cycles lasting between 25 and 35 days who had not been using hormonal preparations for the preceding 3 months. The samples were a subset of those collected, and is detailed in Chapter 3. Endometrial tissue included samples collected from women during the early proliferative phase ( $n=5$ ), mid proliferative phase ( $n=5$ ), late proliferative phase ( $n=5$ ), early secretory ( $n=5$ ), mid secretory ( $n=5$ ) and late secretory phase ( $n=5$ ) of the menstrual cycle. The stage of the menstrual cycle was consistent with the patients reported last menstrual period and was confirmed by histological dating, according to the criteria of Noyes *et al* (Noyes *et al.* 1950). Furthermore, serum samples collected at the time of endometrial tissue collection for determination of circulating oestradiol and progesterone concentrations by RIA were consistent with the designated cycle stage and a significant decline in progesterone concentrations was apparent between biopsies taken in the mid and late secretory phase of the cycle. The samples were stored at  $-80^{\circ}\text{C}$  following collection and were processed to extract RNA as described in previous Chapters (See Chapters 3.3.1; 3.3.2; 3.3.3).

The other main patient group studied in this Chapter included women attending a menstrual problems clinic for treatment of dysfunctional uterine bleeding with the LNG IUS. Endometrial samples were collected from patients attending the clinic with an LNG IUS in-utero for 3 months ( $n=6$ ), 6 months ( $n=3$ ), or greater than 1 year ( $n=4$ ). Samples were frozen immediately upon collection in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  with subsequent RNA extraction as previously described (See Chapters 3.3.1; 3.3.2; 3.3.3).

Previous studies have failed to show the expression of aromatase in the endometrium of patients who have a normal menstrual cycle and no uterine disease. To confirm this finding a random selection of endometrial samples from across the

menstrual cycle were subjected to aromatase immunohistochemistry. The samples were a subset of full thickness endometrial biopsies as described in Chapter 3.1.1.

### **6.2.2 Aromatase Immunohistochemistry**

As detailed in Chapter 3.2.4 the specificity and properties of the antibody for the immunolocalisation of aromatase have been previously described (Turner et al. 2002). This antibody was a gift from Dr P Saunders (MRC, Human Reproductive Sciences Unit, Edinburgh).

Paraffin sections (5µm) were dewaxed and rehydrated through a series of alcohols then washed in distilled water. Tris buffered saline (TBS) was used for all washes and no antigen retrieval step was required. Positive and negative controls were included for human corpus luteum and human placenta. A first non-immune block was performed with normal rabbit serum diluted in TBS/BSA (Diagnostics Scotland, Edinburgh, Midlothian, UK) for 30 minutes at room temperature. Primary antibody incubation was at a 1:50 dilution overnight at 4°C. Rabbit anti-mouse biotinylated secondary antibody was employed diluted with normal rabbit serum to provide a second non-immune block with secondary antibody incubation as above with incubation at room temperature for 60 minutes. The ABC complex used was ABC-HRP and this incubation step was also at room temperature for 45 minutes. Visualisation employed the chromagen 3, 3'-diaminobenzidine (DAKO Liquid, DAKO Corporation, California, USA). Results from immunohistochemistry were descriptive in nature.

### **6.2.3 RNA extraction and real time quantitative RT-PCR**

Frozen samples of endometrium stored at -80°C were first homogenised, and then total RNA was extracted using the commercially available product Trizol (Invitrogen Life technologies Ltd, Paisley, UK). The methods of extraction, quantification, cDNA preparation and quantitative RT-PCR are the same as detailed in Chapter 3.3. All samples were pre treated with RNase free DNase (Invitrogen Life technologies Ltd, Paisley, UK).

Real-time quantitative PCR was performed in an ABI 7700 Sequence Detection System (Perkin-Elmer, CA, USA). Oligonucleotide forward and reverse primers and oligonucleotide Taqman probes for 3 $\beta$ -HSD types 1 and 2, 17 $\beta$ -HSD 5, and 5 $\alpha$ -reductase types 1 and 2 were designed, with the use of Primer Express version 1.0 (Perkin-Elmer Applied Biosystems, Cheshire, UK), from sequences entered in the GenBank database. The sequences for the primers and probes above are detailed in table 3.2. Numeric data from the real time quantitative RT-PCR were analysed by one way ANOVA, followed by Fisher's protected least significant difference test.

## **6.3 Results**

### **6.3.1 Aromatase Immunohistochemistry**

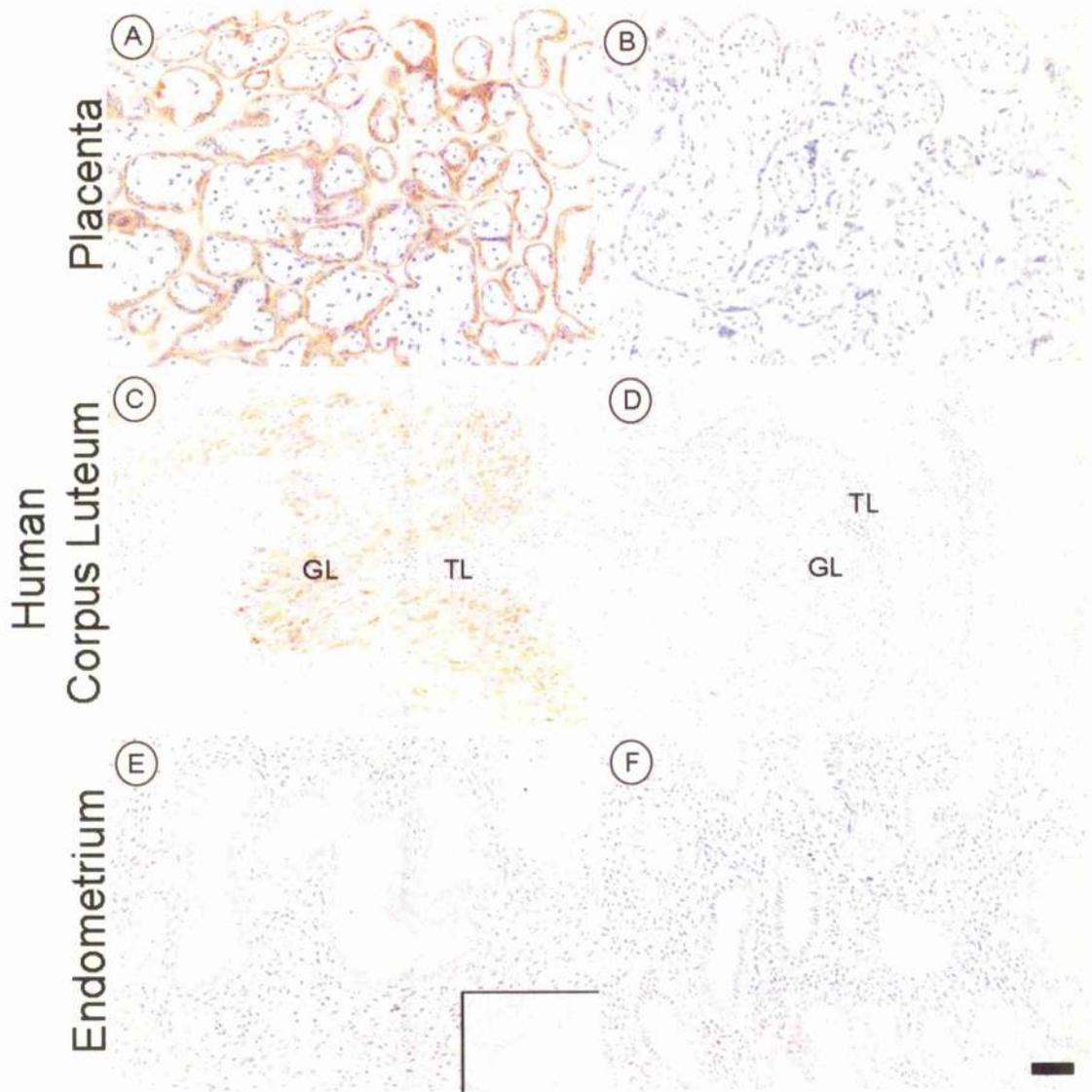
The positive controls for the aromatase immunohistochemistry were human placenta and corpus luteum. Immunoreactivity was noted in human placental syncytiotrophoblast and in the human granulosa lutein cells as would be anticipated (Figure 6.1). The samples of human endometrium did not however show any evidence of positive immunoreactivity (Figure 6.1). Using this new monoclonal antibody we have therefore confirmed the findings of other investigators. As the protein was not expressed in human endometrium no primers or probes were used to look at the expression of aromatase mRNA in human endometrium.

Figure 6.1

Immunohistochemical localisation of Aromatase in human placenta, human corpus luteum, and human endometrium. (x 20 magnification, except corpus luteum images). (A) Human Placenta – immunoreactivity noted in syncytiotrophoblast. (B) Human Placenta – negative control. (C) Human Corpus Luteum (x10 magnification) – immunoreactivity in granulosa lutein region (GL) and absent immunoreactivity in the theca lutein region (TL). As anticipated, aromatase immunoreactivity was in the granulosa cells region as it expresses aromatase whereas theca cells do not. (D) Human Corpus Luteum (x10 magnification) – negative control. (E) Absent immunoreactivity for aromatase in secretory endometrium (negative control inset). (F) Absent immunoreactivity for aromatase in proliferative endometrium. Scale bar 50 µm at x 20 magnification

Reactions Catalysed by Aromatase:

Testosterone	→ Oestradiol
Androstenedione	→ Oestriol



### **6.3.2 Temporal expression of 17 $\beta$ -HSD 5 mRNA in human endometrium and following high dose intra-uterine delivery of levonorgestrel.**

The samples analysed for 17 $\beta$ -HSD 5 revealed highest expression of the mRNA in the mid secretory phase with the fold increase being significantly greater than at any other time point in the menstrual cycle ( $p < 0.05$ ; figure 6.2).

Human endometrium that had been exposed to intra-uterine levonorgestrel for 3, 6 or >12 months *in vivo* were also analysed and compared to an internal mRNA control from the late proliferative phase of the menstrual cycle to calculate the fold increase compared with the menstrual cycle. Endometrium exposed to intra-uterine LNG expressed 17 $\beta$ -HSD 5 mRNA at levels significantly less than the mid secretory phase but not significantly different from any other menstrual cycle stage ( $p < 0.005$ ; figure 6.2).

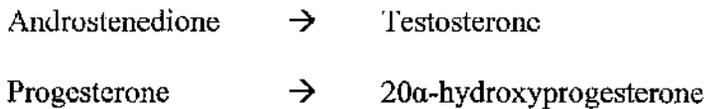
17 $\beta$ -HSD 5 has been widely studied in the human prostate but only one previous study has localised the enzyme to human endometrial glandular epithelium without commenting upon a specific cycle stage. This finding confirms the expression of 17 $\beta$ -HSD 5 at the mRNA level in the endometrium with highest levels found in the mid secretory phase. The antibody to corroborate these data was not commercially available and could not be sourced.

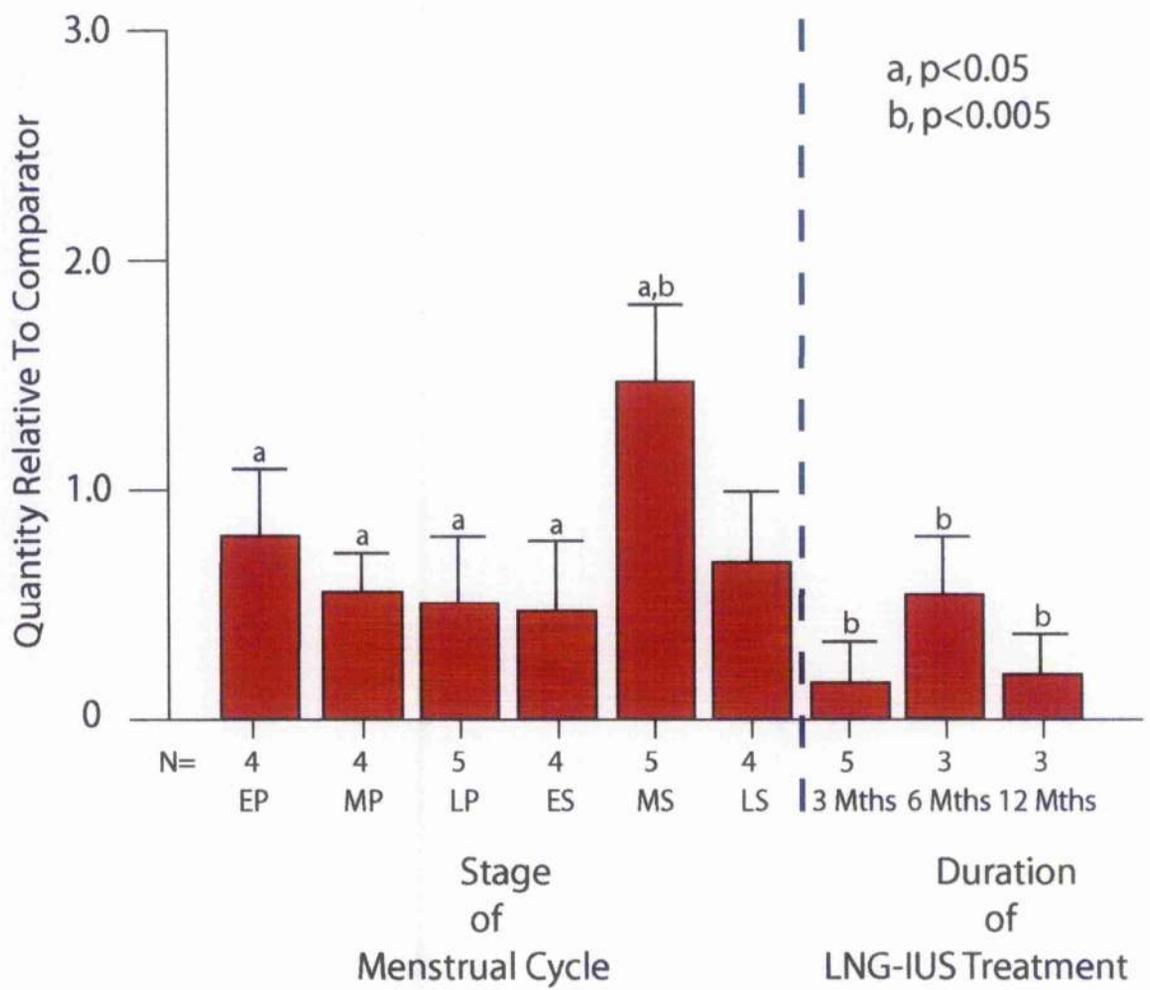
Figure 6.2

Quantitative evaluation of 17 $\beta$ -HSD5 mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of LNG. All endometrial tissue samples were compared to an internal control (comparator) obtained during the early proliferative stage of the cycle.

17 $\beta$ -HSD5 mRNA levels were significantly higher in the mid secretory phase of the menstrual cycle when compared to the proliferative phase, the early secretory and late secretory phases ( $p < 0.05$ ). In endometrium exposed to intra-uterine levonorgestrel, 17 $\beta$ -HSD5 mRNA levels were also significantly lower compared with the mid secretory phase ( $p < 0.005$ ) but not significantly different from any other menstrual cycle phase. EP, early proliferative; MP, mid proliferative; LP, late proliferative; ES, early secretory; MS, mid secretory; LS, late secretory.

Reactions Catalysed by 17 $\beta$ -HSD5:





### **6.3.3 Temporal expression of 3 $\beta$ -HSD 1 and 2 mRNA in human endometrium and following high dose intrauterine delivery of levonorgestrel.**

There is a high degree of homology between types 1 and 2 3 $\beta$ -HSD. In an attempt to provide discrimination in the detection of each subtype, sequences were chosen from the primer express program where there was at least one nucleotide sequence difference in each of the primers and probe from the alternate subtype. This was achieved in both primer probe sets. 3 $\beta$ -HSD 1 contained 3 nucleotides within the forward primer, 2 nucleotides in the reverse primer and 2 nucleotides in the probe that did not correspond to 3 $\beta$ -HSD 2. 3 $\beta$ -HSD 2 contained 1 nucleotide within the forward primer, 3 nucleotides in the reverse primer and 3 nucleotides in the probe that did not correspond to 3 $\beta$ -HSD 1.

The samples analysed for 3 $\beta$ -HSD 1 revealed highest expression of the mRNA in the peri-menstrual stages with the fold increase in the mid to late secretory and early proliferative stages being significantly increased compared to the mid cycle late proliferative and early secretory stages (figure 6.3). The early proliferative and late secretory stages in particular were highly significant compared to the mid cycle stages ( $p < 0.005$ ).

Human endometrium that had been exposed to intra-uterine levonorgestrel for 3, 6 or >12 months in vivo were then analysed for 3 $\beta$ -HSD 1 and compared to an internal mRNA control (comparator) from the late proliferative phase of the menstrual cycle. The LNG exposed endometrium expressed 3 $\beta$ -HSD 1 mRNA with the fold increase at 3 months being significantly greater than any other menstrual cycle stage or treatment duration. Following the initial fold increase in 3 $\beta$ -HSD 1 expression in the first three months a decline in mRNA expression is noted such that by 12 months there is no significant variation in 3 $\beta$ -HSD 1 from the menstrual cycle (figure 6.3).

3 $\beta$ -HSD 2 mRNA showed a similar pattern of expression during the menstrual cycle to that of 3 $\beta$ -HSD 1 with the fold increase in the peri-menstrual stages being significantly greater than the mid cycle stages (figure 6.4). This suggests that either the level of discrimination by the Taqman probes between the two isoforms may not be sufficient, or that both isoforms follow a similar pattern of expression during the menstrual cycle.

Interestingly the data for  $3\beta$ -HSD 2 expression in endometrium from women using the LNG-IUS for 3, 6 or >12 months in vivo do not follow the same pattern as that for  $3\beta$ -HSD 1.  $3\beta$ -HSD 2 mRNA expression from 3 months onwards reveals a maintained significant fold increase in expression when compared to any menstrual stage cycle (figure 6.4).

Figure 6.3

Quantitative evaluation of 3 $\beta$ -HSD1 mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of LNG. All endometrial tissue samples were compared to an internal control (comparator) obtained during the late secretory stage of the cycle.

3 $\beta$ -HSD1 mRNA levels were significantly higher in the mid and late secretory phase as well as the early proliferative phase of the menstrual cycle when compared to the mid cycle, late proliferative and early secretory phase ( $p < 0.005$ ). In LNG-IUS exposed endometrium, 3 $\beta$ -HSD1 mRNA levels at 3 months were significantly higher than all menstrual cycle phases and LNG treatment time points ( $p < 0.001$ ). EP, early proliferative; MP, mid proliferative; LP, late proliferative; ES, early secretory; MS, mid secretory; LS, late secretory.

Reactions Catalysed by 3 $\beta$ -HSD1:

Androstenediol	→	Testosterone
Dehydroepiandrosterone	→	Androstenedione
17 OH Pregnenolone	→	17 OH Progesterone
Pregnenolone	→	Progesterone

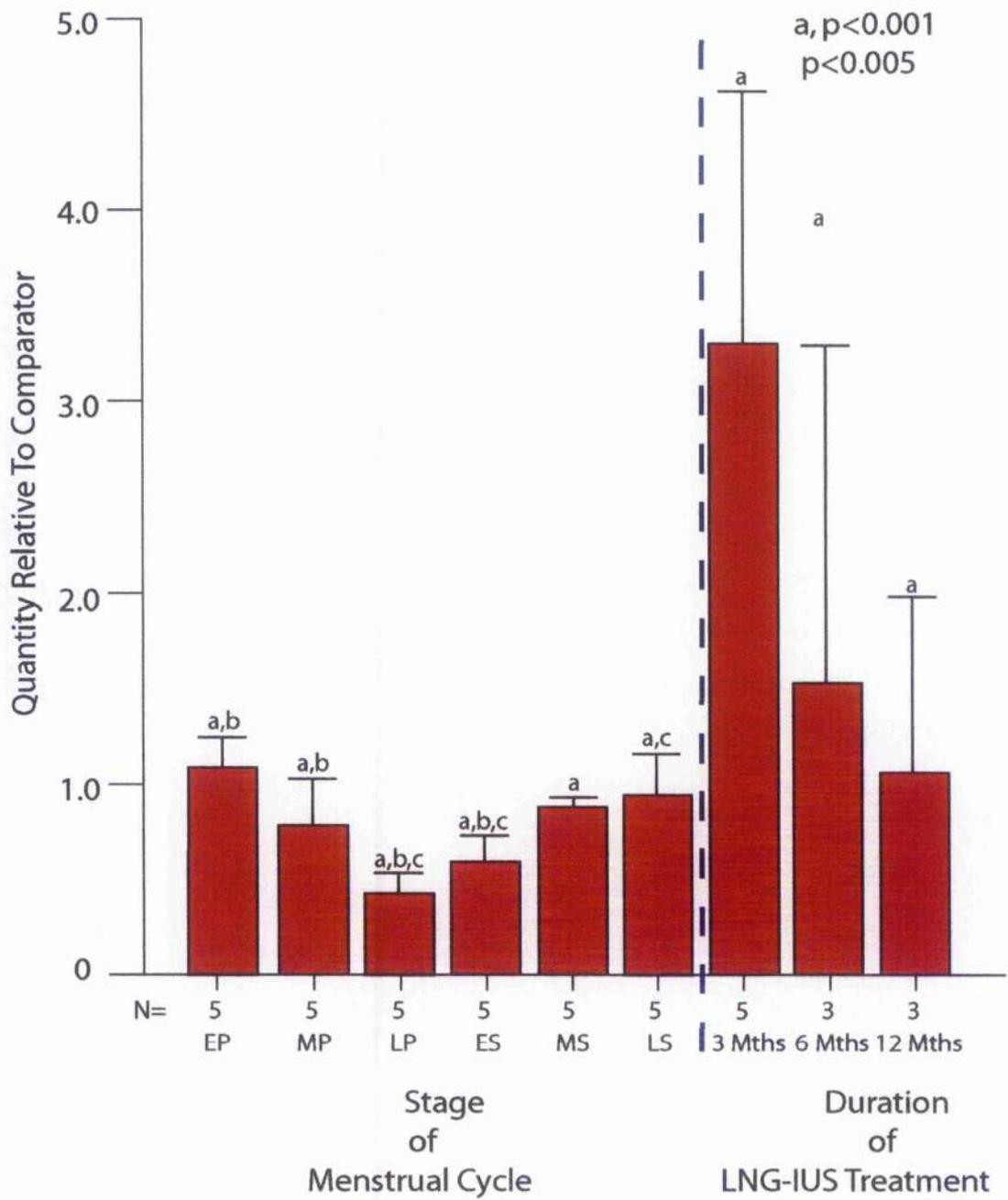


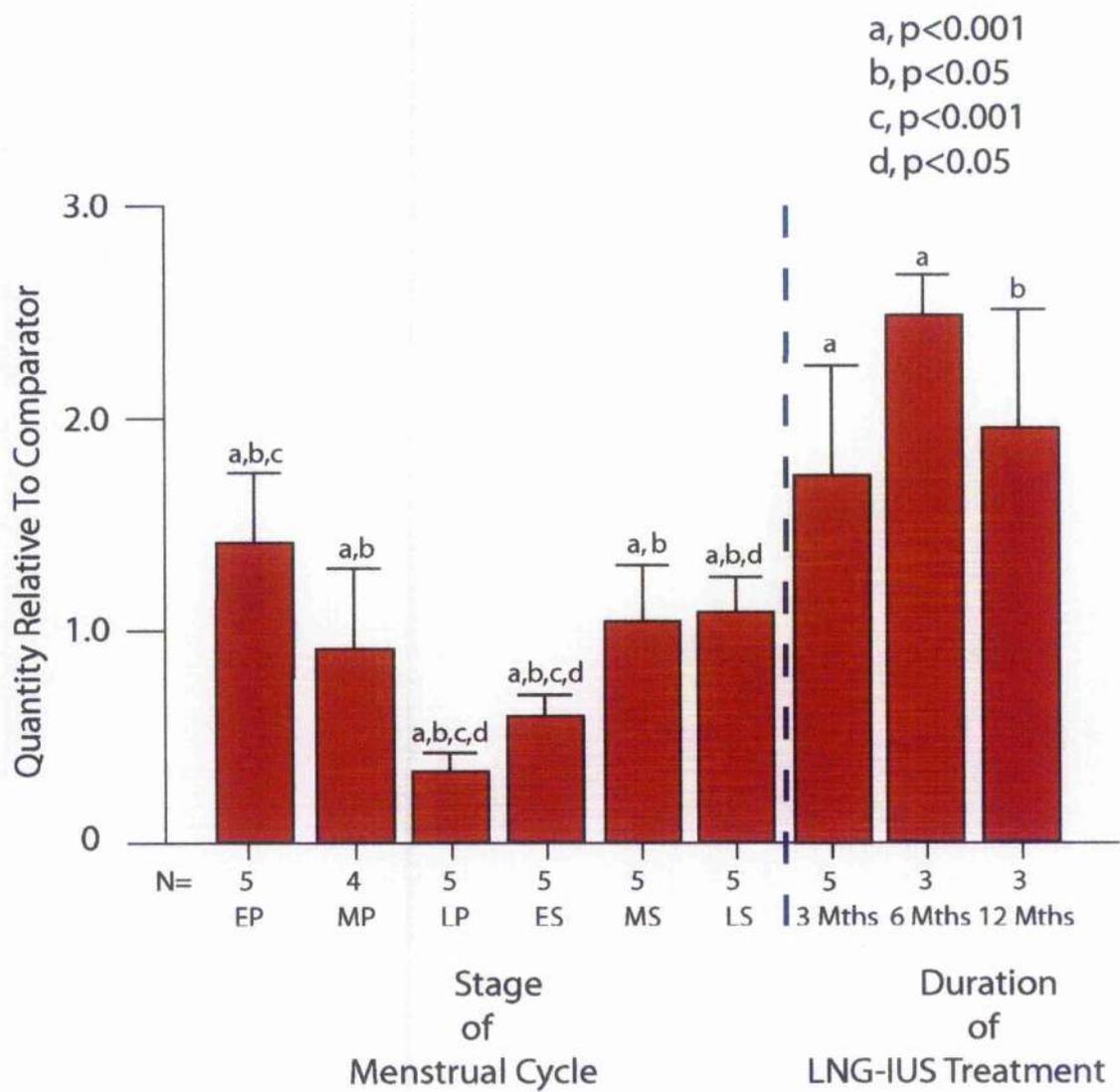
Figure 6.4

Quantitative evaluation of  $3\beta$ -HSD 2 mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of LNG. All endometrial tissue samples were compared to an internal control (comparator) obtained during the late secretory stage of the cycle.

$3\beta$ -HSD 2 mRNA levels were significantly higher in the mid and late secretory phases ( $p < 0.05$ ) as well as the early and mid proliferative phases ( $p < 0.001$ ) of the menstrual cycle when compared to the mid cycle, late proliferative and early secretory phase. In LNG-IUS treated endometrium,  $3\beta$ -HSD 2 mRNA levels at 3, 6 and 12 months were significantly higher than all menstrual cycle phases ( $p < 0.001$ ; significance values for 3 months not shown but similar to those for the 6 and 12 month treatments). EP, early proliferative; MP, mid proliferative; LP, late proliferative; ES, early secretory; MS, mid secretory; LS, late secretory.

Reactions Catalysed by  $3\beta$ -HSD 2:

Androstenediol	→	Testosterone
Dehydroepiandrosterone	→	Androstenedione
17 OH Pregnenolone	→	17 OH Progesterone
Pregnenolone	→	Progesterone



#### **6.3.4 Temporal expression of 5 $\alpha$ -reductase 1 and 2 mRNA in human endometrium and following high dose intrauterine delivery of levonorgestrel.**

There are no reliable antibodies that will discriminate between 5 $\alpha$ -reductase types 1 and 2. Attempts were therefore made to determine enzymatic activity in human endometrium at various menstrual cycle stages through collaboration with Dr Fouad Habib (Reader in Biochemistry and Cell Biology, Department of Oncology, Western General Hospital, Edinburgh, UK) who has published widely on 5 $\alpha$ -reductase in normal and malignant prostate tissues. The assays developed for prostate tissues were not suitable for use in the human endometrial samples that we had collected as a greater volume of tissue was required.

The sequences to detect the mRNA of both 5 $\alpha$ -reductase types 1 and 2 spanned an intron to preclude amplification of genomic DNA and all cDNA samples were prepared with DNase pre-treatment.

The samples analysed for 5 $\alpha$ -reductase type 1 revealed highest expression of the mRNA in the late proliferative stage with the fold increase being significantly greater than in the early proliferative and mid secretory stages with a trend towards being significantly greater than the late secretory stage ( $p < 0.05$ ; figure 6.5).

Endometrium treated with the LNG-IUS for 3, 6 or >12 months in vivo revealed 5 $\alpha$ -reductase type 1 mRNA expression at levels no different from the majority of the menstrual cycle stages with the late proliferative stage still expressing the highest level of 5 $\alpha$ -reductase type 1 (figure 6.5).

5 $\alpha$ -reductase type 2 exhibited an expression pattern similar to that of 3 $\beta$ -HSD 1 and 2 with significantly greater fold increases in the peri-menstrual stages when compared to the mid cycle stages, the late proliferative in particular (figure 6.6).

Endometrium from women with the LNG-IUS for 3, 6 or >12 months produced significantly lower levels of 5 $\alpha$ -reductase type 2 mRNA expression when compared to the peri-menstrual stages of the menstrual cycle but no different from the mid cycle stages (figure 6.6).

Figure 6.5

Quantitative evaluation of 5-alpha reductase 1 mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of LNG. All endometrial tissue samples were compared to an internal control (comparator) obtained during the late secretory stage of the cycle.

5-alpha reductase 1 mRNA levels were significantly higher in the late proliferative phase ( $p < 0.05$ ) of the menstrual cycle when compared to all other menstrual cycle phases except the mid proliferative phase. In LNG-IUS treated endometrium, 5-alpha reductase 1 mRNA levels at 3, 6 and 12 months were significantly lower than the late proliferative phase ( $p < 0.01$ ). EP, early proliferative; MP, mid proliferative; LP, late proliferative; ES, early secretory; MS, mid secretory; LS, late secretory.

Reactions Catalysed by 5-alpha reductase 1:



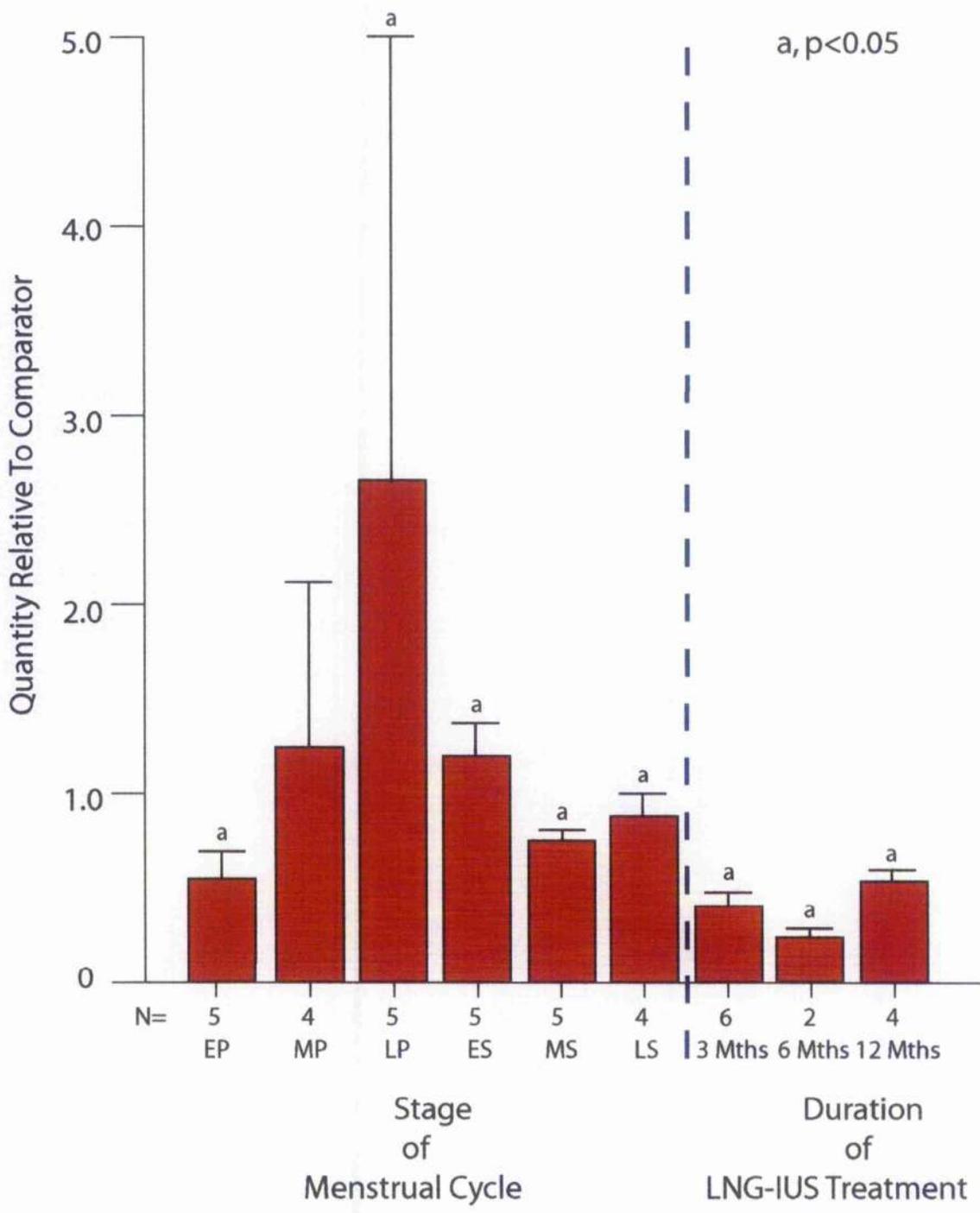


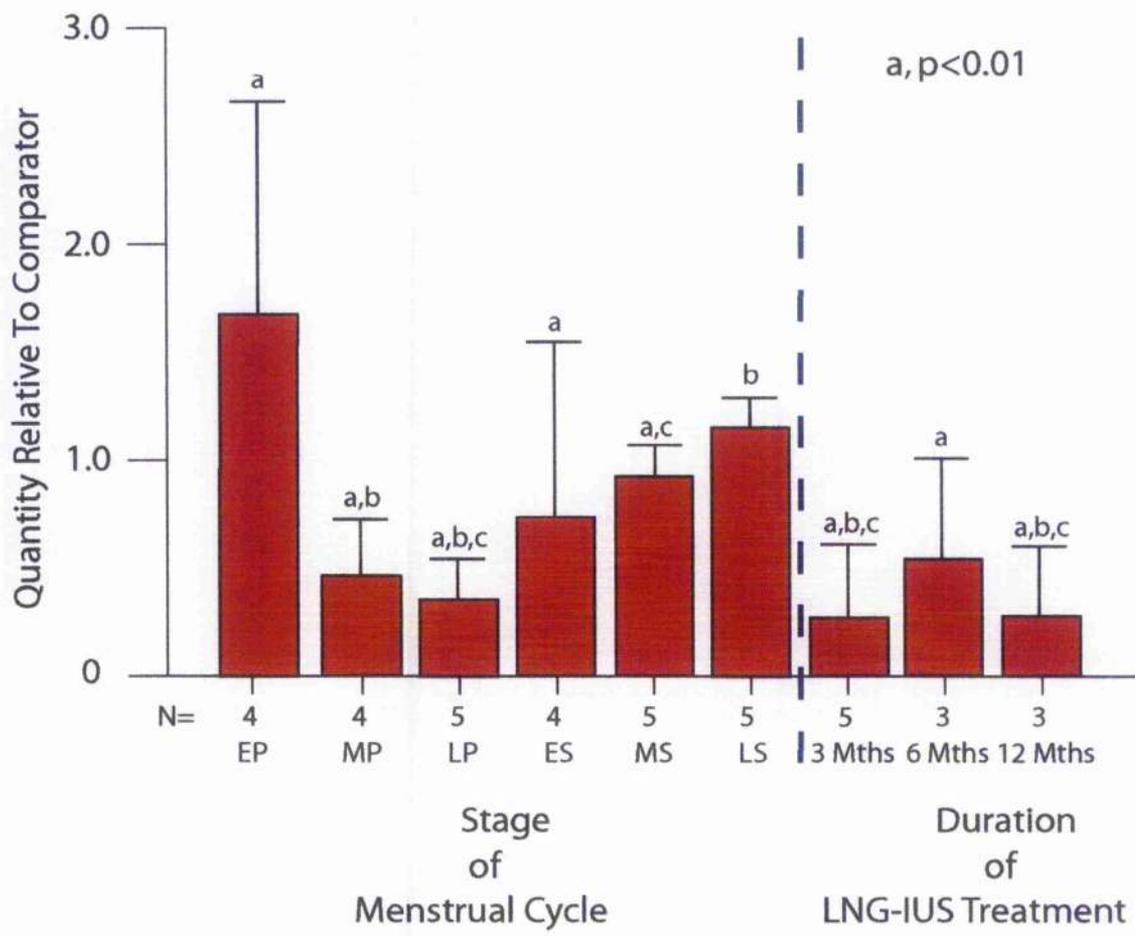
Figure 6.6

Quantitative evaluation of 5-alpha reductase 2 mRNA in endometrium across the stages of the menstrual cycle and following intrauterine delivery of LNG. All endometrial tissue samples were compared to an internal control (comparator) obtained during the mid secretory stage of the cycle.

5-alpha reductase 2 mRNA levels were significantly higher in the early proliferative phase ( $p < 0.01$ ) of the menstrual cycle when compared to all other menstrual cycle phases except the late secretory phase. The late secretory was also significantly elevated compared to the mid and late proliferative phases. In LNG-IUS treated endometrium, 5-alpha reductase 2 mRNA levels at 3, 6 and 12 months were significantly lower than the late proliferative phase ( $p < 0.01$ ). Furthermore, endometrium exposed to LNG for 3 and 12 months expressed significantly lower levels of 5-alpha reductase 2 mRNA compared to the mid and late secretory phases of the menstrual cycle. EP, early proliferative; MP, mid proliferative; LP, late proliferative; ES, early secretory.

Reactions Catalysed by 5-alpha reductase 2:





## 6.4 Discussion

There has not yet been a coordinated analysis of the enzymes that shape the intracrine environment of the endometrium. An understanding of the endometrial intracrine environment will be important to the field of endometrial biology as it will provide further insight to the factors that control the expression of local endometrial factors. The potential importance for such information has been demonstrated in Chapter 5 where levonorgestrel was shown to manipulate this environment by altering the expression of  $17\beta$ -HSD2, contributing further to our understanding of the processes involved in breakthrough bleeding. By extending the range of enzymes analysed we have added to the data available helping to further our understanding of the intracrine factors that affect the function of endometrium through the menstrual cycle and following its exposure to levonorgestrel.

The published data thus far have not supported the presence of aromatase in human endometrium (Bulun et al. 1993). The data presented here employed a novel monoclonal antibody that has been validated and shown to immunolocalise aromatase in primate tissues including human placenta and human ovary (Turner et al. 2002). Using this monoclonal antibody, supported by the inclusion of appropriate control samples, the current findings support the data previously published, i.e., aromatase is not expressed in the endometrium of patients with no concurrent endometrial related pathology. As no aromatase was identified at the protein level, and previous studies have also shown this to be true for mRNA expression (Bulun et al. 1993), real time Taqman analysis for aromatase mRNA was not conducted. This confirmation suggests that any androgenic precursor that may be produced by enzymatic conversion of adrenal precursor substrate will not be converted directly to an oestrogenic form by aromatisation. Consequently it would not be anticipated that aromatase inhibitors would be able to directly manipulate the endometrial intracrine environment.

The enzymes responsible early in the steroidogenic pathway for the synthesis of mineralocorticoids, glucocorticoids and sex steroids include  $3\beta$ -HSD. In humans, the principal location of this enzyme is in the adrenal and gonads (Mason 1993). In the female, dehydroepiandrosterone (DHEA) is produced in the adrenal gland with serum levels some 100-500 times greater than testosterone and 1000-10000

times greater than oestradiol (Labrie et al. 2000). The adrenal is also a source of pregnenolone, a steroid precursor that  $3\beta$ -HSD can convert to progesterone. The adrenal therefore provides an excellent source of substrate for the production of steroids in peripheral target tissue. The presence or absence of  $3\beta$ -HSD in peripheral tissues such as the endometrium will therefore be crucial to a tissue's ability to produce steroid hormones from the abundant adrenal substrate.

Rhee et al. report  $3\beta$ -HSD glandular epithelial immunoreactivity in both secretory and proliferative phase endometrium. They did not subdivide their samples into early, mid or late stages. However, they did note significantly greater immunoreactivity in the secretory phase than the proliferative phase where immunoreactivity was weak (Rhee et al. 2003). Tang et al. also noted activity of  $3\beta$ -HSD to be higher in the secretory phase (Tang et al. 1993). The mRNA data available in the paper by Rhee et al. also suggests that the  $3\beta$ -HSD subtype most likely to be present is type 1. The mRNA data presented herein concurs with those of Rhee et al with higher levels of mRNA being noted in the mid to late secretory phase persisting to the peri-menstrual phase.

Despite being able to identify Taqman probes that were dissimilar, it is not possible to state that there is sufficient discrimination that allows definitive determination of the  $3\beta$ -HSD isoform present in the endometrium. Irrespective of the subtype present, both probes exhibit similar variation through the menstrual cycle. Having determined that  $3\beta$ -HSD is expressed in secretory phase endometrium with mRNA levels highest in the peri-menstrual time frame it remains to be determined how it is regulated and what function this enzyme may have in human endometrium.

Mason et al. (Mason et al. 1993) reviewed the regulation of  $3\beta$ -HSD and suggested that its regulation was tissue-specific involving local mechanisms.  $3\beta$ -HSD mRNA expression is similar to that of  $17\beta$ -HSD2 mRNA with expression at its highest level in the peri-menstrual phase. Our data suggest that  $17\beta$ -HSD2 is up regulated by progesterone and Maentausta et al. also suggest that this enzyme is up regulated by progesterone (Maentausta et al. 1993). Rhee et al. have hypothesised that  $3\beta$ -HSD is progesterone-regulated (Rhee et al. 2003). In the decidual samples analysed by Rhee et al. an up-regulation of  $3\beta$ -HSD was noted, and in the first trimester serum progesterone levels are higher than in the non-pregnant state (Rhee et al. 2003). Tang et al. also showed that proliferative endometrium exposed to the

progesterin medroxyprogesterone acetate *in vitro*, up-regulated 3 $\beta$ -HSD (Tang et al. 1993). The data presented here would also support a role for progesterone in the up-regulation of human endometrial 3 $\beta$ -HSD. In endometrium exposed to high doses of intra-uterine levonorgestrel, both primer probe sets revealed an up-regulation of 3 $\beta$ -HSD mRNA following three months treatment. Up regulation of 3 $\beta$ -HSD 2 favours the formation of androgenic ligands. The androgenic ligands produced may in part explain the mechanism of endometrial atrophy noted with endometrial exposure to high dose intra-uterine LNG-IUS. As described in chapter 4.4, androgens have been shown to have an anti-proliferative effect and data from this study has described a mechanism for the role of androgens in the atrophic changes associated with anti-progesterone use.

As previously stated, the function of endometrial 3 $\beta$ -HSD type 1 is uncertain but it has a high affinity for its substrate (Rheaume et al. 1991) and can convert pregnenolone to progesterone, 17-hydroxypregnenolone to 17-hydroxyprogesterone, DHEA to 4-androstenedione, and 5-androstene-3 $\beta$ ,17 $\beta$ -diol to testosterone (Lachance et al. 1990). In endometrium, it may be involved with the provision of progesterone from pregnenolone and/or the conversion of androstenedione from DHEA. Further sex steroid production would depend upon the presence or absence of alternative metabolizing enzymes such as 17 $\beta$ -HSD 5, 5 $\alpha$ -reductase or aromatase. We have established that aromatase is only expressed in the endometrium of patients with concurrent pelvic pathology such as endometriosis or fibroids. 17 $\beta$ -HSD 5 is known, however, to be expressed in human endometrial glandular epithelium with the stage of the cycle not having been currently determined (Pelletier et al. 1999).

Pelletier et al. used a 17 $\beta$ -HSD 5 antibody to detect the enzyme in a variety of tissues. In the uterus, Pelletier determined that endometrial glandular epithelium of the functional and basal layers exhibited positive immunoreactivity (Pelletier et al. 1999). The enzyme was for a long time misidentified as type 2 3 $\alpha$ -hydroxysteroid dehydrogenase (3 $\alpha$ -HSD) as it is highly labile and it is easily destroyed upon homogenisation (Dufort et al. 1999). It has now been shown that in intact cells there is unidirectional conversion of androstenedione to testosterone in keeping with its 17 $\beta$ -HSD function that is much greater than the 3 $\alpha$ -HSD conversion of dihydrotestosterone to 5 $\alpha$ -androstane-3 $\alpha$ ,17 $\beta$ -diol (3 $\alpha$ -diol) (Dufort et al. 1999). The enzyme is therefore now known as 17 $\beta$ -HSD 5. In addition to 17 $\beta$ -HSD and 3 $\alpha$ -HSD

activity the enzyme also possesses  $20\alpha$ -hydroxysteroid dehydrogenase ( $20\alpha$ -HSD) activity inactivating progesterone to  $20\alpha$ -hydroxyprogesterone. While the enzyme does possess multiple roles, the endometrium has been shown by Labrie et al. to possess higher androgenic  $17\beta$ -HSD activity with the rate of testosterone production by the endometrium only surpassed by placenta, liver and testis (Labrie et al. 1997).

Our results confirm the expression of  $17\beta$ -HSD 5 at the mRNA level with an up regulation of expression in the mid secretory phase. As stated previously there is no commercially available antibody and we could not otherwise source an antibody that would detect  $17\beta$ -HSD 5. In an attempt to see if we could determine the expression of the enzyme, via its  $3\alpha$ -HSD activity, we searched the available literature but no localisation or endometrial enzyme activity studies have been performed. Type 2  $3\alpha$ -HSD mRNA has been demonstrated in the uterus but the cycle stage in which it is predominant was not determined.

The role of  $17\beta$ -HSD 5 in human endometrium has not yet been determined but it is involved in a step that is important for the formation of active sex steroids. Its presence in glandular epithelium is consistent with the findings for other hydroxysteroid dehydrogenases,  $3\beta$ -HSD (Rhee et al. 2003) and  $17\beta$ -HSD 2 (Burton et al. 2003). In the case of  $3\beta$ -HSD mRNA and  $17\beta$ -HSD mRNA and protein, expression has been shown to be maximal in the secretory phase. The same may be true for  $17\beta$ -HSD 5 but as yet we know only that  $17\beta$ -HSD 5 mRNA expression is maximal in the mid-secretory phase and hopefully further data on the immunolocalisation of the protein will be forthcoming in future studies. It would however be credible that  $17\beta$ -HSD 5 would also be expressed at the protein level in the secretory phase glandular epithelium.

To reiterate, the endometrium has been shown by Labrie et al. to possess androgenic  $17\beta$ -HSD activity as evidenced by testosterone production from androstenedione (Labrie et al. 1997). The ability of the endometrium to produce active androgens from precursors is also supported by in vitro data from various authors (Collins et al. 1969; Rose et al. 1978; Hausknecht et al. 1982) In the in vivo situation the expression of  $3\beta$ -HSD would permit the production of androstenedione from DHEA while the expression of  $17\beta$ -HSD 5 would permit the conversion of androstenedione to testosterone. Indeed Bonney et al. showed significant elevations

in the concentration of endometrial DHEA during the secretory phase (Bonney et al. 1984). The expression of 17 $\beta$ -HSD 2, which catalyses oestradiol to oestrone and testosterone to androstenedione, would be a mechanism for the fine balancing of testosterone production should it be required. Puranen et al. suggest that androgenic substrate is the best for 17 $\beta$ -HSD 2 when compared to oestrogen and progesterone and they hypothesise that a possible function for 17 $\beta$ -HSD 2 in the prostate is to protect the tissue from excessive androgen stimulation (Puranen et al. 1999).

A level of complexity is however added with the knowledge that in the secretory phase, serum progesterone levels predominate and there are known progesterone-regulated events important for secretory differentiation. Therefore, it may in fact be that the presence of 3 $\beta$ -HSD (catalysing pregnenolone to progesterone), 17 $\beta$ -HSD2 (catalysing 20 $\alpha$ -dihydroprogesterone to progesterone) and 17 $\beta$ -HSD5 (catalysing progesterone to 20 $\alpha$ -dihydroprogesterone) are more important in balancing progesterone levels in the secretory glandular epithelium. However, neither androgens nor progesterone have significant levels of genomic receptors in the secretory phase glandular epithelium at the time 3 $\beta$ -HSD, 17 $\beta$ -HSD2, and 17 $\beta$ -HSD5 are expressed. The action of the ligands upon genomic sex steroid receptors would therefore need to be via a paracrine mechanism as AR and PR are both present in the secretory phase stromal cells. To further evaluate the likely ligands present to exert an influence upon the endocrine environment we also analysed the endometrial samples for the temporal expression of 5 $\alpha$ -reductase types 1 and 2.

5 $\alpha$ -reductase type 1 is a catabolic enzyme that favours progesterone as a substrate (Andersson et al. 1990) and 5 $\alpha$ -reductase type 2 is an anabolic enzyme that favours dihydrotestosterone production from testosterone (Normington et al. 1992). Ito et al. have reported the expression of 5 $\alpha$ -reductase types 1 and 2 in glandular epithelium of both the proliferative and secretory phases of the menstrual cycle. They do however suggest that expression is weak at all phases. Their study also examined the expression of 5 $\alpha$ -reductase types 1 and 2 in endometrial carcinoma, confirming its expression at the protein and mRNA level. They did not however examine expression of mRNA from the normal menstrual cycle. In our study we employed real time RT-PCR using Taqman probes to identify temporal expression of 5 $\alpha$ -reductase types 1 and 2 during the menstrual cycle. We determined that 5 $\alpha$ -reductase type 1 was expressed maximally in the middle of the cycle whereas maximal

expression of 5 $\alpha$ -reductase type 2 was in the peri-menstrual phase. Of note, the publication by Ito et al. only included 3 patients in each of the early, mid and late proliferative or secretory phases. Their numbers may have been too small to show significant differences in expression between the stages of the cycle. It should also be noted that the positive controls, liver and prostate, employed in the study by Ito et al. express both 5 $\alpha$ -reductase types 1 and 2 and are therefore inadequate to demonstrate specificity of the antibodies. However, Ito et al. do show expression of both reductases in secretory glandular epithelium. This finding of 5 $\alpha$ -reductase expression in glandular epithelium suggests that the secretory glandular epithelium has the ability to function as an intracrine unit controlling ligand availability for paracrine activation of the endometrial stromal cells.

As secretory glandular epithelium is now known to express mRNA and protein for 3 $\beta$ -HSD, 17 $\beta$ -HSD2, 17 $\beta$ -HSD5, and 5 $\alpha$ -reductase types 1 and 2 it has the potential to utilise the relatively abundant levels of DHEA that is produced by the adrenal glands and circulates at far higher concentrations than serum oestradiol or testosterone.

Is the purpose of this intracrine unit to produce active androgens that will affect endometrial function to play a role in successful implantation or partake in the onset of menstruation? Is the purpose of this enzyme complex to balance progesterone expression to allow successful implantation or initiate menstruation should implantation fail to occur?

As the AR is expressed in stromal cells any action of androgens produced by the glandular epithelium are likely to be mediated in a paracrine manner. Androgens have been shown during in vitro and in vivo experiments to have a direct effect upon endometrial stromal cells. In vitro experiments with androgens have shown that they can induce prolactin secretion from endometrial stromal cells in culture (Narukawa et al. 1994). Furthermore, elevated serum androgens are a principal biochemical feature of Polycystic Ovarian Syndrome (PCOS). Data have been published demonstrating an association between PCOS and altered expression of factors important for maintaining a receptive endometrial environment for implantation.

Apparao et al. demonstrated that PCOS patients down regulate alpha (v) beta3 integrin (Apparao et al. 2002), a marker that if down regulated suggests reduced endometrial receptivity (Lessey et al. 1992). In vivo experiments where

uterine flushings were obtained from PCOS patients with elevated serum androgens levels exhibited reduced levels of glycodeclin-A, another factor thought to be important for the establishment of successful conceptus implantation (Okon et al. 1998; Tuckerman et al. 2000). Furthermore, progesterone concentrations appear unrelated to glycodeclin-A levels (Westergaard et al. 1998).

In vitro and in vivo data are also available to illustrate the decreased expression of a further factor believed to be important for endometrial receptivity HOXA-10 (Cermik et al. 2003). Testosterone has been reported as the only sex steroid that diminished uterine expression of HOXA-10. HOXA-10 expression was repressed by in vitro testosterone treatment, an effect that was blocked by flutamide (an androgen receptor antagonist), and in PCOS patients the endometrium of the mid secretory phase expressed significantly less HOXA-10 mRNA compared to fertile controls (Cermik et al. 2003).

Androgens therefore appear to play a role in the control of factors important for the formation of a receptive endometrium that will allow successful conceptus implantation. While serum androgen levels show little variation through the menstrual cycle the presence of an intracrine unit within the glandular epithelium provides a means for local production of androgens that can then be involved in the paracrine control of factors such as prolactin, glycodeclin-A, and HOXA-10. Progesterone is however also known to play a key role in the differentiation of the secretory phase endometrium. It may prove to be that the expression of the enzymes in this intracrine unit serves to provide regulation of ligand for both the AR and PR.

In Chapter 5 the regulation of metabolising enzymes and AR in endometrium exposed to intra-uterine levonorgestrel were explored to provide insight into their regulation. Additionally this provided the opportunity to evaluate the role of high dose local levonorgestrel, released via the LNG-IUS, upon the significant clinical problem associated with LNG-IUS usage, breakthrough bleeding (BTB). We have already shown that AR is down regulated with LNG IUS treatment and  $17\beta$ -HSD 2 is up-regulated. These findings generated the hypothesis that disruption of oestradiol to oestrone ratios in endometrium exposed to intra-uterine levonorgestrel may contribute to BTB. In this Chapter we further assessed the local intracrine environment in relation to the metabolising enzymes  $17\beta$ -HSD 5,  $3\beta$ -HSD types 1 and 2, and  $5\alpha$ -reductase types 1 and 2.

The only significant alterations in enzyme mRNA expression following endometrial exposure to levonorgestrel were the increased expression of  $3\beta$ -HSD and the reduced expression of  $5\alpha$ -reductase2. Type 1 and type 2  $3\beta$ -HSD show an up-regulation in mRNA expression after 3 months treatment with the LNG-IUS. Interestingly from then on, at the 6 month and the greater than 12 month follow up time points,  $3\beta$ -HSD type 1 mRNA levels are not significantly different from the peri-menstrual phase of the cycle levels while  $3\beta$ -HSD type 2 continues to be expressed at a higher level when compared to all stages of the cycle at all time points.  $5\alpha$ -reductase type 2 mRNA was significantly lower than the mid and late secretory phases in addition to the early proliferative phase.

None of the other metabolising enzymes were expressed at levels that were significantly different from the normal menstrual cycle. The differential expression in the  $3\beta$ -HSD subtypes perhaps suggests that the objective of designing two Taqman probes that would differentiate between the types of  $3\beta$ -HSD was achieved. This would therefore also suggest that both subtypes are expressed at significantly elevated levels in the peri-menstrual phase of the cycle. In any event, the levels detected by both probes were elevated at the time point associated with the greatest incidence of breakthrough bleeding. This would add to the evidence that endometrial  $3\beta$ -HSD may be regulated by progesterone to some degree in endometrium. The increased levels of  $3\beta$ -HSD would suggest a tendency towards production of progesterone or androstenedione. There are no reports of aromatase activity or protein expression in levonorgestrel exposed endometrium and given the lack of expression of aromatase in normal endometrium, it would seem unlikely that androstenedione is converted to oestrone in levonorgestrel-treated endometrium. There are studies suggesting that progesterone and medroxyprogesterone acetate can stimulate aromatase activity in endometrial stromal cell cultures but no in vivo data exists (Tseng et al. 1986; Huang et al. 1989). Should aromatase be expressed in levonorgestrel-treated endometrium this may contribute to the hypothesis generated from the finding in the previous Chapter that increased expression of  $17\beta$ -HSD2 would favour higher intracellular levels of oestrone to oestradiol. Additionally the reduced expression of  $5\alpha$ -reductase type 2 would suggest that any precursors would not be likely to be converted to dihydrotestosterone, favouring the formation of oestrogens over androgens. These hypotheses would however require further

substantiation initially with the demonstration of aromatase in endometrium exposed to intra-uterine levonorgestrel, and subsequent clarification of 5 $\alpha$ -reductase protein expression in normal endometrium and endometrium exposed to intra-uterine levonorgestrel.

In summary, the secretory phase endometrium exhibits steroidogenic potential that is greater than the proliferative phase. More specifically, it would appear that the secretory phase glandular epithelium represents an 'intracrine unit' where the expression of key steroidogenic enzymes may play a role in the production of active progesterone or androgens from adrenal precursors. Published data do suggest that androgen production by secretory phase endometrium is significant (Rose et al. 1978; Hausknecht et al. 1982; Labrie et al. 1997). While the precise role of androgen production is unknown, in vitro data suggest that androgens play a role in the expression of factors important for the provision of a receptive endometrium that will provide a platform for successful implantation. Study of endometrial samples from women with the LNG-IUS revealed significant changes in the expression of 3 $\beta$ -HSD and 5 $\alpha$ -reductase type 2. At present, the extrapolation of these data to provide an understanding as to its relevance for BTB will require further study.

# Chapter 7

## General Discussion

## 7.1 General Discussion

To address the hypotheses in this study, it was necessary to provide further clarification with respect to the expression of the androgen receptor and sex steroid metabolising enzymes in human endometrium. The findings of this study provide further clarity in this under researched area and are an important addition to the published literature.

Endometrial tissues from patients with regular menstrual cycles were studied to determine the spatial and temporal expression of Androgen Receptor (AR) in endometrium. Using a monoclonal antibody and objective image analysis, AR was localised to endometrial stromal cells with minimal glandular immunoreactivity. A decrease in expression of AR was noted in the late secretory phase. This localisation and expression pattern is consistent with the localisation of AR mRNA using in-situ hybridisation (ISH). Furthermore, temporal analysis of endometrial tissue utilising quantitative real time RT-PCR (Q-RT-PCR) confirmed a down regulation of AR mRNA in the secretory phase.

It was also important to identify the spatial and temporal expression of sex steroid metabolising enzymes in endometrium. They will determine ligand availability for steroid receptor binding and the net steroid effect on endometrial tissue according to their pattern of expression. Immunohistochemical techniques and Q-RT-PCR were available to study 17 $\beta$ -hydroxysteroid dehydrogenase 2 (17 $\beta$ -HSD 2). 17 $\beta$ -HSD 2 was localised to secretory glandular epithelium with maximal expression in the mid secretory phase. 17 $\beta$ -HSD 2 mRNA revealed a parallel temporal expression to the 17 $\beta$ -HSD 2 protein in keeping with published literature. Q-RT-PCR also revealed significantly higher 17 $\beta$ -HSD 5 mRNA in mid secretory phase endometrium.

Additional sex steroid metabolising enzymes were also analysed using Q-RT-PCR. No methods for detecting protein from this group of enzymes were available at the time of this study. Temporal mRNA analysis demonstrated a significant perimenstrual increase in the expression of both 3 $\beta$ -HSD enzymes and 5 $\alpha$ R 2. 5 $\alpha$ R 1 was significantly increased in the late proliferative phase of the cycle. While evidence of protein expression was not directly available from this study, published

data exists supporting the expression of  $3\beta$ -HSD,  $17\beta$ -HSD2,  $17\beta$ -HSD5, and  $5\alpha$ -reductase types 1 and 2 in secretory glandular epithelium. This study describes this constellation of enzymes as an "intracrine unit". The expression of these enzymes as an intracrine unit in secretory endometrium provides the endometrium with autonomous control over the provision of sex steroid ligands.

Having established the pattern of expression of AR and the sex steroid metabolising enzymes it remained to establish their role in endometrial physiology and pathology. Failure of implantation and recurrent miscarriage are forms of endometrial dysfunction that have been noted in conditions that are associated with higher serum androgen levels. Where such endometrial dysfunction has been noted, genes associated with adverse reproductive outcome have been identified in endometrium and have been shown to be androgen regulated. It would therefore appear that where the ratio of sex steroids lead to a relative androgen excess, the endometrium is adversely affected. The role of locally expressed enzymes in women with a normal reproductive history may therefore be, as originally hypothesised, to provide a stable ratio of sex steroid ligands in endometrium.

Mid and late proliferative phase endometrium show little temporal variation in the expression of enzymes, suggesting there is a relative parity within enzyme expression. The secretory phase however exhibits temporal variation in the expression of sex steroid metabolising enzymes. High levels of serum oestradiol are responsible for the proliferative phase changes but in the secretory phase oestradiol does not exert such an influence. Data from this study and others suggests that progesterone receptor (PR) has a 'braking' effect on oestradiol action in endometrium preventing continued endometrial proliferation leading to endometrial hyperplasia. In the secretory phase, progesterone action is important for endometrial differentiation. Co-incident with the secretory phase elevation in serum progesterone levels there is a significant increase in the expression of  $17\beta$ -HSD 2. The local expression of this enzyme converts local oestradiol and testosterone to the less biologically active oestrone and androstenedione respectively while at the same time providing a route of conversion for the less active  $20\alpha$ -dihydroprogesterone to active progesterone. At this point in the secretory phase, the endocrine ratio favours relatively higher intracellular progesterone and lower intracellular oestradiol and testosterone levels. This effectively maximises the opportunity for progesterone to

play its role in secretory endometrial differentiation. This would also support the data available that suggests elevated androgen levels are associated with failure of implantation and recurrent miscarriage as the presence of 17 $\beta$ -HSD 2 favours a minimisation of the effect of androgens upon endometrial function at this time in the cycle.

To evaluate the role of progesterone in the regulation of endometrial AR, endometrium treated with the anti-progestogen mifepristone (RU486) was also studied. This revealed an up regulation of AR in the glandular and surface epithelial compartments of mifepristone treated secretory endometrium with additional stromal enhancement. This is in keeping with previous data showing oestrogen receptor (ER) and PR up regulation in the same tissue. As PR antagonism up regulates AR expression it may be that down regulation of AR in the late secretory phase is mediated by the elevated serum progesterone levels in the secretory phase. This suggests that AR may in part be regulated by progesterone but data regarding AR regulation is complex and as yet no firm conclusions can be reached.

Reduced late secretory phase AR expression may, however, be a reflection of the dilution in the stromal cell population secondary to the influx of uterine NK (uNK) cells in the late secretory phase. Further studies are required to assess the localisation of AR in uNK cells but it is known that uNK do not express ER $\alpha$  or PR.

The study of mifepristone treated endometrium also presented an opportunity to explain the mechanism of endometrial atrophy associated with mifepristone treatment. Elevated endometrial AR will provide further AR sites for the binding of androgenic ligands that would lead to an enhancement of the effect of androgens. As detailed in chapter 4.4, androgenic agents have been shown to induce endometrial atrophy and androgens have also been shown to have an anti-proliferative effect on the endometrium. Therefore, in mifepristone treated endometrium a direct effect of enhanced stromal AR activation may be an anti-proliferative effect, inducing endometrial atrophy.

Androgenic activity in secretory endometrium is also noted. Temporal variations in the perimenstrual late secretory and early proliferative phases reveal an up regulation of 3 $\beta$ -HSD and 5 $\alpha$ R 2. With both enzymes being expressed at the same time and both producing active androgens, this would provide a means for the local production of dihydrotestosterone (DHT), a more biologically active androgen

than testosterone. The proposed mechanism for this would be Dehydroepiandrosterone (DHEA) conversion to androstenediol by the action of  $17\beta$ -HSD5, up regulated in the mid secretory phase, followed by androstenediol conversion to testosterone by  $3\beta$ -HSD and finally testosterone conversion to DHT by  $5\alpha$ R 2. Secretory phase endometrium has previously been shown to possess androgen steroidogenic activity (Hausknecht et al. 1982). If such an intracrine mechanism for DHT production existed this may be another mechanism to account for the late secretory phase AR down regulation. That is to say, if AR regulation is similar to that of the other sex steroids and it is down regulated by its own ligand then DHT may be responsible for such down regulation. At present, such statements require substantiation with further study and the physiological role of AR and the sex steroid metabolising enzymes remains to be elucidated.

The additional patient groups studied were women using the levonorgestrel releasing intra-uterine system (LNG-IUS). Patients using such methods of contraception may suffer problematic breakthrough bleeding necessitating discontinuation of this treatment. In endometrium exposed to high intra-uterine concentrations of levonorgestrel via the LNG-IUS, AR was significantly down regulated whereas  $17\beta$ -HSD 2 was elevated. Since  $17\beta$ -HSD2 converts oestradiol to the less potent oestrogen, oestrone, the endometrium would have a relatively low intracellular concentration of oestradiol. In such a scenario, oestradiol dependent products of the glands, that may have paracrine actions throughout the endometrium, would thus be suppressed or perturbed at this time. Oestrogen deficiency may therefore be implicated in the mechanism of breakthrough bleeding.

A variation in the expression of some of the other sex steroid metabolising enzymes is also noted, increased expression of  $3\beta$ -HSD and reduced expression of  $5\alpha$ R 2. Neither would be expected to impact upon the intracellular oestradiol concentration. However, if as hypothesised, the ratio of sex steroids is important in maintaining an endometrial equilibrium the altered expression in both would serve to further the imbalance in this ratio with an androgen excess. The mechanism whereby such alterations induce breakthrough bleeding will require further study.

In conclusion, clarification of endometrial AR expression has been an addition to the published literature. Important data regarding the expression of sex steroid metabolising enzymes in human endometrium have also been detailed. The findings of this study will provide a basis to take forward further research to establish what role AR and the androgen steroidogenic activity of secretory endometrium plays in endometrial physiology. Novel data are also presented for the temporal variation in expression of AR and sex steroid metabolising enzymes in endometrium exposed to high dose intra-uterine levonorgestrel. These data also provide an important platform to explore further mechanisms and treatments for the clinical problem of breakthrough bleeding.

## 7.2 Suggestions for Future Study

Further research is required with respect to the expression and regulation of androgen receptor (AR) and sex steroid metabolising enzymes in human endometrial tissues. The purpose of future studies would initially be to provide corroboration of the data presented in this thesis. With corroboration, future study designs could be taken forward to analyse the regulation of AR and sex steroid metabolising enzymes in normal human endometrium. However, in addition to examining such factors in normal endometrium it will be important to study their expression and regulation in dysfunctional endometrium. Dysfunctional endometrium should include endometrium from patients with endometriosis, polycystic ovarian disease, recurrent miscarriage, endometrial hyperplasia and endometrial carcinoma.

Initially I would suggest descriptive studies to provide corroborative data for the expression of the mRNA of sex steroid metabolising enzymes using molecular techniques such as northern blotting or in-situ hybridisation of normal endometrium. There are published data available regarding the expression of several of the sex steroid metabolising enzyme proteins in endometrium. However, there is still a need for further studies to clarify the expression of other key sex steroid metabolising enzymes in normal endometrium at the protein level, as thus far only mRNA data are available. Such studies have been hampered by the lack of available antibodies. Locally available techniques to study 5-alpha reductase ( $5\alpha R$ ) with an enzyme activity assay and 3-beta hydroxysteroid dehydrogenase ( $3\beta$ -HSD) immunolocalisation were available. We did not have the required volume of tissue that was required for the  $5\alpha R$  assay and there was insufficient time within this study to perform the required immunolocalisation experiments for  $3\beta$ -HSD. These are initial experiments that may be performed to establish further data regarding protein expression in endometrium.

Regulation of AR expression in endometrium also requires further study to provide clarity. Initially, it will need to be determined if the significant reduction in late secretory AR is a true down regulation or a dilution effect of secretory phase uterine NK (uNK) cell influx. I would suggest that AR and CD56 co-localisation experiments are performed to determine if the uNK cells expressed AR because

should they not express AR this would remain a possible explanation for the late secretory reduction.

To determine the regulation of AR and sex steroid metabolising enzymes in endometrium I feel a combined in-vitro/in-vivo approach would provide important data. In-vitro endometrial explant culture would allow treatment of proliferative and secretory endometrium with varying doses of sex steroids to assess the dose response of AR and enzyme mRNA in the explant cultures. This would provide some insight into the regulation of these factors. To assess the complex endocrine environment of the endometrium during the menstrual cycle in-vivo analysis would provide the closest approximation to the mechanism of regulation.

Recent data by Slayden et al. has shown that AR blockade with Flutamide, a pharmacological agent that is metabolised to the AR inhibitor hydroxyflutamide, will prevent the atrophic endometrial changes associated with antiprogestin treatment in rhesus macaques (Slayden et al. 2003). I would propose the recruitment of patients with regular menstrual cycles to undergo timed biopsies in the proliferative phase, early secretory, mid secretory and/or late secretory phase of a pre-treatment and treatment cycle, where the treatment is daily flutamide. Samples would then be analysed with optimised immunohistochemical protocols and Q-RT-PCR. This would provide data regarding the effect of AR inhibition on AR, ER, PR, and enzyme expression at the protein and mRNA levels.

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## Appendix I: Source of General Materials

Tissue Collection	Supplier
Neutral Buffered Formalin (NBF)	See Appendix: Recipes
Paraformaldehyde (PFA)	See Appendix: Recipes
Pipelle Suction Curette	Laboratoire CCD, Paris, France.
TP1050 Tissue Processor	Leica Corp., UK.

RNA Extraction	Supplier
Trizol Reagent	Invitrogen Life Tech, Paisley, UK.
Hand Held Homogeniser (Polytron PT 1200B)	Kinematica, Switzerland.
Choloroform	Sigma, Poole, Dorset, UK.
Isopropanol	Sigma, Poole, Dorset, UK.
Ethanol (Molecular Biology Grade)	BDH Laboratory Supplies, Poole, UK.
RNA Storage Solution	Ambion Inc, Austin, Texas, USA.
Phase Lock Gel Tubes	Eppendorf, Hamburg, Germany.

Real Time Q-RT-PCR	Supplier
DNase Amplification Kit	Invitrogen Life Tech, Paisley, UK.
Taqman Reverse Transcription Reagents	AB Applied Biosystems, Cheshire, UK.
Taqman MasterMix	Stratagene, Amsterdam, Netherlands.
Ribosomal RNA Control Reagents	AB Applied Biosystems, Cheshire, UK..
Primers / Probe Sets for Sequences	Biosource, Nivelles, Belgium
ABI Prism 7700	AB Applied Biosystems

<b>Immunohistochemistry</b>	<b>Supplier</b>
Histoclear	Nat. Diagnostics, Atlanta, Georgia, USA.
Phosphate Buffered Saline (PBS)	See Appendix 2
PBS + Tween 20	See Appendix 2
Tris Buffered Saline (TBS)	See Appendix 2
TBS + Tween 20	See Appendix 2
Hydrogen Peroxide Solution 30%	BDH Laboratory Supplies, Poole, UK.
Sodium Citrate	See Appendix 2
Pressure Cooker	Clipso, Tefal, Nottingham, UK.
Avidin/Biotin Blocking Kit	Vector Laboratories, Peterborough, UK.
Non Immune Horse Serum (Vectastain)	Vector Laboratories, Peterborough, UK.
Bovine Serum Albumin	Sigma, Poole, Dorset, UK.
Biotinylated Horse anti-Mouse Antibody	Vector Laboratories, Peterborough, UK.
Avidin Biotin Peroxidase Complex	Vector Laboratories, Peterborough, UK.
3,3' Diaminobenzidine	Dako, Cambridge, UK.
Harris Haematoxylin	Pioneer Research Chemicals, Colchester, UK.
Xylene	BDH Laboratory Supplies, Poole, UK.
Pertex	Cellpath Plc, Hemel Hempsted, UK.
Image Analysis System	Improvision inc., Lexington, MA, USA.

## Appendix II: Recipes for Solutions

<b>4% Paraformaldehyde(PFA)</b>		
<b>Weight/Volume</b>	<b>Chemical Name</b>	<b>Supplier</b>
4g	Paraformaldehyde	Sigma
100ml	PBS (see below)	Sigma
Heat to 60°C		
Store at 4°C and use within 7 days		

<b>4% Neutral Buffered Formalin(NBF)</b>		
<b>Weight/Volume</b>	<b>Chemical Name</b>	<b>Supplier</b>
6.5g	Na <sub>2</sub> HPO <sub>4</sub>	BDH
4.5g	Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	BDH
100ml	40% formaldehyde	BDH
900ml	Distilled Water	

<b>0.01M Phosphate Buffered Saline (PBS) pH 7.4</b>		
<b>Weight/Volume</b>	<b>Chemical Name</b>	<b>Supplier</b>
5 Tablets	PBS	Sigma
1000ml	Distilled Water	

<b>0.01M Phosphate Buffered Saline +Tween (PBST) pH 7.4</b>		
<b>Weight/Volume</b>	<b>Chemical Name</b>	<b>Supplier</b>
5 Tablets	PBS	Sigma
1000ml	Distilled Water	
8g	NaCl	BDH
100µl	Tween 20	Sigma

### 0.5M Tris Buffered Saline (TBS)

Weight/Volume	Chemical Name	Supplier
60.55g	Trizma Base	Sigma
700ml	Distilled Water	

pH to 7.4 and make up to 1000ml with Distilled Water

Dilute 1:10 for working 0.05 Solution and add 8.5g NaCl/litre

### 0.5M Tris Buffered Saline+ Tween 20(TBST)

Weight/Volume	Chemical Name	Supplier
60.55g	Trizma Base	Sigma
700ml	Distilled Water	

pH to 7.4 and make up to 1000ml with Distilled Water

Dilute 1:10 for working 0.05M Solution and add 8.5g NaCl/litre and add 100 µL Tween 20

### 0.1M Sodium Citrate

Weight/Volume	Chemical Name	Supplier
29.41g	Tri-Sodium Citrate	BDH
0.1g	Sodium Azide	Sigma
700ml	Distilled Water	

pH to 6.0 and make up to 1000ml with Distilled Water

Dilute 1:10 for working 0.01M Solution

### Appendix III: Conference Proceedings

1. **Burton KA**, Hillier SG, Habib FK, Mason JJ, Critchley HOD. Spatial and temporal distribution of the androgen receptor in human endometrium. "Fertility 2000" Joint Meeting of the British Fertility Society, Society for the Study of Fertility; and British Andrology Society. August 2000. Edinburgh. **Oral Presentation.** Journal of Reproduction and Fertility: Abstract series 25; Abstract no 113.
2. **Burton KA**, Hillier SG, Habib FK, Mason JJ, Critchley HOD. Localisation of 17 beta-hydroxysteroid dehydrogenase type II (17beta-HSD II) in human endometrium across the menstrual cycle. **Oral Presentation.** Munro-Kerr Society winter meeting. January 2001. Glasgow Royal Infirmary. **1<sup>st</sup> Prize for Best Presentation.**
3. **Burton KA**, Hillier SG, Habib FK, Mason JJ, Critchley HOD. Localisation of 17 beta-hydroxysteroid dehydrogenase type II (17beta-HSD II) in human endometrium across the menstrual cycle. **Oral Presentation.** British Fertility Society. April 2001. Belfast. Human Fertility
4. **Burton KA**, Hillier SG, Habib FK, Mason JJ, Critchley HOD. Modulation of Androgen Receptor and 17 beta-hydroxysteroid dehydrogenase 2 in endometrium treated with the Levonorgestrel releasing intrauterine system. **Poster Presentation.** Blair Bell Society Winter Meeting. London, December 2001