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A role for oestrogen in dynamic interactions between cell types within the human endometrium

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Declaration

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

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Abstract

The human endometrium is a complex multicellular tissue, located within the cavity of the uterus. Its luminal surface is defined by a layer of epithelial cells supported on a multicellular stroma containing fibroblasts, glands (lined by a secretory epithelium), blood vessels (lined with endothelial cells) and several populations of immune cells; the latter includes a unique population of natural killer (uNK) cells. The endometrium undergoes dynamic remodelling across the menstrual cycle in response to fluctuating levels of sex steroids secreted by ovarian cells. The phases of the endometrial cycle include an oestrogen-dominated proliferative phase, a progesterone-dominated secretory phase and menses (endometrial shedding precipitated by falling levels of progesterone). A key feature of the secretory phase is differentiation (decidualisation) of endometrial stromal fibroblasts (ESC) an event characterised by transformation of cell shape, secretion of growth factors/cytokines, angiogenesis/vascular remodelling and an increase in the numbers of resident immune cells. Decidualisation ensures an appropriate nutritional and hormonal environment exists during the establishment of pregnancy. Studies in mice suggest that *de novo* biosynthesis of oestrogen within the uterus may play an essential role in regulation of decidualisation but no data exist for human. Endometrial endothelial and uNK cells both contain oestrogen receptors but the impact of oestrogens on their function has not been explored. In the current studies three questions have been addressed:

1. Is oestrogen biosynthesis a feature of human endometrial stromal cell decidualisation?

2. What is the impact of oestrogen on uNK cell function?

3. What role (if any) does oestrogen play in the interplay between decidual, immune and vascular cells within the human endometrial stroma?

Results obtained provide the first evidence that *de novo* biosynthesis of oestrogens occurs during decidualisation of human ESC. This was attributed to changes in expression patterns of mRNAs encoding proteins that play a critical role in regulation of oestrogen biosynthesis (STAR, CYP11A1, CYP19A1 [aromatase], HSD17B2 [17βHSD2] and STS [steroid sulphatase]). Changes in the pattern of metabolism were confirmed using thin layer chromatography and analysis of concentrations of oestrone (E1) and oestradiol (E2) in culture media. Secretion of E1 and E2 was reduced by addition of an aromatase inhibitor. Data derived from studies described within this thesis also show for the first time that incubation of uNK cells with E2 not only enhanced cell migration but also stimulated secretion of factors that had a significant impact on endothelial cell angiogenesis. These

findings were supported by novel evidence that E2 had a significant impact on expression of genes associated with cell motility and angiogenesis. In addition, factors, including E1/E2, secreted by decidualised stromal cells, stimulated chemotaxis of uNK cells. Future experiments will focus on determining the identity of the angiogenic factors secreted by uNK cells in response to E2 and the mechanisms responsible for uNK cell movement.

In summary, new data presented in this thesis provide evidence that local biosynthesis of oestrogens within the endometrial stroma may play a previously unrecognised role in regulating the function of uNK cells and endometrial endothelial cells in women. These results have implications for treatment of disorders such as infertility, heavy menstrual bleeding and endometriosis.

Presentations relating to this thesis

Endometrial stromal cell decidualisation is associated with generation of an altered steroid microenvironment during the fertile phase of the cycle. Poster presented at Centre for Reproductive Health Research day, Edinburgh, November 2011.

Stromal Decidualization in Women Results in Local Estrogen Biosynthesis. Poster presented at Society for Gynecologic Investigation (SGI) 58th Annual Scientific Meeting, Miami, Florida, USA, March 2011.

The steroid micro-environment generated by stromal decidualisation. Poster presented at International Congress on Hormonal Steroids and Hormones and Cancer (ICHSHC), Edinburgh, UK, September 2010.

A role for local estrogen biosynthesis in stromal decidualisation. Poster presented at SGI 57th Annual Scientific Meeting, Orlando, Florida, USA, March 2010.

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Abbreviations

A4	Androstenedione
ADCC	Antibody-Dependent Cell-Mediated Cytotoxicity
AF1	Activation function 1
AF2	Activation function 2
Ang	Angiopoietin
AP-1	Activator protein 1
APCs	Antigen presenting cell
AR	Androgen receptor
cAMP	cyclic adenosine monophosphate
CD*n*	Cluster of differentiation e.g. CD56
ChIP	Chromatin immunoprecipitation
CM	Conditioned media
COUPTFII	chicken ovalbumin upstream promoter transcription factor II
Ctrl	Control
CXC	chemokine containing two cysteines (C) separated by a single amino acid (X)
DAB	3,3'-Diaminobenzidine
DAPI	4',6-diamidino-2-phenylindole
DARS	Donkey anti rabbit serum
DBD	DNA binding domain
DCs	Dendritic cells
DEC	Decidualised
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E2	17β oestradiol
E1	Oestrone
E1S	Oestrone sulphate
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme linked immunosorbent assay
ER	Oestrogen receptor
ERE	Oestrogen response element
ESC	Endometrial stromal cells
EST	Oestrone sulfotransferase
FAB	Fragment antigen binding
FACS	Fluorescence associated cell sorting
FC	Fold change

FITC	Fluorescein isothiocyanate
FSC	Forward scatter
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GR	Glucocorticoid receptor
HEEC	Human endometrial endothelial cells
hESC	Human Endometrial stromal cells
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigen
HMB	Heavy menstrual bleeding
HOXA10	Homeobox protein Hox-A10
HRP	Horseradish peroxidase
HSD	Hydroxysteroid dehydrogenase
HUVEC	Human umbilical vein endothelial cells
ICI	ICI-182,780, Fulvestrant (Faslodex)
IFN	Interferon
IGFBP	Insulin-like growth factor-binding protein
IL-*n*	Interleukin e.g. IL-15
ITAMs	Immunoreceptor tyrosine–based activation motifs
Ki67	Antigen KI-67
KIRs	Killer-cell immunoglobulin-like receptors
KO	Knockout
LBD	Ligand binding domain
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
MACS	Magnetic-activated cell sorting
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinases
NAD	Nicotinamide adenine dinucleotide
NBF	Neutral buffered formalin
NCRs	Natural cytotoxicity receptors
NK	Natural Killer
NKG	Natural-killer group
OVX	Ovariectomy
P4	Progesterone
Pb	Peripheral blood e.g. pbNK
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PGBS	Gelatin phosphate buffered saline
PGE2	Prostaglandin E2

PKA	Protein kinase A
PLGF	Placental growth factor
PR	Progesterone receptor
PRE	Progesterone response element
PRL	Prolactin
RNA	Ribonucleic acid
RPL	Recurrent pregnancy loss
SDF-1	Stromal-derived factor 1
SERM	Selective oestrogen receptor modulator
SHR	Steroid hormone receptor
Sp1	Specificity protein-1
SSC	Side scatter
StAR	Steroidogenic acute regulatory protein
STS	Steroid sulfatase
T	Testosterone
TBS	Tris-buffered saline
TERT	Telomerase reverse transcriptase
TGF	Transforming growth factor
TLC	Thin layer chromatography
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF	Tumour necrosis factor e.g. TNF-α
TSA	Tyramide signal amplification
uNK	Uterine natural killer cell
VEGF	Vascular endothelial growth factor

Chapter 1

1. Literature review

1.1. The endometrium and the menstrual cycle

The human endometrium is a complex multi-cellular organ that undergoes cyclical remodelling in response to fluctuating levels of sex steroid hormones produced by the ovaries (1). The endometrium lines the uterine cavity and is surrounded by an outer layer of smooth muscle known as the myometrium. The endometrium has epithelial cells on its luminal surface with a multi-cellular stroma containing fibroblasts, blood vessels (lined with endothelial cells), a dynamic immune cell population (including uNK cells and macrophages) as well as glands bounded by a secretory epithelium (2). The endometrium is composed of two layers; the basalis or basal layer which is adjacent to the myometrium and undergoes little obvious structural remodelling in response to hormones across the menstrual cycle, and a layer which includes the luminal epithelium known as the functionalis or functional layer which undergoes cyclical regeneration, proliferation and differentiation in response to fluctuations in ovarian hormones. Regulation of the endometrium by steroid hormones was investigated using primates in a seminal study reported by Good and Moyer in 1968 (3). In this study the investigators varied the ratios of oestrogen (E) and progesterone (P) in ovariectomised rhesus monkeys. They discovered that glandular maturation was favoured by E and stromal maturation modulated by P being favoured by ratios of P>E. Furthermore, glandular maturation was antagonized by high levels of P, highlighting the importance of controlled, balanced hormone levels in mediating both maturation and remodelling of the endometrium during a normal cycle. These findings correlate with the morphological changes and fluctuations in hormone levels observed in the endometrium of women. During their reproductive years hormones circulating in the blood of women are derived predominantly from the ovaries and adrenals (4). Whilst adrenal hormones are relatively constant the growth of ovarian follicles and formation of the corpus luteum after ovulation results in fluctuations in the concentrations of hormones across the menstrual cycle (Figure 1.1).

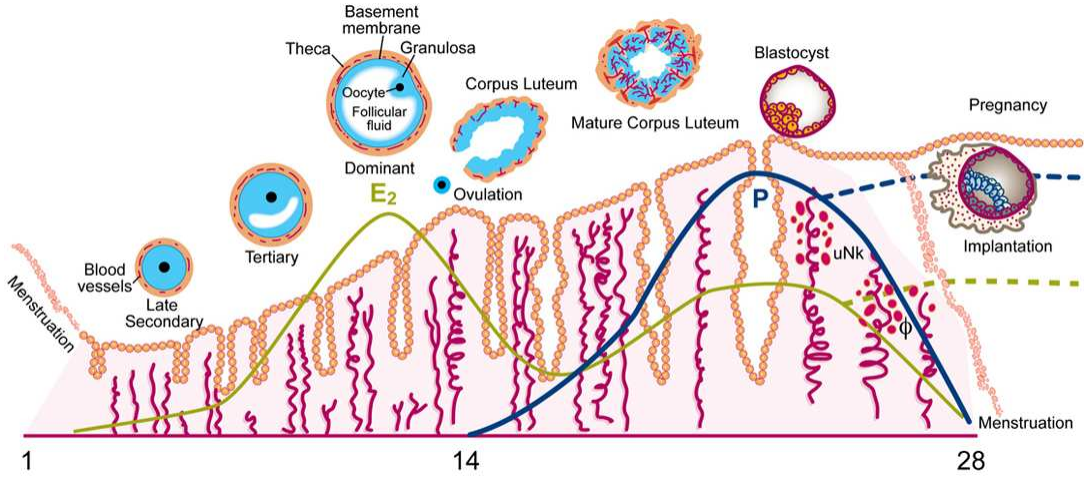

Figure 1.1. Hormone levels through the menstrual cycle. In response to FSH, the dominant follicle enlarges concomitant with increased secretion of oestrogen. Following ovulation (day 14), oestrogen levels rapidly decline and circulating concentrations of progesterone increase secreted by the newly formed corpus luteum. In the absence of pregnancy, the corpus luteum regresses leading to withdrawal of ovarian hormones resulting in the onset of menstruation.

1.1.1. Changes in hormones levels during the menstrual cycle

The secretion of hormones by the ovary is controlled by endocrine feedback loops mediated by the hypothalamic-pituitary-ovarian axis. The hypothalamus is located in the base of the brain and hypothalamic neurons secrete gonadotropin releasing hormone (GnRH) which stimulate gonadotropes in the nearby anterior pituitary to synthesize and secrete follicle stimulating hormone (FSH) and luteinizing hormone (LH) into the circulation. These gonadotropins act via receptors expressed by cells in different ovarian compartments and stimulate steroidogenesis. At menses, a number of primordial follicles begin to enlarge in the ovary, an event stimulated by FSH. As follicles mature granulosa cells adjacent to the wall of the follicle begin to express the enzyme aromatase and these cells therefore develop the capacity to metabolise androgens secreted by adjacent theca cells into oestrogens. Oestrogen increases responsiveness to FSH, eventually leading to the progression of a single, dominant follicle at the expense of the other follicles with the latter becoming atretic. Enlargement of the dominant follicle continues throughout the follicular phase (Figure 1.1.) concomitant with increased secretion of oestrogen. This rise in circulating oestrogen stimulates secretion of LH by the pituitary gonadotropes resulting in a rapid rise in the concentrations in the circulation (pre-ovulatory stage.) During the follicular phase the functional layer of the endometrium regenerates and cells rapidly proliferate in response to rising levels of oestrogen, this is particularly striking in the epithelial cells lining the glands. This phase of

endometrial function is known as the proliferative phase (Figure 1.2.) Circulating levels of oestrogen rise from around 50pg/ml to around 200-300 pg/ml prior to ovulation (5). Oestrogen also induces expression of the progesterone receptor in stromal cells during this stage of the cycle (6-8). Mid-cycle (approximately day 14), the LH surge triggers rupture of the dominant follicle, releasing the ovum and precipitating a rapid drop in ovarian oestrogen (50-100 pg/ml), accompanied by differentiation of granulosa cells resulting in formation of the corpus luteum (see Figure 1.1.). Cells within the corpus luteum synthesize progesterone resulting in a rise in the circulating concentration of progesterone (~8 ng/ml). This, together with biosynthesis of regulatory agents that enhance cyclic AMP (cAMP) concentrations results in profound changes in endometrial architecture including differentiation of stromal fibroblasts and remodelling of the vasculature both of which are essential prerequisites for formation of a functional placenta following implantation of a conceptus (9). The progesterone dominated luteal phase is usually referred to as the secretory phase of the menstrual cycle.

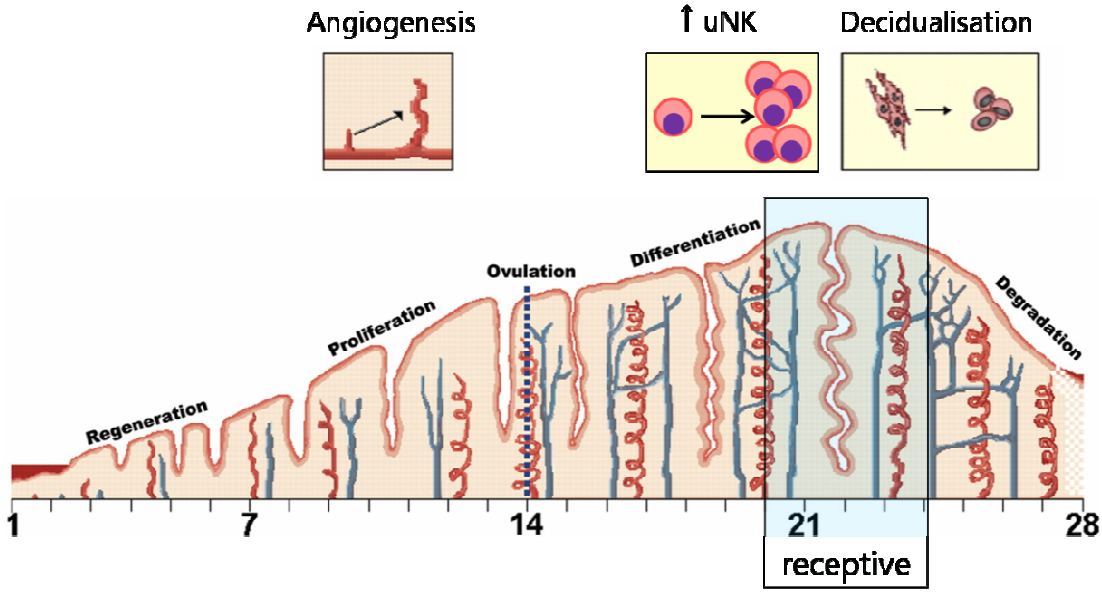

Figure 1.2. Schematic of the morphological changes that take place in the endometrium during the menstrual cycle. The proliferative phase is characterised by growth of the mucosal layer through stromal and epithelial proliferation and angiogenesis. During the receptive phase spiral arterioles become prominent and the first pre-decidual changes take place in peri-vascular stromal cells. Pre-decidual cells are first evident around day 22 and form a solid sheet of decidual cells by day 27. Leukocyte numbers increase during the secretory phase; uNK increases are first evident on day 17. Adapted from (10).

1.2. Histological features of endometrial tissue during the menstrual cycle

Changes in the appearance of the tissue during cyclical remodelling of the human endometrium were detailed in a classic study by Noyes and colleagues who utilised 300 endometrial biopsies that they examined histologically to provide a set of definitive criteria allowing dating endometrial biopsy samples (11); (Figure 1.2.) The descriptions of the endometrium in the following sections are based on those published by Noyes et al (11) and are aligned to an archetypal 28 day cycle (see Figure 1.2.) which begins on day 0 with menstrual shedding, followed by regeneration and then growth during the proliferative phase. Transition from the proliferative to secretory phase following ovulation occurs at day 14 (also LH day 0) and culminates in the formation of decidual cells in the late secretory phase leading to the onset of menses by day 28 (LH+14) in the absence of pregnancy.

1.2.1. The proliferative phase

The proliferative phase of menstrual cycle involves regeneration and growth of the endometrium is responsive to rising concentrations of ovarian-derived oestrogen. On days four to seven of the cycle, the tissue is characterised by a thin luminal epithelium and straight, short, narrow glands, with some glands displaying hallmarks of 'secretory exhaustion' denoted by cuboidal, 'ragged', inactive epithelium as a result of hormone withdrawal. Beneath the luminal epithelium the underlying stroma is compact and the fibroblasts appear to have relatively large nuclei and minimal cytoplasm. By the mid-proliferative phase, days eight to ten, the tissue has a tall columnar epithelium, elongated, curving glands and a less compact stromal compartment due to water imbibition (oedema). The late proliferative phase lasting from days eleven to fourteen and is characterised by an undulating luminal epithelium, and tortuous glands with pseudostratified epithelium. The stroma underlying the surface epithelium is dense with little evidence of oedema. Throughout the proliferative phase, glandular and stromal mitoses are progressively and increasingly prominent consistent with proliferation in these cellular compartments in response to high levels of ovarian oestrogen. Glandular diameter is significantly and positively correlated to peripheral oestradiol levels at this time (5). The epithelial nuclei also become increasingly pseudostratified which culminates in the late proliferative phase and declines once active secretion begins following ovulation. Oestrogen receptors (ER), notably ERα, are required for the uterine proliferative response to oestrogen in mice (12). Specifically, analysis of knockout mice suggests that oestrogen regulates stromal-epithelial interactions via an ERα-dependent signal originating from the stromal compartment. In support of this, Winuthayanon et al reported that in a uterine epithelial cell-specific ERα knock out mouse uterine proliferation was normal (13).

1.2.2. The secretory phase

During the secretory phase, characteristic, progressive changes in endometrial function occur that are distinct from those of proliferative endometrium and are consistent with active secretion by the glandular epithelium and differentiation of the stromal compartment (decidualisation, see Section 1.4.4) both of which occur in an environment of rising progesterone. During the secretory phase the glandular epithelium becomes highly secretory releasing factors such as glycogen into the uterine lumen which support blastocyst survival in the build up to implantation. The function of the luminal epithelium is altered so that it becomes 'receptive' to the blastocyst during the implantation window.

At day fourteen of the cycle, a surge in release of luteinising hormone by cells in the anterior pituitary gland triggers ovulation, leading to a drop in ovarian oestrogen and a rise in progesterone secreted from the newly formed corpus luteum (see Figure 1.2). While no appreciable histological changes occur in the endometrium immediately following ovulation, by day sixteen sub-nuclear vacuolation is prominent in the cells of the glandular epithelium. The number of cells with basal vacuoles has been shown to be negatively correlated to oestrogen levels but positively correlated to progesterone (5). Vacuolation initially has the effect of exaggerating the pseudostratified appearance of the epithelial nuclei, however, by the seventeenth day (LH+3), following increases in the diameter and tortuosity of glands, the pseudostratified appearance abates with nuclei being centrally aligned with vacuoles positioned basally and cytoplasm apically. By the eighteenth day (LH+4), vacuoles have decreased in size, as glycogen is secreted into the lumen and as a result the nuclei return to a more basal position; by the nineteenth day few vacuoles remain. In the absence of pregnancy, glandular secretion diminishes, and secretory 'exhaustion' is apparent by day twenty-four characterised by 'ragged' epithelium with shrunken nuclei.

The mid-secretory phase, is characterised by changes that occur in the stroma, beginning with a marked increase in stromal oedema on day twenty-one (LH+7) which is maximal by day twenty-two (LH+8). Stromal cells appear denser at this time, characterised by relatively large nuclei and little cytoplasm. The spiral arterioles become prominent on the twenty-third day (LH+9) due to the appearance of 'pre-decidual' cells characterised by enlarged nuclei and increased cytoplasm in the peri-vascular area which signifies the beginning of stromal differentiation. Stromal mitoses are evident as proliferation recurs and pre-decidual cells accumulate around arterioles. Stromal differentiation continues with the formation of sub-epithelial pre-decidua on day twenty-five (LH+11) which forms a sheet of well-developed decidual-like cells by day twenty-seven (LH+13). In addition, the differentiation of stromal cells is accompanied by an increase in the number of leukocytes present in the endometrium (see section 1.3) which is maximal by the late-secretory phase and persists in decidua in fertile cycles. Decidual cells persist in pregnancy and form the maternal component of the placenta, the decidua basalis (14). In the absence of pregnancy and consequent demise of the corpus luteum, the decidual endometrium regresses and is shed during menstruation (1, 15).

In addition to remodelling of the endometrial stroma during the secretory phase, changes in the luminal epithelium also occur which facilitate implantation in the presence of blastocyst. This includes epithelial expression of leukaemia inhibitory factor (LIF) and regression of

pinopodes at implantation sites (16, 17). These functional changes are detailed in section 1.4.5.

1.3. Leukocyte populations in the endometrium

1.3.1. Overview

Leukocytes are important in host defence against pathogens and can be found in blood, the lymphatics and in tissues and organs. In pregnancy the maternal uterine mucosa (decidua) is the primary site of contact between semi-allogenic foetal cells and immune-competent maternal cells. The production of cytokines by decidual cells and the signalling environment of the stroma affects the activity of endometrial leukocytes. A variety of immune cells are present in high concentrations in the uterus which through the action of steroids (section 1.5) and cytokines (section 1.7) control the sophisticated immunological regulation of implantation and pregnancy.

CD45 (leukocyte common antigen) positive cells are interspersed in the stroma of the endometrium and increase from 10-15% in the proliferative phase to 20-25% of all endometrial cells in the late secretory phase (18). The major leukocyte population in the endometrium is the uterine natural killer cell (uNK), however significant numbers of macrophages, T cells, B cells, dendritic cells and neutrophils constitute the milieu of immunocompetent cells in the endometrium. In the proliferative phase lymphoid aggregates of T and B cells are detected and *in vitro* CD3+ T cells isolated from proliferative phase endometrium are reported to have cytolytic activity (19). Following the transition from the proliferative to the secretory phase the numbers of uNK cells and macrophages rise. In the decidua of early pregnancy uNK cells are the predominant population, however, there are also increased numbers of T cells, as well as dendritic cells which increase to account for 20% of decidual leukocytes (20); (see Table 1.1).

Cell type	Endometrium		Decidua early pregnancy
	Proliferative phase	Secretory phase	
Lymphocytes			
uNK cells	+	+++	+++++
T cells	+	+	++
B cells	−/+	−/+	−/+
APC			
Macrophages	+	++	+
immature DC	+	+	+++
mature DC	−/+	+	−/+

Table 1.1. Relative frequency of immunocompetent cells in uterine mucosa. Adapted from (20), APC= antigen presenting cell.

1.3.2. Natural killer cells

Natural Killer (NK) cells are lymphocytes that are part of the innate immune system and comprise 15% of all circulating lymphocytes (21). NK cells are effectors of antibody dependent cellular cytotoxicity (ADCC) mediated via CD16 and they do not express CD3 (21). The peripheral blood (pb) contains two main subsets of NK cell, delineated by their expression of two markers; CD56 and CD16. The CD56 antigen is an isoform of the human neural cell adhesion molecule which may mediate interactions between NK cells and their targets (22). CD16 is the low affinity Fcγ receptor III (FcγRIII) expressed on the surface of some NK cell subsets. CD16 mediates cytotoxicity and binds to opsonised (antibody coated) targets and signals through immunoreceptor tyrosine-based activation motifs (ITAMS) to direct ADCC (23).

The two pbNK subsets are characterised as $CD56^{bright}$ $CD16^{-}$ ($CD56^{bright}$) and $CD56^{dim}$ $CD16^{+}$ ($CD56^{dim}$) which are also classified as agranular and granular respectively. $CD56^{dim}$ NK cells constitute around 90% of pbNK cells while $CD56^{bright}$ are the minority subset only consisting around 10% of total pbNK and less than 1% of all lymphocytes (24). A phenotypically distinct population of NK cells has been identified as resident in human endometrium (25). Uterine NK (uNK) cells are found in the endometrium but not the myometrium and appear similar to the minority $CD56^{bright}$ pbNK population as they are $CD56^{bright}/CD16^{-}$ (26) (Figure 1.3). However unlike $CD56^{bright}$ pbNK, the uNK cells are granular and like the majority pbNK population contain Granzyme B and perforin (26). In agreement with differences in phenotype between uNK and pbNK transcriptional analysis

(27) has identified differences between the cell populations. Furthermore, functional capacity of uNK also appears to be distinct from pbNK, with uNK tending to have reduced cytotoxicity and increased cytokine secretion compared to pbNK subsets.

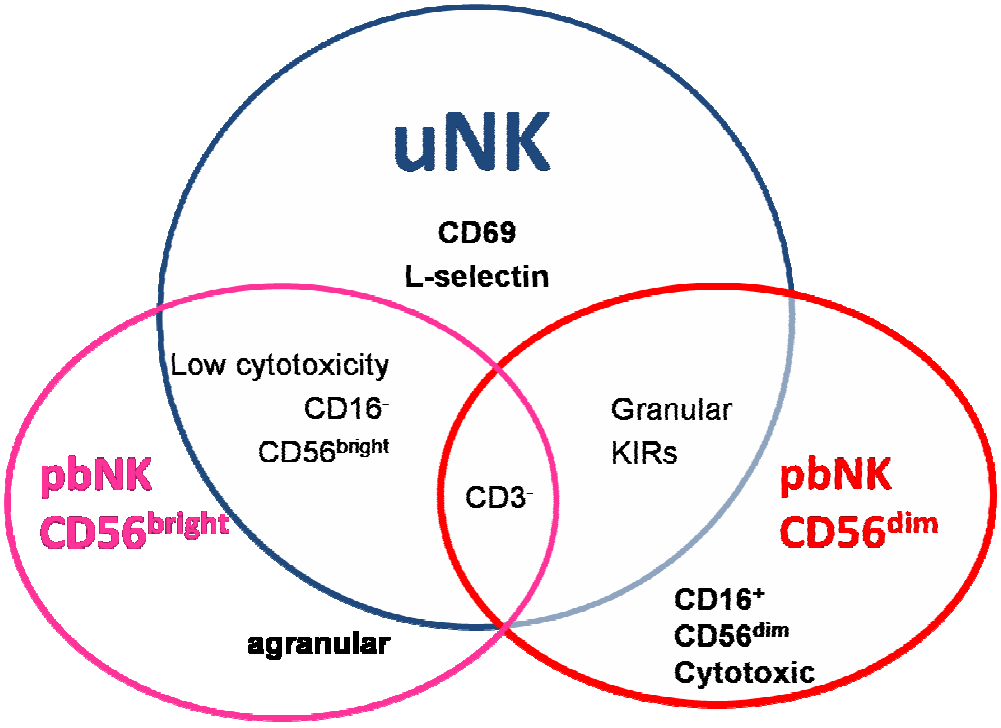

Figure 1.3. Venn diagram summarising the characteristics of NK subsets. Notably, uNK are phenotypically distinct from either population of pbNK although they appear to share some features of both types.

Activation of cytotoxicity in NK cells is thought to be a balance between activating and inhibitory signalling. NK cells express both activating and inhibitory receptors; of which three major superfamilies are expressed. Killer cell Ig-like receptors (KIR) which recognize human leukocyte antigen (HLA)-A, -B and -C are highly expressed in $CD56^{dim}$ pbNK and uNK but there is low expression in $CD56^{bright}$ pbNK (24, 28). CD94 and NKG2 receptors are members of the C-type lectin superfamily and are expressed abundantly in uNK and $CD56^{bright}$ pbNKs but not $CD56^{dim}$ pbNK (24). Finally, natural cytotoxicity receptors (NCRs) are activating receptors expressed in all NK subsets. NCRs transduce intracellular signals that lead to the induction of cytoxicity. Expression of activating receptors; NKp30, NKp44, NKp46, 2B4, and NKG2D have been reported in both uNK and pbNK cells (24, 28). Interestingly, in uNK cells inhibition of cytoxicity can be mediated by the co-receptor 2B4

which is inhibitory in uNK cells due to the low expression of SAP (signalling lymphocyte activation molecule (SLAM)–associated protein) which controls cytotoxicity (29).

1.3.2.1. Origin of uNK population

While $CD56^{bright}$ cells in the peripheral blood are a minority population, uNK cells are present in high numbers in the endometrium and are the major leukocyte population in early pregnancy, constituting 70% of all leukocytes in first trimester decidua (30). The origin of the uNK cell population remains unresolved although it is likely a combination of trafficking from peripheral blood and *in situ* proliferation/differentiation of resident uNK cells/precursors which accounts for changes in tissue resident populations. In support of the idea that uNK cells become tissue-resident as a result of homing of pbNK to the endometrial tissue; there is a high degree of uNK cell homology with pbNK subsets and there is increased vascular permeability in the endometrium coincident with the increase in the uNK cell population. uNK cells also accumulate around spiral arterioles and are abundant in perivascular sites following decidualisation (25). There are increased numbers of circulating $CD56^{bright}$ pbNK cells in women of reproductive age compared to males, which may indicate hormone responsiveness and support recruitment of this subtype to the uterus as precursor to uNK cells (26).

In addition, lineage committed CD34 positive progenitor cells have been identified in decidua and can undergo differentiation into functional $CD56^{+}CD16^{-}$ NK cells in the presence of growth factors or co-culture with stromal cells *in vitro* (31). Notably, decidual CD34 positive progenitor cells express CD45 but not CD56; however expression of CD56 can be induced by factors including IL-15 (31, 32). Strikingly, 40% of CD56-positive uNK cells from late secretory endometrium are Ki67 positive (33) consistent with proliferation of resident populations in response to factors in the uterine environment. Human chorionic gonadotrophin has also been shown to increase the proliferation of uNK which may partially explain increases in number of uNK cells at the time of implantation (34).

1.3.2.2. Regulation of uNK

The uterine environment is thought to play an important role in regulating the function of uNK cells. IL-15 is reported to induce proliferation and recruitment of uNK cells and this cytokine is produced in uterine stromal cells under the influence of progesterone (35, 36). IL-15 is reported to increase CD56 expression in $CD16^{-}$ pbNK and induce a chemokine receptor repertoire similar to that of uNK (37). Chen et al reported that culture of uNK cells with conditioned media from stromal cells obtained at all stages of the menstrual cycle and

decidual stromal cells resulted in an increase in interferon gamma (IFNγ) secretion by uNK. The authors also reported that stromal cell conditioned media reduces uNK cytotoxicity (38).

1.3.2.3. Function of uNK

uNK cells appear to be important in regulating the uterine environment and are associated with a number of processes in normal endometrial function and in early pregnancy. In contrast to pbNK cells, uNK exhibit decreased cytolytic activity and increased secretion of cytokines and growth factors such as IFNγ, tissue necrosis factor alpha (TNFα), transforming growth factor beta 1 (TGF-β1), interleukin 8 (IL-8), CXCL10 and CCL5 (39-41). Secretion of IL-1β, GM-CSF, IL-6, IL-8 and IFNγ increases with gestational age (reviewed in (42)). uNK cells have been shown to induce angiogenesis in *in vitro* and *in vivo* models (39) and they are reported to secrete angiogenic factors such as vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and angiopoeitin 2 (39, 43, 44) all of which may contribute to regulation of formation/maturation of endometrial spiral arteries during the secretory phase of the cycle. In early pregnancy, during development of the placenta, uNK cells accumulate within the vascular wall of uterine arteries and are associated with spiral artery remodelling, a process which is generally complete by 12 weeks gestation (45). Consistent with this, angiogenic growth factor secretion is reported to be decreased at 12-14 weeks compared to 8-10 weeks gestation (43) which suggests the functional role of uNK cells may be dependent on gestational age.

It is thought that uNK cells can modulate immune cell function by secretion of cytokines. Indeed, a bias towards secretion of type 2 cytokines at the expense of type 1 cytokines is thought to be favourable in pregnancy. It is notable that uNK cells are associated with secretion of the type 2 cytokine IL-10 which inhibits synthesis of type 1 cytokines, however, they are also potent producers of the type 1 cytokine IFNγ (46). The balance in cytokine secretion may be influenced by availability of factors such as IL-2 or IL-15 in the local environment which stimulate uNK cells and consequently affect cytokine secretion. Dysregulation of uNK cell cytokine secretion may be important in disorders of pregnancy such as recurrent spontaneous miscarriage; this is associated with increased IFNγ and decreased secretion of IL-10 (47). In summary the presence of numbers of uNK cells are likely to have an impact on the cytokine profile in the endometrium which may be important in immunological regulation of implantation and pregnancy.

Invasion of uterine tissues and spiral arteries by the trophoblast is an essential feature of placentation and successful pregnancy (48) and uNK cells may also be involved in the

regulation of these processes. Inadequate control of trophoblast invasion is associated with disorders of pregnancy such as pre-eclampsia and intra-uterine growth restriction. uNK cells have been reported to be in close association with invading trophoblast cells and may contribute to the regulation of trophoblast invasion in early pregnancy (49). Disorders of pregnancy such as recurrent pregnancy loss (RPL) have been associated with dysregulation of uNK cells but the mechanism is unclear (50). uNK numbers appear increased in RPL and are associated with greater prevalence of CD16+ NK cells (50, 51).

Invading trophoblast cells lack expression of classical class I major histocompatibility complex (MHC) molecules such as HLA-A and -B, which are associated with increased susceptibility to NK cell-mediated cytotoxicity (52). Although decidual uNK cells express a range of receptors that could recognise invading trophoblast cells and isolated uNK cells are cytotoxic against the NK cell target K562 *in vitro*, their activity is severely reduced in comparison to the cytotoxicity of pbNK cells (28). Notably, trophoblast cells express HLA-C, -E and –G which may interact with NK cell receptors and alters their cytotoxicity. Indeed, HLA-E and HLA-G expression protects MHC-1 negative targets from pbNK-mediated cytotoxicity and uNK do not appear to be cytotoxic in the presence of trophoblast antigens due to HLA-G expression on trophoblast cells (53). Consistent with expression of HLA-E and-G, trophoblast cells are not lysed by either uNK or pbNK *in vitro* (54, 55).

The regulation of trophoblast invasion by uNK is probably due to cytokine secretion in response to stimuli provided by adjacent cells. For example, production of IFNγ and VEGF is increased by uNK cells in response to HLA-G (56) and thus secretion of these cytokines would be increased in the presence of trophoblast (HLA-G+) cells. Furthermore, human first trimester trophoblast cells secrete SDF-1 (CXCL12) which is chemoattractant to uNK cells *in vitro* (57) and may therefore encourage uNK and trophoblast cells to become more closely associated. In addition, supernatants recovered from IL-15-stimulated uNK cells increase the invasiveness of isolated cytotrophoblast cells (39) which may in turn be regulated by uNK secretion of IL-8 and CXCL10 (IP-10) in a gestation-dependent manner (39, 58, 59).

1.3.3. Dendritic cells

Dendritic cells (DCs) are a heterogeneous population of leukocytes that are important in immune tolerance and initiation of innate and adaptive immunity. DCs are specialised antigen presenting cells (APCs) that are involved in the initiation and modulation of immune responses through their ability to detect pathogen molecules and secrete cytokines (60, 61). There is emerging evidence that cross-talk between DCs and uNK cells could play a role in

the tolerance of the allogeneic foetus by the maternal immune system (49, 62). Dendritic cells can be plasmacytoid or myeloid in origin. Plasmacytoid DCs are involved in the recognition of viruses (63). Myeloid DCs are involved in the initiation of T cell response and are integral to the maintenance of peripheral immunity (64). Myeloid DCs can be further classified by their maturation state. Mature DCs are CD83+ and cells with this phenotype have been identified in human decidua close to endometrial glands in the basal layer (65). The presence of DC-SIGN+ (dendritic cell specific ICAM-3 grabbing non-integrin, CD209) immature DCs and DEC205+ intermediate DCs have also been demonstrated in human decidua (66, 67). Immature DCs can also be identified by expression of CD1a surface marker and low expression of CD40/CD80/CD86. In human endometrium the density of CD1a+ immature DCs is reported to be comparatively greater than the population of CD83+ mature DCs (61) which may in part be due to migration of DCs to lymphatic tissue upon maturation. Immature CD1a+ DCs increase in number in the basal layer in proliferative and menstrual phase endometrium and influx/proliferation of DCs may be hormonally regulated (61, 65, 66, 68).

1.3.4. Macrophages

The monocyte-macrophage system includes circulating monocytes, their bone marrow precursors, and various tissue macrophages. There are many different cell types in the monocyte-macrophage system and the phenotype of these cells depends on their specific tissue environment (69). Despite phenotypical differences, macrophages share common functionality and play crucial roles in the immune response through phagocytosis of pathogens and modulation of other immune cells through the release of cytokines which act in an autocrine and paracrine manner in the tissue environment to modulate inflammatory responses (70).

Uterine macrophages are tissue-resident leukocytes that arise from peripheral monocytes following extravasation from the circulation and subsequently differentiate into tissue specific macrophages (71). Macrophages are present in the human endometrium and myometrium and their numbers are increased in the late secretory phase coincident with the onset of menstruation (18). Similar to uNK cells macrophages appear to express ERβ and GR but not PR ((72) and unpublished data.) Evidence from mouse models and *in vitro* studies suggest that macrophages are regulated by oestrogens (reviewed in (69)). For example, in mice ovariectomy rapidly reduces macrophage recruitment to the uterus (73). Evidence for direct impacts of sex steroids in regulating macrophage number within the human endometrium is limited however DeLoia (74) reported that administration exogenous

oestrogen resulted in increased abundance of uterine macrophages in cycling women undergoing IVF treatment. Sex steroids may also modulate the functional maturation of uterine macrophages by altering the cytokine milieu of the tissue environment through regulating ER-dependent activation in responsive cells (see section 1.7).

Macrophages are reported to constitute 20-25% of CD45+ cells in decidua (75). In pregnancy decidual macrophages are thought to modulate functional processes at the feto-maternal interface including uterine vascular remodelling (76) and immunotolerance towards foetal antigens (77, 78). Consistent with a role in foetal tolerance a recent study investigating the gene expression profile of human decidual macrophages using microarray analysis revealed a transcriptional profile comprising immunomodulatory and tissue remodelling genes as well as genes related to cell proliferation and metabolism (79). Decidual macrophages are also reported to interact with decidual NK cells (80), and trophoblast cells (81). Decidual macrophages may modulate uNK populations through the secretion of IL-15 (82) (see section 1.3.2.2 and 1.7.5). Macrophages and DCs share common myelomonocytic origin and share common cellular properties and molecular markers. A subpopulation of CD14+ macrophages express the immature DC marker DC-SIGN suggesting there may be a functional overlap between these two subtypes in the decidua (66, 83).

1.4. Functional changes during the menstrual cycle

1.4.1. Menstruation

Menstruation is the shedding of the endometrial tissue. Menstruation occurs in only a few species; humans, some primates, the elephant shrew and some bat species (84, 85). In women, in the absence of pregnancy and the consequent demise of the corpus luteum, falling levels of oestrogen and progesterone trigger a cascade of cellular and molecular changes in endometrial tissue that culminates in shedding of the upper two-thirds of the endometrium (the functional layer) (15). Menstruation is characterised by the demise of all epithelial cells, with the exception of basal glandular epithelial cells, blood vessel necrosis and haemorrhage and sloughing of the tissue (86). Matrix metalloproteinases (MMPs) have the ability to breakdown extra cellular matrix and have a crucial role in menstrual breakdown. MMP expression is increased as a result of hormone withdrawal prior to menstruation (87). In addition, transient hypoxia during menstruation stimulates transcription of genes regulated by hypoxia inducible factor 1 (HIF-1). HIF-1α protein is expressed in the human endometrium in the late secretory and menstrual phases (88). HIF-1α is a transcription factor

that is known to induce VEGF expression (89) which may be important in postmenstrual vascular repair.

In fertile cycles, the terminal differentiation of the endometrial stroma may be considered a protective mechanism, as in humans implantation is aggressive and trophoblast invasion is extensive reaching the inner third of the myometrium (14). The dense decidual stromal layer that is formed as a result of progesterone mediated changes during the secretory phase act as a barrier that prevents excessive invasion. Consistent with this theory, menstruation does not occur in mammals with less aggressive implantation strategies (85). Thus, in the absence of pregnancy and progesterone withdrawal, the terminally differentiated stromal layer is shed in order to allow regeneration of the endometrium for the next cycle. Menstruation is also associated with leukocyte influx and is considered an inflammatory event. Increased infiltration of neutrophils and monocytes, as well as local increases in inflammatory mediators, such as prostaglandins, characterises the breakdown of the functional layer (15, 90). In addition, vasoconstriction of the spiral arteries is triggered during menstruation reported to be mediated in part by prostaglandin F2α (91) which results in hypoxia and precedes bleeding (92).

1.4.2. Endometrial regeneration and proliferation

The human endometrium has a remarkable capacity for scar-free repair following menstruation (93). Following breakdown and shedding of the functional layer of the endometrium during menstruation, the endometrium heals and regenerates a new functional compartment from the basal layer of the endometrium which is not shed at menstruation (94). Re-epithelialisation begins during menses with basal epithelial cells repopulating the luminal surface of the endometrium (95, 96). Although endometrial repair was traditionally thought to be mediated by oestrogen studies in a mouse model of simulated menstruation have indicated that this may be an oestrogen-independent process (97) which would be consistent with healing occurring when oestrogen concentrations are low prior to growth of a new cohort of ovarian follicles. It is postulated that in humans a basal stem cell population is responsible for regeneration of the endometrium although investigations into their identification and regulation are preliminary at this time (98). Regeneration of blood vessels also occurs following menstruation and proliferation of endometrial endothelial cells is at a peak in the mid to late proliferative phase (99). Investigations in ovariectomised mice have revealed that exogenous administration of oestrogen stimulates proliferation of endometrial endothelial cells (100). In a study by Heryanto et al (100) the effect of oestrogen on endothelial proliferation was reported to be dose dependent and was reduced when low, but

not high, amounts of oestrogen were combined with progesterone. Oestrogen also stimulates proliferation of the glandular epithelium and stromal fibroblasts in the functional layer during the proliferative phase. This growth leads to an overall increase in the thickness of the endometrium from approximately 0.5mm to 7mm over a period of approximately 10 days (101).

1.4.3. Angiogenesis in the endometrium

Angiogenesis, the formation of new blood vessels from existing vessels, is a feature of pathologies including cancers (102). Functional angiogenesis is rare in normal tissues apart from those of the reproductive system where it is a feature of the dynamic remodelling of the corpus luteum of the ovary (103) and the endometrium (104). In the human uterus endometrial arterioles arise from arcuate arteries in the myometrium which pass into arterioles in the basal endometrium that directly supply the functional layer (105). Proliferation of endometrial endothelial cells peaks in the mid to late proliferative phase and then again in the secretory phase (99). In addition, endometrial arteries are transformed during decidualisation. The thin walled straight arteries characteristic of the proliferative phase become increasingly tortuous during the secretory phase (106, 107), a phenotype which continues in early pregnancy decidua until remodelling of the vasculature occurs in response to trophoblast invasion (9, 104). Unusually angiogenesis in the endometrium does not seem to involve vasculogenesis from progenitor cells or capillary sprouting but instead appears to involve elongation and enlargement of existing vessels while still retaining the integral structure of the artery (2, 108). Many angiogenic factors have been identified in the endometrium any or all of which may mediate this process. These include; vascular endothelial growth factor A (VEGFA), VEGFC, placental growth factor (PlGF), Angiopoietins (Ang); Ang-1 and Ang-2, as well as the receptors to VEGF and Ang (Tie-1 and Tie-2) (44, 104). In the human endometrium, immunohistochemistry studies have revealed that VEGF is expressed in both the epithelial and stromal compartments and *in vitro* mRNA expression in primary endometrial stromal cells is enhanced by ovarian hormones (109). VEGF is a prime regulator of angiogenesis during decidualisation in mice (110) and angiopoietins act with VEGF to promote angiogenesis (111).

Immunohistochemical experiments have revealed that expression of VEGF by stromal cells is highest in the mid-secretory phase (112) and intense immuno-expression of stromal VEGF is also reported in early pregnancy decidua (112). The pattern of immuno-expression of VEGF in the epithelial cells is similar however staining is absent in the late secretory phase

and less intense in early pregnancy (112). In the same study, VEGF protein was only detected in blood vessels in early pregnancy (112). VEGF mediates its effect via two receptors; the fms-like tyrosine kinase (Flt-1, VEGFR-1) and the kinase insert domain-containing region (KDR: VEGFR-2). In the human endometrium, immunoexpression of VEGFR-1 and VEGFR-2 is detected mainly in vascular endothelial cells (113). VEGFR-1 is detected only in stromal and endothelial cells of early pregnancy, and is absent from stromal and epithelial cells in the normal menstrual cycle (112). However, VEGFR-2 is abundantly detected in epithelial and endothelial cells throughout the menstrual cycle, in early pregnancy and in secretory phase stromal cells (112). Treatment of primary human ESC *in vitro* with combined oestrogen and progesterone enhances mRNA expression of VEGFR-2 (112).

In addition to the angiogenic factors expressed by stromal and epithelial cells, there may also be a role for uNK cells in mediating angiogenesis. It is notable that uNK cells form aggregates around spiral arteries and glands in the late secretory endometrium and decidua (25). Furthermore, cytokine secretion is an important feature of uNK function and secretion of angiogenic factors VEGFC, PLGF, and Ang-2 has been described (39, 44). In addition an increased capacity for network formation by HUVEC (Human umbilical vein endothelial cells) is seen following co-culture with uNK cells (39). The functional impact of uNK may be maturation dependent, as VEGFC secretion by uNK decreases with gestational age during pregnancy (43). Stromal derived factors may also influence uNK production of angiogenic factors, for example, IL-15, which is secreted by ESC, increases VEGFC secretion in uNK cells (44).

Transcription of VEGFA mRNA can be regulated by oestrogen via either ERα or ERβ in human endometrial epithelial and stromal cells (114). Oestradiol-induced angiogenesis is disrupted in ERαKO mice (115) consistent with a role for oestrogen in regulating angiogenesis in the uterus.

1.4.4. Decidualisation

Decidualisation is the differentiation and proliferation of endometrial stromal cells (ESC) that is initiated in the mid to late secretory phase of the menstrual cycle in response to rising levels of progesterone. Stromal differentiation/decidualisation is accompanied by increased angiogenesis and leukocyte infiltration (9) and is unique to species that undergo haemochorial placentation (116). Embryo implantation, placentation and establishment of

pregnancy are dependent on adequate decidualisation. Consequently, failure of decidualisation results in infertility (117, 118).

Differentiation of the stromal compartment is initiated during the mid-secretory phase and is first apparent in cells surrounding the spiral arterioles and thereafter spreads in a wave-like manner through the functional layer completing the pre-decidual transformation of the endometrium (119). Decidualisation is a characterised by the coordinated expression of a specific set of genes, including those encoding growth factors such as prolactin and IGF-binding protein (IGFBP), and has as a defining feature the transformation of fibroblastic stromal cells into 'epithelioid' decidual cells an event apparent during the late secretory phase (119). Decidual cells are much larger than stromal fibroblasts with rounded nuclei, increased numbers of nucleoli, an expanded secretory apparatus, dilated rough endoplasmic reticulum and Golgi apparatus and prominent cytoplasmic accumulation of glycogen (120). Decidual cells persist in pregnancy and form the maternal component of the placenta, the decidua basalis (14).

Progesterone acting through its cognate receptor the progesterone receptor (PR), a member of the superfamily of steroid receptors (see section 1.5), is essential for decidualisation. PR knockout (PRKO) mice are infertile as a result of implantation and decidualisation failure (121). Stromal PR expression is required for progesterone action which drives differentiation of stromal cells and secretion of classical decidualisation markers prolactin and IGFBP (122, 123).

In contrast the functional requirement of oestrogen in decidualisation is less clear. Oestrogen stimulates proliferation of the endometrium and oestrogen priming is required to induce stromal progesterone receptor expression as PR is an oestrogen regulated gene (124). Administration of a pure anti-oestrogen (ICI 182, 780) severely impairs decidualisation in wild type mice suggesting oestrogen priming is important in decidualisation (125). Puzzlingly, the decidualisation response is intact in both ERα and ERβ knockout mice and ovarian oestrogen is not essential for decidualisation (125-127). However, a recent study in mice suggests that *de novo* biosynthesis of oestrogen may also be essential during decidualisation (128) providing an extra dimension to our understanding of the role of oestrogen.

Throughout the secretory phase the endometrial spiral arteries proliferate becoming increasingly coiled and consequently blood flow rate decreases (129). In decidua, functional remodelling of spiral arteries occurs in early pregnancy via interaction with trophoblast cells,

resulting in the formation of large low resistance vessels which permit adequate blood flow to the feto-placental vascular bed (9). Failure of this process is associated with disorders of pregnancy such as intra-uterine growth restriction (IUGR) and pre-eclampsia. Another feature concomitant with decidualisation is the recruitment of leukocytes to the uterus. The number of leukocytes rises from around 5% of uterine cells in the proliferative phase to 25% in the secretory phase. The most prominent of uterine leukocytes is the uterine natural killer cell (uNK) which further increases to account for 75% of total leukocytes in the decidua of early pregnancy. uNK cells and uterine leukocytes are discussed further in section 1.9.

1.4.4.1. Regulation of decidualisation

Primary cells isolated from human endometrium have proven to be an indispensible tool in understanding the regulation of decidualisation. *In vitro* studies using human endometrial stromal cells (ESCs) are commonly used as a model for studying the molecular events which occur during decidualisation and are key to understanding the stromal contribution without compounding factors such as ovarian hormones and cellular cross-talk. ESC can be stimulated to decidualise *in vitro* by addition of progesterone, which on its own is a weak inducer of decidualisation, however its action can be significantly enhanced by addition of cyclic adenosine monophosphate (cAMP) or cAMP inducing agents such as prostaglandin E2 and relaxin (130). cAMP is a ubiquitous second messenger molecule that is generated upon ligand binding to GPCRs (G-protein coupled receptors) and initiates downstream phosphorylation of target molecules, including transcription factors, via the phosphokinase A (PKA) signalling pathway (119, 122, 123, 131). Elevation of intracellular cAMP levels is required for maintenance of the decidual phenotype (132). Successful decidualisation *in vitro* parallels *in vivo* responses and is characterised by increased secretion of classical markers such as IGFBP1 (133) and characteristic changes in cellular morphology, including cytoplasmic aggregates of rough endoplasmic reticulum and secretory granules (130). Synthesized proteins secreted by decidual cells have been identified as decidualisation markers which are used to confirm decidualisation induced *in vitro*. Transcriptional analysis has revealed regulation of genes associated with cytokines, growth factors, nuclear transcription factors, and mediators of the cAMP signal transduction pathway following *in vitro* decidualisation of ESC (134, 135).

1.4.4.2. Biosynthesis by decidual cells

1.4.4.2.1. Prolactin

Prolactin is a peptide hormone which regulates cell growth and differentiation and is an important regulator of the immune system. Prolactin (PRL) is synthesised by decidualised ESC and PRL production is correlated with the extent of stromal decidualisation (136). In non-pregnant endometrium secretion of prolactin can be detected in endometrial explants from the mid-secretory phase onwards coincident with emergence of pre-decidual cells (136). In pregnancy PRL secretion progressively increases until a peak at 20-25 weeks and thereafter declines towards term (137). PRL expression is regulated by progesterone. In women, administration of the synthetic progestin medroxyprogesterone acetate (MPA) increases expression of both prolactin mRNA and protein in secretory phase endometrium (138). Administration of the antiprogestin mifepristone (RU486) reduces concentrations of mRNAs encoding both PRL and the PRL receptor in ESC *in vitro* (139). The distal promoter of human PRL contains a functional oestrogen response element (ERE) and binding site for activating protein-1 (AP-1) suggesting oestrogen may regulate transcription of the PRL gene by both direct and or tethered binding (See section 1.5.4). Studies in T47D breast cancer cells using chromatin immunoprecipitation and gel shift assays have revealed that both ERα and ERβ can directly bind the ERE in the hPRL distal promoter but only ERα interacts with AP-1 proteins at the AP-1 site (140). Prolactin plays an important role in implantation and early pregnancy and prolactin receptor knock-out mice have severely compromised pre-implantation development and implantation failure (141). uNK cells are also known to express the prolactin receptor (142) and thus prolactin has also been associated with immune regulation.

1.4.4.2.2. IGFBP1

The insulin like growth factor system contains several peptide growth factors (IGFs) and specific binding proteins (IGFBPs) that regulate the availability of IGFs at their receptors (143). The IGF family has important roles in growth and apoptosis, metabolism and development (144). IGFBP1 levels are increased in secretory phase endometrium and decidua, coincident with decidualisation (145, 146). Consistent with this IGFBP is considered a marker of decidualisation and its synthesis is used to assess the extent of decidualisation during *in vitro* modelling (147). IGFBP regulates the availability of insulin like growth factors (IGFs) and may have multiple roles in endometrial development and implantation (143). The IGFBP-1 promoter contains 6 promoter regions shown to be functionally relevant *in vitro* including; two glucocorticoid response elements, an insulin

response element (IRE) and cAMP response element (144). Insulin acting at the IRE inhibits transcription of IGFBP-1 however both glucocorticoid and cAMP stimulate IGFBP-1 transcription (144). The expression of mRNA encoding IGFBP-1 in ESC is reported to be promoted weakly by progesterone but more markedly by combined treatment with progesterone and epidermal growth factor (EGF) (147). Induction of IGFBP-1 promoter activity by progestins parallels the induction of IGFBP-1 mRNA reported in decidualised ESC *in vitro* (148) and is reported to be initially slow in the first 4-8 days but then increases exponentially thereafter. The apparent delay in responsiveness to progesterone is thought to be due to indirect action associated with induction of the transcription factor Sp3 which acts to suppress transcriptional repression of the distal IGFBP-1 promoter in decidualised stromal cells (149). Subsequent studies have also identified PR responsive sequences (PREs) in the IGFBP-1 promoter. PR/GR response elements have similar consensus sequences and functional analysis of the IGFBP-1 promoter in ESC has revealed that PR activates the promoter via a sequence termed the PRE1 in decidualised ESC but not undifferentiated ESC (150). The PR exists as two isotypes PR-A and PR-B (151). Studies have reported that ligand activated PR-A is a stronger transactivator of IGFBP-1 than PR-B in ESC (152) consistent with a role in stimulating expression of IGFBP-1. Notably, PR-A is the predominant PR isoform in the endometrial stroma from the midsecretory phase onwards in the endometrium and its expression therefore coincides with the onset of decidualisation and associated IGFBP secretion (153).

1.4.4.2.3. Tissue factor

Tissue factor (TF; also TTF3) is a cell surface glycoprotein involved in blood coagulation cascades that is present in leukocytes (154). TF is thought to maintain vascular haemostasis in the endometrium and is known to induce VEGF production (155, 156). In decidualised ESC expression of TF parallels that of prolactin (157) and it is thus also considered a decidualisation marker. TF expression is controlled by specificity protein (Sp)-1 which itself may interact with progesterone and/or oestrogen receptors (158). Consistent with this interaction, progestins increase TF mRNA and protein concentration in ESC although this is synergistically enhanced by co-stimulation with oestradiol, oestradiol alone does not increase TF transcription (159). Sp-1 mediated enhancement of TF transcription by oestradiol has not been described in ESC however in addition to SP-1 regulation, the TF promoter also contains two AP-1 sites which could be indirectly modulated by ER action, although no such mechanism has been reported (160). Increased production of TF during decidualisation is thought to mediate vascular haemostasis and angiogenesis during remodelling of the uterine

vasculature (155, 158). *In vitro* analysis of steroidal regulation of TF in ESC indicates that steroid withdrawal (i.e. simulation of menstruation) reduces TF mRNA expression which *in vivo* may permit menstrual-associated haemorrhage (161).

1.4.5. Implantation

Implantation involves the attachment of a competent blastocyst to the receptive uterine epithelium which in turn allows for physical and nutritional support by the endometrium (162, 163). Implantation occurs over two distinct phases; attachment and invasion. The conceptus moves from the oviduct to the uterus by ciliary beating (164). The breakdown of the zona pellucida allows 'hatching' of the blastocyst which is followed by apposition and adherence of trophoblast cells to the luminal epithelium of the receptive endometrium (163, 165). This leads to blastocyst mediated breakdown of epithelial cells (166) and there is increased vascular permeability within the stroma underlying the conceptus in association with decidualisation (9). In women, blastocyst implantation occurs following degradation of surface epithelium which facilitates invasion of trophoblast cells into endometrial glandular and decidual compartment (167). In humans invasion is deep with the blastocyst implanting interstitially; the surface epithelium is restored following implantation (163). The trophoblast consists of two cell lineages; the syncitium (syncitiotrophoblast) and the cytotrophoblast. The syncitiotrophoblast is a large multi-nucleated cell type which proliferates to produce more trophoblast cells, further invading the uterine tissue and forms the interface between maternal cells and the cytotrophoblast cells of the conceptus.

The uterine epithelium is normally hostile to the blastocyst and is required to undergo transition to a receptive state in order for implantation to occur. Uterine receptivity occurs over a restricted period of time during each normal cycle (168). While the mechanisms and factors which control the duration of the receptive phase are poorly understood several features of epithelial transition are well described (169). In the non-receptive state the epithelium is repellent to the blastocyst due to long apical microvilli, thick glycocalyx and a high surface charge (168). Receptivity is characterised by the emergence of apical protrusions known as pinopodes which occlude the uterine cavity by bringing opposing epithelia into close apposition (170). The epithelium loses its negative charge and shortening of microvilli and thinning of mucin coat also occur (168). Glycoprotein unmasking at implantation sites correlates with increased blastocyst adhesion (171). The receptive phase lasts approximately 4 days in women usually falling on days 20 to 24 of a standard 28 day cycle (7-11 days post LH surge, Figure 1.2) (172). Following the receptive phase and in the

absence of implantation the uterine epithelium enters a refractory phase and implantation can no longer occur.

Implantation depends on support from the ovarian sex steroid hormones oestrogen and progesterone. While progesterone drives priming of the uterus it is oestrogen that is essential for implantation. Oestrogen acting via oestrogen receptor alpha (ERα) transforms the progesterone primed uterus into a receptive state and the uterine oestrogen metabolite catecholestrogen can activate the blastocyst in a paracrine manner (173). ERα knockout mice are infertile due to implantation failure however implantation is possible in ERβ knockout mice despite reduced ovarian capacity (126, 127). In mice, specific levels of oestrogen are the critical determinant of the window of receptivity; higher levels of oestrogen are associated with aberrant expression of genes normally conducive to implantation, while suboptimal levels do not promote progression from the pre-receptive state (174). Targeted ablation of the progesterone receptor (PR) in mice also results in infertility as a result of implantation and decidualisation failure (121).

Release of cytokines and signalling factors by endometrial epithelial cells has been shown to be essential for normal implantation. In mice exposure to oestrogen increases expression of leukaemia inhibitory factor (LIF) in the glandular epithelium and LIF null mice cannot support implantation (16, 175). In humans, blastocysts express two receptors that bind LIF; LIF-Rβ and gp130, and LIF expression is maximal in the mid-luteal phase in glandular epithelium coincident with the receptive phase (176). Epidermal growth factor (EGF) receptor is expressed in human pre-implantation embryos and stimulation by EGF increases protein synthesis and blastocyst development (177, 178). TGF-β is reported to control trophoblast invasion and promotes angiogenesis and decidualisation (179). Other factors which are essential in mediating implantation are integrins, adhesion molecules which are constitutively expressed in uterine epithelium (163). While the expression of several integrin subunits has been described the subtype αvβ3 appears in both luminal and glandular epithelium at day 19 of the menstrual cycle and expression persists through the secretory phase (180). Unlike in mice, mucin-1 (MUC1) is abundant at the time of implantation in humans yet large molecules such as MUC1 are thought to inhibit implantation due to steric interference (181, 182). It is thought that in humans blastocysts may initially induce epithelial MUC1 expression but subsequently induce cleavage of MUC1 in epithelium during the adhesion stage at the implantation site (171). This paracrine interaction may confer a degree of selectivity such that only suitable, viable blastocysts can implant.

1.5. Sex steroids

1.5.1. Overview

Sex steroid hormones, including; oestrogens, androgens and progestagens, regulate a wide range of physiological functions and are essential mediators of reproductive function. Sex steroids alter cell function following binding to nuclear receptors that are members of a superfamily of ligand-activated transcription factors (http://www.nursa.org/) consisting of more than 48 different nuclear receptors (NR) in humans (183), including orphan receptors for which no natural ligand has been identified (184). These receptors can also be activated by alternative signalling pathways and have been reported to relocate to the membrane (185) or mitochondria (186).

Oestrogens and progestagens are essential to female reproductive function but there is also an emerging role for androgens in regulating distinct gene networks in the endometrium (187). In humans two oestrogen receptors have been identified; ER alpha (ERα, *NR3A1*) and ER beta (ERβ, *NR3A2*) which are the products of two genes (*ESR1* and *ESR2* respectively) located in humans on chromosome 6q25.1 (*ESR1)* and 14q23.2 (*ESR2)* (188). Both *ESR1* and *ESR2* are subject to alternative splicing (reviewed in (189)) and several isoforms have been identified for both ERα (ERα46 and ERα36) (190, 191) and ERβ (ERβ1-5) (192, 193). Progesterone receptor (PR; *NR3C3*) is encoded by a single gene (*PGR*) located on human chromosome 11q22 (194). PR has two main receptor isoforms PR-A and PR-B that differ by molecular weight due to an additional 165 N-terminal amino acids in PR-B and are the result of different promoters on the *PGR* gene (6). In addition, an N-terminal truncated PR-C isoform has been reported which inhibits PR-B transactivation when PR-B and PR-C heterodimerise (195).There is a single androgen receptor (AR, *NR3C4*) encoded by the *AR* gene located on the X chromosome at Xq11-12 (196).

Oestrogens play a fundamental role in the development and normal physiological function of multiple tissue systems and are key regulators of fertility in both males and females (12). Progestins play a central role in the diverse functions which are associated with the establishment and maintenance of pregnancy (197). Androgens are essential regulators of male fertility (198); in addition, ARs are expressed in the ovary (199) and endometrium (200).

1.5.2. Steroid receptor structure

Nuclear steroid receptors contain a conserved arrangement of structural and functional domains (A to F; see Figure 1.4. and Figure 1.5). These include a regulatory N-terminal domain (A/B), a conserved DNA-binding domain which is composed of two zinc fingers (DBD, domain C), a hinge region (D) and a C-terminal domain (domain E/F) that contains peptides involved in ligand binding, receptor dimerisation and nuclear localization (183, 188, 201-203).

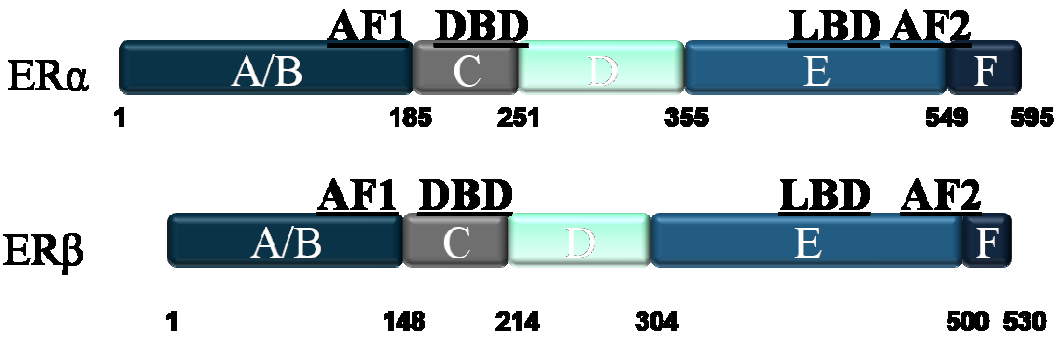

Figure 1.4. Protein structure of the two main ER isoforms; ERα and ERβ. Amino acid sequence and functional regions are shown. Adapted from (204).

The sequences within the N-terminal (A/B) domains of NR3 family members are poorly conserved, for example, there is only 20% sequence homology between human ERα and ERβ. This domain is involved in protein-protein interactions and in transcriptional activation of target gene expression. In ERs, it contains the activation function (AF)-1 region as well as several phosphorylation and sumoylation sites (188, 205) that are downstream targets for growth factor signalling pathways. It has been claimed that the N terminal region of ERβ contains a repressor domain (206). In PR-A and PR-B, AF-1 is also located in the N-terminal functional domain (207) and in PR-B a further regulatory AF-3 region is present in the upstream sequence that is distinct to PR-B (208).

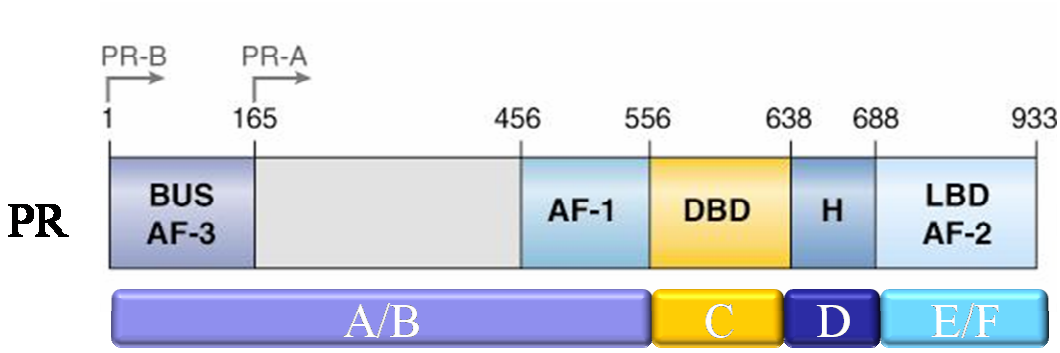

Figure 1.5. Protein structure of progesterone receptor. PR-A and PR-B differ only in the N-terminal amino acid sequence. PR-B has an additional upstream sequence (BUS; B upstream sequence) of 165 amino acids which is absent in PR-A. Adapted from (209).

In these receptors, the ligand binding domain (LBD) also contains an additional transactivation domain known as AF-2, which is responsible for ligand-dependent activation of the receptor. Variations in peptide sequence alter the tertiary structure LBDs in different nuclear receptors and this specifies ligand specific interactions. The LBD of PR, ERα and ERβ , have all been crystallized and their 3D structure has been determined (210, 211). Distinct residues in the LBD distinguish interactions between 3-OH group present in oestrogen and 3-keto group present in other steroids such as progesterone and androgen and this confers ligand specificity for oestrogens (211). Notably, differences in the functional size of the ligand binding pocket as a result of shortened C-terminal sequences and disorientated helix 12 in ERβ isoforms such as ERβ2 and ERβ5 (212) prevents ligand binding. Thus ligand responsiveness to these ERβ isoforms is reported to require heterodimerisation with competent binding partners such as ERα or ERβ1 (212).

1.5.3. Steroid hormone receptor co-regulatory proteins

Steroid hormone receptor (SHR) co-regulators are essential mediators of steroid hormone function. The activities of SHRs are modulated by co-regulatory proteins which can enhance (co-activators) or inhibit (co-repressors) transcription (213). Co-activators are molecules which are recruited to SHRs, usually in a ligand dependent manner, and enhance SHR-mediated transcription. Conversely, co-repressors are typically complexed with unliganded receptors and repress transcription.

The first nuclear receptor co-activator was cloned in 1995, using a yeast two-hybrid system which identified a protein which could enhance transcriptional activity of the human PR

(214). Subsequently, this protein has been termed steroid receptor coactivator-1 (SRC-1); SRC-1 is reported to enhance transcriptional activity of ERs, PR and AR (214, 215). Notably targeted deletion of the SRC-1 gene in mice results in a phenotype which includes reproductive dysfunction characterized by impaired decidualisation response and reduced uterine growth in response to oestradiol in females, and reduced testis weight in males (216). Thus SRC-1 appears to be required for maximal response to steroid hormones *in vivo* and is therefore important in regulating reproductive function.

Prototypical co-repressors were also identified using yeast two-hybrid screening and these include nuclear receptor co-repressor (NCo-R) (217) and silencing mediator of retinoic acid and thyroid-hormone receptors (SMRT) (218). Interestingly, protein expression of SRC-1 (and SRC-2 and -3), and NCo-R has been reported in normal endometrium (219, 220) underlying the importance of these co-regulatory proteins in mediating steroid hormone responses in uterine function.

The association of SHRs with co-regulatory proteins depends largely upon ligand-dependent changes in receptor conformation. Typically ligand binding results in dissociation of co-repressors and recruitment of co-activators. Thereafter the sequential assembly of a multi-protein complex permits recruitment of chromatin remodelling machinery (221, 222). In classical responses to ligand-dependent gene activation local acetyltransferase activity mediated by co-activators results in disruption of nucleosome structure and histone displacement leading to direct interaction of the receptor complex with DNA and transcription (223).

In addition, post translational modifications may also modulate nuclear receptors, their co-regulator complexes and consequently their target gene networks (223). Targeted enzymatic modifications such as; acetylation, methylation, and phosphorylation can all affect nuclear receptors and the function of co-regulatory proteins function and as a result alter transcriptional responses (224). The convergence of other signalling pathways such as growth factor signalling and phosphorylation pathways can mediate PTMs at nuclear receptor complexes adding further complexity to SHR signalling and contribute to cross-talk between responses (225).

1.5.4. Oestrogen receptor dependent signalling

The classical endogenous ligands for both ERα and ERβ are the naturally occurring oestrogens; oestradiol and oestrone, both of which exhibit agonist activity at ERs (226). In

addition, pregnancy associated oestrogens; oestriol and oestetrol, as well as the plant-derived phytoestrogen genistein, are also reported to have activity at ERs (227-229). The compound ICI 182,780 (ICI) acts as an antagonist of both ERα and ERβ. In addition to widely used ER subtype specific agonists including propyl-pyrazole triol (PPT; ERα-specific) and diarylpropionitrile (DPN; ERβ-selective), a wide variety of selective oestrogen receptor modulators (SERMs) are capable of mixed agonist and antagonist activities at ERs dependent on tissue context (230-232). Probably the best known of these is tamoxifen which acts as an antagonist in breast but has mixed agonist/antagonist activity in endometrial tissues associated with stimulation of cell proliferation even in postmenopausal women.

The effects of oestrogens can be mediated through several different pathways (reviewed in (188, 233, 234)). Classically, ligand-activated ERs form homo- or hetero-dimers that interact with consensus sequences known as oestrogen response elements (EREs) within the promoter regions of genes (reviewed in (188)). In addition to full EREs with palindromic sequences, oestrogen receptors can also bind imperfect EREs or ERE half sites leading to transcriptional responses (235). Interestingly, the promoter region of human PGR gene lacks a palindromic ERE but contains an ERE half-site and two Sp1 sites critical to oestrogen responsiveness (236). Genomic studies have also revealed that many oestrogen-responsive genes lack sequences in their promoters that resemble EREs (237) and such genes can be regulated through indirect association of ER with other DNA binding proteins (238). For example, ER complexes can bind promoter regions indirectly through protein-protein interactions with Jun/Fos (at AP-1 response elements) and Sp-1 (at GC-rich Sp-1 motifs) or by interaction with the NFkappa ß pathway (239). The varied mechanisms of ER activation result in distinct ligand and tissue-specific transcriptional responses. Thus, ligand-activated ERs binding via tethered transcription factors may have agonist and antagonist profiles that differ from classical mechanisms of ER action; for example, using a luciferase reporter assay Paech et al reported that ERα tethered via activator protein-1 (AP-1) sites is an E2-dependent transcriptional activator, whereas E2 acting on ERβ at AP-1 sites did not induce transcription of the reporter (240). Interestingly the same authors reported that treatment with oestrogen antagonists such as ICI 182, 780 (Fulvestrant, Faslodex®) increased transcriptional activity of ERβ at AP-1 sites in contrast to the classical antagonist effects of ICI at ERE sites. In addition to AP-1 interaction, both ERα and ERβ can interact with the transcription factor Specificity protein 1 (Sp1.) ERα-Sp1 complexes can be activated by oestrogens, however, little transcriptional activation is reported for Sp1-ERβ complexes (241). Interestingly, the anti-oestrogen ICI 182, 780 which suppresses ERE-dependent transcription is reported to be a potent agonist of ERs when they are tethered to AP-1, SP-1 (237, 242) and STAT-5

transcription factors in the nucleus (243). The affinities of co-factors for ERs can also be subtype specific, for example, thyroid receptor-associated protein 220 (TRAP220) has selective affinity for ERβ (244) while the co-activator SRC-1 is more strongly recruited to ERβ following stimulation with the phytoestrogen genestein (245).

Expression of ERα and ERβ in the human uterus is both temporally and spatially regulated. In the myometrium, concentrations of ERα mRNA are higher than those of ERβ (246). However, in the myometrium of post-menopausal women relative mRNA expression is altered and ERβ expression predominates. During pregnancy there is also a switch to predominance of ERβ over ERα in term myometrium (247). These changes are likely to be related to differences in bioavailable oestrogens but their impacts on myometrial contractility are poorly understood.

In the endometrium both ERα and ERβ proteins are expressed in multiple cell types, including the stromal fibroblasts and epithelial cells. However, ERβ but not ERα is expressed in endothelial cells that line blood vessel walls (248) and in immune cells such as uterine-specific natural killer (uNK) cells (249). During the normal menstrual cycle the pattern of expression in the functional layer of the endometrium of ERα and ERβ changes according to phase. In the oestrogen dominated proliferative phase, there is intense immuno-expression of ERα in the glands and stroma (248) which decreases during the secretory phase following the post-ovulatory rise in progesterone (250). The expression of ERβ isoforms also varies throughout the cycle. While full length ERβ1 mRNA and protein are consistently expressed throughout the cycle, ERβ2 is higher in the proliferative phase and is selectively down-regulated in the glandular epithelium during the secretory phase (250). In decidua of early pregnancy ERα expression is minimal and only detected in nuclei of stromal and epithelial cells however nuclear expression of ERβ is found in all compartments (251). In contrast to the dynamic changes in expression of ERs in the functional layer of the endometrium, immunohistochemical analysis of ERα and ERβ expression has revealed that ER levels are relatively unchanged in the basal layer throughout the menstrual cycle (248).

The uterus is a major target tissue for oestrogen action and stimulation of the functional layer of the endometrium by ovarian oestrogen during the proliferative phase of the menstrual cycle drives uterine proliferation (252). ERα and ERβ are also expressed in the mouse uterus and studies in mice have demonstrated the importance of oestrogen signalling in mediating uterine proliferation. In mice, ovariectomy (ovx) removes the major source of endogenous oestrogens and exogenous administration of E2 stimulates a 3 to 4 fold increase in uterine

wet weight (12). However, following disruption of ER by gene targeting or by co-administration of the ER antagonist ICI 182, 780, the uterotropic effects of E2 observed in wild type mice are abolished, confirming the essential requirement for ER in driving uterine proliferation (12, 253). Oestrogen driven uterine proliferation has been shown to be ERα specific through the use of ER selective ligands (254). For example, Frasor et al reported that in female mice following ovx, administration of the ERα selective agonist PPT stimulates an increase in uterine wet weight and epithelial proliferation that is similar but less potent than E2 (255). However, the ERβ selective agonist DPN did not induce uterine proliferation (255). The authors also reported that co-administration of DPN and PPT reduces the increase in wet weight observed following treatment with PPT alone (255) suggesting a capacity for ERβ to modulate ERα responses in the mouse uterus. Consistent with a key role for ERα in uterine responsiveness, female ERα knock-out (ERαKO) mice exhibit uterine hypoplasia and have uterine weights that are half those of intact controls with a uterine morphology characterized by a sparse distribution of glands (12). Administration of E2 to ERαKO female mice does not induce uterine proliferation (12, 256). Interestingly, the decidual response is intact in ERαKO mice despite the requirement for E2 in inducing artificial decidualisation of wild type mice. Although the decidualisation response is retained, ERαKO female mice are infertile due to implantation failure (126). A uterine epithelial cell-specific ERα knock out mouse model has also been reported and the studies on female mice have demonstrated that proliferation of uterine epithelial cells is mediated by an ERα-dependent proliferative signal from the stroma (13). In contrast to the ERαKO mouse, the uterine weight in ERβKO mice is normal. However, loss of functional ERβ results in reduced epithelial cell differentiation and ERβKO females are hyper-responsive to E2-stimulated uterine proliferation which is consistent with *in vitro* studies suggesting ERβ can act as a negative regulator of ERα mediated proliferation (256).

1.5.5. Progesterone receptor dependent signalling

PRs are expressed in all major physiological systems, and are essential to reproductive function with highest concentrations of PR mRNA being detected in the uterus and ovary (www.nursa.org/10.1621/datasets.02001). PRs are activated by the naturally occurring hormone progesterone, as well synthetic agonists such as medroxyprogesterone acetate (MPA) (257). Progesterone action is inhibited by the PR antagonist RU486 (mifepristone) (258) while selective progesterone receptor modulators (SPRMs), such as J867 (Asoprisnil) (259), have tissue dependent mixed agonist/antagonist actions.

In the absence of hormone, PR is assembled in an inactive multi-protein chaperone complex in the cellular cytoplasm (260). Ligand interaction leads to dissociation of the chaperone complex, receptor dimerisation and modulation of transcription through binding to the regulatory regions in the promoters of target genes (261). PR can bind directly to progesterone response elements (PREs), consensus sequences within the promoter of target genes, to modulate transcription (262). Similar to ERs, PR can also interact with gene promoters via tethering to other transcription factors such as Sp1 (263), Sp3 (264) and the AP-1 complex (265). PR-A and PR-B are functionally unique transcriptional regulators and are capable of regulating distinct responses at promoter regions (266, 267). The protein structure of PR-A contains an AF-1 and AF-2 domain as well as an inhibitory domain (ID) which suppresses transcriptional activity (208, 268). Thus, PR-A acts as a repressor of transcription at a number of different promoters in the presence of both progesterone agonists and antagonists. PR-A is able to act as transdominant repressor, and is reported to modulate the transcriptional activity of PR-B (269) and ERα (270). In addition to the domains contained in PR-A, PR-B also contains a unique AF-3 domain in the 'B upstream sequence' (BUS) of the protein which neutralises the effect of the ID (208), allowing greater transcriptional activity (Figure 1.5) (271). As a result PR-B is the more transcriptionally active PR isoform. PR signalling is modified by interaction with cofactors which can positively or negatively modulate transcription (213, 272). The contribution of cofactors is dependent on their relative abundance which varies in a tissue-specific manner. PR-A and PR-B interact with distinct co-regulatory proteins. This is evidenced by PR-A having a higher affinity than PR-B for the co-repressor SMRT (273) and PR-B having a high affinity for co-activators such as SRC-1 and SRC-2 (273, 274).

In the endometrium, the two PR isoforms appear to have distinct functions. PR-A acts as a transcriptional repressor and has a major role in the endometrium by inhibiting oestrogen-induced proliferation. In contrast, PR-B has an activating role in the endometrium by acting as an endometrial oestrogen agonist (275). This may be important in endometrial pathologies such as endometrial cancers where unopposed oestrogenic stimulation may lead to neoplasia which may in part be a result of altered PR isoform expression (276).

PR isoforms are spatially and temporally regulated in the human endometrium during the menstrual cycle (153). The *PR* gene contains numerous *cis* elements and relies on cooperative interaction of numerous transcription factors to regulate PR gene expression (277). During the menstrual cycle *PR* gene expression is controlled by fluctuating levels of oestrogen and progesterone. During the proliferative phase, *PR* gene expression in the

endometrium is under control of oestrogen which induces PR synthesis (124). Regulation of the *PR* gene by oestrogen is a result of recruitment of ligand activated ERα to a region in the PR promoter which contains an ERE half site and two adjacent Sp1 sites (236, 277) and gel mobility shift assays have shown that ERα greatly enhances the interaction of the transcription factor Sp1 at the PR promoter (278). In female ERαKO mice, PR mRNA is detected at 60% of wild type levels and PR expression cannot be induced by oestrogen treatment, underlining the importance of ERα in mediating PR expression (279). During the secretory phase maximal activation of PR due to elevated circulating levels of progesterone results in reduced proliferation and increased differentiation of the endometrium as a result of negative feedback on expression of ERα (280). Evidence from primate models using an artificially induced menstrual cycle, has shown that progesterone down-regulates expression of both ERα and PR proteins (281). The importance of progesterone in mediating these functions in human endometrium was confirmed in a study in which administration of the progesterone antagonist RU486 to women in the early secretory phase prevented progesterone-induced down regulation of PR and ERα (282). Immunohistochemical studies have revealed that in human endometrium PR-A and PR-B protein are both expressed in the glandular epithelium and stroma in the functional layer of the endometrium during the proliferative phase (283). However, during the secretory phase, overall expression of PR-A and PR-B in the epithelium of the functional layer is decreased compared to the proliferative phase (283) while PR expression is retained in the basal layer (284). PR-B is detected in stromal cells in the functional layer until the mid-secretory phase but thereafter only PR-A is identified (153). Epithelial PR-B in the functional layer is the predominant isoform in the mid-secretory phase but is absent from the epithelium thereafter (153). In mice, immunohistochemical analysis of PR expression has revealed that PR (isoform not specified as antibody used recognized both isoforms) is increased in the luminal epithelium and stroma in the pre-implantation phase and is absent from the uterine epithelium following implantation suggesting an active role for PR during implantation (285). Similarly, temporal expression of PR-B in mid-secretory human endometrial epithelium (i.e. during the implantation window) described previously suggests this isoform may be important in mediating implantation in human.

In the PR knockout (PRKO) mouse both isoforms of PR are ablated and female PRKO mice are infertile due to numerous defects in the hypogonadal-pituitary axis, ovary and uterus (121). For example, in the uterus of PRKO mice, lack of PR expression leads to an impairment of decidualisation and implantation resulting in infertility (121). Steroid hormone receptor cross-talk is essential to mediating normal uterine function, thus, PRKO

mice have abnormal responses to ovarian stimulation and unopposed oestrogen results in uterine hyperplasia, inflammation and hyperproliferation consistent with loss of expression of PR removing the inhibitory effect of PR on expression of ERα in the uterus (286). PR isoform specific ablations have revealed the specific roles of PR-A and PR-B in uterine physiology. Notably, PR-A knockout (PRAKO) mice are infertile and retain uterine defects in decidualisation and implantation present in the global PRKO. PR-A is reported to be essential in mediating down-regulation of the epithelial glycoprotein mucin-1, which is essential for implantation in mice (17) and loss of this PR-A mediated transcriptional regulation may in part account for the implantation phenotype. However, epithelial proliferation following progesterone administration is retained in PRAKO mice suggesting PR-B may be the isoform responsible for mediating this response to progesterone (287). PRBKO mice are fertile suggesting PR-A is the essential isoform for uterine regulation and function (288). Consistent with greater importance of PR-A, in the mouse uterus the ratio of uterine PR-A: PR-B protein is 3:1 (289).

1.5.6. Expression and functional significance of other members of the nuclear receptor superfamily.

In addition to the well defined roles of oestrogen and progesterone receptor signalling outlined in the previous sections, there is emerging evidence of a role for other members of the nuclear receptor superfamily in regulating endometrial function.

Androgens are essential regulators of male fertility (198); in addition, ARs are expressed in female reproductive tissues including the ovary (199) and endometrium (200). ARs are activated by endogenous androgens such as testosterone and the non-aromatisable androgen dihydrotestosterone (DHT) which is a potent AR agonist (290). ARs are expressed in the endometrium throughout the menstrual cycle with peak expression in stromal cells during the proliferative phase (187, 291). AR expression is increased in epithelial cells coincident with progesterone withdrawal (1). In Ishikawa cells, an endometrial adenocarcinoma cell line, AR protein expression can be induced by E or DHT but is down-regulated by MPA or the anti-androgen hydroxyflutamide suggesting progestagens may down-regulate AR expression in the endometrium (292). Consistent with a role for progestagens in mediating down regulation of AR, in women using a levonorgestrel-releasing intra-uterine device lower concentrations of AR mRNA are detected in total endometrial homogenates compared to normally cycling untreated women (293). In a study by Nantermet et al, administration of DHT increased uterine wet weight in ovariectomised rats and microarray analysis revealed

overlapping gene regulation with E2 (294) suggesting androgens may be important in regulating uterine function in humans. Genome-wide expression profiling has revealed that AR regulates distinct gene networks in decidualised human ESC involving cell cycle regulation and cytoskeletal organization (295). In addition, a recent *in silico* analysis revealed a novel androgen regulated gene network in human endometrium associated with regulation of apoptosis (200).

In addition to ligand-activated NR3 subfamily members described previously the orphan oestrogen related receptors (NR3B1-3: ERRα, ERRβ, ERRγ) have important functional roles in regulating reproductive tissues (189, 201). ERRs share significant sequence homology with ERα and ERβ and are capable of binding EREs (296). Binding site recognition experiments have revealed a single consensus sequence that ERRα preferentially binds (ERR response element; ERRE) (297), which has an identical sequence to the consensus binding site of steroidogenic factor 1 (SF-1) (298). ERR monomers and the momomeric orphan nuclear receptor SF-1 preferentially recognise this binding half-site with high affinity (299). Although the ERR LBD exhibits significant amino acid sequence identity with ER LBD, ERRs are unable bind E2 as they lack key Cys residues involved in recognition of oestrogens. Although natural ligands to ERRs are yet to be identified the activity of ERRs can be modulated by synthetic inverse agonists such as diethylstilbesterol (DES) and the ERRα specific inverse agonist XCT790 (296, 300). The LBD in ERR contains an AF-2 domain that can interact with co-factors such as PPARγ and the co-repressor RIP140 (301). ERRs are capable of regulating the activity of other nuclear receptors via transcriptional cross-talk. For example, ERRβ can differentially impact on ERα-dependent gene expression (302) and androgen responsive genes can be down-regulated by inhibition of ERRα (303).

ERRs are expressed in the uterus. ERRα and ERRβ proteins have been localized to cell nuclei within the glandular epithelium, in stromal cells and in endothelial cells in human endometrium at all stages of the cycle (189, 304). Increased expression of ERRα is associated with decidualisation of human ESC *in vitro* and administration of the ERRα inverse agonist XCT790 is reported to inhibit mRNA expression of both the decidualisation marker IGFBP and ERRα itself (305). ERRβ is also expressed in immune cells in the human endometrium as evidenced by co-expression with CD45 (common leukocyte antigen) (304). In uNK cells, macrophages and endothelial cells in the endometrium, ERRβ is co-expressed with ERβ, while during the proliferative phase ERRβ is co-expressed with ERα in epithelial cells (304).

The orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor II (COUPTFII; NR2F2) is expressed in all major physiological systems with high concentrations of mRNA detected in the uterus and ovary (www.nursa.org/10.1621/datasets.02001). COUPTFII null mutants die before embryonic day 10.5 due to cardiovascular defects (306) however conditional ablation of COUPTFII has revealed important roles in reproductive function. Ablation of COUPTFII in the uterus results in defects in implantation and decidualisation (186, 280). Progesterone achieves control of uterine function through stromal and epithelial cross-talk and regulation of PR action is also mediated via other transcription factors such as COUPTFII expressed in the stroma and the signalling factor Indian hedgehog (Ihh) in the epithelium. Furthermore, COUPTFII is reported to be essential for PR mediated down-regulation of epithelial ERα expression which is essential for successful implantation and decidualisation (307). Ihh is a progesterone target that signals downstream to COUPTFII. Conditional knockout of Ihh in the uterine epithelium results in attachment and decidualisation failure underlying the importance of these factors in mediating PR action in the uterus (308).

1.6. Steroid metabolism and synthesis

Steroid biosynthesis occurs in many tissues throughout the body most notably in the gonads (ovary and testis) and the adrenals. There is emerging evidence to support a role for steroid biosynthesis within the normal endometrium in the regulation of fertility; expression of many steroidogenic enzymes has been reported in the endometrial cell types (summarised in Table 1.20). These observations suggest the expression of steroid metabolizing enzymes within tissue compartments in the endometrium can influence the bioavailability of different sex steroids in the local environment and thus influence the control of endometrial function through binding and activating steroid receptors.

	mRNA detection in human endometrium		References
	Stromal cells	**Epithelial cells**	
StAR	+	-	(309)
CYP11A1	+	+	(310)
CYP19A1	Low/absent	Low/absent	(311-313)
17βHSD1	+	+	(312)
17βHSD2	Highest in secretory phase	Highest in secretory phase, stronger than stromal	(312, 314)
17βHSD5	+/-	Highest in early secretory phase	(315, 316)
3βHSD	Not described	Weak in proliferative phase, moderate in secretory phase	(317)
STS	Reduced in late secretory phase	+	(312)
SRD5A1	+/-	All phases	(318, 319)

Table 1.2. Summary of mRNAs encoding proteins involved in steroid biosynthesis within the normal endometrium. For abbreviations see text.

Steroid biosynthesis involves a series of enzyme reactions that convert cholesterol into steroid hormones such as oestrogen and testosterone (see Figure 1.6). Steroidogenic acute regulatory protein (StAR) plays a significant role in steroidogenesis during the transport of cholesterol from the outer to the inner mitochondrial membrane facilitating conversion of cholesterol into pregnenelone as a result of side chain cleavage mediated by cholesterol side chain cleavage enzyme (P450scc, encoded by *CYP11A1* gene). This represents the critical initial steps in steroid hormone biosynthesis (320, 321). The actions of 3β hydroxysteroid dehydrogenase and 17α-Hydroxylase sequentially convert pregnenelone into androgens (via intermediates) which in turn are converted to oestrogens by the aromatase enzyme complex (322). 17β hydroxysteroid dehydrogenases (17βHSD) control the inter-conversion of oestrogens and androgens (323).

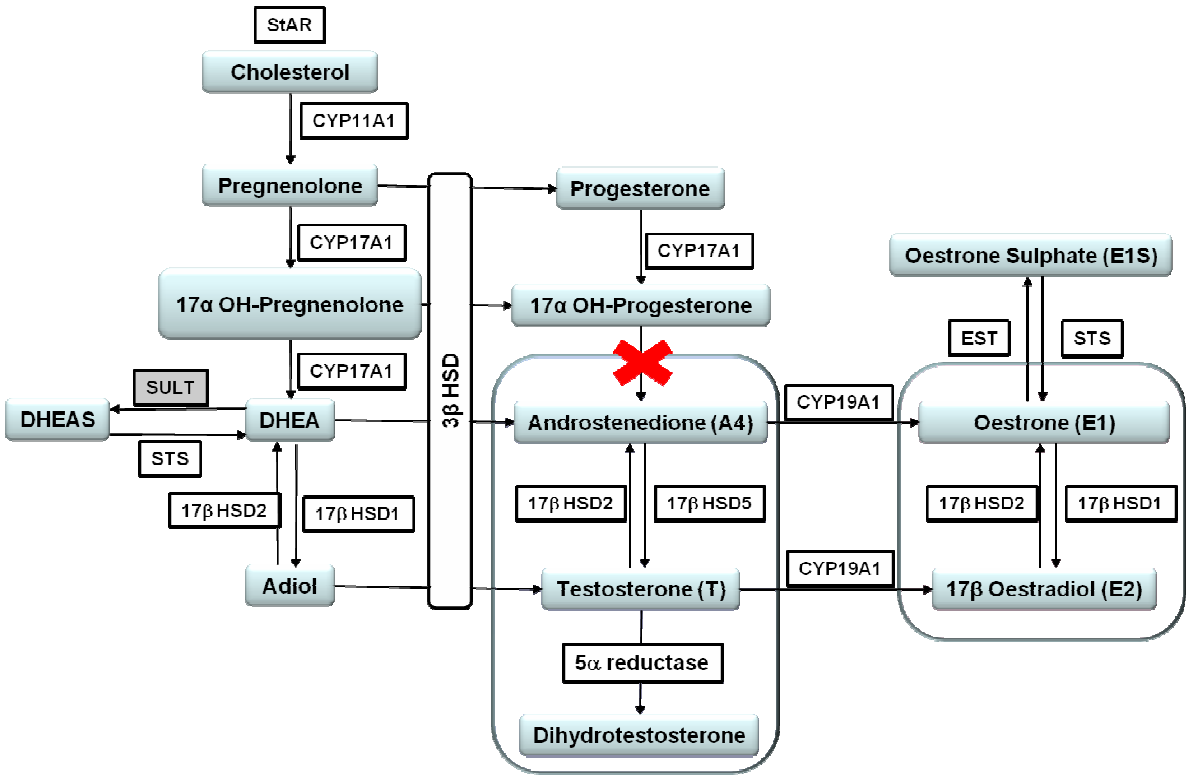

Figure 1.6. Schematic diagram illustrating the steroid metabolism pathway in the context of the endometrium. There is no conversion of 17α-progesterone to A4 in the human endometrium (red cross). EST: sulphotransferase (SULT1E1), STS: steroid sulphatase, SULT: sulphotransferase (SULT1A1).

Following side chain cleavage of cholesterol to pregnenelone by P450scc, 3βHSD catalyses the conversion of pregnenelone to progesterone and also catalyses the conversion of 17α-hydroxypregnenolone to 17α-hydroxyprogesterone and dihydroepiandrosterone (DHEA) to androstenedione (A4) (320). Western blot analysis has revealed 3βHSD protein is present in homogenates of total endometrial tissue (317). In addition, immunohistochemistry studies have revealed that in the human endometrium although some immuno-expression of 3βHSD can be detected in the glandular epithelium during the proliferative phase, expression in is markedly increased in the secretory phase (317).

17α-Hydroxylase (P45017α) is a single enzyme encoded by the *CYP17A1* gene which mediates both 17α-Hydroxylase and 17,20-lysae activities. 17α-hydroxy metabolites of progesterone and pregnenelone undergo cleavage by 17, 20-Lyase action to yield DHEA and A4, steroid precursors to oestrogens and androgens (324, 325). To date, there is no evidence for conversion of 17α-progesterone to A4 in the human endometrium (red cross on pathway, Figure 1.6).

Biosynthesis of oestrogen requires activities of the aromatase enzyme (also known as P450 aromatase, P450arom) complex which contains the aromatase cytochrome P450 and the flavoprotein, NADPH-cytochrome P-450 reductase (326). The aromatase enzyme complex acts by aromatising androgen precursors into oestrogens. In humans the aromatase protein is encoded by a single gene (*CYP19A1*) and expression is regulated by tissue specific promoters (327). Targeted disruption of the *CYP19A1* gene or inhibition of aromatase by inhibitors such as Letrozole (Femara®, Novartis) or Anastrozole (Arimidex®, AstraZeneca) reduces bioavailable oestrogen (328). The *CYP19A1* gene is expressed in a number of cell types throughout the body with key sites being; ovarian granulosa cells, the placental syncitiotrophoblast and the testicular Leydig cells (328). In addition aromatase expression has been localised to extraglandular sites such as the brain, skin and adipose (322), as well as in cancers of breast, endometrium and prostate. Interestingly, adipose tissue is the primary site of oestrogen production in post menopausal women (329).

17β hydroxysteroid dehydrogenases (17βHSD) are an important class of enzymes that catalyse the inter-conversion of oestrogens and androgens (see Figure 1.7) (323). Isozymes of 17βHSD permit conversion of sex steroids in a cellular and isotype specific manner (320).

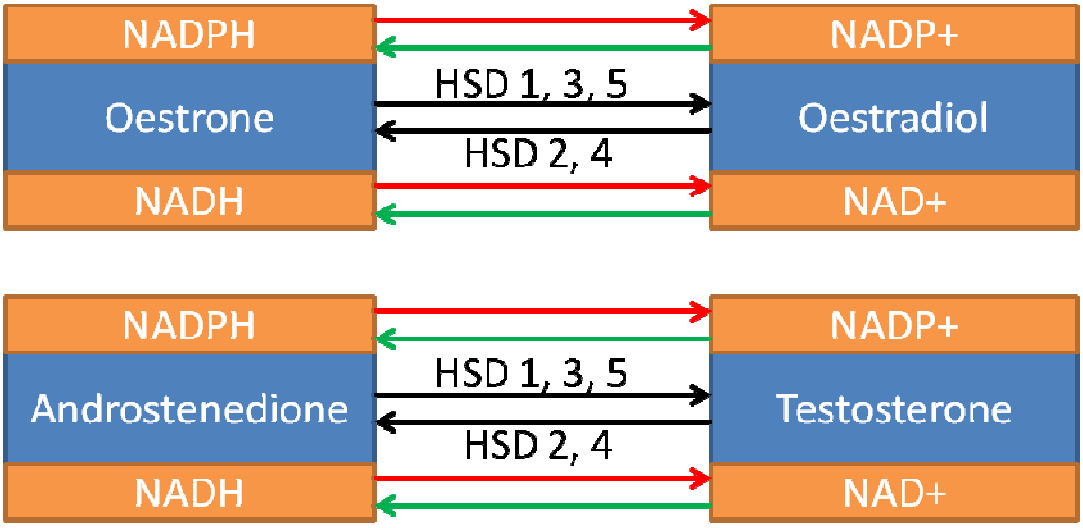

Figure 1.7. Inter-conversion of oestrogens and androgens mediated by 17βHSDs (HSD) and co-factors (orange boxes). Redox reactions of co-factors are indicated by coloured arrows. Green arrows indicates oxidation and red arrows indicate reduction.

17βHSD1 catalyses the reduction of Oestrone (E1) to oestradiol (E2) and androstenedione (A4) to testosterone (T) (323). The proximal promoter of the human 17βHSD1 gene contains

AP-1, Sp-1 and Sp-3 sites (330) and transcription could therefore be affected by steroid receptor interaction (see section 1.5). Expression of 17βHSD1 mRNA is low in normal endometrium although intense protein expression in both stromal and epithelial cells has been detected by immunohistochemistry (312). Concentrations of 17βHSD1 protein do not appear to vary during the menstrual cycle (312) although cAMP treatment increases 17βHSD1 mRNA expression in eutopic endometrial stromal cells from women with endometriosis (313).

17βHSD2 catalyses the oxidation of E2 to E1 and T to A4 (323). 17βHSD2 mRNA expression has been identified in human endometrium and concentrations are highest in the secretory phase of the menstrual cycle (314). Immunoexpression of 17βHSD2 is increased in early and mid-secretory endometrium with positive staining in glandular epithelium and some stromal cells (312). Sp-1 and Sp-3 have been shown to interact with specific motifs in the 17βHSD2 gene promoter to cause up-regulation of 17βHSD2 gene expression in endometrial epithelial cell lines (331). In endometrial explants progesterone induces 17βHSD2 expression. (312)

Androstenedione is reduced to testosterone by 17βHSD3 in the testes and 17βHSD5 in peripheral tissues (332). 17βHSD5 (also known as AKR1C3 due to its aldo/keto reductase activity) is expressed in the endometrium throughout the menstrual cycle with highest concentrations of mRNA detected in the early secretory phase (315). Reports of protein expression have been contradictory, with expression reported to be limited to glandular and luminal epithelium in one study (315) while Hevir et al demonstrated immunopositive staining in both stromal and epithelial cells in another (316).

SRD5A1, also referred to as 5α reductase, is an enzyme responsible for 5α reduction of steroids. 5α reductase has been shown to reduce testosterone to the more potent androgen dihydrotestosterone (DHT) in human endometrium (333) and SRD5A1 has also been shown to be very active in the metabolism of progesterone in the endometrium (334). Reports of protein expression in the endometrium have been contradictory, with expression reported by some to be limited to epithelial cells (318) while others have demonstrated immunopositive staining in both stromal and epithelial cells (319).

Steroid sulphatase (STS) hydrolyses sulphated steroids, which circulate at significant concentrations but are unable to bind and activate steroid receptors, into biologically active steroids (335). STS primarily converts oestrone sulphate to oestrone. STS immunoexpression has been detected in both stromal and epithelial cells in the endometrium across the

menstrual cycle and intensity is reduced in the late secretory phase (312). Sulfotransferase (EST) catalyses the opposite reaction to STS, namely, sulphation (inactivation) of steroids and EST mRNA is detected at all stages of the cycle in total endometrial extracts (312). Furthermore, EST mRNA concentrations are increased by progesterone in endometrial explant culture (312). In normal endometrium, concentrations of mRNAs for STS are higher than EST (336) which suggests the net effect of STS and EST would favour an increase the bioavailability of steroids that are capable of binding and activating steroid receptors in the endometrium. In addition to oestrogen forming activities, STS also has the capacity to hydrolyse DHEA sulphate (DHEAS) to DHEA which may also act as a precursor for the formation of oestrogens and androgens (337).

1.6.1. Steroid metabolism in the endometrium

In addition to measurement of proteins and mRNAs studies on steroid metabolism have revealed an extensive capacity for interconversion between androgens and oestrogens in human endometrial cells and tissue. A study by Collins et al (338) showed that in normal endometrial explants metabolism favours formation of oestrone and androstenedione over oestradiol and testosterone respectively indicative of oxidative 17βHSD activity. Consistent with reactions that favoured oxidative 17βHSD activity, little conversion of oestrone to oestradiol is detected (339). Collins et al also reported that a small percentage of DHEA was transformed into A4, 5α-androstanedione and androsterone (338).

The dynamics of oestrogen and androgen uptake in human endometrial tissue also appears to favour oxidative 17βHSD activity. A study by Gurpide et al (340) investigated the dynamics of sex steroid uptake in endometrial explants perfused with media containing radioactively labelled sex steroids. This study revealed that not only were high levels of oestrogen and androgen were taken up into the endometrial tissue but the net uptake of E2 and T was higher than that of E1 and A4. Interestingly, the authors reported that most of the E1 and A4 that perfused the endometrial tissue was released from the tissue unchanged. Conversely, following tissue uptake, a high proportion of E2 and T was released from the tissue metabolised into E1 and A4 respectively consistent with the oxidative 17βHSD activity reported by Collins et al (340). The fluctuating concentrations in blood perfusing the tissue as a result of changes in steroid production by the ovaries during the menstrual cycle may also influence the rate of metabolism. For example, 17βHSD oxidation of E2 to E1 was reported by Krishnan et al to be higher in the proliferative than secretory phase (341).

However, other studies have suggested that oxidative 17βHSD activity is highest in the secretory phase (342, 343).

The capacity for aromatase activity in endometrial tissue fragments has been described by Tseng et al (344) who showed that incubation of endometrial tissue fragments with labelled testosterone results in formation of E1 (60-90%) and E2 (10-40%) in tissue fragments from proliferative phase endometrium, although the most abundant metabolites seen when the experiment was repeated with secretory phase endometrium were E1S (50-80%) and E1 (10-40%) (344). Further studies from the same laboratory have shown that progesterone stimulates aromatase activity in endometrial stromal cells *in vitro* and this can be potentiated by oestrogen (345). In addition, the synthetic progestin MPA has been shown to stimulate endometrial stromal cell aromatase activity *in vitro*, an effect which could be inhibited administration by the anti-progestin RU486 (346). However, subsequent studies have contradicted these findings and claimed that there is little capacity for aromatase activity in normal endometrial stromal cells (311).

Testosterone is converted to its 5α-reduced metabolite 5α-DHT in human endometrial homogenates cultured with labelled testosterone (333). In endometrial tissue explants perfused with A4, the majority of A4 is metabolised by 5α reduction to androsterone and 5α-androstanedione (347). Endometrial explants infused with DHEAS convert around 16% of DHEAS to DHEA, of which a further 3% is converted to A4 (348). Reduction of A4 to testosterone by 17βHSD activity is also detected in endometrial tissue explant cultures and the rate of this conversion is increased in secretory compared to proliferative endometrium (347, 348).These data suggest an extensive capacity for androgen metabolism in the human endometrium which has received very little attention to date. Interestingly, a recent study has shown that A4 up-regulates aromatase mRNA expression in human endometrial stromal cells and explants (349) underlining the importance of androgen metabolism in modulating the steroid microenvironment in the endometrium.

A recent study revealed an essential requirement for aromatase activity during decidualisation in mice. Administration of the aromatase inhibitor letrozole to ovx, progesterone supplemented, pregnant mice was found to decrease neovascularisation and also blocked the decidualisation reaction (128). These data suggest that *de novo* synthesis of oestrogen is essential during the critical processes of implantation and decidualisation in mice, processes which are both known to be steroid regulated. This is consistent with the concept of ‘intracrinology’ first postulated by Labrie (350, 351). Labrie proposed that the

presence of steroid biosynthetic enzymes in tissues would allow for local conversion of inactive precursors and formation of active steroids thereby creating a tissue microenvironment in which steroids are present at levels which are different to those found in blood as a result of biosynthesis within the gonads and adrenal gland. Local formation of active steroids at high concentrations could have far reaching consequences by increasing the capacity for interaction and activation of steroid receptors resulting in alterations in functional responses. Thus intracrinology could be particularly important in mediating control of endometrial function due to the complex, multi-cellular nature of the tissue. For example, changes in local bioavailability of oestrogens could have autocrine and/or paracrine impacts on several cell types due to the close juxtaposition of stromal, epithelial, endothelial and immune cells, all of which express ERs. Despite the recent findings in mice, a requirement for local oestrogen production during decidualisation in humans is yet to be elucidated.

1.6.2. Changes in steroid metabolism detected in reproductive health disorders.

Aberrant expression of enzymes which mediate steroid metabolism and biosynthesis are features of many reproductive malignancies.

Uterine fibroids (leiomyomas) are benign tumours that arise as a result of aberrant proliferation of uterine smooth muscle tissue and are a common cause of pre-menopausal hysterectomy (352). Increased risk of fibroid development is associated with conditions associated with increased exposure to oestrogens such as obesity, nulliparity as well as breast and endometrial cancer (353). Tissue concentrations of oestrogens are elevated in leiomyomas (354) and oestrogen is thought to drive proliferation of uterine smooth muscle cells via an intracrine mechanism. Sumitani et al have reported that aromatase mRNA and protein is over-expressed in leiomyomas compared to normal myometrium (355). In addition, Sumitani et al have shown that in cultured smooth muscle cells isolated from leiomyomas aromatase expression is immunolocalised to cytoplasmic granules and following stimulation these cells are capable of converting A4 to E1, an effect which was reported to be inhibited by an aromatase inhibitor (355). In the same study, administration of A4 and T but not the non-aromatisable androgen DHT stimulated the growth of cultured leiomyoma smooth muscle cells to the same extent as E2. The stimulatory effect of T on cell growth was shown to be aromatase dependent as it was abolished by co-treatment with an aromatase inhibitor (355). Shozu et al reported that administration of the aromatase inhibitor Fadrozole

(Afema®, Novartis) reduced leiomyoma volume by 71% after 8 weeks of treatment in a patient case report providing *in vivo* evidence for the requirement of local oestrogen biosynthesis in the growth of uterine fibroids (356) .

Endometrial cancer is the most common gynaecological malignancy and unopposed oestrogen exposure predisposes risk of developing the disease (357). Concentrations of oestradiol measured in endometrial cancer tumour tissues are higher than those of disease free endometrium (358). Aromatase is expressed in endometrial cancer tissue and aromatase inhibitors have been used to treat endometrial cancer (359). Rizner et al showed that dysregulation of 17βHSD5 is associated with endometrial cancer with increased mRNA expression in malignant compared to normal endometrium (360). Yamamoto et al reported that both aromatase and STS activity is higher in endometrial cancer tissues preparations compared to normal endometrium (361). High levels of STS mRNA expression have been detected in Ishikawa cells (an endometrial adenocarcinoma cell line) (336) and STS activity has been demonstrated by thin layer chromatography in RL-95 an endometrial carcinoma cell line (335). These data suggest that dysregulation of steroid metabolism is a feature of the pathophysiology of endometrial cancer.

Polycystic ovarian syndrome (PCOS) is an endocrine disorder affecting women of reproductive age and is associated with menstrual cycle disturbances, hyperandrogenism and infertility (362). Excess androgen production in PCOS is clinically associated with amenorrhea, infertility, hirsutism and acne (363). In the human ovarian follicle, androgen production arises from the theca cells which express; StAR, CYP11A1, CYP17A and 3βHSD, the enzymes responsible for converting cholesterol into androgens (364-366). PCOS is associated with dysregulation of steroid metabolism. Nelson et al reported that cultures of theca cells isolated from PCOS ovaries produce higher levels of progesterone, 17α-hydroxyprogesterone and testosterone compared to theca cells from normal ovaries (367). In the same study the metabolism of progesterone, pregenolone and DHEA, all of which are precursors to androgens, was less in PCOS theca cells than theca cells from normal ovaries, which may contribute to excess androgen production due to the increased bioavailability of androgen precursors. A subsequent study by Nelson et al reported that mRNA expression and activity of 17βHSD5, which may convert A4 to T, is similar in normal and PCOS-derived theca cells suggesting 17βHSD5 is not responsible for the increased androgens observed in PCOS (368). However, the activity of 3βHSD and both 17α-hydroxylase and 17,20-lyase activity of CYP17 are reported to be increased in PCOS theca cells (367, 368).

These data suggest an enhanced capacity of PCOS theca cells to convert steroid precursors into T as a result of altered steroid metabolism.

Disorders of uterine bleeding may also be associated with alterations in steroid metabolism (369). Oestrogen is a known regulator of angiogenic factors such as VEGF (114) and in the endometrium steroidal regulation via receptor activation can be altered by changing ligand availability. This may be mediated by 17βHSD2 which converts E2 to the less potent E1, and thus may reduce activation of ERs as a result of decreased local E2 (323). Expression of 17βHSD2 is enhanced by progesterone in the secretory phase and expression decreases following progesterone withdrawal at menstruation or following administration of an antiprogestin (282, 312, 314). The contraceptive levonorgestrel is a synthetic progestin which binds both AR and PR (370). Use of levonorgestrel-releasing intrauterine system (LNG-IUS) is associated with increased 17βHSD2 mRNA expression (consistent with PR mediated up-regulation) within the first 3 months of use which declines thereafter as a result of decidualisation and down-regulation of PR (293). The first three months of LNG-IUS use is often associated with unscheduled breakthrough bleeding and this was proposed by Critchley et al to be due in part to "intracellular oestrogen deficiency" as a consequence of increased 17βHSD2 mRNA expression, leading to vascular fragility (369). This may also be important in heavy menstrual bleeding (HMB), a condition which is characterised by excessive blood loss and is a leading indication for hysterectomy (http://www.nice.org.uk/nicemedia/pdf/CG44FullGuideline.pdf.) Women with HMB appear to have lower VEGF levels than normal women (371). This may be a result of disrupted oestrogen metabolism and signalling. Treatment of HMB with LNG-IUS is highly effective in reducing menstrual blood loss from 3-12 months after insertion (372) possibly as a result of decreased 17βHSD2 expression which may in turn increase intracellular oestrogen stimulating production of VEGF and consequent improvement in vascular stability.

Endometriosis is classified as an oestrogen dependent disease that is characterised by the survival of endometrial tissue lesions in ectopic sites outside the uterus, dysregulated steroid enzyme expression and an increased capacity to produce oestrogens (373-376). Increased expression of aromatase mRNA and protein have been detected in the ectopic endometrium of women with ovarian, peritoneal and deep infiltrating endometriosis (312, 313, 375, 377). In stromal cells isolated from ovarian endometriosis (endometriomas), *CYP19A1* gene expression can be induced by prostaglandin E2 (PGE2) (378) and cAMP (313) both of which are also known to enhance decidualisation (123). In contrast, aromatase is reported to be absent or barely detectable in normal endometrium and only low levels are detected in

eutopic endometrium of women with endometriosis (311, 379). In ovarian endometriosis, 17βHSD1 mRNA concentrations are increased (312, 380) while expression of 17βHSD2 is reported to be down regulated (374, 380). High levels of STS activity have been correlated with severity of peritoneal endometriosis (381) and increased *SRD5A1* gene expression in ovarian endometriosis (316). Hevir et al reported a slight increase in 17βHSD5 mRNA expression between endometriotic tissue and normal endometrium (316).

Women with congenital adrenal hyperplasia type V are deficient in 17α-Hydroxylase due to autosomal recessive mutation of the *CYP17A1* gene. The reproductive capacity of these women is severely reduced due to chronically elevated serum progesterone which suppresses endometrial proliferation (382).

1.6.3. Summary

In summary, the human endometrium expresses many of the enzymes required for biosynthesis of sex steroids and studies have shown that human endometrium has the capacity for sex steroid metabolism, most likely by the oxidative activity of 17βHSD enzymes acting on oestrogens and androgens and 5α reductase acting on androgens and progesterone. The relative expression and activities of steroid metabolising enzymes can alter the local bioavailability steroids and thus their ability to regulate endometrial function through activation of steroid hormone receptors. Dysregulation of enzymes which mediate steroid metabolism are important features of the pathophysiology of reproductive malignancies. Aberrant expression of aromatase and local oestrogen biosynthesis are established features of uterine fibroids, endometriosis and endometrial cancer and as a result aromatase inhibitors have been utilised in clinical treatment of these disorders. There is emerging evidence from animal studies to suggest that aromatase activity may also be important in regulating decidualisation and neovascularisation (128) however a similar role in the human uterus is yet to be described.

1.7. Cytokines in the endometrium

Cytokines are regulatory peptides or glycoproteins with pleiotropic effects that typically alter tissue function in a paracrine and/or autocrine manner. Cytokines play key roles in regulation of reproduction with impacts on ovarian follicular development, endometrial development, implantation and trophoblast invasion. Cytokines are known to regulate uterine immune cells, including the uNK population, and their potential regulation by steroid hormones is detailed in the following section.

1.7.1. CXCL12/SDF-1

Stromal derived factor 1, SDF-1 (CXCL12) is a proliferative and chemotactic factor for lymphocytes as well as endothelial cells and haematopoietic progenitor cells (383-385). Tsutsumi et al. showed that expression of SDF-1 in human endometrial stromal cells is induced by E2 but this is blocked with the addition of the ER antagonist ICI or the progestin MPA (386). Progesterone has also been shown to inhibit E2-mediated increases in expression of SDF-1 by rat uterine cells (387). Although information about the pattern of SDF-1 expression in the endometrium is limited, recent evidence suggests it may be present in the epithelium; a recent study by Laird et al described positive immunoexpression of CXCL12 in the endometrial epithelium which was not altered with menstrual phase (388). In contrast, Kitaya et al. reported positive immunoexpression in decidua and intravascular trophoblasts but not decidual stroma or in endometrium (389). CXCL12 mRNA has also been reported in decidual stromal but not epithelial cells (37). CXCL12 is reported to increase the migration of pbNK and uNK cells (37) as well as the specific migration of CD16- NK cells (390). The receptor for CXCL12, CXCR4, is expressed on the surface of uNK cells (390) and throughout the endometrium particularly in epithelium and blood vessels walls but weak staining is also reported in the stroma (388). Laird et al reported that concentrations of mRNAs encoding CXCR4 are higher in endometrial biopsies from early proliferative phase compared to late proliferative and secretory phase (388). The CXCL12/CXCR4 signalling pathway is also reported to positively regulate oestrogen-regulated gene transcription (391). Using MCF-7 breast carcinoma cells Sauve et al reported that treatment with SDF-1 increased ER transcriptional activity and expression of ER target genes including SDF-1 itself (391).

1.7.2. Interleukin-1

Interleukin-1 (IL-1) is a pleiotropic cytokine which exists in two isoforms; IL-1α and IL-1β, both of which act via a common receptor IL-1R. IL-1 is produced by many cell types including fibroblasts, endothelial cells, keratinocytes, smooth muscle cells as well as cells in the endometrium and decidua (392). In the endometrium, IL-1β mRNA concentrations are maximal in the late secretory phase with the protein detected in stromal cells, macrophages and endothelial cells (393, 394). Serum levels of IL-1 are also increased at this time (395). IL-1α and IL-1β both inhibit decidualisation of endometrial stromal cells *in vitro* (396, 397). For example, IL-1β inhibits the production of prolactin and IGFBP-1 when added to media bathing ESC stimulated to decidualise *in vitro* (396). Paradoxically, IL-1β mRNA

concentrations are reported to be increased in decidualised stromal cells which suggests IL-1β may modulate the degree of decidualisation via an autocrine feedback mechanism in ESC (396). IL-1β protein is also abundant in the uterus during early pregnancy and its immunoexpression is detected in syncitiotrophoblast, cytotrophoblast, decidual stromal cells and activated macrophages which may indicate a role in establishment of pregnancy (398). Indeed, secretion of IL-1β by cultured cytotrophoblast cells is reported to be directly proportional to their invasiveness possibly by modulating increases in matrix metalloproteinase activity in trophoblast cells (399).

IL-1β may also regulate immune populations in the uterus, in addition to expression in macrophages, IL-1 is reported to stimulate uNK cells to increase secretion of GM-CSF *in vitro* (400). Okada et al have demonstrated that IL-1β inhibits progesterone mediated IL-15 secretion by endometrial stromal cells *in vitro* during decidualisation (401) which could have an impact on uNK cell populations *in vivo* as IL-15 is essential for uNK migration and proliferation (401). IL-1β also increases secretion of monocyte chemoattractant protein-1 (MCP-1) by first trimester decidual stromal cells which may also influence the recruitment of macrophages to the uterus (402). In addition, IL-1 increases endometrial stromal cell expression of IL-8, which is an important mediator of angiogenesis and leukocyte chemotaxis (403). Low levels of E2 are reported to decrease amounts of IL-1β detected in whole blood (404), whereas pharmacological doses of E2 (10^{-6}M) are reported to increase IL-1 secretion by peripheral blood mononuclear cells (405). E2 has also been shown to increase secretion of IL-1β by rat macrophages (406). Furthermore, oestradiol inhibits IL-1β mediated responses in uterine epithelial cells through down-regulation of IL-1R type 1 (407). In summary, regulation of IL-1β by sex steroid hormones could have important impacts on the control of reproductive processes which could directly influence decidualisation and indirectly modulate immune populations by altering the cytokine milieu.

1.7.3. Interleukin-8

Interleukin-8 (IL-8) has a functional role in promoting both angiogenesis and in chemotaxis of leukocytes; particularly neutrophils (408, 409). It acts via the surface membrane receptors CXCR1 and CXCR2. IL-8 is expressed in perivascular cells in late secretory phase endometrium (410) and its mRNA is reported to be highest in tissue extracts prepared from late-secretory and mid-proliferative phase endometrium (411). IL-8 is reported to increase proliferation of endometrial stromal cells (412) and production of IL-8 in endometrial epithelial and stromal cells is induced by IL-1α and TNFα (403). Progesterone enhances the

IL-1 mediated increase in concentrations of IL-8 mRNA in ESC (403), however progesterone treatment alone is reported to reduce IL-8 secretion in endometrial explants (413). IL-8 secretion by uNK cells is reported to increase the invasiveness of extra-villous trophoblast cells treated with uNK conditioned supernatants in an *in vitro* model; an effect which could be partially abrogated by addition of an IL-8 neutralising antibody to the uNK supernatant (59). Interestingly, treatment of ESC with leukocyte conditioned media is reported to up regulate IL-8 gene expression (414).

1.7.4. Interkeukin-11

Interkeukin-11 is a pleiotrophic cytokine which acts on a range of different cells and tissues including haematopoietic cells, the nervous system and gastrointestinal tract (415). IL-11 signals via a heterodimeric receptor complex comprising the signalling component gp130 and the IL-11 receptor α chain (IL-11Rα) (416). In the human endometrium, IL-11 and IL-11Rα are expressed in all cell types and immunoexpression of IL-11 is reported to be highest in decidualised stroma cells in secretory phase (417, 418). In addition, *in situ* hybridization studies have revealed that gp130 is detected throughout the cycle restricted to the luminal and glandular epithelium of the human endometrium (16). Targeted mutation of IL-11Rα in mice results in infertility due to impaired decidualisation during blastocyst implantation (118). Expression of IL-11 and IL-11Rα also enhances progesterone mediated decidualisation of human endometrial stromal cells (419), and neutralisation of IL-11 by an anti-human IL-11 antibody inhibits the secretion of IGFBP and prolactin during *in vitro* decidualisation (419). Furthermore, IL-11 is also increased following cAMP-induced decidualisation and is enhanced by relaxin and prostaglandin E2 via a cAMP/PKA-dependent signalling (420). IL-11 is considered essential to differentiation of the endometrium and is one of few factors identified as being essential for implantation. Altered expression of IL-11 is also a feature of pathophysiologies, for example, loss of IL-11 expression in glandular epithelium is a feature of infertility associated with endometriosis (421). Studies in mouse models suggest IL-11 may also influence immune populations and notably IL11Rα null mice uNK cells are virtually absent from implantation sites (422). However, the impact of IL-11 on uNK populations in human endometrium is unknown.

1.7.5. Interleukin-15

Interleukin-15 (IL-15) is a cytokine which can regulate both proliferation and activation of NK cells (423). Okada et al (424) reported that IL-15 protein is secreted by endometrial stromal cells *in vitro* following treatment with progesterone. In the same study the authors

also reported that progesterone induces IL-15 mRNA expression in a dose-dependent manner. Progesterone combined with oestradiol also increased IL-15 mRNA expression however treatment with E2 alone did not (424). In addition, expression of mRNAs encoding IL-15 are reported to be induced by cAMP, PGE2 and IFNγ in endometrial stromal cells *in vitro* (35) suggesting a complex interplay of signals. Verma et al demonstrated that IL-15 induces proliferation of isolated uNK cells (423) while in an *in vitro* migration assay IL-15 enhanced recruitment of CD16- pbNK cells (425). IL-15 may be important in decidualisation as IL-15 mRNA and protein expression is reported to be increased during the secretory phase of the menstrual cycle and in first trimester decidua where it is prominent in perivascular sites surrounding spiral arterioles (36, 426). Evidence from studies in mouse models suggests IL-15 is important in immune cell recruitment and endometrial remodelling in response to implantation and decidualisation. For example, IL-15 is reported to be essential for uNK homing to implantation sites in mice (427) and IL-15 null mice are NK-cell deficient, additionally, these IL15 deficient mice have decidual abnormalities with arterial pathologies (428, 429). In humans, bone marrow derived CD34+ progenitor cells can be differentiated into CD56+ NK cells following *in vitro* culture with IL-15 (32). IL-15 is also reported to increase CD56 expression in CD16- pbNK and induce a chemokine receptor repertoire similar to uNK (37) underlying the importance of this cytokine in uNK function. In addition, IL-15 is reported to enhance in vitro expansion of regulatory T cells (Tregs), which are enriched in decidua, a response which was synergistically increased when administered in combination with IL-2 (430, 431).

1.7.6. Interleukin-18

The expression of the interleukin-18 (IL-18) system; comprising IL-18, IL-18 binding protein (IL18-BP) and IL-18 receptor (IL-18R) has been reported in human endometrium (432). IL-18 is structurally similar to IL-1 cytokines and requires cleavage by IL-1 converting enzyme (also known as caspase-1) to generate a biologically active form (433). IL-18BP is the natural inhibitor of IL-18 which neutralizes IL-18 effector functions (434). IL-18 has pro-inflammatory properties and induces production of IL-1β, TNFα, chemokines and prostaglandins (435). Expression of mRNAs encoding IL-18, IL-18BP and IL-18R has been reported in total RNA extracted from endometrial tissue however expression did not vary across the menstrual cycle in this study (432). In isolated endometrial cell cultures, Western blot analysis revealed that IL-18 protein is present in cultured endometrial epithelial cells but not stromal cells (432). Interestingly, in the same study, IL-18BP was detected in cell isolated from both cellular compartments (432). IL-18 appears to be steroid regulated in

humans as mRNA expression of IL-18 and IL-18BP are decreased following addition of exogenous ovarian hormones in women undergoing steroid substituted cycles as part of IVF embryo transfer protocols (436). Ledee et al (436) reported a comparison of mid luteal biopsies from natural cycles compared to mid luteal biopsies from the same patient following a steroid substituted cycle, i.e. 14 days exogenous oestradiol, followed by combined oestradiol and progesterone from days 14 to 21 with biopsy on day 21. The comparison of date matched endometrial tissue from a natural cycle and a steroid substituted cycle revealed that treatment with exogenous steroids significantly reduced the expression of mRNAs encoding IL-18 and IL-18BP (436) suggesting supraphysiological levels of oestrogen and progesterone may down-regulate IL-18 and IL-18BP mRNA expression *in vivo*. In a study comparing secretory phase biopsies from women who failed to become pregnant after repeated IVF cycles with secretory phase biopsies from fertile controls, the numbers of $CD56^{bright}$ cells were significantly greater in infertile women and these differences were significantly and positively correlated with IL-15 and IL-18 mRNA expression levels, (437). These data suggest that steroid concentrations and cytokine expression may be important in regulating uNK cells in successful reproductive function.

1.7.7. Interferon gamma

Interferon γ (IFNγ) is expressed in many cell types and is a potent stimulator of cytokine secretion by endometrial stromal cells (438). Immunohistochemistry studies in the human endometrium have revealed that secreted IFNγ protein is detected throughout the stroma and is concentrated beneath luminal epithelium and adjacent to glandular epithelium that is proximal to the lumen (439). In the same study the authors reported that detection of IFNγ did not vary with menstrual cycle phase or between pre-menopausal and post menopausal samples of endometrium suggesting that IFNγ secretion is not affected by fluctuations in ovarian hormone concentrations. T cells are reported to secrete IFNγ (440). In human endometrium, lymphoid aggregates, which predominantly contain T cells, were initially thought to be a likely source of IFNγ in the endometrium (439, 441, 442). However, immunohistochemistry co-expression studies have demonstrated no dual positive staining of IFNγ positive cells in the human endometrium with antigens to T, macrophage or NK cell markers and the authors concluded that IFNγ positive cells in the endometrial stroma were likely to be polymorphonuclear cells based on nuclear morphology and surface marker staining (439). However, uNK cells isolated from decidua express mRNAs encoding IFNγ and secreted IFNγ is detected in cell culture supernatants from this cell type (40) calling into question the data from immunostaining. In NK-deficient mice, spiral artery remodelling is

impaired; however administration of IFNγ was able to rescue this phenotype (443) consistent with a role for NK-derived IFNγ in regulating spiral arteries in this species. In addition, IFNγ directly regulates gene expression in human uterine microvascular endothelial cells (444) and also reported to directly decrease the proliferation of human endometrial epithelial cells *in vitro* (445). NK-derived IFNγ may also mediate maturation of dendritic cells in the human endometrium (62) and IFNγ from decidual uNK cells is reported to inhibit migration of cytotrophoblast cells *in vitro* (446). In activated murine spleen cells as well as cultured T-cell line transiently transfected with oestrogen receptor treatment with E2 increases concentrations of IFNγ mRNA consistent with transcriptional regulation of the gene promoter (447). In addition, studies in mice with disrupted ERα and ERβ genes have revealed that ERα but not ERβ is required for enhanced E2 mediated Th1 cell responsiveness, which includes secretion of IFNγ (448).

1.7.8. Monocyte chemotactic protein-1/CCL2

Monocyte chemotactic protein-1 (MCP-1; CCL2) is β cytokine which is a potent chemoattractant and activator of immune cells including monocytes, macrophages, T cells, basophils, mast cells and NK cells (449). *In vitro* incubation of human cells has revealed that MCP-1 is secreted by endothelial cells, fibroblasts, monocytes and lymphocytes (450, 451). In the human endometrium, immunopositive staining for MCP-1 is detected in epithelial and endothelial cells but not stromal cells (452). Cultures of human uterine epithelial cells are reported to secrete MCP-1 and epithelial cell conditioned media has been shown to increase the chemotaxis of monocytes (453). Immunohistochemistry studies have demonstrated intense staining of MCP-1 in perivascular areas in human endometrium and decidua which *in vivo* may enhance chemotaxis of immune cells to the endometrium by stimulating extravasation of leukocytes at these sites (454). Expression of MCP-1 is reported to be inhibited by sex steroids in cultures of endometrial stromal cells *in vitro* with treatment with E2 inhibiting expression of MCP-1 mRNA and protein (455). Progesterone also decreases MCP-1 mRNA and protein in cultured ESC but to a lesser extent than E2 (455). In mice genetically ablated for CCR2, the MCP-1 receptor, the number and distribution of uNK cells in pregnancy were similar to those observed in wild-type mice suggesting MCP-1 is dispensable for the migration and distribution of uNK at least during murine pregnancy (456).

1.7.9. Macrophage inhibitory protein-1β/CCL4

Macrophage inhibitory protein-1β (MIP1β; CCL4) protein and mRNA have both been detected in the human endometrium throughout the menstrual cycle with expression being highest during the secretory phase (457). Consistent with increased expression in the secretory phase, CCL4 mRNA is reported to be induced by progesterone in cultured endometrial stromal cells (457). However, in cultures of endometrial tissue slices, E2 is reported to have no impact on concentrations CCL4 mRNA (458). CCL4 is reported to be chemoattractant to CD16- pbNK cells (459) and FACS analysis has demonstrated that human $CD56^{+}CD16^{-}$ pbNK cells express CCR5, the CCL4 receptor (460). Using an *in vitro* migration assay and a neutralization assay with specific blocking antibodies, Kitaya et al (425) reported that CCL4, CXCL9 and CXCL10 from secretory phase endometrium selectively recruited CD16- pbNK cells. Ablation of CCR5 does not appear to have an impact on the number and distribution of uNK cells in mice (456). However, a recent gene array study on human endometrial tissue identified CCL4 as being up-regulated during the period of endometrial receptivity and early pregnancy (461). CCL4 may therefore have importance in the establishment of pregnancy in human, possibly by regulating the numbers of uNK cells at the implantation site by enhancing selective recruitment of CD16- pbNK cells to the uterus.

1.8. General conclusions and aims of the study

The endometrium is a multi-cellular tissue that is the source of a variety of signalling molecules including steroid hormones, cytokines and growth factors which act in an intracrine, autocrine or paracrine manner to regulate reproductive processes. In the endometrium implantation, decidualisation, immune cell trafficking and remodelling of the feto-maternal interface during pregnancy are orchestrated by the action of these signalling factors. A variety of immune cells are present in high concentrations in the uterus but the predominant uNK cell is known to be a major contributor to local control of the uterine environment and is believed to play an essential role in the successful establishment and maintenance of pregnancy.

The human endometrium expresses many of the enzymes required for biosynthesis and metabolism of sex steroids. There is emerging evidence to suggest that aromatase activity may be important in normal uterine function and a recent study in mice suggested that *de novo* biosynthesis of oestrogen within the uterus may play an essential role in regulation of

decidualisation and neovascularisation (128), however a similar role in the human uterus is yet to be described. The regulation of expression of steroidogenic enzymes in the endometrium is likely to be important in controlling the local bioavailability of sex steroid hormones which can in turn activate steroid hormone receptors and mediate control of endometrial function. The functional changes that occur in the endometrium during decidualisation including angiogenesis and leukocyte recruitment are essential to implantation and establishment of pregnancy but their control is poorly understood. Cytokine expression and secretion by different cell types in the endometrium may be influenced by the concentrations of sex steroid hormones. Cytokines produced within endometrial tissue may act on multiple cell types and may influence the recruitment/differentiation of immune cell populations, such as uNK cells, and in the formation and function of the vascular compartment. Endometrial endothelial and uNK cells both contain the oestrogen receptor beta isoforms but the impact of oestrogens on their function has not been explored. We hypothesize that decidualisation of the endometrium results in an altered local steroid environment characterised by secretion of high concentrations of oestrogens which consequently influence the dynamic interactions of cell populations within the endometrium.

In the current studies three questions have been addressed:

1. Is oestrogen biosynthesis a feature of human endometrial stromal cell decidualisation?

2. What is the impact of oestrogen on uNK cell function?

3. What role (if any) does oestrogen play in the interplay between decidual, immune and vascular cells within the human endometrial stroma?

The present study utilised primary human endometrial stromal cells, immortalised endometrial stromal cells (SHT 290) and uNK cells isolated from primary human first trimester decidua. Following *in vitro* decidualisation the expression of mRNAs encoding enzymes important in steroid synthesis and metabolism was assessed using Taqman Q-RT-PCR in ESC. Secretion of the decidualisation marker IGBFP-1 and the presence of oestrogens in culture supernatants was determined by ELISA and the enzyme activity of undecidualised and decidualised SHT 290 cells was assessed using thin layer chromatography. Isolated uNK cells were treated with or without oestradiol for 2 hours and differential gene expression assessed using Taqman Q-RT-PCR. Migration and chemotaxis of uNK cells was assessed in response to E2 and decidual conditioned media respectively.

Human endometrial endothelial cells were cultured with conditioned media from uNK cells treated with or without oestradiol for 24 hours and network forming capacity was measured.

New data presented in this thesis provide evidence that local biosynthesis of oestrogens within the endometrial stroma may play a previously unrecognised role in regulating the function of uNK cells and endometrial endothelial cells in women. These results have implications for treatment of disorders such as infertility, heavy menstrual bleeding and endometriosis.

Chapter 2

2. Materials and Methods

2.1. Chemicals, ligands and inhibitors

Unless otherwise specified all chemicals and standard laboratory reagents were obtained from Sigma (Sigma-Aldrich Company Ltd, Dorset, UK).

2.1.1. Steroid hormones

17β-Oestradiol (E2, $C_{18}H_{24}O_2$) is a natural oestrogenic ligand. E2 (Sigma, Cat. No. E-8875) was reconstituted in dimethyl sulfoxide (DMSO) to a stock concentration of 10^{-2}M from which a range of dilutions in phosphate buffered saline (PBS) were made and stored at -20^{o}C. E2 was most commonly used at a final concentration of 10^{-8}M.

Progesterone (P4, $C_{21}H_{30}O_2$) is the major naturally occurring human progestagen. P4 (Sigma, Cat. No. P-0130) was reconstituted in DMSO to a stock concentration of 10^{-2}M from which a range of dilutions in PBS were made and stored at -20^{o}C. P4 was used at a final concentration of 10^{-6}M to induce decidualisation.

Testosterone (T, $C_{19}H_{28}O_2$) was reconstituted in ethanol to a stock concentration of 10^{-2}M from which a range of dilutions in PBS were made and stored at -20^{o}C. T (Sigma, Cat. No. T-1500) was used at a final concentration of 10^{-8}M.

2.1.2. Cyclic AMP

8-Bromoadenosine 3',5'-cyclic monophosphate (cAMP) (Sigma, Cat. No. B5386) was reconstituted at 100 mg/ml in H_2O and stored at -20^{o}C. A final concentration of 0.1 mg/ml was used to induce decidualisation.

2.1.3. Selective Inhibitors

Anastrozole (Arimidex®, AstraZeneca) is an aromatase inhibitor (462). Anastrozole was reconstituted in DMSO and diluted in PBS to be used at a final concentration of 10^{-6}M and 10^{-5}M. Stocks were stored at -20^{o}C.

ICI 182 780 ($C_{32}H_{47}F_5O_3S$) is a pure oestrogen receptor antagonist (Faslodex®, AstraZeneca). This antagonist has been shown to block the activity of both ERα and ERß (463). In the studies presented in this thesis ICI 182 780 (Tocris, Cat. No. 1047) was

reconstituted to a concentration of 10^{-2}M in DMSO and diluted in PBS to be used at a final concentration of 10^{-5}M and 10^{-6}M. Stocks were stored at -20^{o}C.

STX64 ($C_{14}H_{15}NO_5S$) is an irreversible inhibitor of steroid sulphatase (464). STX64 (Sigma S1950) was reconstituted in DMSO at a concentration of 5mg/ml and diluted in PBS to be used at final concentration of 0.5μg/ml. Stocks were stored at -20^{o}C.

2.2. Patients and Samples

2.2.1. Tissue collection

Human endometrial tissue specimens (n=27) were obtained from women undergoing surgery for non-malignant gynaecological conditions. Biopsies were collected with an endometrial suction curette and processed as described in section 2.2.2 and 2.3.1. All patients were of reproductive age, had regular menstrual cycles between 25-35 days and had not received exogenous hormones or used an intrauterine contraceptive device in the three months prior to surgery. After collection histological staging was performed on fixed tissue sections stained with haematoxylin and eosin by an expert histologist and peripheral hormone levels were evaluated as previously reported (250). Endometrial samples from which stromal cells were isolated were determined to be in the proliferative phase based on the patient history at the time of collection and histological staging.

Decidual samples (n=117) were obtained from women undergoing surgical termination of pregnancy procedure (STOP) at 8-12 weeks gestation (dated from woman's reported last menstrual period). All women had an ultrasound scan to confirm viability of pregnancy and gestational age. All material from suction curettage was collected and decidual tissue was identified and separated from specimens using sterile forceps and washed in sterile saline prior to tissue processing.

Local ethical committee approval was granted and written informed patient consent was obtained prior to tissue collection by a dedicated research nurse. (Ethical approval held by Professor H.O.D. Critchley; LREC/05/51104/12 and LREC/10/51402/59.)

2.2.2. Tissue processing

Tissue samples were divided equally and placed in the following; neutral buffered formalin (NBF) for subsequent processing into paraffin wax (see section 2.12.1), RNAlater (samples stored at -80^{o}C) or PBS for isolation of cell populations (see section 2.3). Tissues placed in PBS were either stored at 4^{o}C overnight or processed immediately.

2.3. Cell Separation

2.3.1. Isolation of primary human endometrial stromal cells

Endometrial tissue samples were washed in PBS and minced into $1mm^3$ fragments using surgical blades. The tissue was digested in collagenase type IV (1 mg/ml; Sigma, C5138) and DNAse (0.1 mg/ml; Sigma, DN25) for 80 minutes at 37°C. Repeated passage through an 18 gauge needle was used to aid tissue dispersion. Tissue was suspended in 10ml of RPMI 1640 medium (Sigma, Cat. No. R0883) and cells pelleted by centrifugation (115 x g, 3 minutes). The cells were resuspended in 10mls of RPMI 1640 media (Sigma) supplemented with 10% foetal calf serum (Invitrogen, Cat. No. 10082-147), 1% Penicillin/Streptomycin (Sigma, Cat. No. P-4333) and 5μg/ml gentamycin (Sigma). The suspension was sequentially passed through 70 μm (BD Falcon, 352350) and 40 μm (BD Falcon 352340) cell strainers in order to separate stromal cells from glands. The human endometrial stromal cells were subsequently maintained *in vitro* under conditions described in section 2.4.1.

2.3.2. Isolation of lymphocytes

Total decidual tissue was minced into $1mm^3$ fragments using surgical blades and residual blood clots were removed. The tissue was digested overnight at room temperature under agitation in 20mls of RPMI media (RPMI (phenol free), NEAA 5mls, 20% FCS, 5mls Pen/Strep, HEPES (50μL) and Na Pyruvate 0.01mM with 1ml of collagenase type IV (2 mg/ml; Sigma) and 125μL of DNAse (0.1 mg/ml; Sigma, DN25). After digestion, a further 30mls of media was added and the mixture left for 5 minutes to allow for sedimentation. The supernatant was sequentially passed through 70μm and 40μm cell strainers. The filtrate was then plated on a large Petri dish for 1 hour to allow for separation of adherent cells.

The cell suspension was then collected in 50ml Falcon tube and centrifuged at 870 x g for 5 minutes. The resulting cell pellet was resuspended in 15ml of PBS supplemented with 2% FCS and 0.1% NaN_3. The cell suspension was carefully overlaid on to 15 ml of Histopaque 1077 (Sigma, Cat. No. 10771) ensuring the two layers did not mix and then centrifuged at 1094 x g for 20 minutes with no brake and at the slowest acceleration speed. Cells at the interface (lymphocytes) were collected and washed in 20 ml PBS. The cell suspension was then centrifuged at 870 x g for 5 minutes. The resulting pellet was resuspended in 10 ml PBS for cell counting using a FastRead™ cell counter (ImmuneSystems, UK).

2.3.3. Isolation of Uterine natural killer cells

Uterine natural killer (uNK) cells were isolated with CD3 negative selection and CD56 positive selection using the appropriate antibody-coated magnetic microbeads and the MACS® system (Miltenyi Biotech, Germany).

Cells were depleted by negative selection using magnetic separation to remove CD3+ cells. The lymphocytes were counted and the suspension centrifuged for 5 minutes at 870 x g. The cell pellet was then resuspended in 80µL of MACS buffer per 10^7 total cells. 20µL of CD3 microbeads (Miltenyi, 130-050-101) were added per 10^7 cells and the mixture was incubated on ice for 20 minutes. An LD column (Miltenyi, 130-042-901), for cell depletion, was placed in the magnetic field of MACS separator. The column and pre-separation filter were primed by passing two 1ml volumes of MACS buffer. The cell suspension was then added to the column followed by two 1ml washes of MACS buffer. The total effluent that contained the unlabelled cells (CD3-) was collected and used for CD56 positive selection. The cells were counted and the suspension centrifuged for 5 minutes at 870 x g. The cell pellet was subsequently resuspended in 80µL of MACS buffer per 10^7 total cells and 20µL of CD56 microbeads (Miltenyi, 130-050-401) were added per 10^7 cells. The mixture was incubated on ice for 20 minutes. LS column (130-042-401), for cell selection, was placed in the magnetic field of MACS separator. The column was primed by passing three 1ml washes MACS buffer. The cell suspension was added to the column followed by 3mls wash of MACS buffer. The column was removed from the magnetic field and placed in a suitable collection tube. Magnetically labelled cells (CD56+) were flushed from the column by addition of five 1ml washes of MACS buffer into a fresh tube. The resulting cell suspension was CD56+, CD3- and was immunophenotyped as uNK cells using flow cytometry.

2.4. Cell Culture

2.4.1. Primary human endometrial stromal cells

Primary human endometrial stromal cells (hESC) were isolated from biopsies of healthy endometrium as detailed in section 2.3.1. Cells were seeded in 6 well plates at a density of $2x10^5$ cells per well. Cells were maintained for a maximum of 4 passages and cultured in RPMI 1640 media (Sigma, Cat. No. R0883) supplemented with 10% heat-inactivated foetal calf serum (FCS; Invitrogen, Cat. No. 10082-147), 10mL/L Penicillin/Streptomycin (10,000 units penicllin and 10mg Streptomycin per ml solution, Sigma, Cat. No. P-4333), 2mM L-glutamine (Sigma, Cat. No. G-7513), and 2.5µg/ml fungizone (Invitrogen, Cat. No. 15290-018). These cells were transferred to phenol red-free RPMI 1640 medium (Sigma, Cat. No.

R7509) containing 10% charcoal-stripped FCS (CSFCS, section 2.4.4) and otherwise supplemented as detailed above for 48 hours prior to use in experimental assays. Prior to decidualisation hESCs were transferred to serum depleted media (as above but 2% CSFCS). Cells were maintained at 37^{o}C under 5% CO_2 in air.

2.4.2. Immortalised human endometrial stromal cells

The human TERT- immortalised stromal cell line, SHT 290, was obtained from Dr. David Kaufman, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, NC, USA. This cell line is derived from a patient with normal endometrium and has been immortalised by expressing a transduced human telomerase reverse transcriptase (TERT). This cell line was originally reported to retain steroid hormone receptor expression and responsiveness as well as the capacity to decidualise (465). In the current study maintenance of a phenotype similar to that of primary hESC was confirmed (see Chapter 3).

SHT 290 cells were maintained in RPMI 1640 media (Sigma, Cat. No. R0883) supplemented with 10% heat-inactivated foetal calf serum (Invitrogen, Cat. No. 10082-147), 10mL/L Penicillin/Streptomycin (Sigma, Cat. No. P-4333), 2mM L-glutamine (Sigma, Cat. No. G-7513), and 2.5µg/ml fungizone (Invitrogen, Cat. No. 15290-018). These cells were transferred to phenol red-free RPMI 1640 medium (Sigma, Cat. No. R7509) containing 10% charcoal-stripped FCS (CSFCS, section 2.4.4) and otherwise supplemented as detailed above for 48 hours prior to use in experimental assays. Cells were seeded in 6 well plates at a density of $2x10^5$ cells per well for experimental assays. Prior to decidualisation SHT 290 cells were transferred to serum depleted media (as above but 2% CSFCS). Cells were maintained at 37^{o}C under 5% CO_2 in air.

2.4.3. Primary human uterine natural killer (uNK) cells

Isolated uNK cells were cultured at a density of $1x10^6$ cells per ml in phenol red-free RPMI 1640 medium (Sigma, Cat. No. R7509) supplemented with 10% CSFCS, 10mL/L Penicillin/Streptomycin (Sigma, Cat. No. P-4333), 2mM L-glutamine (Sigma, Cat. No. G-7513), and 2.5µg/ml fungizone (Invitrogen, Cat. No. 15290-018). Cells were maintained *in vitro* for a maximum of 24 hours at 37^{o}C under 5% CO_2 in air.

2.4.4. Charcoal stripping of serum

Charcoal-stripped FCS (CSFCS) was prepared by heat inactivating serum at 57^{o}C for 30 minutes, followed by mixing with activated charcoal (Sigma, C3345) overnight on a stirrer at

4°C. Excess charcoal was removed by centrifugation and supernatant was then passed through 0.2 µm filters, aliquoted and stored at -20°C.

2.5. Chemotaxis assay

The chemotaxis of uNK cells was assessed using Ibidi µ-Slide Chemotaxis^{3D} chamber slides (Ibidi, 80326, supplied by Thistle Scientific, Uddingston, UK). Isolated uNK cells were suspended in a collagen matrix and response to different chemotaxis stimuli was measured using time lapse microscopy and recorded using Axiovert 200 Inverted Fluorescent Microscope from Zeiss.

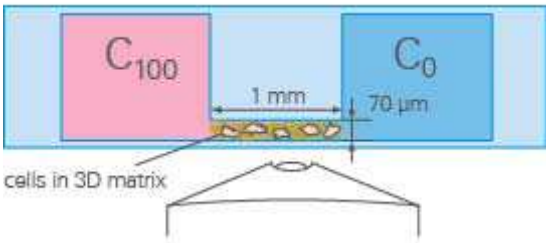

*Figure 2.1. Schematic diagram of cells in 3D matrix subject to chemotaxis gradient (+/-). Cells were viewed using inverted microscope (*http://www.ibidi.com*).*

Isolated uNK cells were suspended in RPMI 1640 phenol red free media (R7589, Sigma) supplemented with 10% charcoal stripped foetal calf serum, 200mM L-Glutamin and 10ml/L Penicillin/Streptomycin at a density of $3x10^9$ cells/ml. A 1.6 mg/ml collagen gel was prepared from 5 mg/ml bovine collagen type I (A10644-01, GIBCO, supplied by Invitrogen, Paisley UK), 10x MEM (M-0275, Sigma) and 7.5% $NaHCO_3$ (S8761, Sigma) and mixed carefully with the uNK cell suspension avoiding air bubbles during mixing. The collagen gel cell suspension was then filled into the middle chamber of the µ-Slide and placed in an incubator (37°C, 5% CO2) for 30 minutes to allow for gelation of the collagen matrix. Three chambers were seeded and two controls set up; -/- containing chemoattractant free media in both reservoirs and +/+ containing chemoattractant media in both reservoirs to account for influence of random migration (chemokinesis.) Finally a chamber with chemoattractant-free media in one reservoir and chemoattractant containing media in the other (+/-) was prepared thereby forming a chemotaxis gradient across the chamber (Figure 2.1). Chemoattractant-free media was conditioned media recovered from undecidualised endometrial stromal cells (stromal conditioned media). Chemoattractant media was media recovered from decidualised stromal cells (decidual conditioned media).

Cells were imaged every 5 minutes for 4 hours using Axiovert 200 Inverted Fluorescent Microscope from Zeiss and image stacks analysed using ImageJ (NIH.gov) and the manual tracking plug-in (http://rsbweb.nih.gov/ij/plugins/track/track.html). The movement of cells was tracked and recorded and measurements were quantified using Ibidi Chemotaxis and Migration Tool 2.0 software. The analysis software provided output values for a number of criteria, detailed in Figure 2.2. Briefly, the average end point of all cells (centre of mass) is an estimation of the overall migration of the cell population. The centre of mass is a single point and the coordinates of the centre of mass can be either positive or negative depending on the direction of the cell movement. The length of the centre of mass is the direct distance between the point of origin and the centre of mass coordinates. The centre of mass is a strong parameter for evaluating chemotaxis. Other measurements of cell movement include the accumulated distance, i.e. the total distance a cell has moved irrespective of direction, the Euclidean distance (direct distance between point of origin and end point) and velocity of cells.

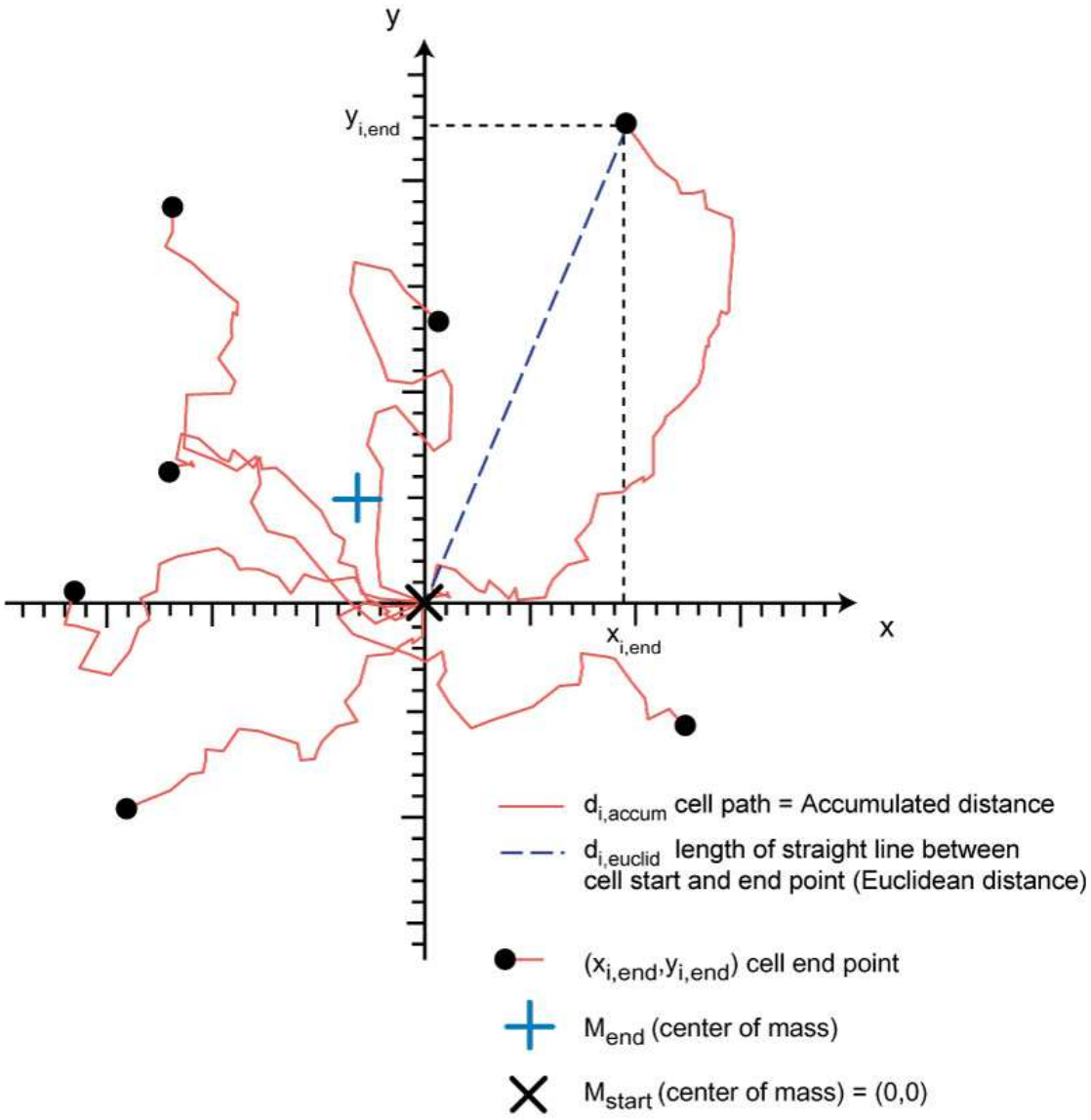

Figure 2.2. Definitions in trajectory plots. "i" is the index of different single cells. (http://www.ibidi.com)

2.6. Migration assay

Migration of uNK cells was assessed using a modified transwell migration assay. Isolated uNK cells were suspended in culture media; RPMI 1640 phenol red free media (R7589, Sigma) supplemented with 10% CSFCS, 1% Penicillin/Streptomycin (Sigma, Cat. No. P-4333), 1% L-glutamine (Sigma, Cat. No. G-7513), and 0.5% fungizone (Invitrogen, Cat. No. 15290-018), at a density of $1x10^6$ cells/ml. Cells were treated with vehicle control, 10^{-8}M E2, 10^{-6}M ICI or combination of E2 and ICI for 1 hour prior to assay. Following treatment, cells were pelleted and resuspended in culture media prior to the migration assay. A 1.6mg/ml collagen gel was prepared from 5mg/ml bovine collagen I (GIBCO/Invitrogen, A10644-01),

10x MEM (Sigma, M-0275) and 7.5% $NaHCO_3$ (Sigma, S8761) and 200µL of the gel was pipetted onto the bottom of the well in a 24 well plate and placed in the incubator (37^{o}C, 5% CO2) for 30 minutes to allow for gelation of the collagen matrix. The collagen gel was considered a neutral surface that would allow adherence of uNK cells without acting as a chemoattractant and was prepared as described immediately prior to migration assay. In the same well, 800µL of serum free RPMI 1640 phenol red free media was added over the top of the collagen matrix. Cell culture inserts with 5µm membrane pore (Costar/Corning, 3421, NY, USA) were then placed into the well into which 200µL of uNK suspension was added. Cells were treated in duplicate. Cells were left in the incubator to migrate for 1 hour. Following migration cell culture inserts were removed and cells in the lower chamber were counted. Migrated cells were counted by imaging 8 random fields using Axiovert 200 Inverted Fluorescent Microscope (Zeiss) and the mean number of migrated cells was calculated for each treatment.

2.7. Angiogenesis assay

Isolated uNK cells were cultured at a density of $1x10^6$ cells per/ml for cell treatment in phenol red-free RPMI 1640 medium (Sigma, Cat. No. R7509) supplemented with 10% CSFCS, 1% Penicillin/Streptomycin (Sigma, Cat. No. P-4333), 1% L-glutamine (Sigma, Cat. No. G-7513), and 0.5% fungizone (Invitrogen, Cat. No. 15290-018). Cells were cultured for 24 hours at 37^{o}C under 5% CO_2 in air. Cells were treated with 10^{-8}M E2, 10^{-6}M ICI or combination of E2 and ICI for 24 hours. Following treatment, cells were pelleted and the supernatant, uNK conditioned media (CM), was stored at $^{-}$80^{o}C until assay. uNK cell CM was thawed immediately prior to assay and warmed to 37^{o}C.

Telomerase immortalised human endometrial endothelial cells (HEEC, Gift from Dr. Graciela Krikun, Department of Obstetrics, Gynecology and Reproductive Science, Yale University, New Haven, CT, USA) (466) were plated at a density of 25,000 per transwell insert (inserted into 24 well plates) which had been coated with growth factor reduced matrigel (BD Biosciences, Oxford, UK) and allowed to solidify prior to cell plating. uNK cell CM was added to the bottom chambers and cells incubated overnight at 37ºC with 5% CO_2. Each treatment was set up in duplicate. After 16 hours incubation HEECs were fixed with ice cold methanol for 20 minutes and then stained with hematoxylin. The formation of networks was visualised using Axiovert 200 Inverted Fluorescent Microscope (Carl Zeiss, UK). Three fields of vision were captured at 5x magnification for each well. Network formation was quantified using ImageJ (NIH.gov) and verified by counting branch points.

2.8. RNA extraction

Extraction of RNA from cell monolayers extracts was performed using RNAeasy® mini kit (Qiagen, Crawley, UK). Biological samples were collected in lysis buffer and homogenized using QIAshredder® spin columns (Qiagen, Crawley, UK). Ethanol was added to lysate to provide appropriate binding conditions, and the sample was then applied to an RNeasy® mini spin column. Total RNA bound to the membrane and contaminants were washed away through a series of wash steps. On column DNAse digestion was performed to remove contaminating DNA using a Qiagen RNase-Free DNase kit (Cat. No.79254, Qiagen, Crawley, UK). RNA was then eluted in 30μL RNAse-free water.

2.8.1. RNA quantification using Nanodrop®

The total concentration of RNA was determined by measuring the absorbance of eluted RNA samples using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA samples were then standardised to concentration of 100ng/μL.

2.9. Preparation of cDNA using SuperScript® VILO™ synthesis kit.

Reverse transcription of RNA to form a single stand of complementary DNA (cDNA) was carried out using the SuperScript VILO cDNA synthesis kit (InVitrogen, Paisley, UK) according to the manufacturer's instructions. Briefly, each reaction (final volume 20μL) contained a final concentration of 1x VILO reaction mix, 0.125x Superscript enzyme mix and 100ng of RNA. Samples were incubated under the following conditions: 25^{o}C for 10minutes, 42^{o}C for 60 minutes, 85^{o}C for 5minutes in a thermal cycler (MJ Research PTC-200 Thermo Cycler, BC-MJPC200).

2.10. Quantitative-Real Time-PCR (Taqman® Method)

Concentrations of mRNAs in samples under test were determined by reverse transcription followed by quantitative-real time polymersase chain reaction (qRT-PCR) performed using an ABI machine and the Taqman® Method.

2.10.1. Preparation of Taqman reaction mix using the Universal Probe Library™

Quantitative Real Time Taqman® PCR is an automated, high throughput variation of conventional PCR. The Taqman® method employs the use of fluorogenic probes from the

Universal Probe Library™ (Roche, West Sussex, UK) to enable quantification of DNA content based on a measure of fluorescence detection after each PCR cycle. The Taqman® reaction mix was prepared using the Universal Probe Library™ (UPL).

The UPL is a set of 165 short hydrolysis probes (8-9 nucleotides in length) that are labelled with fluorescein (FAM) at the 5' end and a dark quencher dye at their 3'end.

These short probes are based on locked nucleic acids (LNAs) as they have a 'locked' ribose ring with a methylene bridge where the 2'-O atom is connected to the 4'-C atom. LNAs have the ability to bind to their target sequences despite their short length as they contain the six common nucleobases (T, C, G, A, U, mC) that appear in RNA and DNA. The resultant specificity of these short LNA-probes facilitates distinction between transcripts by using a probe that can only bind to a specific transcript based on the primer pairs used. Primers were designed using the Roche online 'probe finder' assay design method at https://www.roche-appliedscience.com/sis/rtpcr/upl/ (see section 2.15).

2.10.2. Quantitative real time Taqman® PCR

Taqman relies on two principles; fluorescence resonance energy transfer (FRET) and 5'nuclease activity of the Taq polymerase. Taqman probes consist of a fluorophore covalently attached to 5'- end of the oligonucleotide probe (6-carboxyfluorescein, FAM) and a quencher at the 3'-end. Taqman probes amplify DNA regions to specific set of primers. During each PCR cycle the Taq polymerase extends the primer and the probe is cleaved and degraded. Fluorescence emission occurs upon cleavage of the probe as degradation breaks proximity and relieves the quencher affect on the fluorophore. Thus, fluorescence detected is directly proportional to fluorophore release and hence the amount of DNA in the PCR.

Each reaction was prepared using the qPCR Supermix with Premixed ROX kit (11795-01K; Invitrogen), conducted in duplicate and plated in a 96-well MicroAmp Fast Optical reaction plate (Applied Biosystems). Real time PCR amplification was performed using the Applied Biosystems® 7900HT Fast Real-Time PCR System using the reaction mix detailed in Table 2.10. The Taqman® reaction contained passive reference ROX, internal control gene 18s (VIC-conjugated) and gene of interest (FAM-conjugated).

Reagent	Final Concentration	Vol./15µL reaction (µL)
2x Express Supermix	1x	7.5
Forward Primer 20µM	200nM	0.15
Reverse Primer 20µM	200nM	0.15
Probe 10µM	100nM	0.15
18S	(10nM primer, 40nM probe)	0.1125
H_20		5.4375
cDNA		1.5

Table 2.1. Reaction mixtures for Taqman analysis

2.10.3. Analysis of computational output

Fluorescence was detected by ABI7900 sequence detection system and processed as an amplification plot. The threshold cycle (Ct) value is the point on the exponential curve at which the level of fluorescence reached is considered statistically greater than background levels. The Ct value is directly proportional to the amount of PCR product formed and a change of one-fold Ct value represents a two-fold change in the initial cDNA concentration. Analysis was performed by comparative ΔΔCt method where fold change is relative to a reference sample such as an untreated control as well as an internal control. The reporter was standardised to an internal control (18s ribosomal RNA) to correct for fluctuations between samples ΔCt = FAM Ct – 18S VIC Ct. This value was then expressed as change relative to reference sample ΔΔCt = ΔCt sample – ΔCt reference sample. Fold change was then calculated using the equation $2^{-\Delta\Delta Ct}$.

2.10.4. Primer/probe Validation

To validate use of the primer/probe mix, log ng mRNA values were plotted against the average of the Ct values.

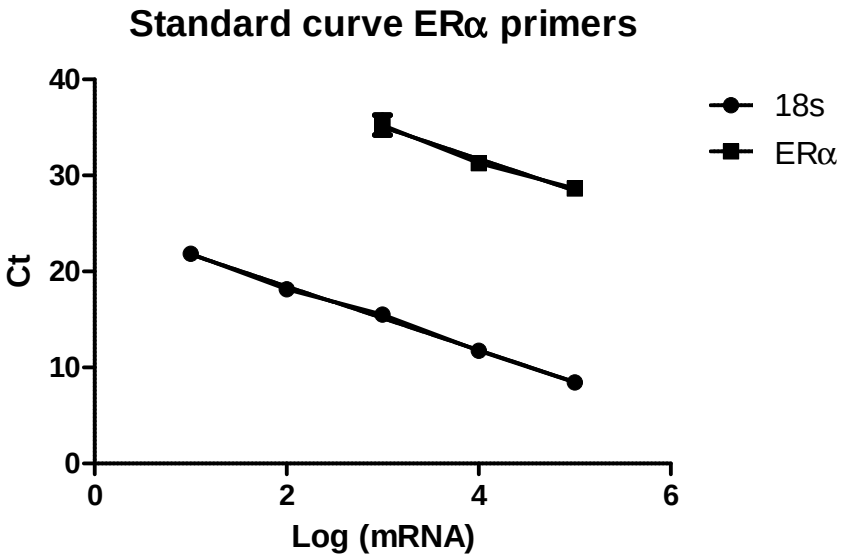

Figure 2.3. Validation of gene of interest primer/probe usage relative to 18S primer/probe control mix. 18s, R^2=0.996, 99.5% efficiency, ERα R^2=0.96, 99.5% efficiency

2.10.5. RT^2 PCR profiler arrays

The RT^2 profiler PCR arrays (SABiosciences, Frederick, MD, USA) are designed to analyse a panel of genes related to a specific biological function. This platform combines the performance of real time PCR with the multi gene profiling capabilities of a microarray. Each array contains a panel of 96 primer sets for a set of 84 functionally focussed genes, together with five housekeeping genes and three RNA and PCR quality controls.

RNA samples from uNK cells were retrotranscribed using RT^2 single strand cDNA synthesis kit. Samples from 3 sets of treated uNK cells were analysed for differential gene expression following E2 treatment. RNA samples were converted to first strand cDNA using the RT^2 first strand kit (SABiosciences, Frederick, MD, USA). Briefly, 300ng/ml RNA was mixed with 1x genomic DNA elimination mixture and made up to final volume of 10μL with nuclease-free H_2O per sample. SYBR green dye binds with all types of double stranded DNA, therefore complete removal of genomic DNA is essential for accurate expression data. The contents were mixed and briefly centrifuged before being incubated at 42^{o}C for 5 minutes, then cooled on ice for at least 1 minute. The RT cocktail was prepared as follows for each reaction:

RT Cocktail	x1 rxn	x6 rxn
BC3 (5x RT Buffer 3)	4µL	24µL
P2 (Primer & External control Mix)	1µL	6µL
RE3 (RT Enzyme Mix 3)	2µL	12µL
H_2O	3µL	18µL
Final Volume	10µL	60µL

Table 2.2. RT cocktail mixture for cDNA synthesis

10µL of RT cocktail was added to each treated RNA sample and mixed by pipette. The samples were then incubated for 15 minutes at 42^{o}C and the reaction was then stopped by heating to 92^{o}C for 5 minutes. 20µL of cDNA synthesis mix was diluted by addition of 91 µL of H_2O and samples held on ice or stored at -20^{o}C until RT PCR was performed. Frozen samples were thawed on ice immediately prior to use. The RT PCR reaction mixture was as follows:

Reagent	Volume
2X SABiosciences RT^2 qPCR Master Mix	1350µL
Diluted first strand cDNA synthesis reaction	102µL
H_2O	1248µL
Total Volume	2700µL

Table 2.3. RT PCR reaction mixture for each 96 well array plate.

25µL of the experimental mixture was added to each well of the 96 well array plate using a multi-channel pipette to minimise intra-well variability. Following addition of samples, plates were sealed and held on ice until use. Prior to RT PCR plates were centrifuged for 1 minute at room temperature 1000g to remove bubbles. Real time PCR amplification was performed using the Applied Biosystems® 7900HT Fast Real-Time PCR System PCR using RT^2 Real-Timer SyBR Green/ROX PCR Mix kit according to manufacturer's instructions. Using an ABI 7900HT the following two-step cycling program was set up:

Cycles	Duration	Temperature
1	10 minutes	95°C
40	15 seconds	95°C
	1 minute	60°C

Table 2.4. Thermal cycling program for PCR amplification.

SABiosciences RT^2 SYBR Green Master Mix utilises Hot-start Taq DNA polymerase, which prevents polymerase activity before heat activation and thereby reducing amplification of non-specific PCR products. SYBR green fluorescence was detected and recorded during the annealing step in each cycle and the threshold Ct for each well was calculated. As an additional quality control, a melt curve program was run after every cycling program for each array plate. A first derivative dissociation curve for each well was recorded and checked for single peaks at temperatures greater than 80°C. Wells which did not meet these criteria were excluded from analysis.

PCR array data analysis was performed using the SABiosciences web portal (http://www.SABiosciences.com/pcrarrayanalysis.php). Data analysis was performed using the ΔΔCt method (Section 2.10.3).

2.11. Enzyme-linked Immunosorbant Assay

Enzyme-linked immunosorbent assay (ELISA) is method used to detect and quantify target proteins in biological samples. In the present studies two forms of ELISA were utilized.

2.11.1. Two-site sandwich ELISA.

Using a 'sandwich' ELISA the target antigen, usually a soluble protein, is quantified using matched antibody pairs (Figure 2.4).

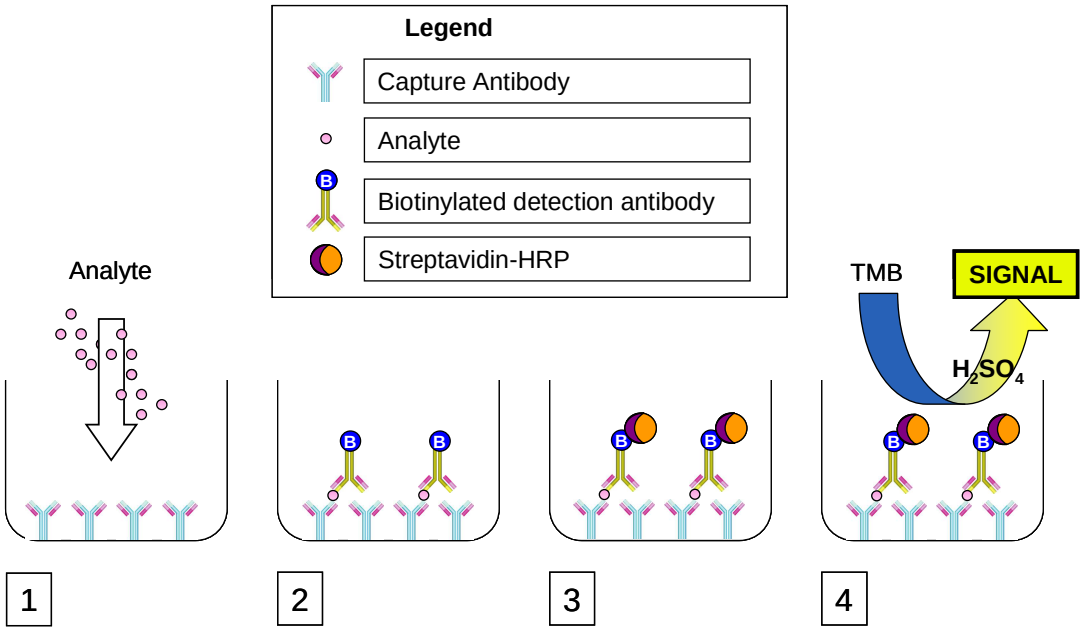

Figure 2.4. Schematic diagram of general protocol for sandwich ELISA. 1. Plate is coated with capture antibody and sample (analyte) is added. 2. Any antigen present (pink) binds the capture antibody. The biotinylated detection antibody then binds antigen-capture antibody complexes. 3. Streptavidin-HRP binds any biotinylated detection antibody present. 4. 3,3',5,5'-Tetramethylbenzidine (TMB) substrate is added and converted to signal by HRP-linked antibody complexes. Reaction is stopped by the addition of H2SO4 which results in a colour change from blue to yellow. The colour signal produced is proportional to the amount of antigen present in the sample.

The ELISA methodology was used for detection of human insulin-like Growth Factor Binding Protein 1 (IGFBP-1) in cell culture (reagents from DuoSet ELISA development kit, R&D Systems, Minneapolis, MN, USA). Cell growth media was collected following *in vitro* treatment of cells and stored at -80°C to preserve protein integrity and thawed immediately prior to the assay.

2.11.1.1.1. Protocol

Clear, flat-bottomed 96-well microplates (Corning/Costar) were coated with 100µL/well of capture antibody diluted to working concentration of 4.0µg/ml in PBS. Plates were incubated overnight at 4^{o}C on a rocker. After incubation, capture antibody solution was aspirated and each well washed 3 times with wash buffer (0.05% Tween20 in PBS). Complete removal of liquid and bubbles from wells was achieved by blotting plates against paper towel at each wash step which ensured good assay performance. After washing, plates were blocked by adding 300µL of blocking buffer (5% Tween20 in PBS) to each well and incubated for a minimum of 1 hour at room temperature to prevent non-specific binding and reduce

background response. The aspiration/wash step was then repeated and 100µL of sample or standards were added to each well. Standards were made from recombinant human IGFBP-1 reconstituted in reagent diluent (5% Tween20 in PBS) to produce a high standard of 2000pg/ml from which two-fold serial dilutions were made to produce a seven point standard curve. Samples and standards were incubated overnight at 4^{o}C on a rocker. Free, specific protein in the samples spontaneously bound to the capture antibody coated to the base of the wells in the plate. The aspiration/wash step was repeated prior to addition of a biotinylated detection antibody, diluted in reagent diluent with 2% heat inactivated normal goat serum to a working concentration of 400ng/ml (100µL/well). Plates were covered and incubated for two hours at room temperature, after which the plate was washed as described previously. The plate was then incubated with 100µL per well of Streptavidin-HRP solution (1:200 in PBS) for 20 minutes in the dark at room temperature. Following a final wash step 100µL of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added to each well and incubated for 20 minutes in the dark at room temperature. TMB forms a blue product in the presence of peroxidase enzymes. The colour reaction was stopped by addition of 50µL/well of 2N H_2SO_4. The addition of sulfuric acid changed the TMB yellow. Optical density of each well was measured immediately using a microplate reader set to 450nm with wavelength correction set to 540nm. This subtraction corrects for optical imperfections in the plate and thus produces more accurate readings. The delta absorbance was recorded as final values and concentrations were then calculated from the standard values.

2.11.1.1.2. Standard curve

Standards were assayed in duplicate and average responses were plotted as a standard curve with concentration along the x-axis and mean absorbance on the Y-axis. Data was linearised by plotting log concentration against log optical density. A best fit line was determined by regression analysis using GraphPad Prism and used to calculate concentrations of the unknown samples. A standard curve was generated for each set of samples assayed.

2.11.2. Competitive ELISA

In competitive ELISA unlabelled antigen present in samples, standards and controls compete for binding with enzyme-labelled antigen (conjugate) for a limited number of target antibody binding sites in the microwells of the assay plate. Conjugated antigen will bind to the target antibody wherever binding sites are not occupied by unlabelled antigen. Washing removes unbound materials. Wherever conjugated antigen is bound enzyme substrate reacts with the conjugate (enzyme-labelled antigen) to produce a colour reaction. Thus, in competitive ELISA the intensity of colour formed is inversely proportional to the concentration of

antigen in the sample. The enzymatic reaction is terminated by addition of stopping solution and optical density of each well measured using a spectrophotometer.

2.11.2.1. Oestradiol ELISA

To determine the amount of oestradiol in culture supernatants a competitive ELISA was performed using oestradiol-6-HRP conjugate (in house preparation) and oestradiol 6 antibody (Fitzgerald Industries International, Concord, MA, USA.)

Immuno 96 MicroWell™ Solid Plates (NUNC, supplied by Scientific Laboratory Supplies, Nottingham, UK) were coated with 100µL/well of 1:200 DARS (Donkey anti rabbit serum) in coating buffer (100mM Bicarbonate solution) and incubated overnight in at 4^{o}C on a rocker. After incubation, coating solution was aspirated and each well washed 3 times with wash buffer (0.05% Tween20 in PBS). Complete removal of liquid and bubbles from wells was achieved by blotting plates against paper towel at each wash step which ensured good assay performance. After washing, plates were blocked by adding 300µL of blocking buffer (1% Gelatin in PBS) to each well and incubated for a minimum of 1 hour at room temperature to prevent non-specific binding and reduce background response. Plates were washed a further three times and 100µL of standards/unknowns added to the plate. 50µl of oestradiol-6-HRP conjugate 1:40,000 in PGBS (0.1M Phosphate Gelatin Buffered Saline, pH 7.4) and 50µl of antibody at dilution of 1:150,000 in PGBS was added and incubated for 3 hours at room temperature or overnight at 4^{o}C. Solutions were aspirated and plates washed four times. This followed by addition of 100ul of TMB substrate solution to each well and incubation in the dark for 20 minutes. The colour reaction was stopped by addition of 50µL/well of 2N H_2SO_4. Optical density of each well was measured immediately using a microplate reader set to 450nm with wavelength correction set to 540nm. This subtraction corrects for optical imperfections in the plate and thus produces more accurate readings. The delta absorbance was recorded as final values and concentrations were then calculated from the standard values.

2.11.2.2. Oestrone ELISA

Oestrone in cell culture supernatants was detected using Estrone ELISA kit (BioVendor, Czech Republic) and quantified against calibrator standard set. Briefly, 50µL of samples, controls and calibrators were added into corresponding wells of a rabbit anti-estrone antibody coated microwell plate. This was followed by addition of 100µL of conjugate solution containing Estrone-biotin and avidin-horse radish peroxidase (at a concentration of 1:100 diluted in assay buffer) to each well and incubation for 1 hour on a plate shaker. After incubation, sample and conjugate solution mix was aspirated form the plate and each well

washed 3 times with wash buffer. Complete removal of liquid and bubbles from wells was achieved by blotting plates against paper towel at each wash step to ensure good assay performance. The colour reaction was initiated by addition of 150µL of TMB substrate to each well, followed by incubation on plate shaker for 10-15 minutes. The colour reaction was stopped by addition of 50µL/well of stopping solution (1M H_2SO_4). Optical density of each well was measured immediately using a microplate reader set to 450nm with wavelength correction set to 540nm. This subtraction corrects for optical imperfections in the plate and thus produces more accurate readings. The delta absorbance was recorded as final values and concentrations were then calculated from the calibrator standard values.

2.12. Immunohistochemistry

Immunohistochemistry is the localisation of specific proteins of interest (antigens) in tissue sections through the binding of labelled antibodies to small, unique regions (epitopes) of the antigen under investigation. Antigen-antibody interactions can be visualised by secondary interaction with a marker such as a fluorescent dye or an enzyme complex which reacts with a chromogen substrate. Immunohistochemistry makes it possible to visualise tissue localisation and distribution of a specific antigen within a cell or tissue.

Immunohistochemistry was conducted on human endometrial and decidual tissue sections in accordance with standard lab protocols to establish the presence and localisation of proteins of interest.

2.12.1. Tissue processing

Tissues were collected and fixed in 4% neutral buffered formalin (NBF) overnight before being transferred to 70% ethanol prior to being embedded in paraffin wax according to standard methods by the histology team at the MRC Human Reproductive Sciences Unit, Edinburgh. Wax-embedded tissue blocks were cooled on ice to ensure rigidity of the wax and ease of sectioning. 5µm sections were cut using a microtome (Leica RM2135, Germany) and mounted onto coated glass slides (VWR, Poole, UK) and dried overnight in an incubator at 55^{o}C to ensure complete adhesion to the slide.

2.12.2. Dewaxing

To ensure optimal antibody interaction with the tissue complete removal of wax was carried out. Tissue sections were dewaxed in xylene (two 5 minute washes) and rehydrated at 20 second intervals through graded alcohol before being washed in water.

2.12.3. Antigen Retrieval

Antigen retrieval was performed by pressure cooking in 0.05M Glycine/EDTA buffer (pH 8) or 0.01M citrate buffer (pH 6) for 5 minutes at full pressure and then allowed to stand for 20 minutes. This ensures any 'masking' of epitopes due to formation of cross linkages is disrupted and allows greater binding of the antibody to the tissue antigen. Glycine/EDTA buffer was considered a more suitable buffer for endometrial tissue as it gave cleaner and stronger staining.

2.12.4. Blocking to promote specific signal amplification

To reduce non-specific antibody binding and to promote specific signal amplification the following blocking stages were utilised.

2.12.5. Methanol peroxidase block

To prevent endogenous peroxidases in the tissue section reacting with chromogen substrate endogenous peroxidase activity was blocked by washing slides in 3% (v/v) hydrogen peroxide in methanol for 30 minutes at room temperature. Following the block slides were washed in tap water and then Tris-buffered saline (TBS; 0.05M Tris-HCL, pH 7.4, 0.85% NaCl) for two 5 minute washes.

2.12.6. Serum block

To prevent non-specific binding of the secondary antibody serum from the species in which the secondary antibody was raised was diluted 1:4 in TBS solution containing 5% bovine serum albumin (BSA) was added to the slides and incubated for 30 minutes at room temperature.

2.12.7. Avidin-Biotin block

The use of biotinylated secondary antibodies in the immunohistochemistry process relies upon the strong affinity of avidin and streptavidin for biotin. To avoid avidin-peroxidase complexes binding to endogenous biotin in the tissue an avidin biotin blocking kit was used (Vector Laboratories, Peterborough, UK). Sections were incubated with avidin-D for 15 minutes, followed by two 5 minute washes in TBS and then biotin for 15 minutes, followed by a further two 5 minute washes.

2.12.8. Primary antibodies

Primary antibodies were diluted in blocking serum at an appropriate dilution and added to the slides which were incubated in a humidified chamber and overnight at 4^{o} C. Optimal dilutions were obtained for each antibody outlined in Table 2.5. Negative controls were conducted for each antibody where primary antibody was replaced with blocking serum alone.

Source	Primary antibody	Species raised	DAB dilution	Fluorescence Dilution
Sigma Aldrich	ACTR3	Mouse	1:50	N/A
Gift from Prof. Evan Simpson, Prince Henry's Institute, Clayton, Australia.	Aromatase	Rabbit	N/A	1:250
Thermo Scientific	CD10	Mouse	1:200	N/A
Zymed	CD56	Mouse	1:400	1:2500
R&D	CXCR4	Mouse	1:500	1:80
Vector	ERα	Mouse	1:200	1:1000
Serotec	ERβ	Mouse	1:200	1:1000
Ab frontier	PTMA	Mouse	1:500	N/A
Bioquote	SOD2	Rabbit	1:200	1:400

Table 2.5. Primary antibodies and dilutions used in immunohistochemistry studies.

2.12.9. Secondary Antibodies

Following two 5 minute washes in TBS, appropriate biotinylated secondary antibody in blocking serum were added to the slides and incubated for 30 minutes at room temperature in a humidified environment.

2.12.10. Antigen Detection

2.12.10.1. DAB immunohistochemistry

Following 2 further washes in TBS, streptavidin-HRP (horseradish peroxidase) diluted 1:1000 in TBS was applied for 30 minutes at room temperature. A final TBS wash preceded antibody localisation determined by liquid DAB substrate chromogen system (DAKO, High Wycombe, UK). The chromogenic substrate 3,3'-diaminobenzidine (DAB) was oxidised by any antigen bound antibody HRP complexes resulting in the development of brown deposit at antigen sites (positive staining). DAB development was monitored microscopically and

the reaction was stopped by washing in water. Slides were counterstained in Harris' haematoxylin (causes cell nuclei to turn blue) followed by washes in acid alcohol (10 seconds) and Scotts tap water (30 seconds.) Slides were then rehydrated through graded alcohols, washed in H_20 and finally immersed in xylene before being mounted with coverslips in pertex glue (Cell Path, Hemel Hempstead, UK).

2.12.10.2. Tyramide signal amplification fluorescence immunohistochemistry

Immunofluorescent immunohistochemistry using tyramide signal amplification (TSA) enables the identification of multiple antigens in the same tissue section and detection of colocalised target antigens. Under ideal conditions the primary antibodies utilised in TSA are from distinct species and the fluorescent detection dyes have discernable excitation/emission spectra with minimal overlap. Fluorescent immunohistochemistry utilised phosphate buffered saline (PBS) as wash buffer in place of TBS. The slides were protected from photobleaching by covering in foil.

Following antigen retrieval and hydrogen peroxide block (as described above) the slides were washed in water and then twice in PBS for 5 minutes. The slides were serum blocked for 30 minutes at room temperature before incubation overnight with primary antibody diluted at appropriate dilution (see Table 2.5) Following washes in PBS, slides were incubated with a species appropriate peroxidase-conjugated (with attached FAB fragments) secondary antibody for 30 min at room temperature. Slides were washed in PBS and incubated for 10 min with TSA kit (PerkinElmer, Massachusetts, USA) using Tyramide-cyanine 3 diluted 1:50 in substrate. The red fluorophore (cyanine 3) was usually used for the first primary antibodies as the green fluorophore (fluoroscein) was more prone to photobleaching and diminished signal when used first. After washing in PBS the slides were microwaved in boiling 0.01M citrate buffer for 2.5 minutes and then allowed to stand for a further 30 minutes to block cross-reactivity. The slides were then blocked in NGS/PBS/BSA and incubated overnight with the second primary antibody. Incubations with appropriate secondary antibody and TSA kit were performed as described above. The green fluorophore (fluorescein) was used for the second primary antibody. Following washes in PBS the slides were counterstained with DAPI diluted 1:1000 in PBS and mounted with coverslips in permafluor (Immunotech, Marseille, France).

2.12.11. Image analysis

DAB positive sections were imaged using PROVIS microscope (Olympus Optical, London UK) with an attached Canon DS126131 camera. Fluorescent images were examined using Zeiss LSM 510 Meta-Confocal microscope (Carl Zeiss, Hertfordshire, UK).

2.13. Flow Cytometry

Flow cytometry allows simultaneous analysis of multiple parameters of individual cells within a heterogeneous cell mixture. Cell suspensions are passed through the laser beam of a flow cytometer one cell at a time by a fluidics system which uses hydrodynamic focussing to ensure single cell detection. As each cell is passed through the laser the refraction pattern of the light is recorded by an electronic detection device. The light scatter created by cells passing through the laser provides information about the cell. Forward scatter (FSC) is roughly proportional to the volume/size of the cell. The internal complexity of cells such as shape of the nucleus and granularity of cells can be discerned from side scatter (SSC). Incubation with fluorescence-conjugated antibodies can be used to further describe the cell population by the detection of emission spectra of cells as they pass through the laser. Flow cytometry was used to describe and identify uNK cells from tissue extractions by immunophenotyping and viability testing using flow cytometry (see Section 4.4.2).

2.13.1. Preparation of samples.

Following isolation of uNK cells from human decidual tissues (see section 2.3.3) cells were suspended in FACS buffer (PBS, containing 1% FCS and 0.1% NaN_3) at a concentration of 500,000 cells per ml.

2.13.2. Conjugated antibody staining

Cell suspensions were stained with antibodies conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) in order to immunophenotype cell populations. These fluorescent conjugates were chosen as their emission spectra do not overlap and therefore dual detection could be carried out. Single stains were set up to define the parameters for scanning the samples (see Table 2.60) together with unstained samples and isotype controls. Single and double stained samples were utilised to fully describe the sample cell population. Cell suspensions were centrifuged for 2 minutes at to pellet cells which were consequently resuspended in 100µL of FACS buffer. Conjugated antibodies at an appropriate dilution were then added and cells incubated in the dark for 30 minutes. Following incubation cells were washed by adding 1ml of FACS buffer and centrifuged for 2 minutes at 1080 x g. The

cell pellet was resuspended in 1ml of FACS buffer centrifuged again. Finally the cell pellet was resuspended in 500µL of FACS buffer for scanning. Cells were placed on ice and protected from light until scanning was completed.

Antibody	Conjugate	Ig	Species	Manufacturer	Product code	Dilution
CD3	RPE	IgG2a	Mouse	AbD Serotec	MCA2184PE	1:50
Isotype control	RPE	IgG2a	Mouse	AbD Serotec	MCA929PE	1:50
CD16	RPE	IgG1κ	Mouse	BD Pharmingen	555407	1:50
Isotype control	RPE	IgG1κ	Mouse	BD Pharmingen	555749	1:50
CD56	FITC	IgG1κ	Mouse	Abcam	Ab48521	1:20
Isotype control	FITC	IgG1κ	Mouse	Abcam	Ab18435	1:20

Table 2.6. Conjugated antibodies and isotype controls used in flow cytometry studies.

2.13.3. Propidium Iodide staining

To assess cell viability the capacity for cells to incorporate the fluorescent DNA-binding probe propidium iodide (PI) was measured. Non-viable cell membrane proteins become porous and they can readily incorporate PI. The degree of positive PI staining directly correlated to the proportion of non-viable cells. Viable cells are intact and do not incorporate the PI, thus the proportion of dye excluded cells gives an approximation of the percentage viability. PI (Sigma, 81845) was added to the cell suspension, incubated for 5 minutes in the dark and fluorescence measured using flow cytometer.

2.13.4. BD FACScan cytometry

Sample cell suspensions were measured using Becton Dickinson FACScan™ automated flow cytometer (Becton Dickinson, Oxford, UK). The FACScan™ system analyses cells as they pass through a focused laser beam one cell at a time in a moving fluid stream and at a rate of up to 2000 events per second. The FACScan™ system consists of a fixed 488nm Argon laser and three-colour detection capabilities. Samples were assessed at high flow rate (60µL/min of sample through flow cell). Five parameters are available which describe the characteristics of cells in the sample suspension; forward scatter (FSC) which gives an indication of cell size/volume, side scatter (SSC) which gives an indication of cell granularity/internal complexity and fluorescence measurements; FL-1, FL-2 and FL-3 for measuring the extent of positive staining in cells by fluorochrome-conjugated antibodies. Measurements of FSC and SSC were recorded as well as FL-1 for detection of fluorescein

isothiocyanate (FITC)-conjugated antibodies and FL-2 for phycoerythrin (PE)-conjugated antibodies and propidium iodide (PI).

2.13.5. FACS analysis

FACS samples were analysed using FlowJo (Tree Star Inc, OR, USA)

2.14. Thin Layer Chromatography (TLC)

Thin Layer chromatography is a method for separating a mixture of compounds based on their size and polarity. In the method employed, metabolised derivatives of tritiated steroids were measured along with the parent compound to give an indication of steroid metabolism in stromal cells. Stromal cells were cultured with media containing 'cold' steroid (10^{-8} M final concentration) as well as 'hot' steroid, which was the same steroid but labelled with tritium sufficient to give 100,000 counts per ml, and incubated for various time periods. Following incubation for the appropriate amount of time, cell culture media was aspirated and mixed with 9ml dichloromethane (CH_2Cl_2) in a conical bottomed glass vial. The media and solvent were vortexed and allowed to stand for a minimum of two hours to allow steroid to dissolve into the solvent phase. The vials were then centrifuged at 868 x g for 15 minutes to ensure clear separation of the aqueous (cell culture media) and solvent (dichloromethane) layers. The upper aqueous layer was then discarded using suction pump at radioactive sink. The solvent layer was then dried using a sample drier under nitrogen gas on a heat block set to 40^{o}C. The chromatography tank was prepared by adding 150ml of running solvent (Chloroform/ethanol; 92/8%) and allowed to equilibrate for 1 hour before use. Dried samples were reconstituted in 100µL spotting mix, which contained 'cold' steroid mix and dichloromethane. The Aluminium Silica coated TLC plate (Alugram, SIL G/UV_{254}, Macherey Nagel, Germany) was prepared by marking an origin line 20mm from the bottom of the plate on which samples were spotted at even intervals. Sample spotting involved careful dropping of sample onto the plate allowing drying between each additional spot of the sample and keeping the spot area as small as possible. This allowed the sample to be concentrated onto a small area which would be distinct from other samples on the plate. Following spotting of samples the plate was placed in the chromatography tank for approximately 1hour 30 minutes or until the solvent front reached the end of plate. Finally the plate was removed and allowed to dry in the fume hood before being placed on TLC scanner to record plate readings. Distribution of radioactivity on TLC plates was evaluated by radiometric scanning (Bioscan system 200 image scanner, Bioscan, Washington, DC, USA) and recorded using WinScan software, Lablogic software.

2.15. Tritiated water assay

Aromatase activity was determined using the tritiated water release method (467) using ^{3}H-androstenedione as the substrate ([1β-^{3}H]-androstenedione, Perkin Elmer Life Science, Boston; specific activity 23.5 Ci/mmol). In the method employed, the extent of substrate aromatisation was quantified by measuring the amount of tritiated water released. As a consequence of ^{3}H-androstenedione aromatization, the tritium tracer is released in the form of tritiated water (^{3}HOH). Released ^{3}HOH is isolated following solvent extraction of steroids which removes any remaining ^{3}H-androstenedione and thus leaves only ^{3}HOH as the only detectable tracer in the sample. The relative quantity of ^{3}HOH is measured using a beta counter and this is then quantitated against reference blanks/controls to determine the specific activity of aromatase in samples.

Cells were incubated with 150pmol/ml androstenedione (A4) substrate (30pmol 'Hot' A4 i.e. ^{3}H-androstenedione and 120pmol 'Cold' A4) at 37°C for 2hrs. Following incubation, cells were incubated on ice for 10 minutes and then medium was removed and 1ml of 30% trichloroacetic acid (TCA) was added to the media. Cells were then washed twice in PBS and stored at -20^{o}C until protein concentration was determined by Lowry method (468).

4ml of Chloroform ($CHCl_3$) was added to samples, which were vortexed and incubated overnight on a shaker to extract steroids. Samples were centrifuged at 1811 x g for 5 minutes and 1 ml of the upper (aqueous) phase transferred to a new glass vial. To this 2ml of charcoal/dextran solution (Charcoal dextran, Sigma C6241; 5%:0.5% v/w in H_2O) was added and the sample was vortexed thoroughly. The samples were then centrifuged at 1811 x g for 30 minutes. 1 ml of supernatant was then transferred to scintillation vials containing 10 mls of scintillation fluid (ProSafe FC, Meridian Biotech, Epsom, Surrey, UK), the samples were counted with a beta counter (Packard Tri-carb 2100TH, liquid scintillation analyzer) and the specific activity calculated.

2.16. Primers

Gene name	Forward Primer	Reverse Primer	UPL probe number
Homo sapiens adrenomedullin (ADM)	gcctgcccagacccttat	gtagcgcttgactcggatg	57
Homo sapiens aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) (AKR1C3)	tgggttccgccatatagatt	tcgatgaaaagtggaccaaa	68
Homo sapiens androgen receptor (AR)	gccttgctctctagcctcaa	gtcgtccacgtgtaagttgc	14
Homo sapiens angiopoietin 2 (ANGPT2)	tgcaaatgttcacaaatgctaa	aagttggaaggaccacatgc	75
Homo sapiens angiopoietin 4 (ANGPT4)	caagctgaccgacatggag	atctgggcatccattcttga	17
Homo sapiens ARPS actin-related protein 3 homolog (yeast) (ACTR3)	tggtgtcactcatgtcattcc	atatctcgtcctgcgattgg	41
Homo sapiens caveolin 2 (CAV2)	ggctcaactcgcatctcaa	cgtatttgctgatttcaaagagg	46
Homo sapiens chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1) (CXCL12)	ttgacccgaagctaaagtgg	ccctctcacatcttgaacctct	80
Homo sapiens coagulation factor III (thromboplastin, tissue factor) (F3)	ggagaaaggggaattcagaga	gggagttctccttccagctc	51
Homo sapiens CXCR4 (stromal cell derived factor 1 receptor)	ttaagcgcctggtgactgtt	gcccatttcctcggtgtag	47
Homo sapiens cytochrome P450, family 11, subfamily A, polypeptide 1 (CYP11A1), nuclear gene encoding mitochondrial protein, transcript variant 1, mRNA	ccagacctgttccgtctgtt	gaagttctgggtgtatatgtcagc	85
Homo sapiens estrogen receptor 1 (ESR1)	ttactgaccaacctggcaga	atcatggagggtcaaatcca	24
Homo sapiens estrogen receptor 2 (ER beta) (ESR2)	gctcctgtcccacgtcag	tgggcattcagcatctcc	62
Homo sapiens glycoprotein hormones, alpha polypeptide (CGA)	tgctgtgtagctaaatcatataacagg	tcaagacagcacttggtaaaaca	59
Homo sapiens hydroxysteroid (17-beta) dehydrogenase 2 (HSD17B2)	cctggaggcttcctaacaaa	gtcctggccgtagtcttcct	10
Homo sapiens insulin-like growth binding protein 1 (IGFBP1)	aatggattttatcacagcagacag	ggtagacgcaccagcagagt	58
Homo sapiens interleukin 15 (IL15)	cagatagccagcccatacaag	ggctatggcaaggggttt	46
Homo sapiens interleukin 15 receptor, alpha (IL15RA)	acaacccccagtctcaaatg	tgccgtcgttactgtggag	37

Homo sapiens IQ motif containing GTPase activating protein 1 (IQGAP1)	ctagaaacaccagccaccagt	tcacggatagcacgtctctg	38
Homo sapiens peripheral myelin protein 22 (PMP22)	ttctcatcatcaccaaacgaa	gctgaagatgatcgacaggat	49
Homo sapiens progesterone receptor (PGR)	tttaagagggcaatggaagg	cggattttatcaacgat	11
Homo sapiens prolactin (PRL)	aaaggatcgccatggaaag	gcacaggagcaggtttgac	18
Homo sapiens prothymosin, alpha (gene sequence 28) (PTMA)	cgaaatcaccaccaaggact	ctccccattttcctcattctc	34
Homo sapiens Rho-associated protein kinase (ROCK)	acatttgactggaaataaaga	gctcgagttgcagggttaga	88
Homo sapiens steroid sulfatase (microsomal), isozyme S (STS)	cggaagtaatgggatctataaagg	aacgaaggatgcctggaac	12
Homo sapiens steroid-5-alpha-reductase, alpha polypeptide 1 (3-oxo-5 alpha-steroid delta 4-dehydrogenase alpha 1) (SRD5A1)	catgttcctcgtccactacg	aacataatcgccattgtacacg	59
Homo sapiens steroidogenic acute regulatory protein (STAR), nuclear gene encoding mitochondrial protein, transcript variant 1	ggcatccttagcaaccaaga	actttgtccccattgtcctg	11
Homo sapiens superoxide dismutase 2, mitochondrial (SOD2)	ctggacaaacctcagcccta	tgatggcttccagcaactc	22
Homo sapiens vascular endothelial growth factor A (VEGFA)	tgcccgctgctgtctaat	tctccgctctgagcaagg	1

Table 2.7. Table of oligonucleotide sequences used in RT-PCR analysis.

Chapter 3

3. The steroid microenvironment generated by decidualisation of endometrial stromal cells.

3.1. Introduction

The endometrium is a dynamic tissue which undergoes cyclical regeneration and remodelling in response to ovarian sex steroids. Decidualisation is the differentiation and proliferation of endometrial stromal cells (ESC) initiated in the mid to late secretory phase of the menstrual cycle in response to rising levels of progesterone and is accompanied by angiogenesis and leukocyte infiltration (9). In women decidualisation occurs independent of the presence of a conceptus. Decidualisation of stromal cells provides an appropriate nutritional and hormonal environment that is essential during the establishment of pregnancy. The uterine preparation for pregnancy requires concerted control of receptivity, implantation and decidualisation. Indispensable to the synchronous control of these processes are the ovarian steroid hormones oestrogen and progesterone.

Decidualisation occurs in an environment of rising progesterone during the secretory phase of the menstrual cycle (1). Stromal differentiation begins in cells surrounding the spiral arterioles approximately 8 days following the luteinizing hormone surge (LH+8) and spreads in a wave-like manner through the uterus (2, 119). Decidualisation is a continuum which features coordinated expression of decidual specific genes, secretion of growth factors such as prolactin and IGF-binding protein (IGFBP) and culminating in the transformation of fibroblastic stromal cells into ‘epithelioid’ decidual cells in the late secretory phase (119). Decidual cells persist in pregnancy and form the maternal component of the placenta, the decidua basalis (14). In the absence of pregnancy and consequent demise of the corpus luteum, circulating progesterone levels fall and the decidual endometrium regresses and is shed during menstruation (1, 15).

Progesterone acting through its cognate receptor (PR) is essential for decidualisation. PR knockout (PRKO) mice are infertile as a result of implantation and decidualisation failure (121). Stromal PR expression is required for progesterone action which drives differentiation of stromal cells and secretion of classical decidualisation markers prolactin and IGFBP (122, 123).

In contrast, the functional requirement of oestrogen in decidualisation is less clear. Oestrogen drives proliferation of the endometrium and oestrogen priming is required to induce stromal progesterone receptor expression. Administration of the anti-oestrogen ICI 182780 severely impairs decidualisation in wild type mice suggesting oestrogen may be important in decidualisation (125). Despite this the decidualisation response is intact in both ERα and ERβ knockout mice and ovarian oestrogen is not essential for decidualisation (125-127).

Steroid biosynthesis occurs in many tissues throughout the body most notably in the gonads; ovary and testis, and adrenals and involves a series of enzyme reactions that convert cholesterol into steroid hormones such as oestrogen and testosterone (see Figure 3.1). The activity of steroidogenic acute regulatory protein (StAR) and cholesterol side chain cleavage enzyme (encoded by *CYP11A1* gene) mediate the critical initial steps in steroid hormone biosynthesis (320, 321) (See section 1.6 and Figure 3.1).

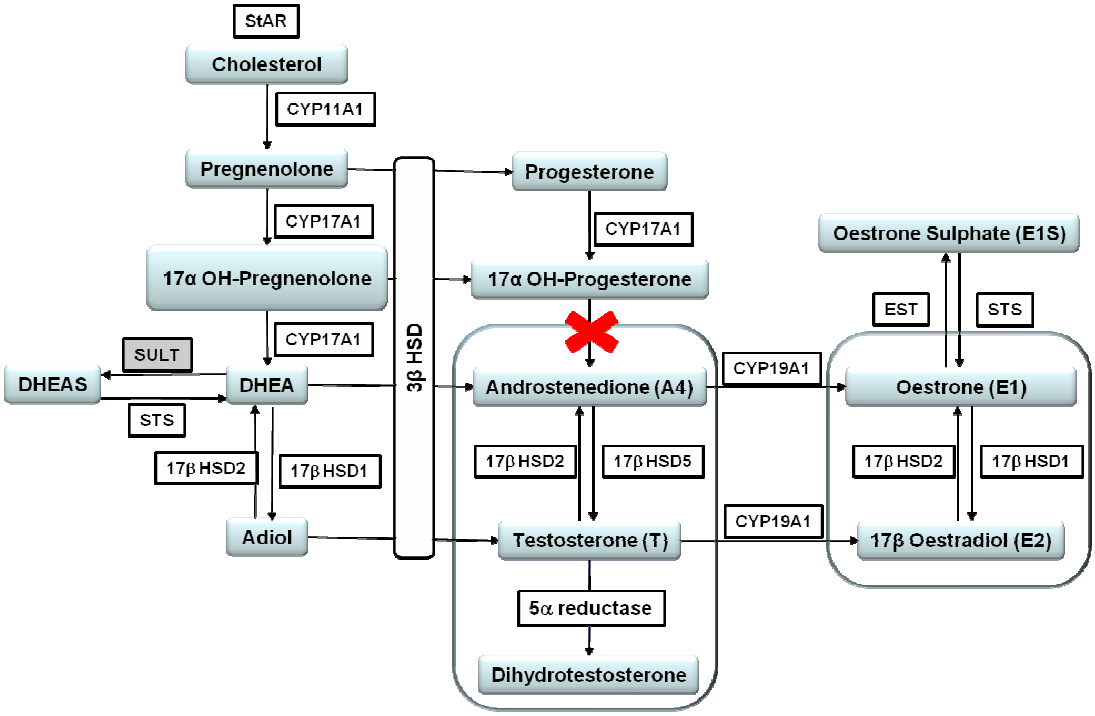

Figure 3.1. Schematic diagram illustrating the steroid biosynthesis pathway from cholesterol to formation of androgens (blue section) and oestrogens (pink section). The steroid enzymes which were studied in ESC (white boxes) are shown in the context of steroid metabolism in the human endometrium.

The critical step in biosynthesis of oestrogens involves the activity of the aromatase enzyme complex which acts by aromatising androgen precursors to oestrogens (328). In humans aromatase is encoded by a single gene (*CYP19A1*) and expression is regulated by tissue specific promoters (327). 17β hydroxysteroid dehydrogenases (17βHSD) are an important class of enzymes that catalyse the inter-conversion of oestrogens and androgens [13]. Isozymes of 17βHSD permit conversion of sex steroids in a cellular and isotype specific manner (320). Additionally, steroid sulphatase (STS) hydrolyses sulphated steroids, which are abundant in the circulation but are inactive at steroid receptors, into biologically active steroids (335) and may be an additional source of bioavailable oestrogens. The endometrium is known to express a wide range of enzymes responsible for the metabolism and conversion of steroids (See Figure 3.1, Table 3.1).

	Expression in Endometrium		**References**
	Stromal	**Epithelial**	
StAR	+	-	(309)
CYP11A1	+	+	(310)
CYP19A1	Not detected	Not detected	(311-313)
17βHSD1	+	+	(312)
17βHSD2	Highest in secretory phase	Highest in secretory phase, stronger than stromal	(312, 314)
17βHSD5	+/-	Highest in early secretory phase	(315, 316)
3βHSD	Not described	Weak in proliferative phase, moderate in secretory phase	(317)
STS	Reduced in late secretory phase	+	(312)
SRD5A1	+/-	All phases	(318, 319)

Table 3.1. Summary of mRNAs encoding proteins involved in steroid biosynthesis within the normal endometrium. See text for abbreviations.

Studies on steroid metabolism in human endometrial tissue explants have revealed a high capacity for inter-conversion between androstenedione and testosterone as well as oestrone and oestradiol. In normal endometrium the reaction favours formation of oestrone and androstenedione over oestradiol and testosterone respectively (338). Steroidogenic enzymes also appear to be under cyclical control in the endometrium. 17βHSD2 mRNA expression has been identified in human endometrium and expression is highest in the secretory phase of the menstrual cycle (314). 17βHSD5 (also known as AKR1C3 due to its aldo/keto reductase activity) is expressed in the endometrium throughout the menstrual cycle with highest mRNA detection in the early secretory phase (315). STS immunoexpression has been detected in both stromal and epithelial cells in the endometrium across the menstrual cycle and is reduced in the late secretory phase (312). Levels of 17βHSD1 do not appear to vary

over the menstrual cycle (312). Endometrial expression of enzymes with oestrogen forming capacity such as 17βHSD1, 17βHSD2 and STS has been reported (Table 3.1).

The role of aromatase in normal endometrium is poorly understood. Initial reports (345, 346, 469) described aromatase activity in ESC and epithelial glands from normal endometrium. However subsequent studies have failed to describe CYP19A1 mRNA expression in normal endometrium (311, 375). Little is known about the potential for the endometrium to synthesise steroid hormones and this tissue has classically been thought of as a steroid target and site of steroid metabolism but not biosynthesis (312, 338).

Endometriosis is a chronic gynaecological disorder that is characterised by the presence of uterine endometrial tissue at ectopic sites outside of the normal location (373). Endometriosis is an oestrogen dependent disease that is associated with aberrant expression of steroidogenic enzymes (376). Increased expression of aromatase is reported in ectopic endometrium of women with endometriosis and decreased expression of 17βHSD2 is reported in endometriomas (313, 374, 375). Since endometriosis is an oestrogen-dependent disease aberrant expression of aromatase is considered to be critical in its pathophysiology. Thus, aromatase inhibitors have been used in the treatment of endometriosis as a means of impairing oestrogen formation (462). Stromal cells from endometriotic lesions also have reduced decidualisation capacity *in vitro* and dysregulation of steroidogenic enzymes in response to decidualising stimuli is also reported (313, 470).

In addition to the pivotal role of ovarian progesterone (1, 121) in regulating decidualisation a recent study in mice suggests that *de novo* biosynthesis of oestrogen may also be essential during decidualisation (128). In mice local aromatase activity is essential to decidualisation and establishment of pregnancy, however to date there are no data from other species. We hypothesise that local biosynthesis of oestrogen occurs during decidualisation in women and that non-pathological changes in steroidogenic enzymes may be a feature of differentiation of stromal cells as part of the functional changes in the uterine environment required for establishment of pregnancy. This chapter describes preliminary investigations into the role of oestrogen biosynthesis and metabolism during *in vitro* decidualisation of endometrial stromal cells.

3.2. Aims of the Chapter

1. To determine whether decidualisation of human endometrial stromal cells is associated with changes in local steroid metabolism.

2. To determine the impact of inhibitors of steroidogenic enzymes on decidualisation and steroid biosynthesis.

3.3. Methods

3.3.1. Primary human endometrial stromal cells

Primary human endometrial stromal cells (hESC) were isolated from biopsies (n=27) of healthy endometrium as detailed in section 2.3. Cells were seeded in 6 well plates at a density of $2x10^5$ cells per well. hESC were maintained in RPMI 1640 media (Sigma, Cat. No. R0883) supplemented with 10% heat-inactivated foetal calf serum (FCS; Invitrogen, Cat. No. 10082-147), 10mL/L Penicillin/Streptomycin (10,000 units penicillin and 10mg Streptomycin per ml solution, Sigma, Cat. No. P-4333), 2mM L-glutamine (Sigma, Cat. No. G-7513), and 2.5µg/ml fungizone (Invitrogen, Cat. No. 15290-018) for a maximum of 4 passages. Cells were transferred to phenol red-free RPMI 1640 medium (Sigma, Cat. No. R7509) containing 10% charcoal-stripped FCS (CSFCS) and otherwise supplemented as detailed above for 48 hours prior to use in experimental assays. Prior to decidualisation hESC cells were transferred to serum depleted media (as above but 2% CSFCS) for 24 hours. Cells were maintained at 37°C under 5% CO_2 in air. Table 3.2-3.5 detail the treatment regimens utilised. Treatments were completed in duplicate and each experiment repeated 3-8 times with hESC isolated from separate endometrial biopsies.

Treatment name	Treatment	Final concentration	Incubation period
Control	DMSO	Equivalent 10^{-6}M	6, 8 days
DEC	8-Br-cAMP	0.1 mg/ml	6, 8 days
	Progesterone	10^{-6}M	

Table 3.2. Experimental regimen for experiments 3.4.1, 3.4.2, 3.4.3, 3.4.4 and 3.4.7.

Treatment name	Treatment	Final concentration	Incubation period
Control	DMSO	Equivalent 10^{-6}M	1, 2, 4, 8 days
cAMP	8-Br-cAMP	0.1 mg/ml	1, 2, 4, 8 days
cAMP + P4	8-Br-cAMP	0.1 mg/ml	1, 2, 4, 8 days
	Progesterone	10^{-6}M	

Table 3.3. Experimental regimen for experiments 3.4.5 and 3.4.8.

Treatment name	Treatment	Final concentration	Incubation period
Control + Vehicle	DMSO	Equivalent 10^{-5}M	8 days
Control + 1μM AI	anastrozole (AI)	1μM AI	8 days
Control + 10μM AI	anastrozole (AI)	10μM AI	8 days
DEC + Vehicle	8-Br-cAMP	0.1 mg/ml	8 days
	Progesterone	10^{-6}M	
	DMSO	equivalent 10^{-5}M	
DEC + 1μM AI	8-Br-cAMP	0.1 mg/ml	8 days
	Progesterone	10^{-6}M	
	Anastrozole (AI)	1μM	
DEC + 10μM AI	8-Br-cAMP	0.1 mg/ml	8 days
	Progesterone	10^{-6}M	
	Anastrozole (AI)	10μM	

Table 3.4. Experimental regimen for experiment 3.4.9.

Treatment name	Treatment	Final concentration	Incubation period
DEC	8-Br-cAMP	0.1 mg/ml	1, 2, 4, 8 days
	Progesterone	10^{-6}M	
DEC + STX	8-Br-cAMP	0.1 mg/ml	1, 2, 4, 8 days
	Progesterone	10^{-6}M	
	STX64	5μg/ml	

Table 3.5. Experimental regimen for experiment 3.4.10.

3.3.2. Immortalised human endometrial stromal cells (SHT290)

The human TERT- immortalised stromal cell line, SHT 290 (Gift from Dr. David Kaufman, Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, NC, USA.), was maintained under the same culture conditions as hESC (see section 2.4.22.4.2) however unlike hESC, SHT 290 cells could be maintained for multiple passages. SHT 290 cells were seeded in 6 well plates at a density of $2x10^5$ cells per well for experimental assays. Prior to decidualisation SHT 290 cells were transferred to serum depleted media Tables 3.6-3.8 detail the treatment regimens utilised. Treatments were completed in duplicate and each experiment repeated 3 times.

Treatment name	Treatment	Final concentration	Incubation period
Control	DMSO	Equivalent 10^{-6}M	8 days
DEC	8-Br-cAMP	0.1 mg/ml	8 days
	Progesterone	10-6M	

Table 3.6. Experimental regimen for experiment 3.4.1.

Treatment name	Treatment	Final concentration	Incubation period
Control	DMSO	Equivalent 10^{-6}M	6 days
DEC	8-Br-cAMP	0.1 mg/ml	6 days
	Progesterone	10-6M	

Table 3.7. Experimental regimen for experiment 3.4.6.

Treatment	Final concentration	Incubation period
oestrone + ^{3}H-oestrone	10^{-8}M	2, 4, 8, 16, 24 h
oestradiol + ^{3}H-oestradiol	10^{-8}M	2, 4, 8, 16, 24 h
testosterone + ^{3}H-testosterone	10^{-8}M	2, 4, 8, 16, 24 h
androstenedione + ^{3}H-androstenedione	10^{-8}M	2, 4, 8, 16, 24 h

Table 3.8. Experimental regimen for experiment 3.4.6. Cells were treated as in table 3.7 followed by time course shown.

3.3.3. Immunohistochemistry

Immunohistochemistry was conducted on human endometrial tissue sections to establish the presence and localization of CD10 (stromal cell marker). Tissues were collected and fixed in 4% NBF and embedded in paraffin wax. 5µm sections were cut and mounted onto coated glass slides (VWR, Poole, UK). Antigen retrieval was performed using 0.05M Glycine/EDTA buffer (pH 8) followed by methanol peroxidase block, serum block, and avidin-biotin block. Primary antibody to CD10 was diluted 1:200 in blocking serum (normal goat serum, TBS, BSA), added to sections and incubated overnight. Slides were incubated with goat anti-mouse biotinylated secondary antibody followed by streptavidin-HRP before final antibody detection using 3,3'-diaminobenzidine (DAB). Slides were counterstained in Harris' haematoxylin. DAB positive sections were imaged using PROVIS microscope (Olympus Optical, London UK) with an attached Canon DS126131 camera.

3.3.4. Immunocytochemistry

Isolated stromal cells (SHT290 and hESC) were grown on chamber slides (Ref no: 354102, BD Biosciences, Belgium) at an initial seeding density of $1x10^{4}$ cells/ml and immunodetection of CD10 and aromatase was determined using immunocytochemistry. Following culture, cells were fixed in ice-cold methanol and endogenous peroxidase blocked in 0.15% H_2O_2 in methanol for 30 minutes. Following washes in PBS, cells were permeablised by incubation in 0.2% IGEPAL, 1% BSA, 10% NGS in PBS for 20 minutes followed by serum block for 30 minutes. Thereafter cells were incubated overnight at 4^{o}C with primary antibody. Antigen detection was performed as described in section 2.12.

3.3.5. RNA extraction

Extraction of RNA from cell monolayers was performed using RNAeasy® mini kit (Qiagen, Crawley, UK) as described in section 2.8. Samples were collected in 350µl of lysis buffer per well of 6-well plate and homogenized using QIAshredder® spin columns (Qiagen, Crawley, UK).

3.3.6. Preparation of cDNA using SuperScript® VILO™ synthesis kit.

Reverse transcription of RNA to form a single stand of complementary DNA (cDNA) was carried out using the SuperScript VILO cDNA synthesis kit (InVitrogen) as described in section 2.9. Samples were incubated under the following conditions: 25^{o}C for 10 minutes, 42 ^{o}C for 60 minutes, 85 ^{o}C for 5 minutes in a thermal cycler.

3.3.7. Quantitative-Real Time-PCR (Taqman® Method)

Concentrations of mRNAs in samples was determined by reverse transcription followed by quantitative-real time polymersase chain reaction performed in an ABI machine using the Taqman® Method (Section 2.10).

Each reaction was prepared using the qPCR Supermix with premixed ROX kit (11795-01K; Invitrogen) and conducted in duplicate and plated in a 96-well MicroAmp Fast Optical reaction plate (Applied Biosystems). Real time PCR amplification was performed using the Applied Biosystems® 7900HT Fast Real-Time PCR System using reaction mix described in section 2.10.2 under the following cycling conditions: 95^{o}C for 10 minutes, followed by 40 cycles of 95 ^{o}C for 15 seconds and 60 ^{o}C for 1 minute.

3.3.8. Human insulin-like Growth Factor Binding Protein 1 enzyme-linked immunosorbent assay (ELISA)

The concentration of human Insulin-like Growth Factor Binding Protein 1 (IGFBP) protein in cell culture supernatants was determined by two-site sandwich ELISA as described in section 2.11.

The ELISA was performed using reagents from IGFBP1 DuoSet ELISA development kit (R&D Systems, Minneapolis, MN, USA). Cell growth medium was collected following *in vitro* treatment of cells (sections 3.3.1 and 3.3.2) and stored at -80°C to preserve protein integrity and thawed immediately prior to the assay. Optical density of each well was

measured immediately using a microplate reader set to 450nm with wavelength correction set to 540nm. Concentration of IGFBP1 in cell culture supernatants was calculated from the standard curve. Data was linearised by plotting log concentration against log optical density and unknowns determined by regression analysis using GraphPad Prism (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com).

3.3.9. Oestrone ELISA

Oestrone in cell culture supernatants was detected using an Estrone ELISA kit (BioVendor, Czech Republic) and quantified against a calibrator standard set as described in section 2.11.2.2. Optical density of each well was measured immediately using a microplate reader set to 450nm with wavelength correction set to 540nm. The delta absorbance was recorded as final values and concentrations were then calculated from the standard values. Unknowns were determined by non-linear regression analysis using GraphPad Prism (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The specificity of the oestrone ELISA kit is described in the table below.

Steroid	**% Cross-Reactivity**
Estrone	100
Estrone-3-Sulfate	4.9
17β-Estradiol	2.2
Estrone-3-Glucuronide	1.2
17β-Estradiol-3-Glucuronide	0.14
Androstenedione, Cholesterol, Corticosterone, Cortisol, Cortisone, DHEAS, Diethylstilbesterone, Estriol, Estradiol-Sulfate, Progesterone, 17-OH Progesterone, Testosterone.	0.1

Table 3.9. Specificity of oestrone ELISA kit as described in manufacturers instructions. The detection limit was 10 pg/ml

3.3.10. Oestradiol ELISA

Oestradiol in cell culture supernatants was detected by competitive ELISA using oestradiol-6-HRP conjugate (in house preparation) and oestradiol-6 antibody (#20R-ER005, Fitzgerald Industries International, Concord, MA, USA) as described in section 2.11.2.1.

Immuno 96 MicroWell™ Solid Plates (NUNC, Roskilde, Denmark) were coated with 100µL/well of 1:200 DARS (Donkey anti rabbit serum) in coating buffer (100mM Bicarbonate solution, pH 9.6) and incubated overnight at 4^{o}C on a rocker. After incubation

coating solution was aspirated and each well washed 3 times with wash buffer (0.05% Tween20 in PBS). After washing, plates were blocked by adding 300µL of blocking buffer (1% Gelatin (Sigma, G9382, Dorset, UK) in PBS) to each well and incubated for a minimum of 1 hour at room temperature. Plates were washed a further three times and 100µL of standards/unknowns added to plate. 50 µL of oestradiol-6-HRP conjugate 1:40,000 in PGBS and 50 µL of oestradiol 6 antibody at dilution of 1:150,000 in PGBS was added and incubated for 3 hours at room temperature or overnight at 4^{o}C. Solutions were aspirated and plates washed four times. 100ul of TMB substrate solution was added to each well and incubated in the dark for 20 minutes. The colour reaction was stopped by addition of 50µL/well of 2N H_2SO_4. Optical density of each well was measured immediately using a microplate reader set to 450nm with wavelength correction set to 540nm. The delta absorbance was recorded as final values and concentrations were then calculated from the standard values. Unknowns were determined by non-linear regression analysis using GraphPad Prism (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The specificity of the oestradiol-6 antibody is described in the table below.

Steroid	**% Cross-Reactivity**
Estradiol	100
Estrone	<2
Estriol	<0.15
Testosterone	<0.01
DHEA	<0.01
Androstenedione	0

Table 3.10. Specificity of oestradiol-6 antibody as described in manufacturers instructions. The detection limit of E2 ELISA was 10 pg/ml.

3.3.11. Thin Layer Chromatography (TLC)

SHT 290 cells were cultured with medium containing unlabelled steroid (10^{-8} M final concentration) as well as tritium-labelled steroid, sufficient to give 100,000 counts per ml, and incubated for 2, 4, 8, 16 or 24 hours. Following incubation for the appropriate amount of time, cell culture media were aspirated and mixed with 9 ml dichloromethane (CH_2Cl_2) in a conical-bottomed glass vial. The media and solvent were vortexed and allowed to stand for a minimum of two hours to allow steroid to dissolve into the solvent phase. The vials were then centrifuged at 868 x g for 15 minutes to ensure clear separation of the aqueous (cell culture media) and solvent (dichloromethane) layers. The upper aqueous layer was then discarded by suction pump aspiration at a designated radioactive sink. The solvent layer was

then dried using a sample drier under nitrogen gas on a heat block set to 40°C. The chromatography tank was prepared by adding 150ml of running solvent (Chloroform/ethanol; 92%/8%) and allowed to equilibrate for 1hr before use. Dried samples were reconstituted in 100µL spotting mix, which contained unlabelled steroid mix and dichloromethane. Samples were spotted onto an Aluminium Silica coated TLC plate (Alugram, SIL G/UV_{254}, Macherey Nagel, Germany) which was placed in the chromatography tank for approximately 90 minutes or until the solvent front reached the end of the plate. Finally the plate was removed and allowed to dry in the fume hood before being placed on TLC scanner to record plate readings (see Figure 3.20 and Figure 3.3). Distribution of radioactivity on TLC plates was evaluated by radiometric scanning (Bioscan system 200 image scanner, Bioscan, Washington, DC, USA) and recorded using WinScan software, Lablogic software.

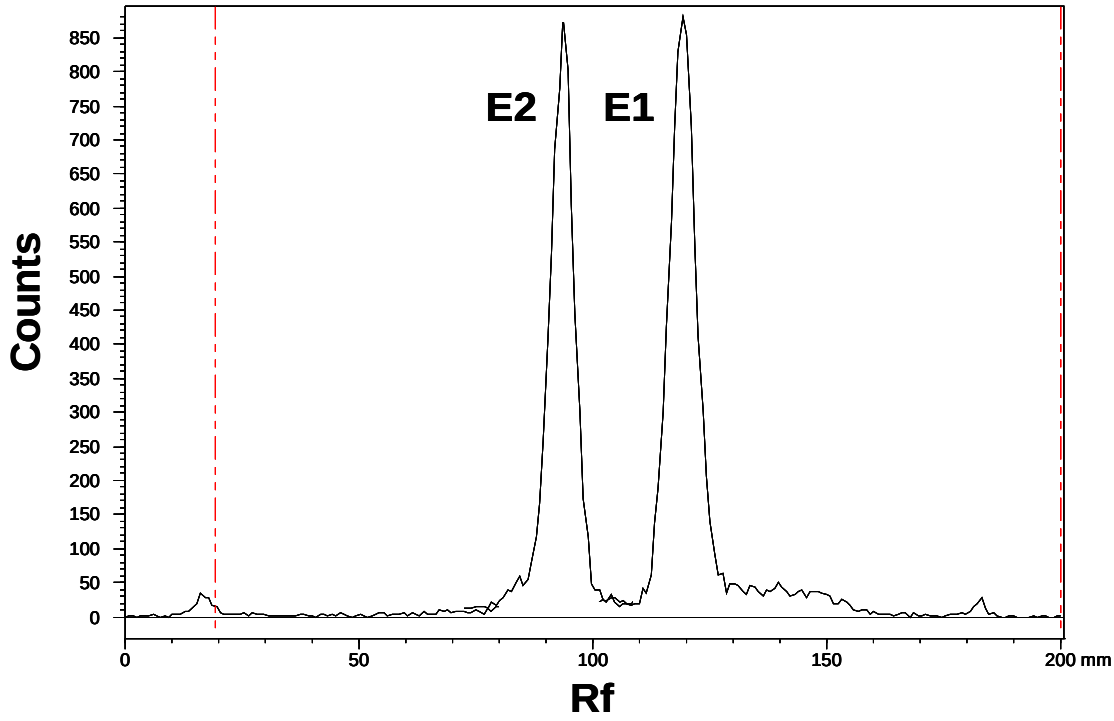

Figure 3.2. Oestrogen TLC standards. ^{3}H-Oestrone (E1) and ^{3}H-Oestradiol (E2) tracers give Rf values of ~120 and ~95 respectively. The retention factor (Rf) values (X axis) give a measure of the distance the spotted analyte has travelled along the plate which directly correlates to the physical properties of the analyte such as polarity. Arbitrary counts (Y axis) are the measured radioactivity of the tracer which is greatest where the analyte is most concentrated on the plate i.e. the point on the plate to which the analyte has travelled. Under consistent assay conditions analytes are detected at the same positions on the TLC plate and can be identified based on the Rf values of standards.

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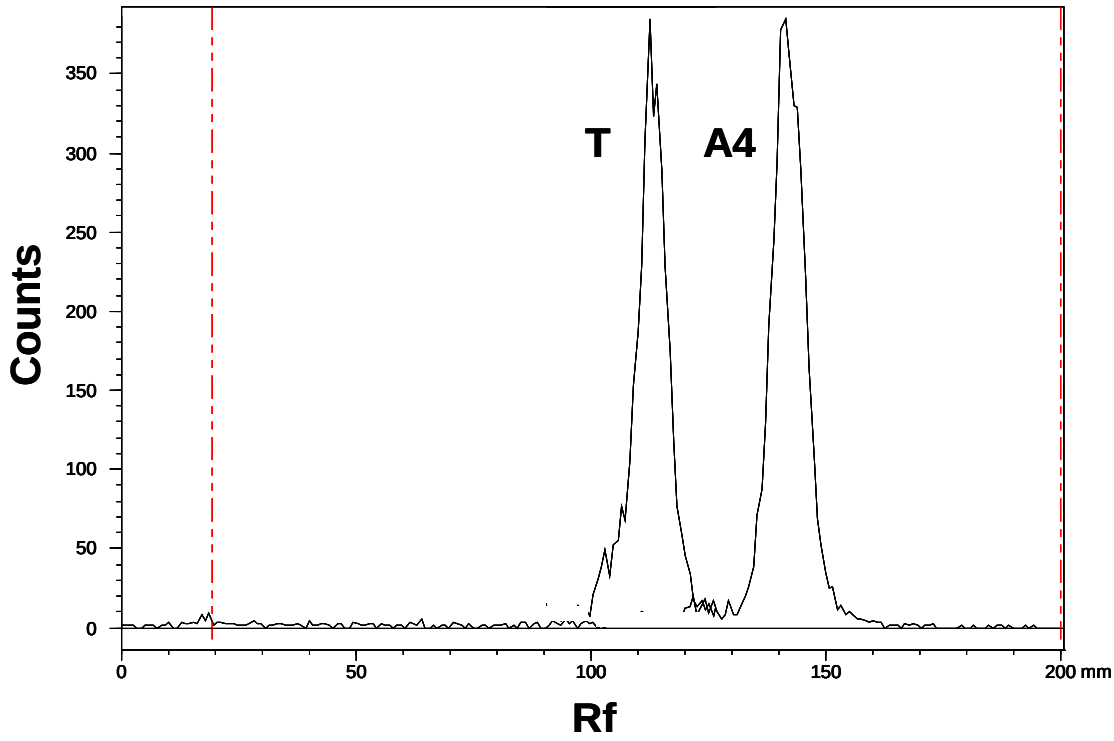

Figure 3.3. Androgen TLC standards. ^{3}H-testosterone (T) and ^{3}H-androstenedione (A4) tracers give Rf values of ~112 and ~145 respectively. The retention factor (Rf) values (X axis) give a measure of the distance the spotted analyte has travelled along the plate which directly correlates to the physical properties of the analyte such as polarity. Arbitrary counts (Y axis) are the measured radioactivity of the tracer which is greatest where the analyte is most concentrated on the plate i.e. the point on the plate to which the analyte has travelled. Under consistent assay conditions analytes are detected at the same positions on the TLC plate and can be identified based on the Rf values of standards.

3.3.12. Tritiated water assay

To determine aromatase activity in hESC a tritiated water assay was performed (see section 2.15). hESCs were cultured in 6 well plates as described previously (section 2.4). Decidualised cells were cultured in decidualising media (cAMP and P4, see section 3.3.1) for up to 6 days prior to assay. Prior to assay all cells were transferred to serum-depleted phenol red-free medium.

Cells were incubated with 150pmol/ml androstenedione (A4) substrate (30pmol 'Hot' A4 i.e. ^{3}H-A4 and 120pmol 'Cold' A4) at 37°C for 2hrs. Following incubation, cells were incubated on ice for 10 minutes and then medium was removed and 1ml of 30% trichloroacetic acid was added to the media.

4ml of Chloroform ($CHCl_3$) was added to the samples, which were vortexed and incubated overnight on a shaker to extract steroids. Samples were centrifuged at 1811 x g for 5 minutes and 1 ml of the upper phase transferred to a new glass vial. To this, 2ml of charcoal/dextran solution (5%:0.5% v/w in H_2O, Charcoal dextran; Sigma C6241) was added and samples were vortexed thoroughly. The samples were then centrifuged at 1811 x g for 30 minutes. 1 ml of supernatant was then transferred to scintillation vials containing 10 mls of scintillation fluid and finally the activity of samples was measured using a beta counter.

3.3.13. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The criterion for significance for all tests was set at $p<0.05$.

3.4. Results

3.4.1. Primary human ESC and immortalised SHT290 cells are both CD10-positive and exhibit characteristic responses to decidualising stimuli.

Expression of CD10 was investigated in order to confirm that isolated human endometrial stromal cells (hESC) and SHT290 cells were phenotypically identical to endometrial stromal cells. CD10 is normally expressed in endometrial stromal cells (471, 472) (see Figure 3.4 A, B) and its expression distinguishes stromal cells from glandular epithelial cells. When immunocytochemistry was performed on primary hESC and SHT290 cells using chamber slides, intense positive cytoplasmic staining was observed in both cell types (Figure 3.4, D, E and G, H respectively).

To confirm that both primary hESC and SHT290 retained the capacity to differentiate cells were treated according to standard protocol utilizing cAMP and progesterone (132, 305, 473) for 8 days. Decidualisation was assessed by measuring the expression of mRNAs encoding the classical decidualisation marker IGFBP-1. Secretion of IFGBP-1 protein was detected by ELISA. After 8 days, consistent with the expected response to cAMP and progesterone, the morphology of both cell types was changed from a classical elongated fibroblast into cells with an ‘epithelial’ appearance (Figure 3.5, A and B). Consistent with the observed change in cellular morphology, expression of IGFBP-1 mRNA was significantly up-regulated in decidualised ESC compared to non-decidualised ESC ($p<0.0001$, $n=4$) (Figure 3.5, C). IGFBP-1 protein secretion paralleled mRNA expression changes and was increased in

decidualised ESC ($p<0.0001$, n=4) (Figure 3.5, D). The response of SHT290 was similar to isolated primary ESC (Figure 3.5, E and F) confirming they represented a suitable model system (465).

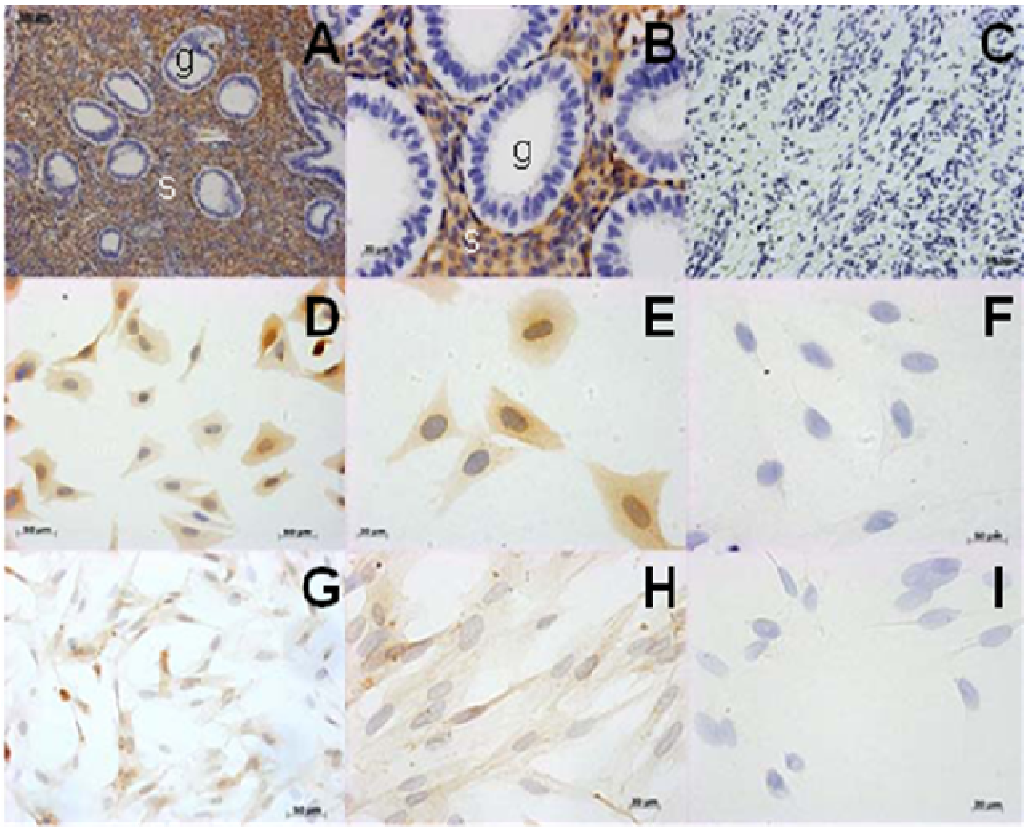

Figure 3.4. Immunodetection of CD10 in endometrial tissue sections and on isolated primary endometrial stromal cells and SHT cells. On sections of normal endometrium (A, B) immunoexpression of CD10 was intense (indicated by brown staining) in stromal fibroblasts (s) but epithelial cells were immunonegative and no staining was detected on the luminal surface (not shown) or the lining of the glands (g). Primary ESC (D, E) and immortalised SHT290 (G, H) both expressed CD10 confirming stromal phenotype. Negative controls were conducted in the absence of primary antibody for endometrium, stromal fibroblasts, and SHT290 (C, F, and I respectively).

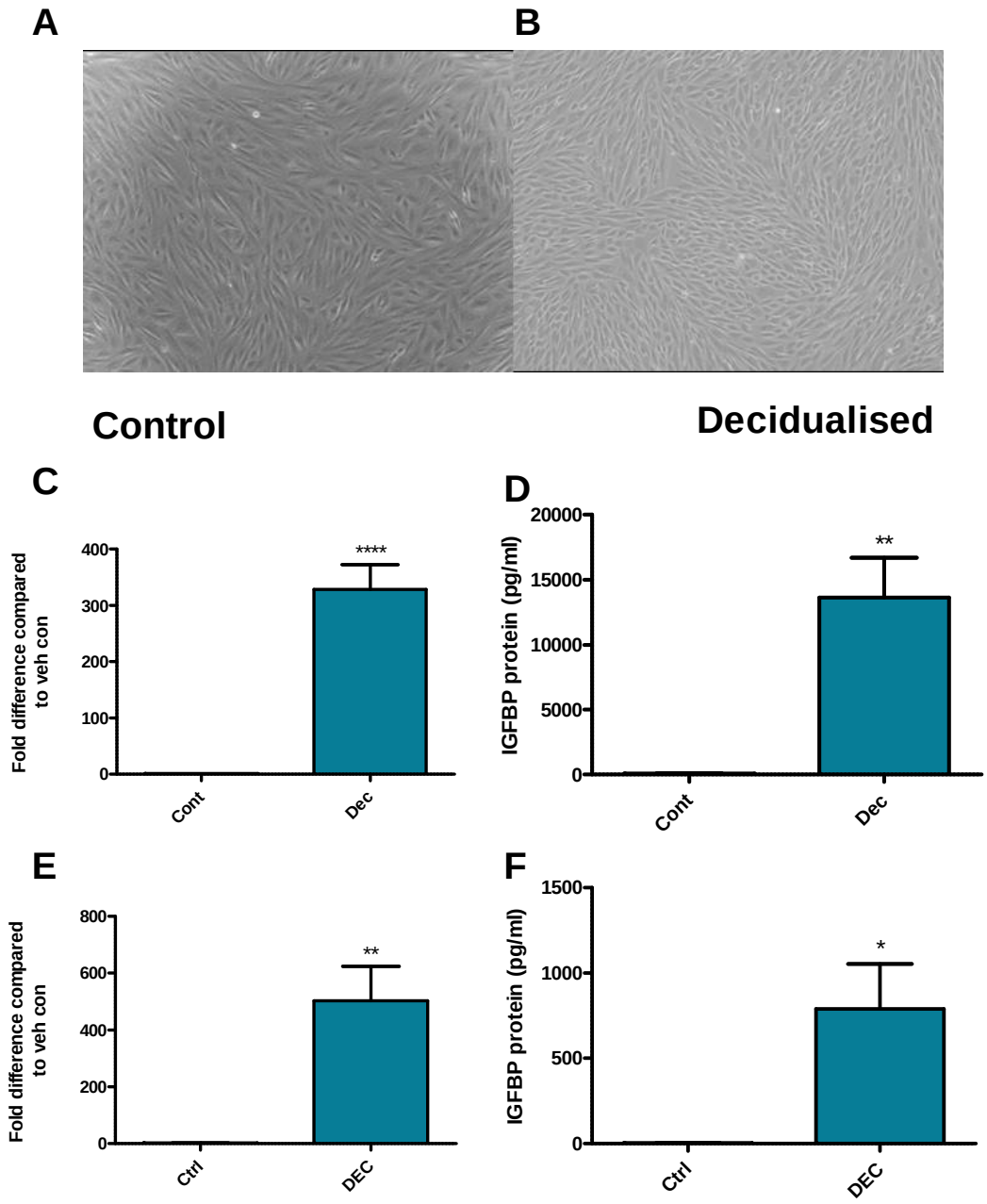

*Figure 3.5. Incubation of primary ESC induces a characteristic response according to a classical decidualisation protocol of 8 days treatment with 8-bromo cyclic adenosine monophosphate (cAMP) and progesterone (P4) in serum-depleted media. Changes in cell morphology observed in ESC upon decidualisation (A,B). Stromal cells change from elongated fibroblast morphology to more rounded epithelial phenotype. Expression of IGFBP mRNA (C) and secretion of IGFBP protein (D) in control (Ctrl) and decidualised (DEC) endometrial stromal cells (n=4, **** p<0.0001, ** p<0.01). Expression of IGFBP mRNA (E) and protein (F) was similarly upregulated in SHT cells (n=3, * p<0.05, ** p<0.01, veh control= vehicle control.) Statistical analysis was performed using an unpaired t-test.*

3.4.2. Human endometrial stromal cells express sex steroid receptors and their expression is altered in response to decidualisation.

To address whether changes in the local steroid environment could have a functional impact on decidualised and non-decidualised ESC expression of sex steroid receptors mRNAs was measured (Figure 3.6). The two key receptor isoforms of the oestrogen receptor, oestrogen receptor α (ERα) and oestrogen receptor β (ERβ) as well as androgen receptor (AR) and progesterone receptor (PR) are all expressed in the stromal compartment of the endometrium throughout the menstrual cycle (250, 291, 474). Expression of mRNAs encoding for sex steroid receptors were detected in both undecidualised and decidualised ESC. Expression of ERα ($P<0.001$, $n=4$), AR ($P<0.01$, $n=4$) and PR ($P<0.001$, $n=4$) mRNA was decreased in decidualised ESC. Expression of ERβ mRNA was increased in decidualised ESC ($P<0.01$, $n=4$).

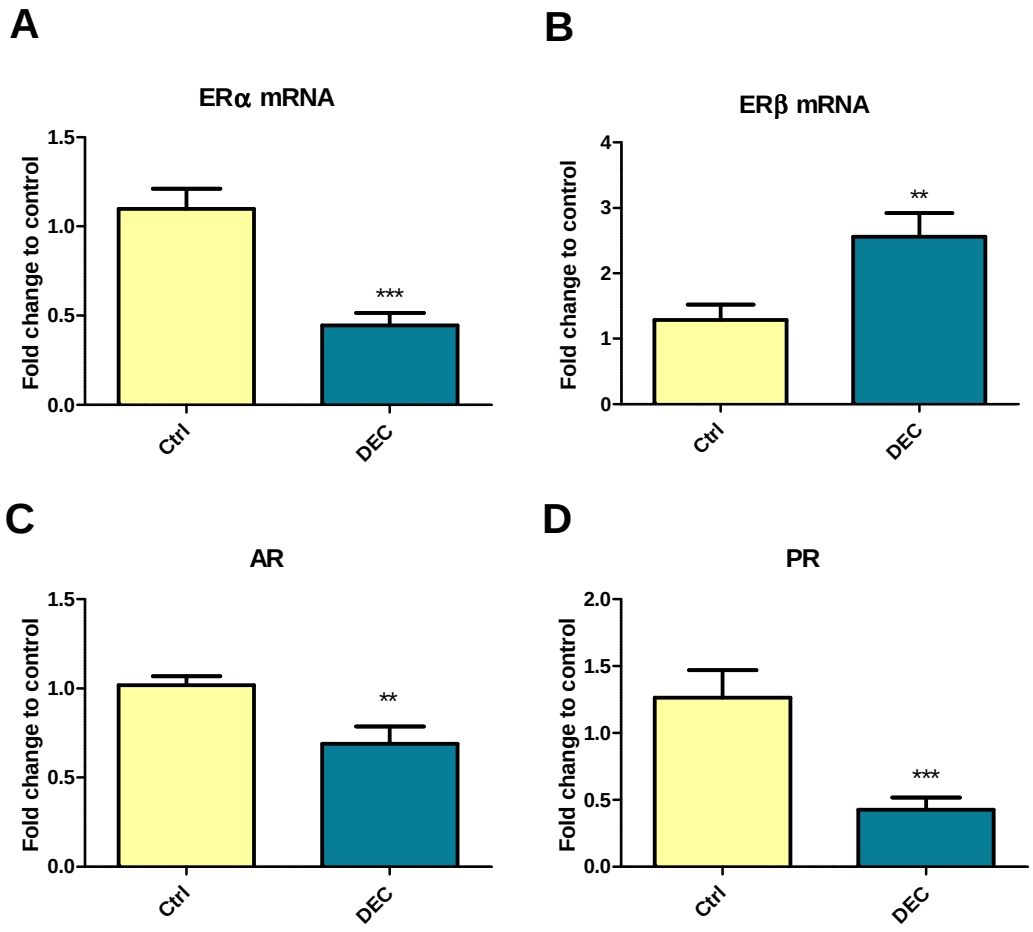

*Figure 3.6. Expression of sex steroid receptors; oestrogen receptor alpha (ERα), oestrogen receptor beta (ERβ), androgen receptor (AR) and progesterone receptor (PR) in primary ESC (ctrl) and in ESC exposed to decidualising media for 8 days (DEC). Expression of ERβ mRNA was increased in DEC cells whereas expression of ERα, AR and PR mRNA was decreased relative to control primary ESC. Expression of ERα (A), ERβ (B), AR (C) and PR (D) mRNA in non-decidualised and decidualised stromal cells as determined by Taqman qRTPCR (n=4 ** p<0.01; *** p<0.001). Statistical analysis was performed using an unpaired t-test.*

3.4.3. Decidualisation of ESC results in altered expression of mRNAs encoding enzymes associated with steroid metabolism.

To assess whether decidualisation of stromal cells might be associated with changes in intracellular steroid metabolism the concentrations of mRNAs encoding key metabolising enzymes in primary ESC and cells decidualised *in vitro* (8 days treatment regime) were

investigated. To assess whether cells could support *de novo* steroid synthesis expression of steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme (CYP11A1) were both measured.

StAR regulates cholesterol transport within mitochondria a key rate-limiting step in steroid biosynthesis and although expression of StAR mRNA was decreased in decidualised ESC (Figure 3.7 A, $p<0.01$, $n=4$) it was still detectable. CYP11A1 catalyses the first step in steroid hormone production by converting cholesterol to pregnenelone. Expression of CYP11A1 mRNA was not significantly different ($p=0.2106$) between decidualised and non-decidualised ESC (Figure 3.7 B).

Biosynthesis of oestrogen requires aromatase (encoded by *CYP19A1* gene) which acts by aromatising androgen precursors to oestrogens. Expression of CYP19A1 mRNA was increased in response to decidualisation (Figure 3.8 A, $p<0.01$, $n=4$) however a large number of cycles were required to detect this transcript ($Ct>35$). 17β hydroxysteroid dehydrogenase 1 (HSD1) catalyses the conversion of oestrone to oestradiol. Expression of 17βHSD1 mRNA was detected in both decidualised and non-decidualised ESC but no significant difference in expression was observed (Figure 3.8 B, $p=0.4632$, $n=4$). 17βHSD2 catalyses the conversion of oestradiol to oestrone and testosterone to androstenedione (323). Expression of 17βHSD2 mRNA was significantly higher in decidualised ESC than non-decidualised ESC with a striking 30-fold change in expression (Figure 3.8 C, $p<0.01$, $n=4$). 17βHSD5 catalyses the conversion of androstenedione to testosterone. Decidualisation did not significantly change the expression of 17βHSD5 (Figure 3.8 D, $n=4$).

Steroid sulphatase (STS) converts oestrone sulphate to oestrone. STS mRNA expression was highly and significantly up-regulated in decidualised ESC (Figure 3.9 A, $p<0.05$, $n=4$). The potential for conversion of androgens was also investigated by measuring of expression of 5α reductase (SRD5A1) mRNA. SRD5A1 is responsible for converting testosterone to dihydrotestosterone and expression of 5αR was significantly increased in decidualised ESC (Figure 3.9 B, $p<0.001$, $n=4$).

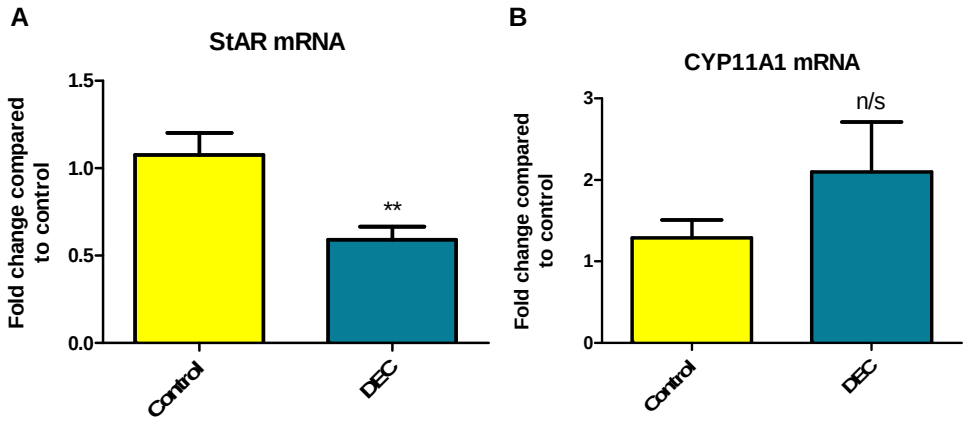

Figure 3.7. The expression of mRNAs encoding enzymes responsible for de novo synthesis of steroids were measured following decidualisation. CYP11A1 mRNA expression was not significantly changed although there was a trend toward increased expression following decidualisation. StAR mRNA expression was decreased after 8 days. Expression of steroidogenic enzymes in primary human ESC following 8 days in vitro decidualisation. ***A*** *StAR, n=4 ** p<0.01.* ***B*** *CYP11A1, n=4, n/s (not significant), p=0.2106. Cells were treated with control (DMSO) or decidualising media (DEC) for 8 days in serum-depleted phenol red-free medium. Statistical analysis was performed using an unpaired t-test.*

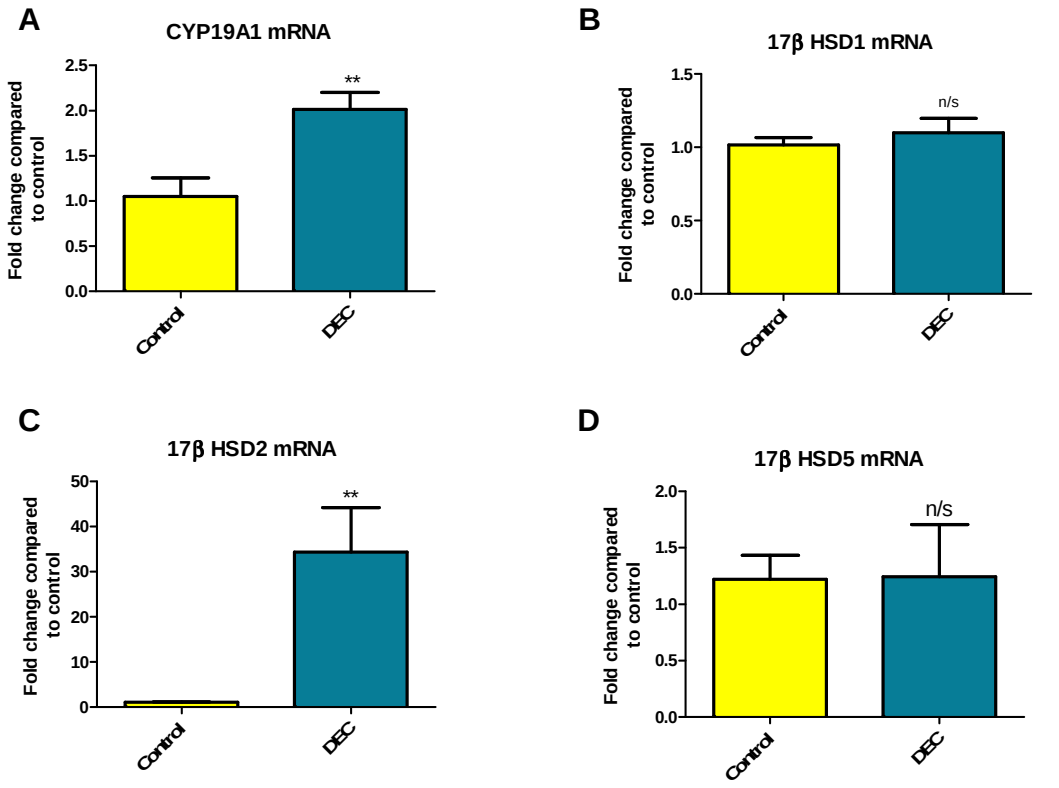

*Figure 3.8. The expression of mRNAs encoding enzymes responsible for conversion of androgens to estrogens (CYP19A1) and interconversion of androgens and oestrogens (17βHSD1, 17βHSD2 and 17βHSD5) were assessed. Following decidualisation, expression of mRNAs encoding CYP19A1 and 17βHSD2 were both significantly increased but 17βHSD1 and 17βHSD5 mRNAs remained unchanged. Expression of steroidogenic enzymes in primary human ESC following 8 days in vitro decidualisation. A CYP19A1 (aromatase); B 17β hydroxysteroid dehydrogenase (HSD) 1; C 17βHSD2; D 17βHSD5 (n=4, ** p<0.01, n/s not significant). Expression of mRNA determined by Taqman qRTPCR. Cells were treated with control (DMSO) or decidualising media (DEC) for 8 days in serum-depleted phenol red-free medium. Statistical analysis was performed using an unpaired t-test.*

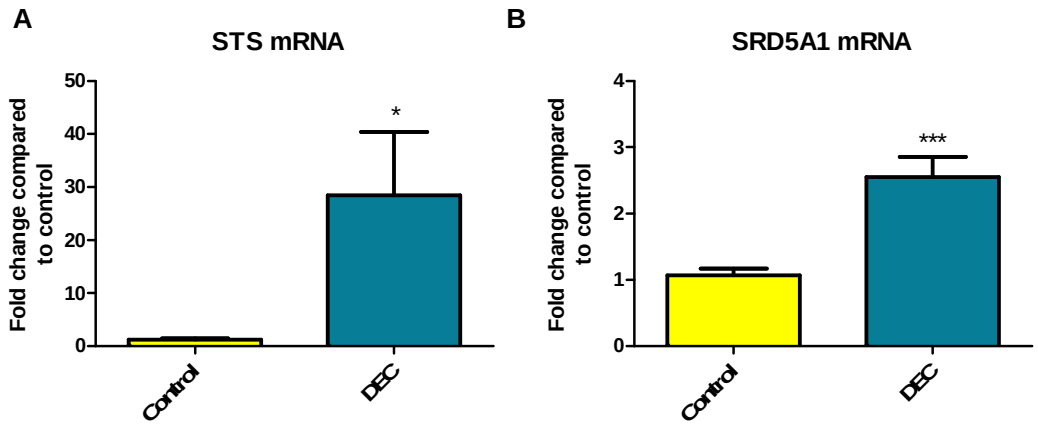

Figure 3.9. Expression of mRNAs encoding enzymes responsible for conversion of sulphated oestrogens (STS) or androgens (SRD5A1) were both increased by decidualisation. Expression of steroidogenic enzymes in primary human ESC following 8 days in vitro decidualisation. ***A*** *STS;* ***B*** *SRD5A1 (n=4 * P<0.05; *** p<0.001). Expression of mRNA was determined by Taqman qRTPCR. Cells were treated with control (DMSO) or decidualising media (DEC) for 8 days in serum-depleted phenol red-free medium. Statistical analysis was performed using an unpaired t-test.*

3.4.4. Decidualised hESC express aromatase protein

To assess whether decidualisation might be associated with increased expression of aromatase protein in primary hESC, the expression of aromatase was investigated using immunocytochemistry. In agreement with previously described mRNA expression data (section 3.4.3), aromatase was readily detected by immunocytochemistry in decidualised hESC (Figure 3.10, A) but absent in undecidualised hESC (Figure 3.10, B). In decidualised hESC, characteristic staining for aromatase was detected; positive punctate cytoplasmic staining was observed which was absent in the negative control. Antibody specificity was confirmed using positive control placental tissue sections in which aromatase staining was localized to the syncytiotrophoblast (Figure 3.10, C).

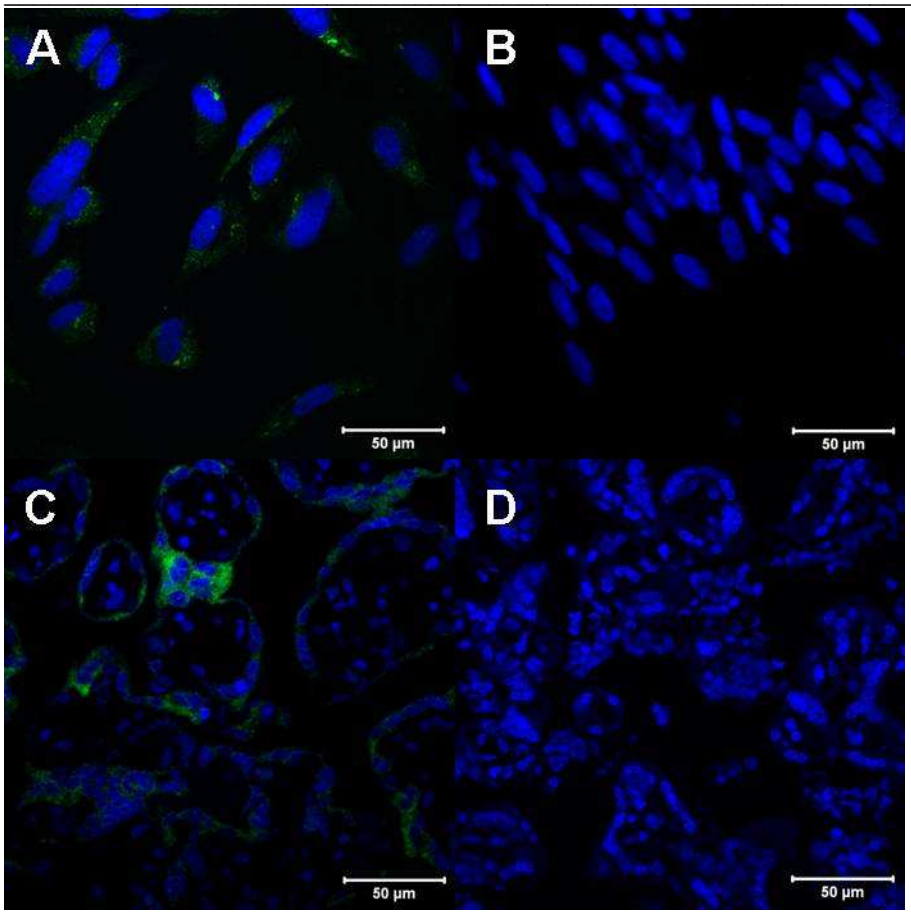

Figure 3.10. Immunodetection of aromatase in placental tissue sections and on isolated primary endometrial stromal cells. Positive cytoplasmic staining for aromatase (green staining) was detected in isolated decidualised human endometrial stromal cells (A), however this was absent in undecidualised ESC (B). On sections of placenta (C) aromatase (green staining) was detected in the syncytiotrophoblast which was absent in the negative control (B). Nuclear counterstain DAPI (blue staining). Scale bar 50 µm.

3.4.5. Expression of mRNAs encoding enzymes associated with biosynthesis and conversion of sex steroids exhibit time-dependent changes in gene expression in response to decidualisation.

To explore whether changes in steroid metabolism as a result of decidualisation were dynamic, an additional study was undertaken using cells exposed to either cAMP alone or cAMP plus progesterone for 1 to 8 days. In this experiment the concentration of StAR mRNA in response to treatment was found to be time dependent with a significant increase compared to controls at 1 and 2 days (Figure 3.11 A, $p<0.001$, $n=4$) a result that contrasted with that of CYP11A1 mRNA which was only significantly increased at 8 days in those cells incubated with both cAMP and P4 (Figure 3.11 B, $p<0.01$, $n=4$) compared with controls.

Expression of 17βHSD2 mRNA showed a time dependent increase that was significant at 8 days in cells treated with cAMP alone ($P<0.05$) or cAMP and P4 ($P<0.001$) with a non-significant trend after 4 days of treatment (Figure 3.11 C). Expression of STS mRNA was only increased after 8 days of incubation and in agreement with results for 17βHSD2 mRNA this was true of cells incubated with either cAMP alone or cAMP in combination with P4 (Figure 3.11 D, $p<0.001$, $n=4$).

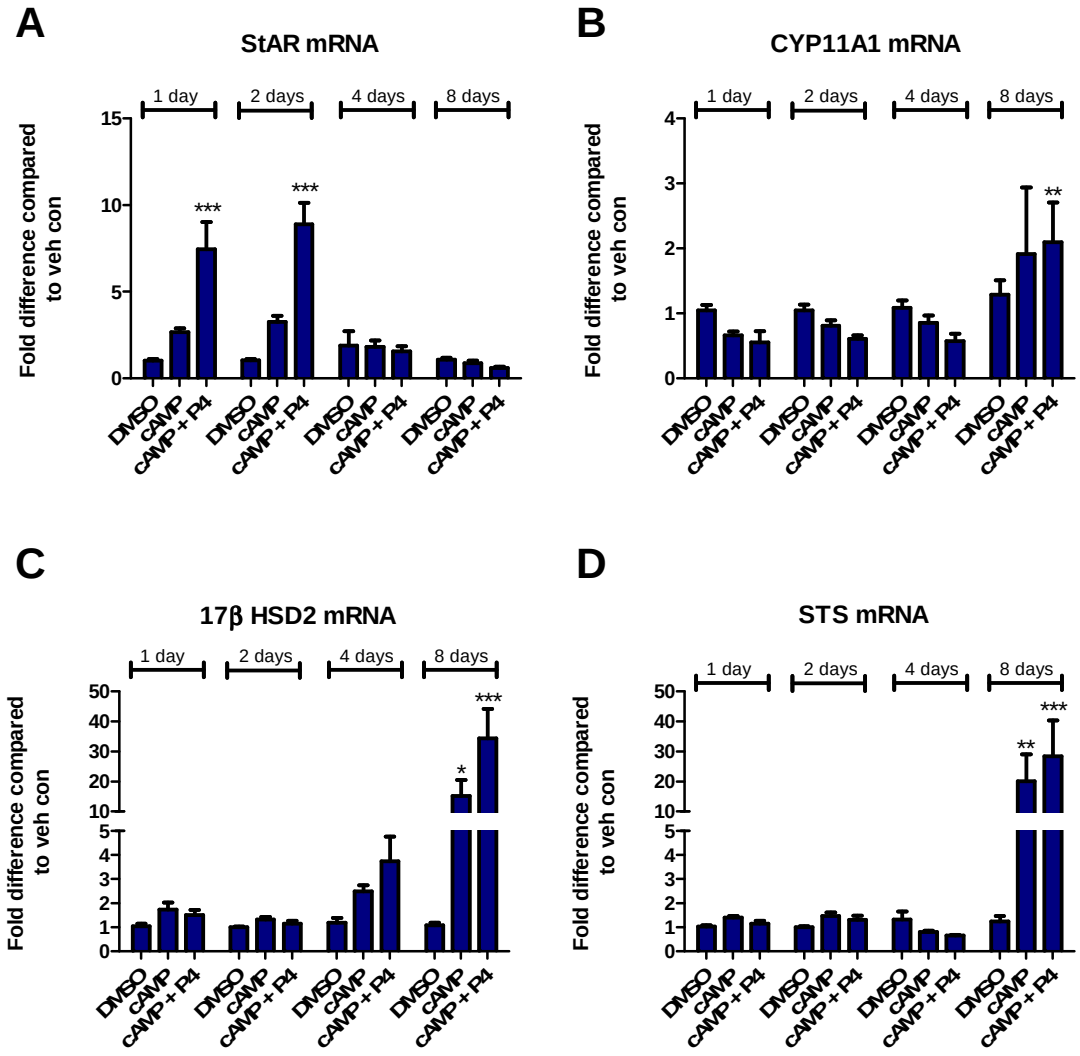

Figure 3.11. Time-dependent changes in expression of mRNAs encoding enzymes responsible for de novo steroid biosynthesis (StAR and CYP11A1) as well as enzymes that facilitate interconversion of androgens and oestrogens (17βHSD2), and conversion of sulphated oestrogens (STS). Concentrations of StAR mRNA was significantly increased at early time points (1 and 2 days) but returned to normal thereafter. Conversely CYP11A1 mRNA was only significantly increased at 8 days. 17βHSD2 and STS mRNAs were both highly and significantly expressed at 8 days but not at earlier time points. Expression of enzymes associated with biosynthesis of sex steroids throughout in vitro decidualisation determined by Taqman qRTPCR. ***A*** *Steroidogenic acute regulatory protein (StAR);* ***B*** *CYP11A1;* ***C*** *17β HSD2;* ***D*** *steroid sulfatase (STS) (n=4 * P<0.05; ** p<0.01; *** p<0.001). Cells were treated with control (DMSO), cyclic adenosine monophosphate (cAMP) or cAMP plus progesterone (P4) for 1, 2, 4 or 8 days in serum-depleted phenol red-free media. Statistical analysis was performed using a Two-way ANOVA.*

3.4.6. Analysis by thin layer chromatography reveals dynamic changes in steroid metabolism associated with decidualisation.

To compare the functional activity of steroid enzymes in ESC with their decidualised counterparts, metabolism of tritiated steroid hormones was assessed using thin layer chromatography (TLC). These studies were conducted using the SHT290 cell model. The activity of the 17βHSD2 enzyme was quantified by determining the metabolism of ^{3}H-oestradiol or ^{3}H-testosterone to oestrone (E1) and androstenedione (A4) respectively.

In control (non-decidualised) SHT cells greatest conversion of ^{3}H-oestradiol occurred within the first 2 hours and there was 100% conversion to E1 after 16 hours (Figure 3.12 G). The metabolism of ^{3}H-oestradiol was comparatively reduced in decidualised SHT cells. Percentage conversion of ^{3}H-oestradiol tended to be less in decidualised SHT cells and was significantly reduced in decidualised SHT cells compared to normal SHT cells after 8 hours (Figure 3.15 A, n=4, $p<0.05$). Rate of conversion, determined by calculating the proportion of ^{3}H-oestradiol metabolised per minute, was also consistently lower in decidualised SHT cells and was significantly reduced at 2 hours (Figure 3.16 A, n=4, $p<0.01$).

A similar pattern was observed for metabolism of ^{3}H-testosterone in normal and decidualised SHT cells (Figure 3.13). Percentage conversion of ^{3}H-testosterone tended to be lower in decidualised SHT cells (Figure 3.15 B). Rate of conversion was also consistently lower in decidualised SHT cells and was significantly reduced at 2 hours (Figure 3.16 B, n=4, $p<0.05$).

Overall the rate of metabolism, as determined by percentage conversion of substrate (nmoles) over time in minutes, appeared to be reduced in decidualised SHT cells compared to control SHT cells and was significantly lower after 2 hours for both E2 and T (Figure 3.16 A and B).

The activity of 17βHSD1 was be investigated by addition of ^{3}H-oestrone to cells however no metabolism was observed in either decidualised or control (non-decidualised) SHT cells (Figure 3.14 A, B). Similarly no apparent 17βHSD5 activity (metabolism of ^{3}H-androstenedione) was detected in either control (non-decidualised) or decidualised SHT cells (Figure 3.14 C, D). Aromatase activity could not be assessed using TLC.

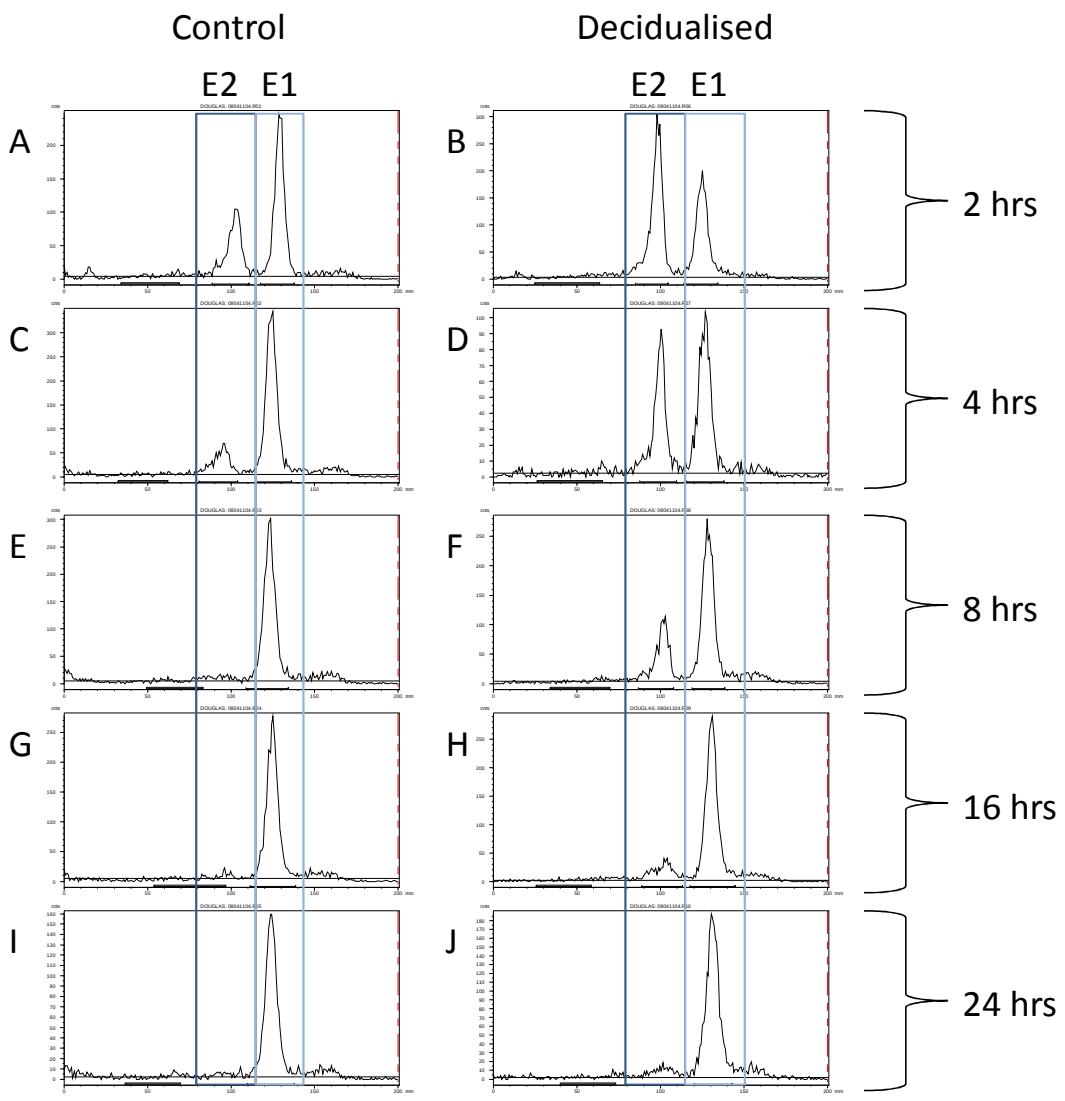

Figure 3.12. Metabolism of ^{3}H-oestradiol (E2) to oestrone (E1) is less rapid in decidualised cells. Metabolism of ^{3}H-E2 was measured after 2, 4, 8, 16 and 24 hours in undecidualised (A, C, E, G, and I) and decidualised stromal cells (B, D, F, H, and J). The proportion of E2 (first peak) converted to E1 (second peak) increases over time and is fully converted by 16 hours in undecidualised cells (G). The rate of conversion is slower in decidualised cells (Figure 1.12A) and E2 is not fully converted by 24 hours (J). Cells were treated with control or decidualising media for 6 days and then metabolism of ^{3}H-E2 was measured by TLC. Chromatographs shown representative of 1 of 3 replicates of assay.

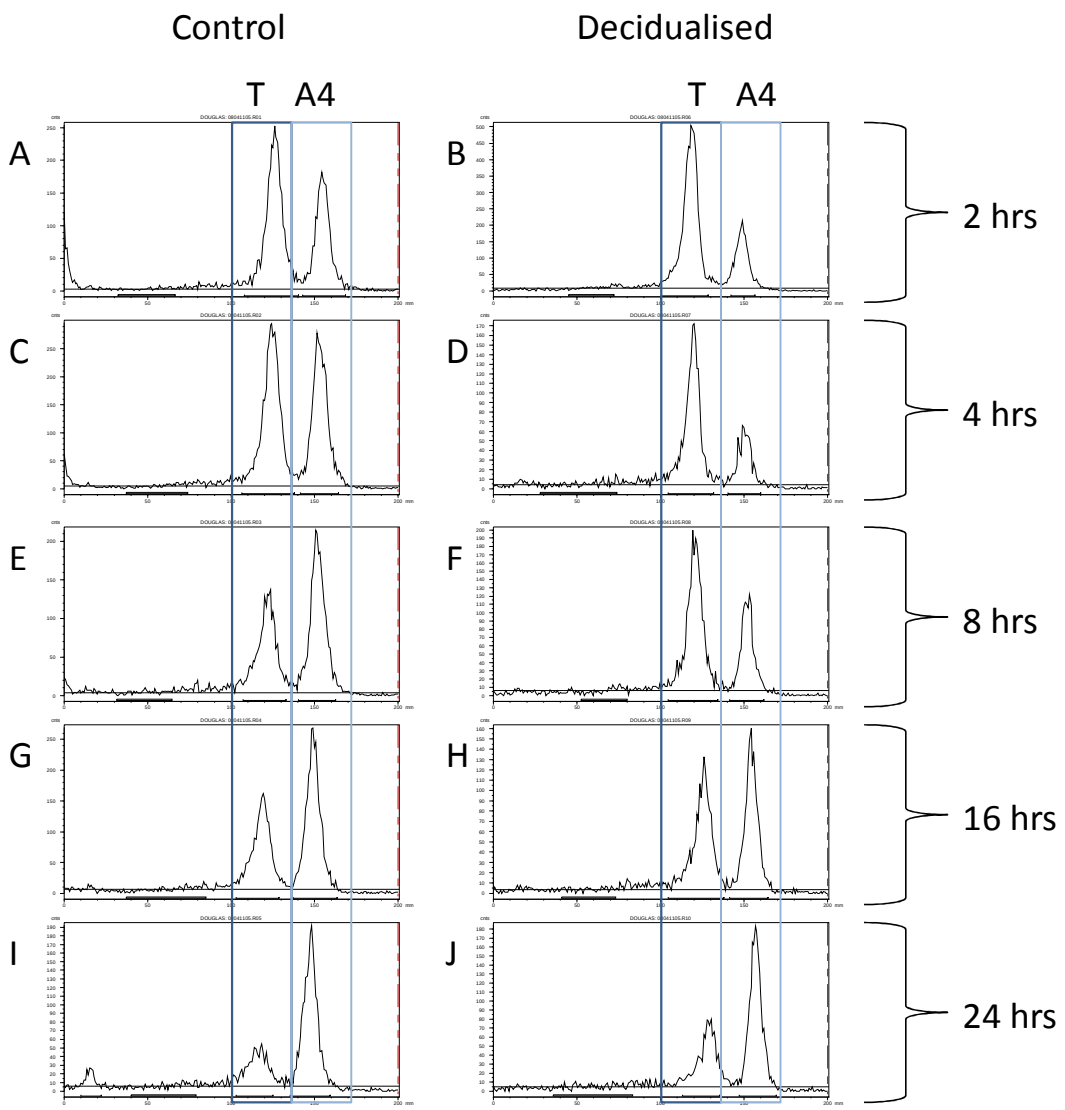

Figure 3.13. Metabolism of ^{3}H- testosterone (T) to androstenedione (A4) is less rapid in decidualised cells. Metabolism of ^{3}H-T was measured after 2, 4, 8, 16 and 24 hours in undecidualised (A, C, E, G, and I) and decidualised cells (B, D, F, H, and J). The proportion of T (first peak) converted to A4 (second peak) increases over time and is 75% after 24 hours in undecidualised cells (I). The rate of conversion is slower in decidualised cells and less T is converted at each time point compared to undecidualised cells (Figure 1.12B). Cells were treated with control or decidualising media for 6 days and then metabolism of ^{3}H-T was measured by TLC. Chromatographs shown are representative of 1 of 3 replicates of assay.

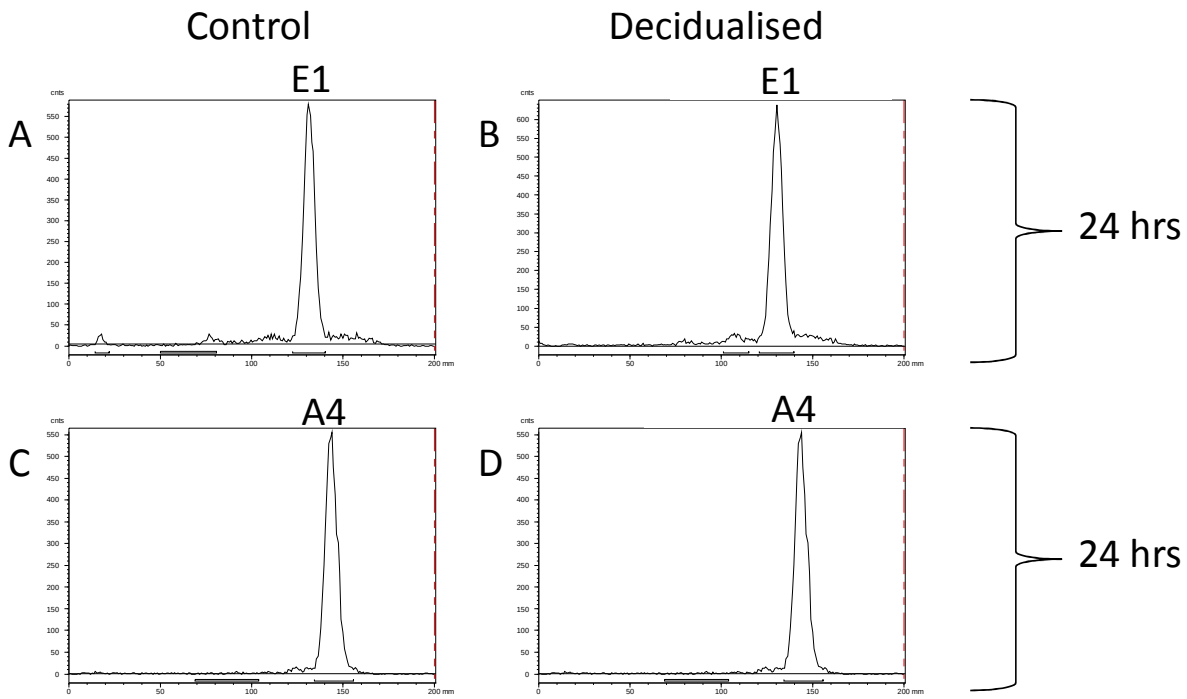

Figure 3.14. Metabolism of ^{3}H-estrone (E1) and ^{3}H- androstenedione (A4) was not detected in control or decidualised cells. Metabolism of ^{3}H-E1 and ^{3}H-A4 was measured after 2, 4, 8, 16 (not shown) and 24 hours. No metabolism of ^{3}H-E1 or ^{3}H-A4 was detected in undecidualised (A, C) or decidualised (B, D) cells after 24 hours. Cells were treated as described in Figure 1.9.

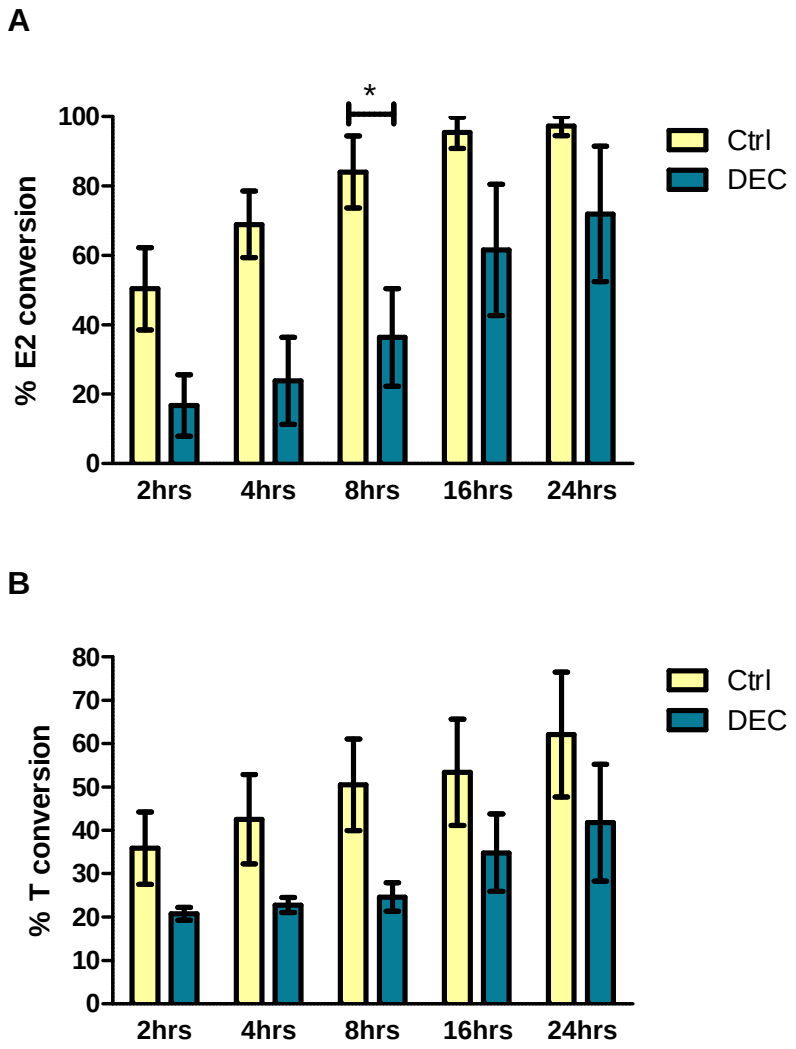

*Figure 3.15. Summary of average percentage conversion of ^{3}H-E2 to E1 (A) and ^{3}H-T to A4 (B) in control and decidualised cells. The proportion of each metabolite was expressed as a percentage of the total metabolite detected. The amount of converted steroid, either E1 or A4, gives an indication of the degree of metabolism of the original labelled steroid; ^{3}H-E2 or ^{3}H-T. The proportion of converted steroid at each time point in control and decidualised cells is shown. At all time points metabolism appears reduced in decidualised cells and metabolism of ^{3}H-E2 is significantly less after 8 hours in decidualised cells (n=4, * p<0.05, Two-way ANOVA).*

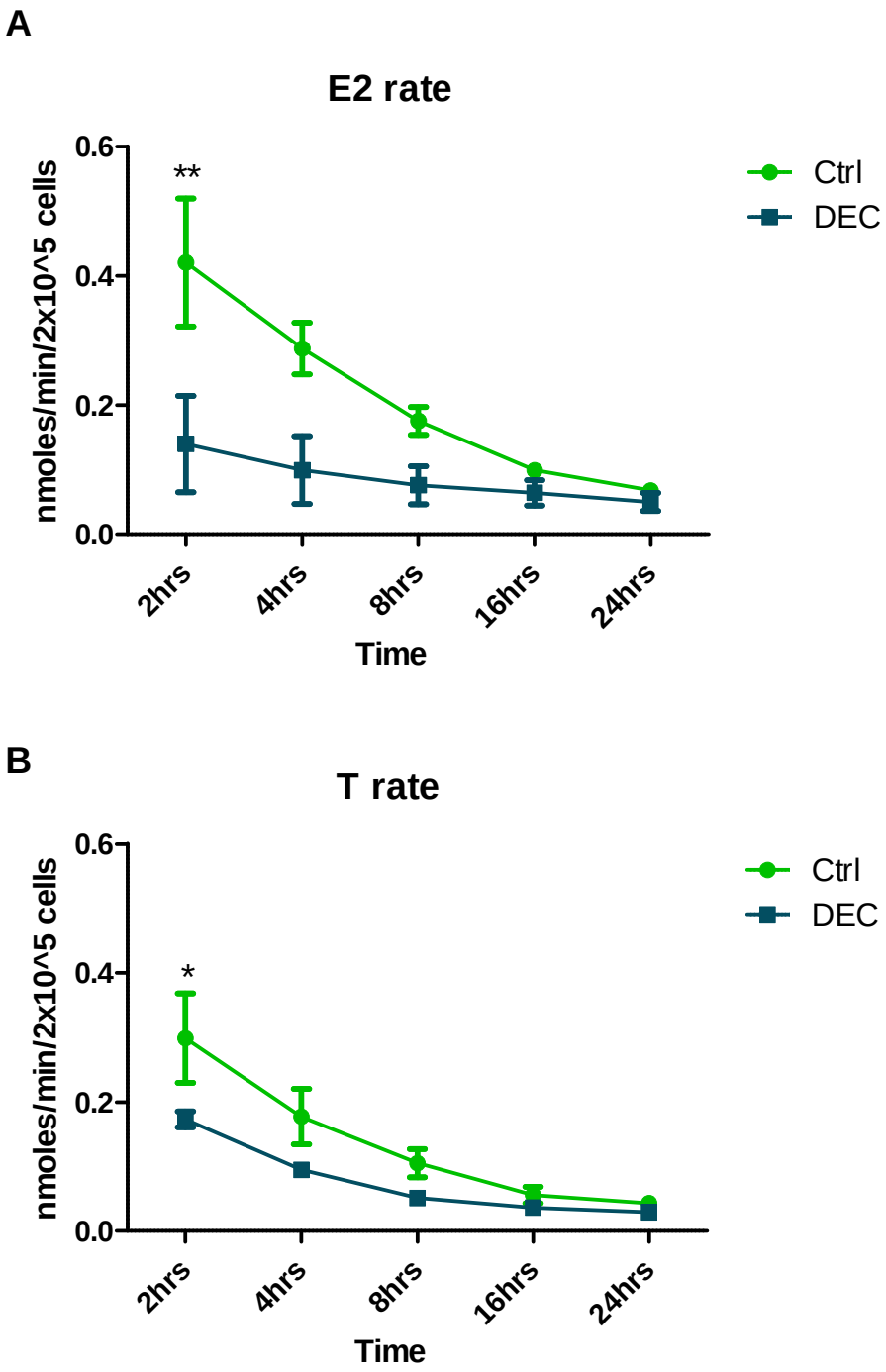

*Figure 3.16. Rate of conversion of ^{3}H-E2 to E1 (A) and ^{3}H-T to A4 (B) in control and decidualised cells. The rate of conversion was calculated by expressing the proportion of converted steroid over time. The rate of conversion appears fastest at early time points and tends to be suppressed in decidualised cells. At two hours rate of conversion of E2 (n=4, ** p<0.01, Two-way ANOVA) and T (n=4, * p<0.05, Two-way ANOVA) is significantly lower in decidualised cells. At all time points metabolism appears reduced in decidualised cells.*

3.4.7. Decidualisation of hESC results in increased aromatase activity.

To assess whether the increased expression of aromatase protein and mRNA as a result of decidualisation might be associated with an increase in aromatase enzyme activity in hESC, a tritiated water assay was utilised.

In undecidualised hESC, stimulation for 24 hours with cAMP and P4 (decidualisation stimulus) resulted in a significant increase in aromatase activity (Figure 3.17 A, $p<0.001$). Furthermore, in hESC which had been decidualised prior to the assay, stimulation with cAMP and P4 also resulted in a significant increase in aromatase activity (Figure 3.17 B, $p<0.001$). Basal aromatase activity was significantly higher in decidualised cells (Figure 3.17 C, $p<0.001$) and the stimulatory effect of cAMP and progesterone was enhanced in cells which were pre-decidualised (Figure 3.17 D, $p<0.001$).

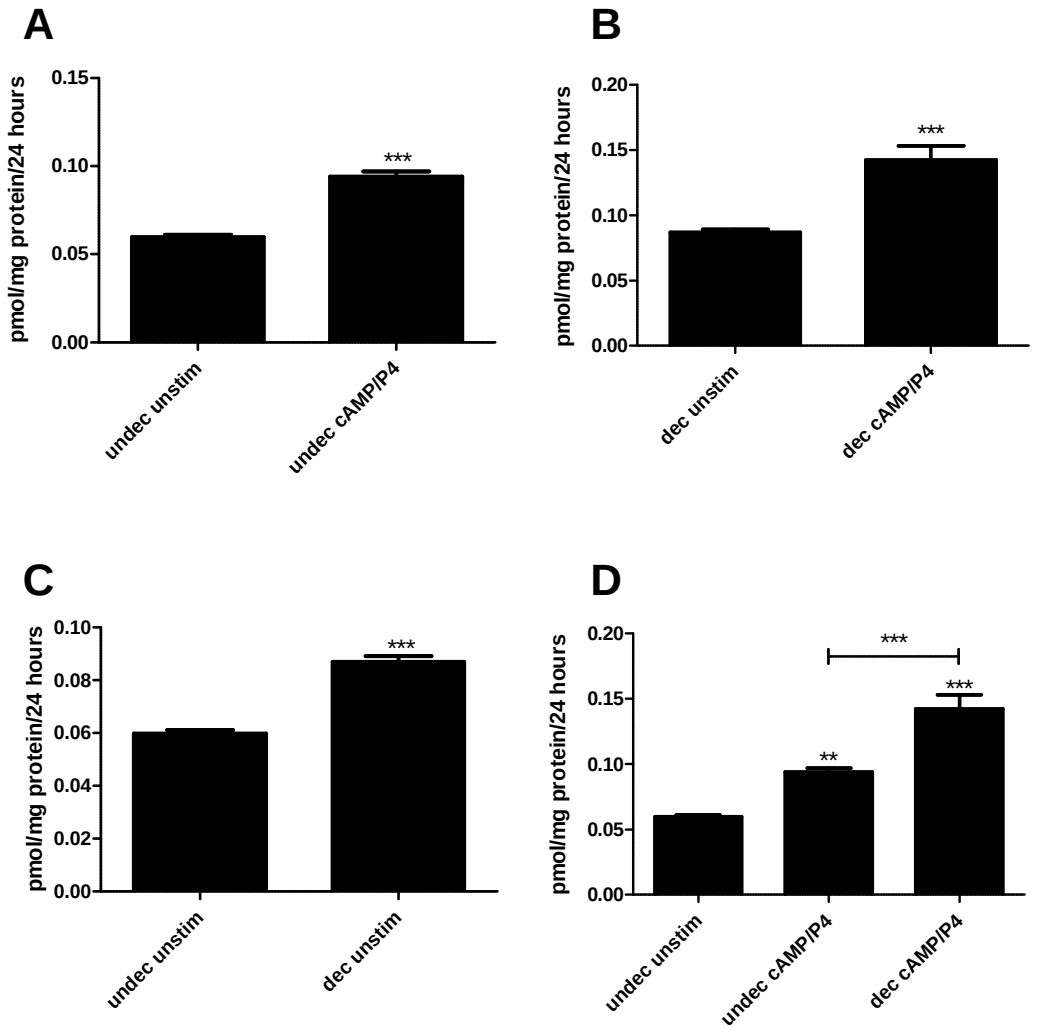

*Figure 3.17. Decidualisation induces aromatase activity in human endometrial stromal cells. Stimulation with cAMP and progesterone for 24 hours significantly increased aromatase activity in undecidualised (undec, A) and decidualised (dec, B) stromal cells. Basal aromatase activity was significantly higher in decidualised cells (C) and the stimulatory effect of cAMP and progesterone was greater in cells which were pre-decidualised (D).unstim=unstimulated, undec=undecidualised, dec=decidualised. *** p<0.001; n=3-5 replicates. Statistical analysis was performed using an unpaired t-test and one-way ANOVA.*

3.4.8. Oestrogen secretion by hESC is increased by decidualisation.

To investigate whether changes in expression of genes implicated in steroid biosynthesis resulted in secretion of oestrone (E1) or oestradiol (E2), concentrations in the cell culture supernatants were determined by ELISA. At all time points examined (days 1-8) the

concentration of E1 was significantly higher (Figure 3.18 A; $p<0.0001$, n=4) in media recovered from ESC incubated with cAMP and P4 than in those remaining in control media or media with the addition of cAMP. A time-dependent increase in concentration of E2 was detected in media of cells treated with cAMP and P4 and it was significantly higher than that of control or cAMP treated cells at all time points up to and including day 8 (Figure 3.18 B; $p<0.001$, n=4). In media recovered from cells incubated with cAMP and P4, concentrations of E1 are significantly higher than those of E2 at 1 ($p<0.0001$, n=4), 2 ($p<0.0001$, n=4), and 4 days ($p<0.001$, n=4) but there is no significant difference between the concentrations of E1 and E2 at 8 days (Figure 3.19). To compare the response to treatment at different time points concentrations of IFGBP1 protein were measured in the same samples. In contrast to the results obtained in the steroid ELISAs a superficial increase in IGFBP protein product was detected in response to incubation with cAMP alone as well as cAMP and P4. Secretion of IGFBP1 was highest at 8 days when it was the same in cAMP treated samples as those with the addition of P4 (Figure 3.18 C; $p<0.001$, n=4).

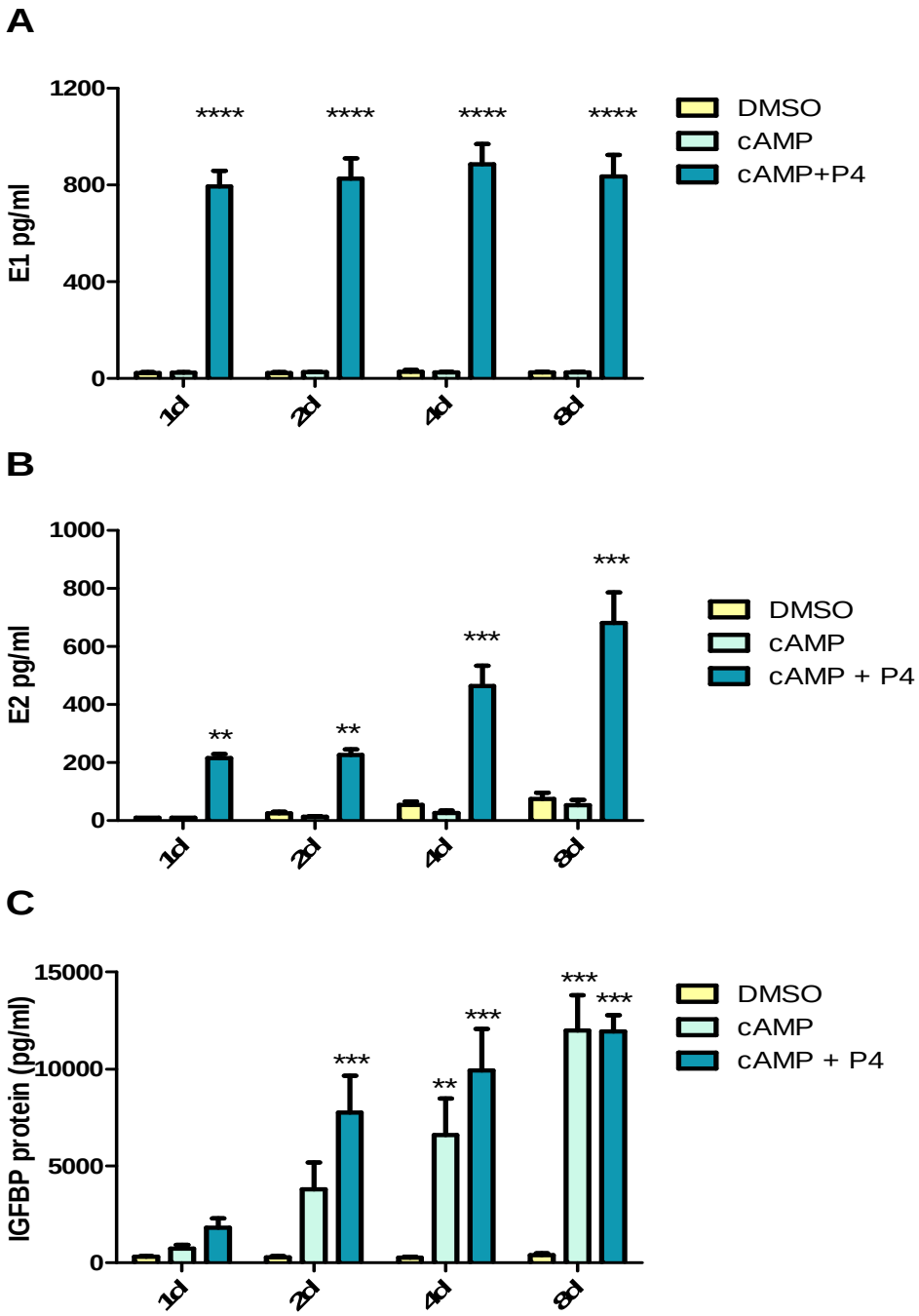

*Figure 3.18. Incubation of hESC cells with cAMP and P4 resulted in secretion of E1 and E2. Treatment with cAMP alone was sufficient to stimulate IGFBP secretion but treatment with both cAMP and progesterone (P4) was required for secretion of E1 and E2. Cells were treated for 1, 2, 4, or 8 days with vehicle (DMSO), cAMP alone or cAMP in combination with P4. Concentrations of E1 (**A**), E2 (**B**), or IGFBP protein (**C**) was determined by ELISA (n=4, ** p<0.01; *** p<0.001, Two-way ANOVA).*

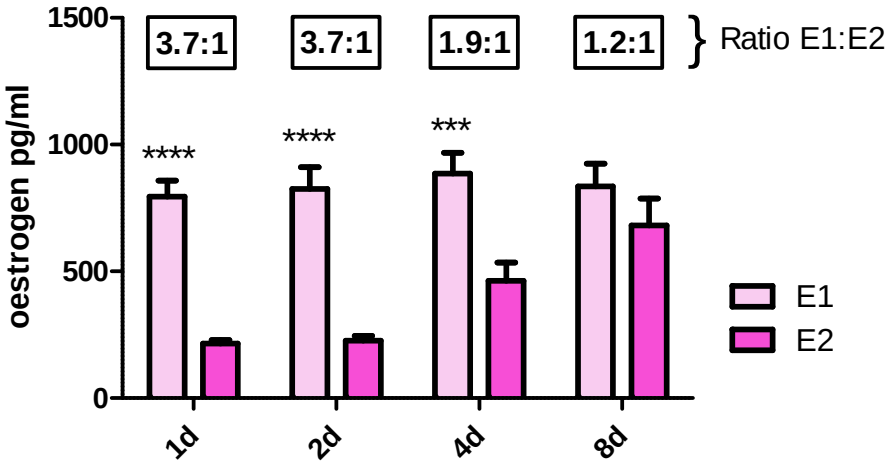

*Figure 3.19. Incubation of ESC with cAMP plus P4 was associated with biosynthesis of E1 and a time-dependent increase in secretion of E2. E1 concentrations were significantly higher than those of E2 on days 1, 2, and 4. The concentrations of E1 and E2 approached equality after 8 days. Ratio of E1:E2 is shown in white boxes for each time point. Cells were treated for 1, 2, 4, or 8 days with cAMP in combination with P4. Concentrations of E1 and E2 were determined by ELISA (n=4, Two-way ANOVA, *** p<0.001; **** p<0.0001).*

3.4.9. The impact of aromatase inhibition on decidualisation.

The aromatase enzyme plays a crucial role in the conversion of androgens to oestrogens. The studies presented above have demonstrated that decidualised human ESC exhibit a capacity to synthesise and secrete both E1 and E2 consistent with *de novo* steroid biosynthesis and aromatase enzyme activity. To determine whether aromatase activity was essential for biosynthesis of E1 and E2 studies were undertaken using two concentrations of the aromatase inhibitor anastrozole (Arimidex®, AstraZeneca ZD1033). The aromatase inhibitor anastrozole was administered to non-decidualised and decidualised ESC. Addition of anastrozole did not have a significant impact on secretion of IGFBP by ESC incubated with cAMP and P4 for 8 days with only a minor reduction when co-incubated with the higher concentration of anastrozole (10μM) (Figure 3.20).

Incubation of hESC with 10μM anastrozole significantly reduced E1 biosynthesis in decidualised stromal cells (Figure 3.21; $p<0.01$, n=4); although E1 was not significantly reduced when cells were co-incubated with 1μM Anastrozole the mean concentration of E1 was 30% lower than that of the vehicle control. Biosynthesis of E2 was significantly reduced when cells treated with cAMP and P4 were co-incubated with anastrozole. Notably addition of 1μM anastrozole reduced E2 production by approximately 10% (Figure 3.22, $p<0.05$,

n=3) but the impact was more marked in the presence of 10μM anastrozole (Figure 3.22, $p<0.001$, n=3) which reduced oestradiol by around 27% (figure 1.16).

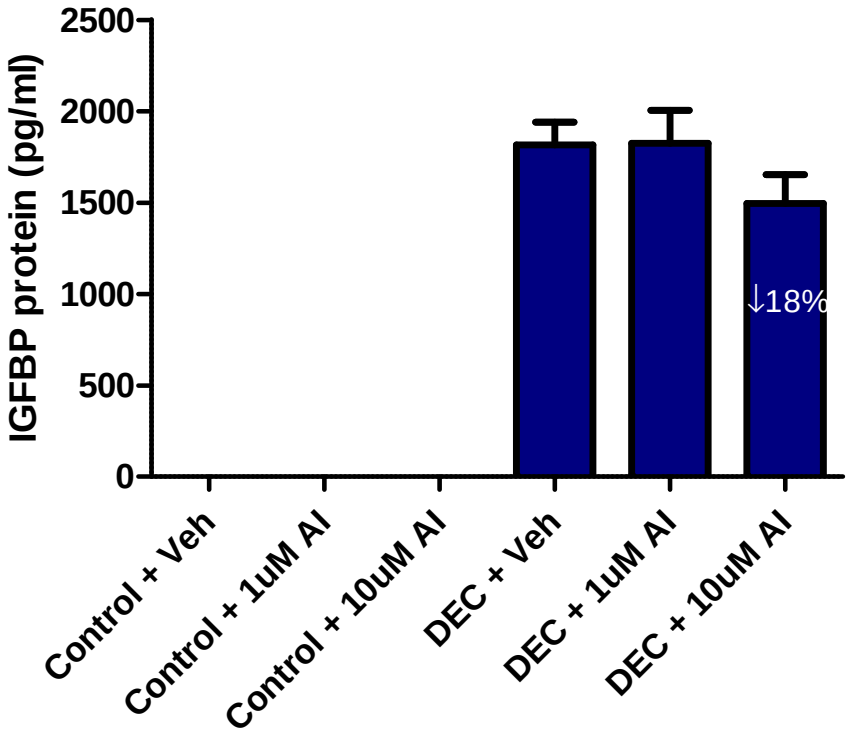

Figure 3.20. Incubation of cells with the aromatase inhibitor anastrozole did not have a significant impact on secretion of IGFBP. The impact of anastrozole (AI) on IGFBP production was assessed by decidualising cells (DEC) for 8 days in the presence of vehicle, 1μM AI or 10μM AI. Undecidualised cells were subject to the same treatment regimen to serve as a control. IGFBP was not detected in any of the control samples. Treatment with 10μM AI resulted in a slight non significant decrease in IGFBP secretion in decidualised cells (p=0.1368, n=4, DEC Veh vs Dec 10μM AI, T test). Secretion of IGFBP protein was determined by ELISA. Percentage reduction of mean DEC control value shown in white.

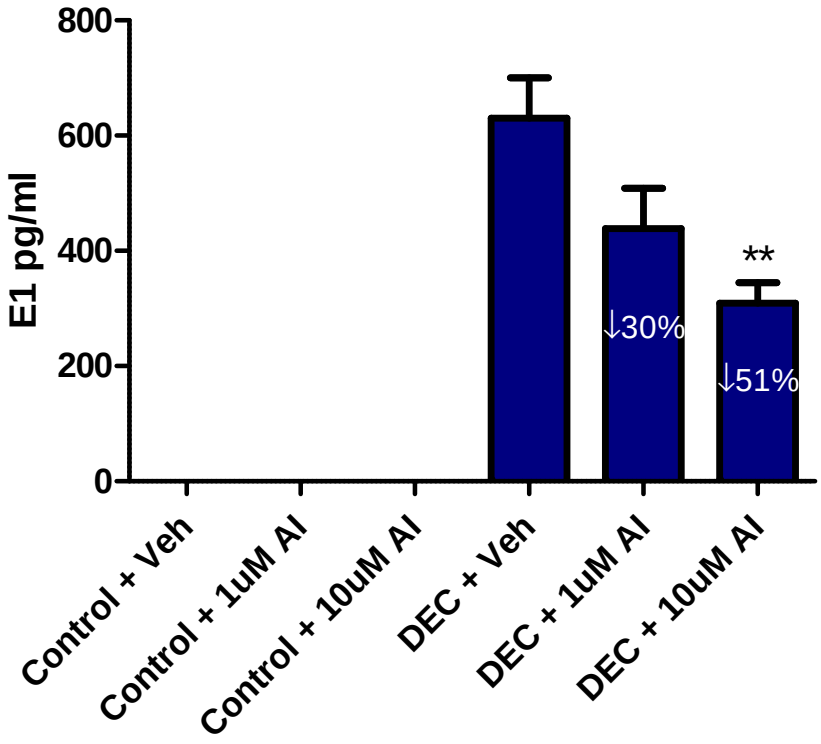

*Figure 3.21. Inhibition of aromatase significantly reduced secretion of oestrone (E1) by decidualised stromal cells (DEC). The impact of the aromatase inhibitor anastrozole (AI) on E1 production was assessed by decidualising cells for 8 days in the presence of vehicle, 1μM AI or 10μM AI. Undecidualised cells were subject to the same treatment regimen to serve as a control. E1 was not detected in any of the control samples. n=4, ** p<0.01, One-way ANOVA. Percentage reduction of mean DEC control value shown in white.*

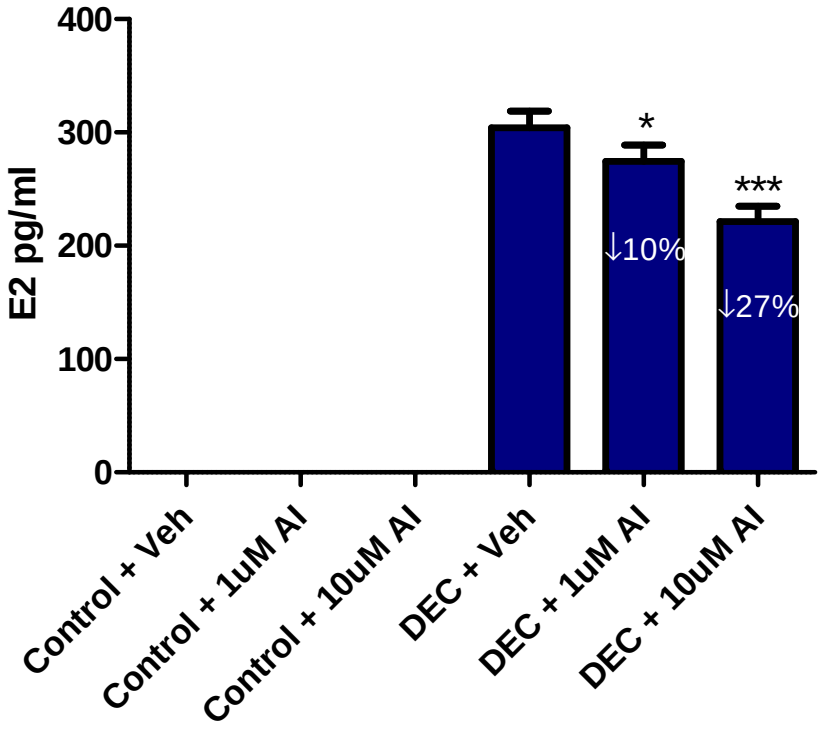

*Figure 3.22. Inhibition of aromatase significantly reduced oestradiol (E2) secretion by decidualised stromal cells. The impact of the aromatase inhibitor anastrozole on E2 production was assessed by decidualising cells for 8 days in the presence of vehicle, 1uM anastrozole (AI) and 10uM AI. Undecidualised cells were subject to the same treatment regimen to serve as a control. E2 was not detected in any of the control samples. n=3, * p<0.05, *** p<0.001, One-way ANOVA. Percentage reduction of mean DEC control value shown in white.*

3.4.10. Inhibition of steroid sulphatase has a significant impact on decidualisation.

Steroid sulphatase (STS) catalyzes conversion of oestrone sulphate (E1S) to E1. To investigate whether the activity of STS might contribute biosynthesis and secretion of E1 ESC were incubated with the STS inhibitor STX64. During decidualisation expression of STS mRNA was increased after 8 days (see section 3.4.3). Unexpectedly, secretion of IGFBP by decidualised ESC was reduced in cells incubated with STX64 for 1 to 4 days; by day 8, IGFBP secretion had recovered to control levels possibly due to incomplete inhibition of STS activity (Figure 3.23; $p<0.05$, $n=4$). Following the same treatment paradigm the effect of STX64 on oestrogen biosynthesis was also investigated. STS inhibition did not have a significant impact on the production of E2 (not shown) however concentrations of E1

in culture media were significantly reduced at 2 days (Figure 3.24; $p<0.05$, n=4) with a non-significant trend on days 1 and 4.

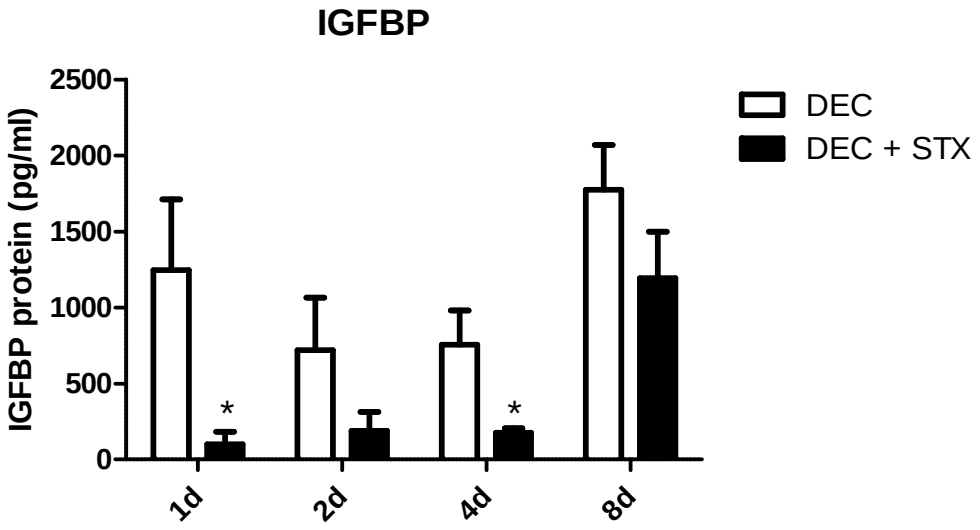

*Figure 3.23. Secretion of IGFBP by decidualised stromal cells was reduced by incubation of cells with the steroid sulfatase inhibitor STX64. Steroid sulfatase (STS) is the enzyme responsible for the conversion E1S to E1. IGFBP secretion was significantly decreased in decidualised stromal cells treated with STX64 at 1 and 4 days tended to be decreased at 2 days. The impact of the STS inhibitor STX64 on IGFBP production over time was assessed by decidualising cells for 1, 2, 4, and 8 days +/- STX64. IGFBP secretion was determined by ELISA. n=3, * p<0.05, unpaired T-test.*

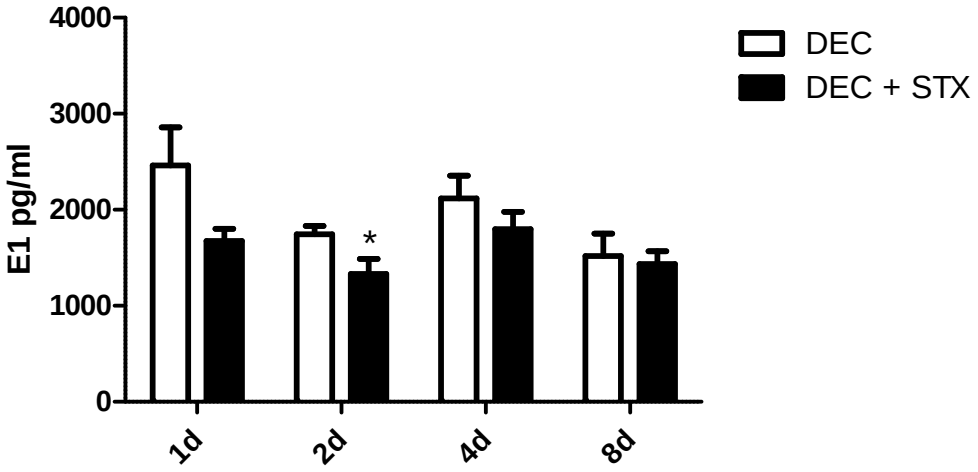

*Figure 3.24. Secretion of oestrone by decidualised stromal cells is reduced following inhibition of steroid sulfatase by STX64. STS is differentially expressed over time during decidualisation. Conversion of E1S to E1 may contribute to levels of secreted oestrogen as a result of decidualisation. E1 secretion was tended to be decreased in decidualised stromal cells treated with STX64 at early time points and was significantly decreased at 2 days. The impact of the STS inhibitor STX64 on E1 production over time was assessed by decidualising cells for 1, 2, 4, and 8 days +/- STX64. E1 secretion was determined by ELISA. n=3, * p<0.05, unpaired T-test.*

3.5. Discussion

The current study sought to determine whether local biosynthesis of oestrogen occurs during decidualisation in women by investigating oestrogen biosynthesis and metabolism using an *in vitro* model of decidualisation.

In the studies presented herein we report novel evidence that supports a role for local biosynthesis of oestrogen during decidualisation (see Figure 3.25). We have shown that *in vitro* decidualisation of primary endometrial stromal cells is associated with time-dependent changes in expression of genes encoding proteins important in steroid biosynthesis including transient up-regulation of *StAR* and *CYP11A1* as well as increased expression of aromatase mRNA and protein. We detected a change in enzyme activity in decidualised cells which appeared to result in reduced 17βHSD2 activity and an increase in aromatase activity. Secretion of both E1 and E2 by primary ESC was confirmed using ELISA, with increasing production of E2 associated with progressive decidualisation of cells. Secretion of E1 and E2 was partially ameliorated by inhibitors of aromatase and STS. There was also evidence to

suggest that treatment of cells with STX64 (an STS inhibitor) resulted in reduced biosynthesis of E1 as well as secretion of the decidualisation marker protein IGFBP1.

3.5.1. Evidence for biosynthesis of oestrogens

Principal to the biosynthesis of oestrogen is the enzyme complex aromatase. Aromatase (encoded by *CYP19A1* gene) acts by aromatising androgen precursors to oestrogens and is required for biosynthesis of oestrogen. Bulun et al reported that aromatase mRNA expression could not be detected in endometrium from normal women (311) however expression is reported to be increased in endometriosis (lesions and eutopic endometrium) and endometrial cancer (312, 376, 475).

In the current study, decidualisation of ESC for 8 days resulted in doubling in expression of CYP19A1 mRNA while CYP19A1 mRNA was barely detectable in undecidualised ESC consistent with previous reports (311). Generally, CYP19A1 transcripts were detected at a very low copy number (Ct>35) however there was a consistent, significant difference in expression after 8 days decidualisation treatment compared to control. Interestingly, high levels of 17βHSD1 protein expression have been reported in the endometrium despite low reported levels of mRNA expression (312). Consistent with this, protein expression analysis revealed that aromatase protein was detected in decidualised hESC that was absent in undecidualised hESC. Furthermore, utilisation of a tritiated water assay revealed that decidualisation was associated with an increase in aromatase enzyme activity. In addition to increased aromatase mRNA and protein expression as well as the increased aromatase activity described in our study, we consequently detected high levels of secreted oestrogens in matched supernatants from ESC. Furthermore, secretion of oestrogens could be significantly diminished following treatment with the selective aromatase inhibitor anastrozole (see later). Taken together these results suggest the presence of functional aromatase in decidualised ESC.

In stromal cells from women with endometriosis (eutopic endometrial stromal cells from women with endometriosis) and stromal cells derived from ovarian endometriomas, CYP19A1 expression can be induced by prostaglandin E2 (PGE2) (378) and cAMP (313, 378) both of which have been implicated in enhancing the process of decidualisation (123). In humans, aromatase expression is regulated by tissue specific promoters (327) and aromatase expression stimulated by cAMP and PGE2 in endometriosis appears to be via promoter II (378). Further analysis of the factors which promote aromatase expression in

decidualising ESC may help to elucidate the transcriptional mechanisms governing expression of CYP19A1 in decidualised ESC.

The interconversion of E1 and E2 is mediated via 17β hydroxysteroid dehydrogenases (17βHSD), an important class of enzymes that control interconversion of androgens and oestrogens (Figure 3.1) [13]. 17βHSD2 catalyses the oxidation of E2 to E1 and T to A4 (323). Expression of 17βHSD2 mRNA and protein has previously been described in human endometrium with expression being highest in the secretory phase (314) associated with positive immunoexpression in glandular epithelium and some stromal cells (312). Sp-1 and Sp-3 have been shown to interact with specific motifs in the *HSD17B2* gene promoter resulting in up-regulation in expression of *HSD17B2* gene in endometrial epithelial cell lines (331). In endometrial explants progesterone induces 17βHSD2 expression (312). In the current investigation we report that incubation of ESC with cAMP and P4 had a striking and highly significant impact on expression of 17βHSD2 gene expression, while treatment of cells with cAMP alone also induced a significant increase in mRNA concentrations suggesting changes in expression as a result of PKA-dependent signaling (313). Thus decidualising stimuli are sufficient to increase mRNA expression of 17βHSD2 which would suggest metabolism may be directed toward the formation of E1 and A4 in response to decidualisation.

Interestingly, expression of 17βHSD isotypes which catalyze the formation of E2 and T i.e. the opposite reaction to 17βHSD2, were unchanged following decidualisation of ESC. Expression of 17βHSD1 mRNA is low in normal endometrium although prominent protein expression in both stromal and epithelial cells has been described by immunohistochemistry (312). Levels of 17βHSD1 mRNA do not appear to vary over the menstrual cycle (312) but cAMP treatment is reported to increase concentrations of 17βHSD1 mRNA expression in eutopic endometrial stromal cells from women with endometriosis (313). The proximal promoter of the human 17βHSD1 gene contains AP-1, Sp-1 and Sp-3 sites (330) and transcription could therefore be affected by steroid receptor activation. In the current study expression of 17βHSD1 mRNA was identical in non-decidualised and decidualised stromal cells consistent with observations across the menstrual cycle. Thus stimulation with cAMP or the presence of steroids in culture media did not have an impact on 17βHSD1 mRNA expression.

A4 is reduced to T by 17βHSD3 in the testes and 17βHSD5 in peripheral tissues (332). 17βHSD5 is expressed in the endometrium throughout the menstrual cycle with highest

mRNA detection in the early secretory phase. Dysregulation of 17βHSD5 has been reported to occur in endometrial cancers with increased expression in malignant tissue compared to normal endometrium (360). In the current study, consistent with the expression of 17βHSD1, 17βHSD5 mRNA expression was identical in non-decidualised and decidualised stromal cells.

An additional source of oestrogens could be from the action of steroid sulphatase (STS). STS hydrolyses sulphated steroids, which are abundant in the circulation but are inactive at steroid receptors, into biologically active steroids (335). STS primarily converts oestrone sulphate (E1S) to oestrone (E1) which may be a potential source of oestrogen production in the endometrium. STS immunoexpression has been detected in both stromal and epithelial cells in the endometrium across the menstrual cycle and is reduced in stromal compared to epithelial cells in the late secretory phase (312). In normal endometrium mRNA expression of STS is higher than sulfotransferase, the enzyme which catalyzes sulphation of steroids, the opposite reaction to STS (336). In the present study, STS mRNA expression was significantly and highly increased by either cAMP alone or in combination with P4 at 8 days. Expression of sulfotransferase mRNA could not be detected (data not shown). In addition to oestrogen forming activities, STS also has the capacity to hydrolyse dihydroepiandrosterone sulphate (DHEAS) to dihydroepiandrosterone (DHEA) which in turn could also be a potential route to oestrogen and androgen formation during decidualisation (337) (see Figure 3.10). The metabolism of androgens could also impact on biosynthesis of oestrogens, since androgens are the substrate for aromatase. SRD5A1, also known as 5α reductase, is an enzyme responsible for 5α reduction of steroids. 5α reductase has been shown to reduce testosterone to the more potent androgen dihydrotestosterone (DHT) in human endometrium (333). Reports of protein expression in the endometrium have been conflicting, with expression reported by some to be limited to epithelial cells (318) while others have demonstrated immunostaining in both stromal and epithelial cells in normal endometrium (319). Increased SRD5A1 mRNA expression is associated with ovarian endometriosis (316). In the studies reported in this chapter, SRD5A1 mRNA expression was increased in endometrial stromal cells treated with cAMP and to a greater extent following treatment with both cAMP and progesterone after 8 days of treatment. While enzyme activity was not measured using TLC, increased expression may affect the availability of bioactive androgens and consequently oestrogens.

To assess whether cells could support *de novo* steroid biosynthesis, the expression of steroidogenic acute regulatory protein (StAR) and cholesterol side-chain cleavage enzyme

(CYP11A1) were both measured and were found to be regulated in a time dependent manner in decidualised ESC. StAR mRNA expression was increased at early time points suggesting an acute response to decidualisation stimulus which would facilitate the delivery of cholesterol to the inner mitochondrial membrane for conversion by the constitutively active CYP11A1 (321). StAR is reported to be expressed in stromal but not epithelial cells in the endometrium and its expression can be increased by prostaglandin E2 (PGE2). Increased expression in endometriotic stromal cells has also been reported (309) and cAMP treatment is reported to increase StAR mRNA expression in endometrial stromal cells (313). In the current study significant increases in StAR and CYP11A1 mRNA expression were observed only with combined treatment of progesterone and cAMP, however, the trend was reproduced in cells treated with cAMP alone to a lesser extent suggesting progesterone may enhance previously reported cAMP responses.

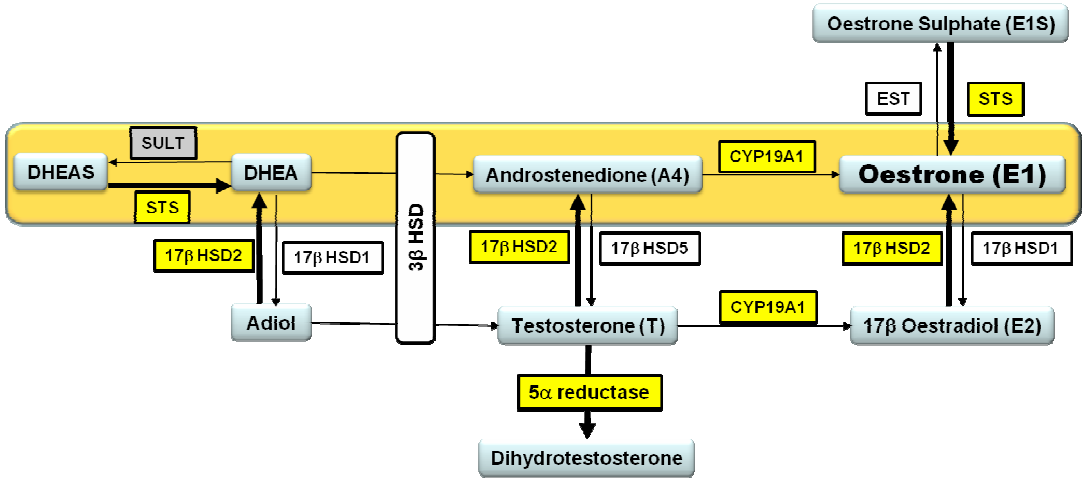

Figure 3.25. Summary of changes in steroid biosynthesis in decidualised ESC. Increased concentrations of mRNAs encoding STS, 17βHSD2, CYP19A1 and SRD5A1 (5α reductase) were detected in decidualised ESC (yellow boxes) which may result in an increase in the formation of oestrogens, in particular oestrone.

In the current experiment changes observed in mRNAs encoding proteins involved in steroid biosynthesis in response to a decidualising stimulus appeared to favour formation of oestrogens as mRNAs encoding both aromatase and STS were up regulated (Figure 3.8 and 3.9). In addition, decidualisation resulted in increase in aromatase activity and aromatase protein was detected in decidualised hESC (Figure 3.10 and 3.17). Furthermore, interconversion of androgens and oestrogens appeared likely to favour oxidative activity as there was a striking increase in expression of 17βHSD2 but no change in expression of

17βHSD1 or 17βHSD5. This was confirmed using TLC wherein conversion of E2 to E1 and T to A4 was demonstrated but not the reverse reactions. These results are consistent with a study by Collins et al which showed that in normal endometrium metabolism favours formation of E1 and A4 over E2 and T respectively (338). The same study showed that DHEA could be metabolized to androstenedione, 5α-androstanedione and androsterone in human endometrium albeit in small quantities (338). The capacity for endometrial conversion of DHEA adds weight to the potential for hydrolysis of DHEAS by STS action to contribute to the formation of androgens and oestrogens. The dynamics of oestrogen and androgen uptake by human endometrium also appears to favour oxidative 17βHSD activity. For example, a study by Gurpide et al. revealed a high level of oestrogen and androgen uptake in perfused endometrium with net uptake of E2 and T being higher than that of E1 and A4. Following steroid uptake by endometrial tissue E1 and A4 was released unmetabolised from the tissue whereas a high proportion of E2 and T was released metabolized to E1 and A4 respectively, consistent with oxidative 17βHSD activity (340).

In the data presented, despite the striking increase in 17βHSD2 mRNA detected in the decidualised cells, 17βHSD2 enzyme activity appeared decreased compared to undecidualised ESC when measured by TLC. Conversion of E2 by decidualised ESC was significantly decreased after 8 hours as compared to that observed in parallel experiments using non decidualised cells. The rate of conversion was significantly lower for both E2 and testosterone in decidualised cells after 2 hours. This apparent contradiction to the mRNA expression changes observed may be due to changes in the metabolic demands of the decidualising tissue that may favour other reactions which could not be detected by TLC, such as aromatase activity, or may simply indicate that mRNA changes do not accurately reflect activity of the enzyme. It is also conceivable that this effect may be an artifact of the assay resulting from increased unlabelled substrate that is produced from decidualised cells making the metabolism appear slowed as proportionally less labeled substrate is converted. Differentiation of stromal cells could also result in changes in availability of cofactors such as NAD and NADP which would also have an impact on reaction rates and which metabolism reactions are favoured.

Consistent with changes in concentrations of mRNA encoding steroidogenic enzymes and altered metabolism as detected by TLC, E1 and E2 were readily detected in media recovered from decidualised ESC. Oestrogens were not detected in undecidualised ESC nor were they detected in cells treated with cAMP alone. Consistent with this, aromatase protein was only detected in decidualised ESC. Interestingly while concentrations of E2 increased in a time-

dependent manner the total concentration of E1 was the same at all time points which may be indicative of higher 17βHSD2 activity and contribution from STS. While the evidence presented above would be consistent with *de novo* biosynthesis of E1 and E2, it is conceivable that the amounts of E1 and E2 detected in the culture media may also reflect metabolism of progesterone as E1 and E2 were only detected in media recovered from cells in which progesterone was part of the treatment. Previous studies have reported no evidence for 17α-hydoxylase activity in progesterone metabolism in the endometrium, and that metabolism of progesterone is predominantly via 5α reduction (334) which would not be consistent with metabolism directed to oestrogen formation. This may however explain the increased mRNA levels of SRD5A1 observed in this study. If the oestrogens detected where solely metabolites of progesterone then the amounts detected would likely be proportional to the amount of progesterone added and thus total oestrogen amount would be roughly equivalent at each time point. This was not the case. Furthermore there is anecdotal evidence to suggest oestrogens may be detected from cells transferred to control media after treatment (i.e. without progesterone) as E2 was detected up to two days following decidualisation in treated cells transferred to control media (n=1, data not shown). To clarify the contribution, if any, of progesterone metabolism to the amount of oestrogen produced the levels of progesterone should be assayed throughout the time course as well as metabolism investigated by TLC.

3.5.2. The impact of inhibitors

Treatment with the aromatase inhibitor anastrozole significantly reduced both the amount of E2 (27% reduction) and E1 (51% reduction) detectable in culture media. Aromatase inhibitors have been used in the treatment of endometriosis and in the first reported use of this therapy by Takayama, anastrozole reduced circulating E2 levels by 50% (462) while the aromatase inhibitor letrozole is reported to completely inhibit uterine oestrogen production in ovariectomised mice (128). Furthermore, in a recent study by Das et al (476), the concentration of letrozole utilized to inhibit aromatase activity in mouse uterine stromal cells was between 20 and 50 μM, concentrations which were much greater then those used in the present study. It is conceivable that letrozole may be a more effective aromatase inhibitor than anastrozole and its use in this model, perhaps at higher concentrations than those used in this study, may deliver more complete inhibition of oestrogen production, consistent with responses observed in the mouse.

STS activity can be inhibited by the non-steroidal inhibitor STX64 (667 COUMATE) (381, 464). In the current study, STX64 had no effect on E2 production suggesting that STS action does directly impact E2 formation. There is little evidence from either mRNA expression or TLC to suggest that there is conversion of E1 to E2 in endometrial stromal cells, which may explain why STS inhibition had no effect on E2 production as any hydrolysed E1S would not be further converted from E1 to E2. STX64 tended to reduce E1 formation but only after 1 and 2 days treatment. Thus, STS may contribute to the high levels of E1 observed but only at early time points. Paradoxically, STS mRNA was significantly and highly up regulated at 8 days but inhibition of STS had no effect on either E1 or E2 production at this time point. This may suggest a role for STS in ongoing function of the decidua or may simply indicate that inhibition was insufficient when expression is so highly increased. We detected high levels of E1 at all time points but found that the ratio of E1 to E2 was roughly equivalent by 8 days. As E2 levels increase the levels of E1 remain at a similar level which does not suggest that E2 synthesis is from 17βHSD reduction of E1 or are at least not to the detriment of E1 levels. This would be consistent with the lack of 17βHSD1 enzyme activity determined by TLC reported herein.

3.5.3. The role of the local steroid environment

The current studies have demonstrated that human endometrial stromal cells express sex steroid receptors and their expression is altered in response to decidualisation. These observed changes in steroid receptor expression are consistent with those reported in the literature in secretory phase endometrial stromal cells (291, 477, 478) indicating recapitulation of the *in vivo* response in the endometrium. Changes in steroid receptor expression may be mediated by steroid receptor cross-talk, for example, PR controls the down-regulation of epithelial ERα via COUPTFII which is essential for successful implantation and decidualisation in mice (280, 479). In addition, increased expression of ERβ in the stromal compartment is likely to be important as changes in the ERα: ERβ ratio can impact transcriptional responses to oestrogen by the formation of heterodimers (480). We reported that as a result of local steroid biosynthesis, high concentrations of secreted oestrogens were detected in supernatants from decidualised stromal cells which exceed peak circulating levels (300pg/ml in proliferative phase (481)) and are likely to mediate significant effects in the local environment during decidualisation. It is also conceivable that there may be significant quantities of androgens, as precursors to oestrogen formation, present in the stromal environment as a result of increased local steroid biosynthesis. This

may be particularly relevant as androgen receptor has been shown to regulate distinct processes such as cytoskeletal organization and cell cycle regulation in the decidualising endometrium (295) and a recent *in silico* analysis revealed a novel androgen regulated gene network in human endometrium associated with regulation of apoptosis (200).

A significant degree of steroid receptor regulation is likely to mediate decidualisation of endometrial stromal cells via altered receptor expression and in addition to control by ovarian hormones may be influenced by locally produced sex steroids.

In the present study a definitive impact of inhibition of steroid biosynthesis on decidualisation could not be observed. Inhibition of aromatase and STS appears to reduce IGFBP secretion in decidualising cells however this was not sufficient to completely ameliorate secretion or reverse decidual phenotype. However, IGFBP was significantly decreased by STX64 at 1 and 4 days and tended to be decreased at 2 days. This trend in decreased IGFBP secretion at early time points following STS inhibition is associated with modest decreases in E1 at similar time points. To our knowledge this is the first evidence of inhibition of decidualisation markers by steroid enzyme inhibitors in human endometrial stromal cells and may indicate an importance for E1 during decidualisation.

In a mouse model the aromatase inhibitor letrozole was shown to block the stromal differentiation process (128) highlighting that aromatase is essential in decidualisation in mice. In our study inhibition of aromatase or STS did not completely block steroid synthesis however a more complete depletion of E1 and E2 synthesis may yield a more significant decrease in IGFBP. Inhibition of both aromatase and STS at all time points may have a more pronounced impact on decidualisation and may help to elaborate the specific roles of E1 and E2 during decidualisation of human endometrial stromal cells.

Despite inhibition of aromatase and STS not having a large impact on decidualisation, the presence of such high levels of oestrogens during decidualisation suggests a high capacity for modifying the uterine environment. Oestrogen is known to be essential in implantation and decidualisation in mice and is the critical determinant of the window of uterine receptivity. In addition, down regulation of ERα is essential for successful implantation (126, 174). Several other factors which may be important in mediating decidualisation have been identified as a result of studies in mice. Targeted knockout of LIF (175), HOXA10 (482), COX2 (483) and IL-11R (118) all result in decidualisation defects. Interestingly many of these factors are under transcriptional control by oestrogen. However, expression of these factors was found to be normal in ERαKO mice (126) suggesting oestrogen acting via ERα

does not regulate these factors during decidualisation in mice. Despite this, it is conceivable that increased local oestrogen may influence expression of these factors during decidualisation in human.

While implantation triggers decidualisation in mice, in humans decidualisation occurs in the absence of conceptus and thus implantation. However in fertile cycles the uterine preparation for pregnancy requires synchronous control of receptivity, implantation and decidualisation. Impaired decidualisation leads to disruption of embryo-maternal interactions in sporadic and recurrent pregnancy loss highlighting the necessity for coordinated control of implantation and decidualisation in the human (484). However the mechanisms which control these processes are poorly understood in humans. In this study we have shown that local biosynthesis of oestrogen is a feature of *in vitro* decidualisation of ESC and that partial inhibition of oestrogen biosynthesis may inhibit decidualisation. It is unknown whether local oestrogen biosynthesis may affect implantation or whether it is essential to decidualisation in humans. However locally produced oestrogen is likely to influence the implantation site and process of decidualisation which may involve modulation of angiogenesis and leukocyte recruitment (Discussed in chapter 5).

3.5.4. Summary

In the current study decidualisation of ESC resulted in altered expression of steroid metabolizing enzymes, expression of aromatase mRNA and protein as well as transient up-regulation of StAR and CYP11A1, enzymes responsible for *de novo* biosynthesis of steroids. Changes in expression in steroidogenic enzymes were accompanied by high levels of E1 and E2 detected in cell culture supernatants from decidualised cells consistent with oestrogen biosynthesis. There was an accompanying change in enzyme activity in decidualised cells which resulted in increased aromatase activity and appeared to result in reduced 17βHSD2 activity, which was in contrast to the observed mRNA changes. Oestrogen production could be partially ameliorated by inhibitors of aromatase and STS. Inhibition of E1 formation via STS reduced the expression of the decidualisation marker IGFBP1 suggesting an importance for local oestrogen in decidualisation. The production of E1 exceeded that of E2. Consistent with this aromatase inhibition had a greater impact on E1 production suggesting aromatase mediated conversion from androstenedione to E1 is the more prominent reaction in decidualised ESC.

Aromatase expression and oestrogen biosynthesis has previously been associated with disease states of the endometrium such as endometriosis and endometrial cancer. However,

herein is described expression of aromatase and oestrogen biosynthesis as a result of decidualisation of ESC, a model of normal endometrial function. Interestingly, while we report no change in 17βHSD1 expression and increased expression of 17βHSD2 as a result of decidualisation, in endometriosis the expression pattern is reversed i.e. there is increased 17βHSD1 and decreased 17βHSD2 expression. Thus if decidualised ESC share the capacity for oestrogen biosynthesis with endometriotic cells, their distinction appears to be in the expression of 17βHSD isotypes and thus capacity to form different oestrogens.

The results described herein provide a novel description of the regulation of oestrogen biosynthesis and metabolism during *in vitro* decidualisation of endometrial stromal cells. While further elaboration of the impact of inhibitors is required to elucidate the full impact of locally produced oestrogens during decidualisation, this novel and important description of the steroid microenvironment generated by decidualisation can provide a framework from which comparative dysregulation in disease paradigms can be measured. This work highlights, for the first time, the concept of the endometrium as a steroidogenic tissue in a non-diseased state.

Chapter 4

4. The impact of oestrogen receptor-dependent signalling on gene expression in uNK cells.

4.1. Introduction

Human endometrium contains several leukocyte populations, the composition of which changes during the menstrual cycle, and in pregnancy. Approximately 30% of cells in the stromal compartment in late secretory phase non-pregnant endometrium are natural killer cells (25) (uNKs) that are phenotypically and functionally distinct from the main population of peripheral blood NK cells (pbNKs) (24, 27). The number of uNK cells rises to account for 75% of all leukocytes in first trimester decidua (75) .

4.1.1. Natural killer immune cell populations

Natural Killer (NK) cells are lymphocytes that are part of the innate immune system and comprise 15% of all circulating lymphocytes (21). NK cells are effectors of antibody dependent cellular cytotoxicity via CD16 and they do not express CD3. The peripheral blood (pb) contains two main subsets of NK cell, usually classified according to their expression of the surface markers CD56 (also known as NCAM) and CD16, as $CD56^{bright}$ $CD16^{-}$ or $CD56^{dim}$ $CD16^{+}$ also classified as agranular and granular respectively. The $CD56^{dim}$ population constitute around 90% of pbNK cells while $CD56^{bright}$ cells are in the minority representing ~10% of total pbNK and <1% of all lymphocytes (24).

A phenotypically distinct population of NK cells has been identified as resident in human endometrium (25). These so called uterine NK (uNK) cells are found in the endometrium but not the myometrium and appear similar to the minority $CD56^{bright}$ pbNK population as they are $CD56^{bright}/CD16^{-}$ (26). However unlike $CD56^{bright}$ pbNK, the uNK cells are granular and like the majority pbNK population contain Granzyme B and perforin (26). In agreement with differences in phenotype between uNK and pbNK (Figure 4.1) transcriptional analysis (27) has identified differences between the cell populations. Notably, compared to pbNK, uNK exhibit decreased cytolytic activity and increased secretion of cytokines and growth factors such as interferon gamma (IFNγ), tissue necrosis factor alpha (TNFα), transforming growth factor beta 1 (TGF-β1), interleukin 8 (IL-8), CXCL10 and CCL5 (39-41) and they are reported to secrete angiogenic cytokines such as vascular endothelial growth factor (VEGF), placental growth factor (PLGF) and angiopoeitin 2 (39, 43, 44).

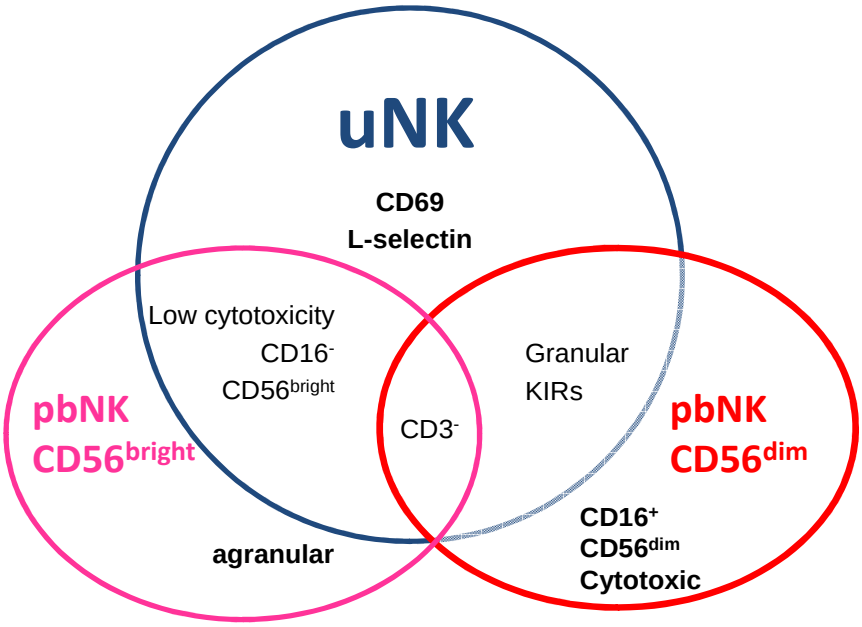

Figure 4.1. Venn diagram summarising the characteristics of NK subsets. Notably, uNK are phenotypically distinct from either population of pbNK although they appear to share some features of both types (27, 39, 75).

4.1.2. Origin and function of uNK

uNK cells are abundant in the uterus. While $CD56^{bright}$ cells in the peripheral blood are a minority population, uNK cells are abundant in the endometrium and are the major leukocyte population in early pregnancy, constituting 70% of all leukocytes in first trimester decidua (30). However uNK cell numbers appear to regress as pregnancy progresses suggesting their role may be most significant during early pregnancy. It has been reported that uNK cells play an important role in having functional significance in many processes during early pregnancy including immune tolerance at the feto-maternal interface, regulation of trophoblast invasion, spiral artery remodelling and angiogenesis (reviewed in (41)). The interaction between uNK cells and invading trophoblast cells is reported to influence placentation and the establishment of pregnancy in humans (485, 486). Maternal-immune interactions are essential to optimal reproductive success and thus defects in this interaction are associated with disorders of pregnancy (41). The origin of the uNK cell population remains unresolved. Two main theories abide; trafficking from peripheral blood or *in situ*

proliferation/differentiation of resident uNK cells/precursors. Evidence supporting both theories exists.

Precursor uNK cells may be trafficked from the peripheral blood and proliferate and differentiate *in situ*. In support of the idea that uNK result from homing of pbNK to endometrial tissue, there is a high degree of uNK homology with pbNK subsets and there is increased vascular permeability coincident with the increase in the uNK cell population. uNK cells also accumulate around spiral arterioles and are abundant in perivascular sites following decidualisation (25). There are increased numbers of circulating $CD56^{bright}$ NK cells in women of reproductive age compared to males suggesting this subtype may be recruited to the uterus as precursor to uNK cells (26).

In addition, lineage committed CD34+ progenitor cells have been identified in decidua and can undergo differentiation into functional CD56+CD16- NK cells in the presence of growth factors or co-culture with stromal cells (31). Decidual CD34+ progenitor cells express CD45 but notably, not CD56; however expression of CD56 is induced with differentiation by factors including IL-15 (31, 32) consistent with differentiation within the decidual uterine environment.

uNK cells are present in small numbers in proliferative and early secretory endometrium but are increased in late secretory endometrium and decidua. Stromal conditioned media from secretory phase endometrium and decidua increases proliferation of uNK cells. Strikingly, 40% of CD56-positive uNK cells from late secretory endometrium are Ki67 positive (33) consistent with proliferation of resident uNK cells in response to factors in the uterine environment. Notably, human chorionic gonadotrophin (hCG) has been shown to increase the proliferation of uNK cells which may partially explain increases in the number of uNK cells at the time of implantation (34).

4.1.3. Regulation of uNK cells

The regulation of uNK cells by sex steroid hormones is poorly understood. Premenarche and postmenopause uNK cell numbers are sparse suggesting steroid regulation. Leukocytes constitute 5% of uterine cells in the proliferative phase but increase to 25% of total cells in the late secretory phase (25, 487). The number of uNK cells changes under the influence of ovarian hormones with low numbers during the proliferative phase of the menstrual cycle but a pronounced increase during the secretory phase following the post-ovulatory rise in progesterone and coincident with decidualisation of the uterine stroma. uNK cells do not express progesterone receptor (PR) or oestrogen receptor (ER) α, but do express ERβ and

glucocorticoid receptor (GR) (249). Thus any influence of progesterone would be indirect and the influence of oestrogen could only be via ERβ-dependent receptor signalling. The uterine environment is thought to play an important role in regulating the function of uNK cells. Interleukin-15 (IL-15) is a cytokine which can regulate both proliferation and activation of NK cells (423). Okada et al reported that IL-15 protein is secreted by endometrial stromal cells *in vitro* following treatment with progesterone. Verma et al demonstrated that IL-15 induces proliferation of isolated uNK cells (423) while in an *in vitro* migration assay IL-15 enhanced recruitment of CD16- pbNK cells (425). IL-15 can also increase CD56 expression in CD16- pbNK and induce a chemokine receptor repertoire similar to that of uNK (37). Chen et al reported that culture of uNK cells with conditioned media from endometrial stromal cells isolated from different stages of the menstrual cycle and first trimester decidua influenced uNK cell function (38). The authors reported that stromal cell conditioned media from secretory phase and first trimester decidua significantly induced proliferation of uNK cells (38). Conditioned stromal cell media also increased interferon-γ (IFNγ) secretion by uNK cells irrespective of menstrual cycle phase. In addition NK cytotoxicity was reduced by stromal conditioned but this effect was not specific to endometrial phase (38).

These findings indicate that the uterine environment may play a critical role in determining the functional characteristics that distinguish uNK cells form peripheral blood counterparts. There is accumulating evidence for an indirect role of progesterone on uNK but the role of oestrogen is less clear.

4.1.4. Evidence that oestrogen may influence function of uNK cells

Deloia et al. reported that treatment of peri-menopausal women with oral oestrogen increased total uterine leukocytes detected by immunohistochemistry in endometrial biopsies. The authors reported a significant increase in uNK cells and macrophages detected in the endometrium while T cell numbers were unaffected by oestrogen treatment (74). Following ovarian stimulation during *in vitro* fertilization (IVF) treatment, supraphysiological levels or sex steroids, including oestrogen, are detected in the circulation. Lukassen et al reported that as a result of this treatment, higher leukocyte levels were detected in the endometrium of treated women and a shift towards increased $CD56^{bright}$ to $CD56^{dim}$ NK cells was observed in the endometrium while T cell numbers were unaffected (488). This effect was specific to the uterus as the authors reported no change in peripheral blood NK populations (488). In ovariectomised women oestrogen and progesterone are both

required for the presence of uNK cells in the endoemetrium, consistent with absence premenarche and postmenopause (489). Mouse NK cells are recruited to the pre-implantation uterus via oestrogen and progesterone signaling, an effect which is reversed by antagonism of oestrogen and progesterone receptor (490). Oestradiol increases NK cell number and decreases cytoxicity in mouse pbNK cells (491).

Oestrogen induces protein inhibitor 9, a granzyme B inhibitor in YT cells, a human NK cell line (492, 493), which may be a mechanism by which uNK cells retain granularity but lack cytotoxic activity in the endometrium. The CD16 promoter contains several ERE sites and several Sp1 consensus sequences (494) and control of expression and in turn cytoxicity could be in part controlled by oestrogen via CD16. Kitaya et al. demonstrated that neither progesterone nor oestradiol affected proliferation or cytotoxicity of uNKs isolated from decidua, or secretory and proliferative endometrium (495). In the same study, *in vitro* treatment of uNK cells isolated from secretory phase endometrium and first trimester decidua with IL-15, oestradiol or progesterone did not affect secretion of IFNγ or tissue necrosis factor α (TNFα) detected by ELISA (495).

An indirect role of for oestrogen may also be important in uNK function. uNK cells express high levels of CXCR3 and CXCR4 (389). Oestrogen induces CXCL10 and CXCL11 expression in the endometrium, cytokines which interact with CXCR3 and may activate NK cell migration (458). CXCL10 protein is increased in secretory phase endometrial explants the time at which uNK numbers are increased (458). Oestradiol induces SDF-1 production in endometrial stromal cells (386) which may further mediate migration of uNK via CXCR4 expression.

4.1.5. Summary

The function of uNK cells is affected by both the endometrial environment and the circulating levels of sex steroids which are important in mediating proliferation and/or differentiation of uNK precursors in the endometrium. Oestrogen seems to be involved in trafficking of NK cells to the uterus and conferring uNK phenotype however the direct functional impact of oestrogen on uNK cells is unresolved.

The studies presented in Chapter 3 documenting biosynthesis of oestrogens by endometrial stromal cells as well as previous studies from our laboratory documenting expression of ERβ in this cell type (249) formed the backdrop to studies described in this chapter exploring the impact of oestrogens on gene expression/function of human uNK cells.

4.2. Aims

1. To isolate and culture pure and viable populations of uNK cells and confirm ER expression.
2. To investigate changes in gene expression in isolated uNK cells following *in vitro* treatment with oestradiol.

4.3. Methods

4.3.1. Tissue

Decidual samples were obtained from women requesting surgical termination of pregnancy (STOP) between 8-12 weeks gestation. All women had an ultrasound scan to confirm viability of pregnancy and gestational age. All material from suction curettage was collected and decidual tissue was identified and separated from specimens using sterile forceps and washed in sterile saline prior to tissue processing. Local ethical committee approval was granted and written informed patient consent was obtained prior to tissue collection by a dedicated research nurse. (Ethical approval held by Professor H.O.D. Critchley; LREC/05/51104/12 and LREC/10/51402/59.)

4.3.2. Isolation of lymphocytes

Decidual cell preparations were prepared by an adapted standard protocol (26, 27). Briefly, total decidual tissue (n=22, see section 2.30) was minced into $1mm^3$ fragments using surgical blades and residual blood clots were removed. The tissue was digested overnight at room temperature under agitation in 20 ml of RPMI media (RPMI (phenol free), NEAA 5mls, 20% CSFCS, 5mls Pen/Strep, HEPES (50ul) and Na Pyruvate 0.01mM with 1ml of collagenase type IV (2 mg/ml; Sigma) and 125 µl of DNAse (0.1 mg/ml; Sigma). After digestion a further 30mls of media were added and the mixture left for 5 minutes to allow for sedimentation. The supernatant was sequentially passed through 70 µm and 40 µm cell strainers. The filtrate was then plated on a large Petri dish for 1 hour to allow for separation of adherent cells.

The cell suspension was collected in a 50 ml falcon tube and centrifuged at 870 x g for 5 min. The resulting cell pellet was resuspended in 15 ml PBS supplemented with 2% FCS and 0.1% NaN_3. The cell suspension was carefully overlaid on to 15 ml of Histopaque 1077 (Sigma, Cat. No. 10771) ensuring the two layers did not mix and then centrifuged at 1094 x g for 20 minutes with no brake at the slowest acceleration speed. Cells at the interface (lymphocytes) were collected and washed in 20 ml PBS and centrifuged at 870 x g for 5 min. The resulting pellet was then resuspended in 10 ml PBS for cell counting.

4.3.3. Purification of uterine natural killer cells

Uterine natural killer (uNK) cells were isolated with CD3 negative selection and CD56 positive selection using the appropriate antibody-coated magnetic microbeads and the MACS system (Miltenyi Biotech, Germany) in a protocol adapted from Vassiliadou and Bulmer (496).

Cells were depleted by negative selection using magnetic separation to remove CD3+ cells and thus deplete any T or NKT cells (a subset of T cells which are $CD3^{+}CD56^{+}$) in the cell suspension. The lymphocytes were counted and the suspension centrifuged for 5 minutes at 870 x g. The cell pellet was then resuspended in MACS buffer plus 20 µL of CD3 coated microbeads (Miltenyi, 130-050-101) per 10^7 cells and the mixture was incubated on ice for 20 minutes. An LD column (Miltenyi, 130-042-901), for cell depletion, was placed in the magnetic field of MACS separator. The column and pre-separation filter were primed by passing 2 x 1ml of MACS buffer. The cell suspension was then added to the column followed by 2x 1ml washes of MACS buffer. The total effluent which contained the unlabelled cells (CD3-) was collected and used for CD56 positive selection of uNK cells. The cells were counted using a FastRead™ cell counter (ImmuneSystems, UK) and the cell pellet was then resuspended in MACS buffer plus 20µL of CD56 microbeads (Miltenyi, 130-050-401) per 10^7 cells. The mixture was incubated on ice for 20 minutes. LS column (Miltenyi, 130-042-401), for cell selection, was placed in the magnetic field of MACS separator. The column was primed by passing 3 x 1ml of MACS buffer. The cell suspension was added to the column followed by 3ml wash of MACS buffer. The column was removed from the magnetic field and placed in a suitable collection tube. Magnetically labelled cells (CD56+) were flushed from the column by addition of 5 x 1ml of MACS buffer into a fresh tube. The resulting cell suspension was CD56+, CD3- and was immunophenotyped as uNK cells using flow cytometry (see section 4.3.5.)

4.3.4. uNK culture conditions

Isolated uNK cells were cultured at a density of $1x10^6$ cells per/ml for cell treatment in phenol red-free RPMI 1640 medium (Sigma, Cat. No. R7509) supplemented with 10% CSFCS, 1% Penicillin/Streptomycin (Sigma, Cat. No. P-4333), 1% L-glutamine (Sigma, Cat. No. G-7513), and 0.5% fungizone (Invitrogen, Cat. No. 15290-018). Cells were cultured up to a maximum of 24 hours at 37^oC under 5% CO_2 in air. Cultured uNK were incubated either with vehicle control (DMSO), 10^{-8}M E2, 10^{-6}M ICI or combined E2 and ICI for 2 or 24 hours.

	Research number	Tissue number	Lab number	GESTATION
Sample 1	7762	554	CD1040D	<8 wks
Sample 2	7644	385	CD774D	8 wks 1 day
Sample 3	7633	380	CD763D	8 wks 4 days
Sample 4	5721	703	CT1205D	8 wks 6 days
Sample 5*	5696	446	CD480D	9 wks
Sample 6*	7648	387	CD778D	9 wks 2 days
Sample 7*	5650	417	CD722D	9 wks 5 days
Sample 8*	7663	397	CD731D	9 wks 6 days
Sample 9*	7721	545	CD999D	9 wks 6 days
Sample 10*	7640	383	CD770D	10 wks 1 day
Sample 11	6415	524	CD713D	10 wks 5 days
Sample 12	6418	527	CD716D	10 wks 5 days
Sample 13	7688	537	CD966D	10 wks 5 days
Sample 14	6413	523	CD798D	11 wks 2 days
Sample 15	7647	386	CD777D	11 wks 2 days
Sample 16	7642	384	CD772D	11 wks 2 days
Sample 17	6417	526	CD715D	11 wks 5 days
Sample 18	7652	390	CD782D	11 wks 5 days
Sample 19	5667	440	CD451D	10 wks 0 days
Sample 20	5669	441	CD453D	10 wks 1 days
Sample 21	7697	540	CD975D	10 wks 2 days
Sample 22	7701	542	CD979D	11 wks 2 days

*Table 4.1. Decidual samples from which uNK where isolated for treatment. Gestation in weeks (wks) and days based on ultrasound scan and date of last menstrual period recorded by a research nurse. Samples which were clustered for analysis in section 4.4.7 are indicated by *, samples 19-22 were those used in section 4.4.8.*

4.3.5. Flow Cytometry

The immune-phenotype of isolated uNK cells was confirmed using flow cytometry. Briefly, uNK cells isolated from human decidual tissues were suspended in FACS buffer (PBS, containing 1% FCS and 0.1% NaN_3) at a concentration of $5x10^5$ cells/ml. Cell suspensions were stained with antibodies conjugated to fluorescein isothiocyanate (FITC) (CD56) or

phycoerythrin (PE) (CD3 or CD16). Single stains were used to define the parameters for scanning the samples together with unstained samples and isotype controls. Single and double stained samples were utilised to fully describe the sample cell population. Cell pellets were resuspended in 100 μl of FACS buffer and incubated with conjugated antibodies for 30 minutes in the dark. Following incubation cells were washed by adding 1 ml of FACS buffer and then resuspended in 500ul of FACS buffer for scanning. Labelled cells were placed on ice and protected from light until scanning was completed.

4.3.6. Propidium Iodide staining

To assess cell viability the capacity for cells to incorporate the fluorescent DNA-binding probe propidium iodide (PI) was measured. Non-viable cell membrane proteins become porous and they can readily incorporate PI. Viable cells are intact and do not incorporate the PI, thus the proportion of dye excluded cells gives an approximation of the percentage viability. PI was added to the cell suspension, incubated for 5 minutes in the dark and fluorescence measured using flow cytometer.

Flow cytometry was performed using a Becton Dickinson FACScan flow cytometer (Bectin Dickinson, Oxford, UK). Forward scatter, side scatter and fluorescence measurements were recorded and analysed using FlowJo software (Tree Star Inc, OR, USA).

4.3.7. Immunohistochemistry

Immunohistochemistry was conducted on human decidual tissue sections in accordance with standard laboratory protocols to establish the presence and localisation of proteins of interest (Full details see section 2.9). Decidual tissue was collected and fixed in 4% NBF and embedded in paraffin wax. 5μm sections were cut and mounted onto coated glass slides (VWR, Poole, UK). Antigen retrieval was performed using 0.05M Glycine/EDTA buffer (pH 8) followed by a methanol peroxidise block, serum block, and avidin-biotin block. Primary antibodies were diluted in blocking serum (normal serum, TBS, BSA), added to tissue sections and incubated overnight. Primary antibody dilutions are detailed in 0. Slides were incubated with appropriate biotinylated secondary antibody followed by incubation with streptavidin-HRP before final antibody detection using 3,3'-diaminobenzidine (DAB). Slides were counterstained in Harris' haematoxylin. DAB positive sections were imaged using a PROVIS microscope (Olympus Optical, London UK) with an attached Canon DS126131 camera. DAB detection immunohistochemistry was also preformed on cytospins

of isolated uNK cells. $2x10^5$ cells were applied to glass slides using centrifugal force and presence of CD56 was detected.

Immunofluorescent immunohistochemistry using Tyramide signal amplification (TSA) enables the identification of multiple antigens in the same tissue section and detection of colocalised target antigens and was used to identify co-expression of the uNK marker CD56 with other proteins of interest.

Following antigen retrieval and hydrogen peroxide block, slides were washed in water and then twice in PBS. The slides were serum blocked for 30 min at room temperature before incubation overnight with primary antibody diluted at appropriate dilution (see Table 4.2) Following washes in PBS, slides were incubated with appropriate peroxidase (with attached FAB fragments) secondary antibody for 30 min at room temperature. Slides were washed in PBS and incubated for 10 min with TSA kit (PerkinElmer, Massachusetts, USA) using Tyramide-cyanine 3 diluted 1:50 in substrate. The red fluorophore (cyanine 3) was usually used for the first primary antibodies as the green fluorophore (fluoroscein) was more prone to photobleaching and diminished signal when used first. After washing in PBS the slides were microwaved in boiling 0.01M citrate buffer for 2.5 min and then allowed to stand for a further 30 min to block cross-reactivity. The slides were then blocked and incubated overnight with the second primary antibody. Incubations with appropriate secondary antibody and TSA kit were performed as described above. The green fluorophore (fluorescein) was used for the second primary antibody. Following washes in PBS the slides were counterstained with DAPI diluted 1:1000 in PBS and mounted with coverslips in permafluor (Immunotech, Marseille, France). Fluorescent images were examined using a Zeiss LSM 510 Meta-Confocal microscope (Carl Zeiss, Hertfordshire, UK).

Source	Primary antibody	Species raised	DAB dilution	Fluorescence Dilution
Zymed	CD56	Mouse	1:400	1:2500
Bioquote	SOD2	Rabbit	1:200	1:400
Sigma Aldrich	ACTR3	Mouse	1:50	N/A
Ab frontier	PTMA	Mouse	1:500	N/A
R&D	CXCR4	Mouse	1:500	1:80
Vector	ERα	Mouse	1:200	1:1000
Serotec	ERβ	Mouse	1:200	1:1000

Table 4.2. Primary antibodies and dilutions used in immunohistochemistry studies.

4.3.8. Cytospins

Suspensions of isolated uNK cells were adhered to glass slides using a Shandon cytospin 2. Following isolation of uNK, $2x10^6$ cells/ml were suspended in PBS and 100 µL of cell suspension added to each slide. Slides were spun at 24.2 x g for 3 minutes and then allowed to air dry for 20 minutes at room temperature. Cells were fixed in 90% acetone/10% methanol for 20 minutes and then air dried for a further 10 minutes. Immuno-detection of CD56 protein (diluted 1:50) was performed on cells immediately following fixation. Endogenous peroxidase activity was blocked by incubating slides in 0.15% H_2O_2 in methanol for 30 minutes at room temperature. Following TBS washes cells were serum blocked for 30 minutes at room temperature. Thereafter protocol was identical to 4.3.7.

4.3.9. RNA extraction and Taqman Q-RT PCR

Extraction of RNA from cell extracts was performed using RNAeasy® mini kit (Qiagen, Crawley, UK). Biological samples were collected in 350µl of lysis buffer per well of 6-well plate and homogenized using QIAshredder® spin columns (Qiagen, Crawley, UK). On column DNAse digestion was performed to remove contaminating DNA. RNA was eluted in 30µl RNAse-free water and quanitified using a Nanodrop® ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). RNA samples were standardised to concentration of 100ng/µl prior to cDNA preparation. Reverse transcription of RNA to form a single stand of complementary DNA (cDNA) was carried out using the SuperScript VILO cDNA synthesis kit (InVitrogen) according to the manufacturer's instructions. Briefly, each reaction (final volume 20µl) contained a final concentration of 1x VILO reaction mix, 0.125x Superscript enzyme mix and 100ng of RNA. Samples were incubated under the

following conditions: 25^{o}C for 10 minutes, 42 ^{o}C for 60 minutes, 85 ^{o}C for 5 minutes in a thermal cycler. Concentrations of mRNAs in samples were determined by reverse transcription followed by quantitative-real time polymersase chain reaction performed in an ABI machine using the Taqman® Method (Section 2.3). Each reaction was prepared using the qPCR Supermix with premixed ROX kit (11795-01K; Invitrogen) and conducted in duplicate and plated in a 96-well MicroAmp Fast Optical reaction plate (Applied Biosystems). Real time PCR amplification was performed using the Applied Biosystems® 7900HT Fast Real-Time PCR System using reaction mix described in Table 4.3.

Reagent	**Final Concentration**	**Vol./15µl reaction (µl)**
2x Express Supermix	1x	7.5
Forward Primer 20µM	200nM	0.15
Reverse Primer 20µM	200nM	0.15
Probe 10µM	100nM	0.15
18S primer/probe mix	10nM primer, 40nM probe	0.1125
H20		5.4375
cDNA		1.5

Table 4.3. Reaction mixture for Taqman analysis.

Gene	Accession number	Forward Primer	Reverse Primer	UPL probe number
ACTR3	Nm_005721	tggtgtcactcatgtcattcc	Atatctcgtcctgcgattgg	41
CAV2	Nm_001233	ggctcaactcgcatctcaa	cgtatttgctgatttcaaagagg	46
CGA	Nm_000735	tgctgtgtagctaaatcatataacagg	tcaagacagcacttggtaaaaca	59
CXCL12	Nm_000609	ttgacccgaagctaaagtgg	Ccctctcacatcttgaacctct	80
CXCR4	NM_003467	ttaagcgcctggtgactgtt	Gcccatttcctcggtgtag	47
ESR1	Nm_000125	ttactgaccaacctggcaga	Atcatggagggtcaaatcca	24
ESR2	Nm_001437	gctcctgtcccacgtcag	Tgggcattcagcatctcc	62
IL15RA	NM_002189	acaacccccagtctcaaatg	Tgccgtcgttactgtggag	37
IL15RA	NM_000585	cagatagccagcccatacaag	Ggctatggcaaggggttt	46
IQGAP1	Nm_003870	ctagaaacaccagccaccagt	Tcacggatagcacgtctctg	38
PMP22	Nm_153321	ttctcatcatcaccaaacgaa	gctgaagatgatcgacaggat	49
PTMA	Nm_001099285	cgaaatcaccaccaaggact	Ctccccattttcctcattctc	34
ROCK	NM_005406	Acatttgactggaaataaaga	Gctcgagttgcagggttaga	
SOD2	Nm_001024465	ctggacaaacctcagcccta	Tgatggcttccagcaactc	22
TTF3	Nm_001993	ggagaaaggggaattcagaga	Gggagttctccttccagctc	51

Table 4.4. Table of oligonucleotide sequences used in RT-PCR analysis.

4.3.10. Metacore

Metacore (http://www.genego.com/metacore.php) is an integrated knowledge database of manually curated human protein-protein, protein-DNA and protein-compound interactions used in pathway analysis of gene lists. Bioinformatic analysis was used to analyse the shortest path interactions with ERβ and candidate genes were carried out using Metacore.

4.3.11. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The criterion for significance for all tests was set at $p<0.05$.

4.4. Results

4.4.1. Uterine natural killer (uNK) cells are present in large numbers in human decidua.

To document numbers of uNK cells in tissue samples of decidua used for cell isolation, sections were immuno-stained with anti-CD56 (Figure 4.2). Consistent with previous findings (25, 249) CD56 positive cells were abundant within the decidual stroma with some being closely adjacent to blood vessels (Figure 4.2, B). Qualitative assessment of uNK numbers suggested no difference between 8 and 10 weeks gestation.

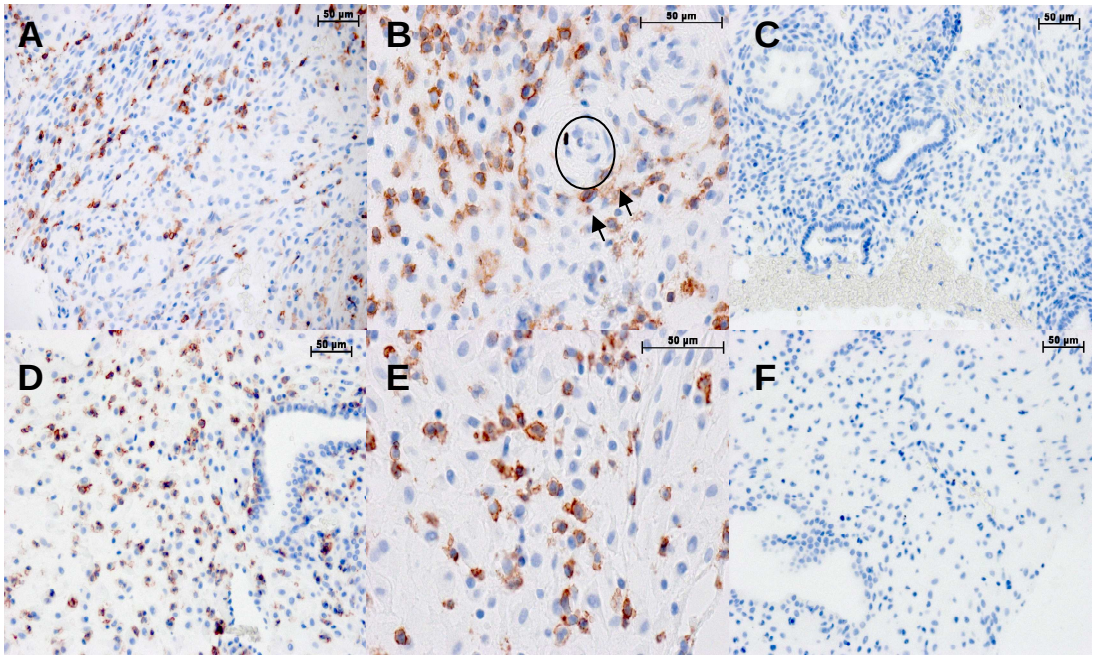

Figure 4.2. Immunolocalisation of CD56 to sections of human first trimester decidua from 8 weeks gestation (A, B) and 10 weeks gestation (D, E). Note CD56+ cells (arrowheads) were abundant, distributed throughout the stromal compartment and often found adjacent to blood vessels (circled). Negative controls were conducted in the absence of primary antibody (C and F). Scalebar equivalent to 50µm.

In agreement with findings from non-pregnant endometrium (249) double fluorescent immunohistochemistry revealed co-localisation of the CD56 surface marker with nuclear staining for ERβ but not ERα (Figure 4.3). Notably both ER subtypes could be detected in the stromal cells in decidua. In the tissue environment as well as in isolated cell populations uNK are ERβ positive and ERα negative. The absence of ERα suggests any direct impacts of oestrogens present within the local environment will be mediated by ERβ-dependent changes in gene expression.

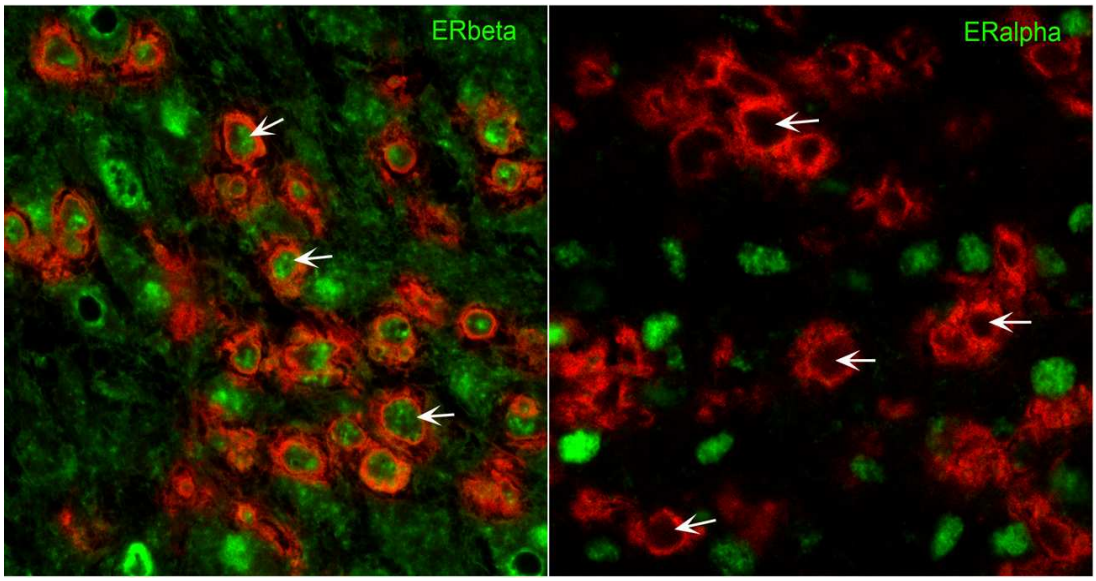

Figure 4.3. Decidual uNK cells are immunopositive for ERβ but not ERα. Immunohistochemistry shows co-localisation of ERβ but not ERα expression with CD56. In human first trimester decidua expression of CD56 (red staining) colocalised with ERβ expression (green staining, white arrows, left panel). ERα expression could be detected in stromal cells but not in CD56 positive uNK cells (white arrows, right panel).

4.4.2. uNK cells isolated from decidual tissue using MACS have high purity and viability.

To provide enough cells for experiments uNK cells were isolated from first trimester decidua using the quadro MACS system and the phenotype of the isolated cells investigated using flow cytometry. This analysis revealed a population that was highly enriched for CD56+ cells (Figure 4.4 C, ungated population >94%). Double staining of cells with CD56 and either CD3 or CD16 confirmed that the isolated cell population expressed the uNK phenotype with expression $CD56^{+}$ $CD3^{-}$ $CD16^{dim}$ (Figure 4.6). Positive staining was validated using isotype matched Ig-conjugated controls and was found to be antibody specific (Figure 4.5).

The viability of isolated uNK cells was tested using the propidium iodide assay (497) to confirm overall viability following isolation and to further determine viability following incubation for 2 and 24 hours. Viability was routinely found to be greater than 94% in the ungated cell population (Figure 4.7, A and C). Following incubation for two hours viability was >91% (Figure 4.7, B and D) and interestingly >79% after 24 hours despite no growth

factors or cytokines being added to supplement the incubation media (Figure 4.8). This was important in order to distinguish changes in gene expression as being mediated by the oestrogen treatment and not by supplemental factors.

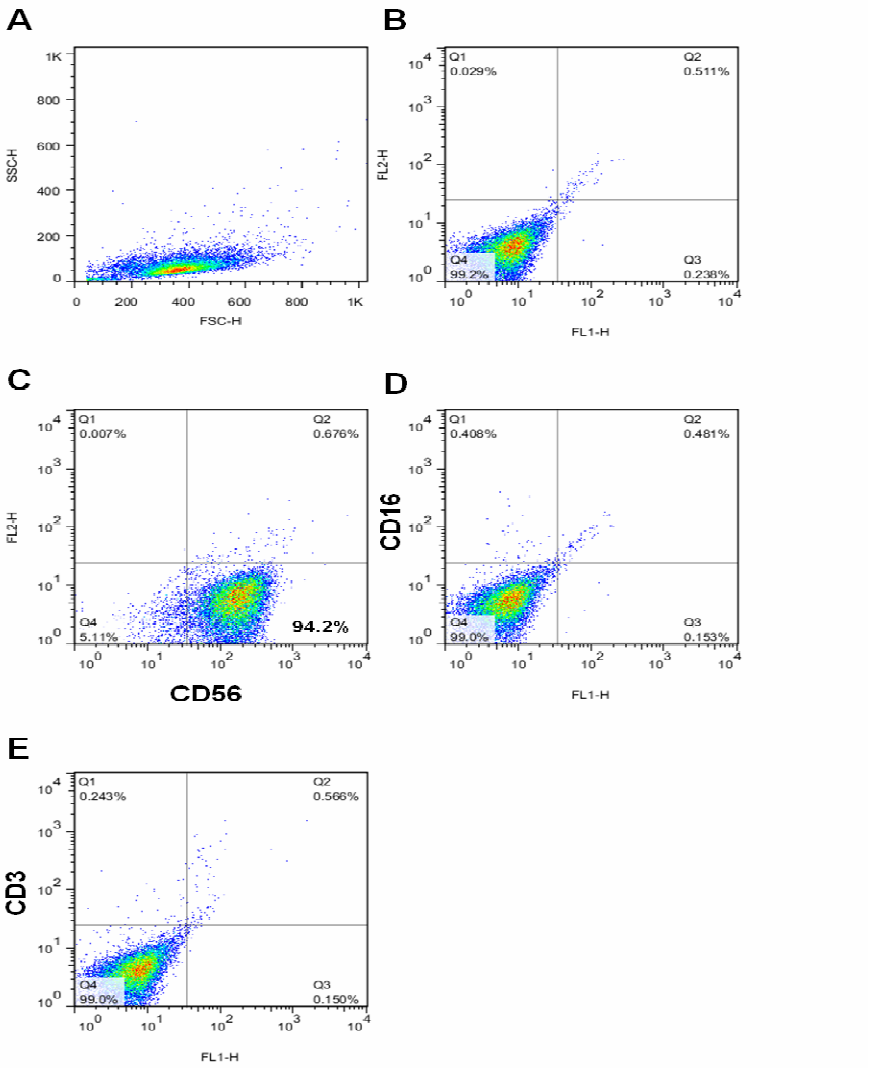

Figure 4.4. Purified uNK cells were CD56 positive, CD3 negative and CD16 negative. Forward scatter and side scatter values for the cell population (A) as well as unstained fluorescence levels are shown (B). Isolated cells were incubated with conjugated fluorescent antibodies and fluorescence intensity detected using BD FACScan. The whole ungated population was measured and >94% of the cells were CD56 positive (C).Greater than 99% of uNK were CD16 (D) and CD3 (E) negative.

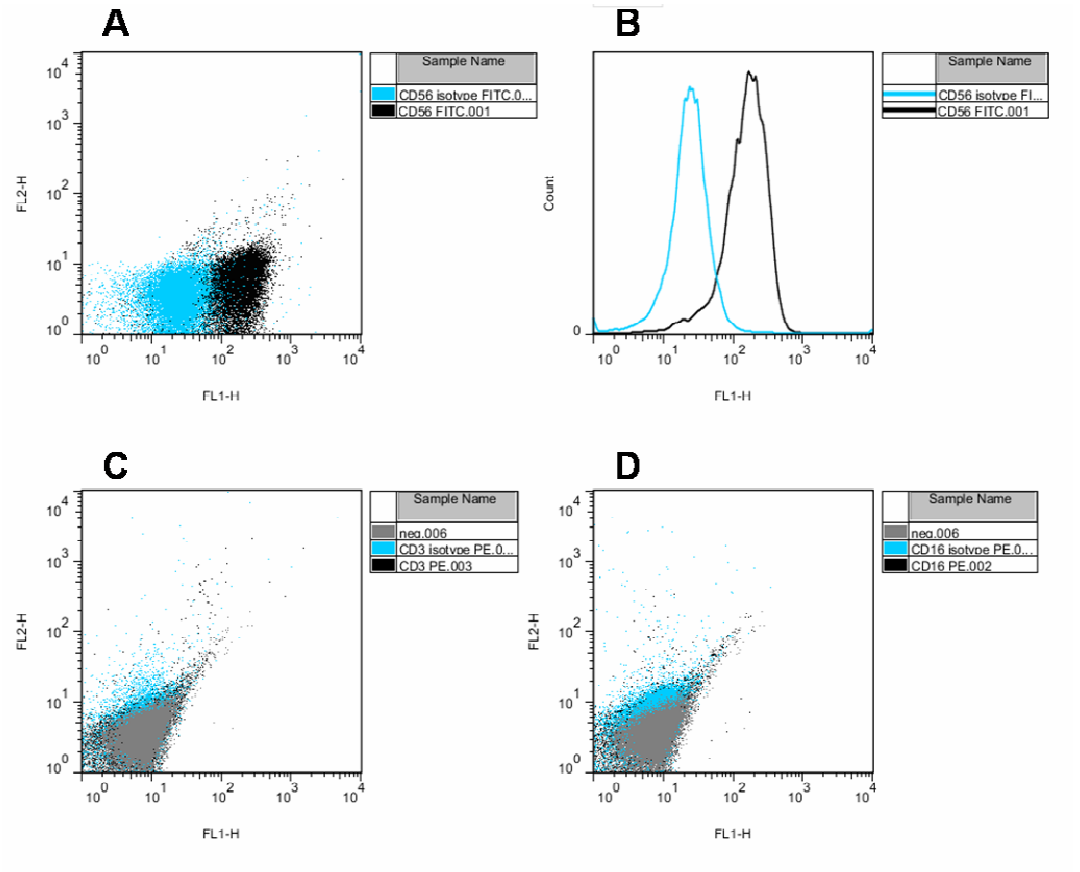

Figure 4.5. Isotype controls confirm specific antibody expression. Ig-matched fluorescence conjugated controls were incubated with the isolated cells. Positive CD56 fluorescence was confirmed to be antibody specific (A, B) demonstrated by increased fluorescence intensity following CD56 staining (black) that is distinct from the Ig-matched control (blue). There was no difference in fluorescence intensity between unstained (grey dots), antibody stained (black dots) and Ig-matched fluorescent conjugated controls (blue dots) for either CD3 (C) or CD16 (D) confirming lack of expression.

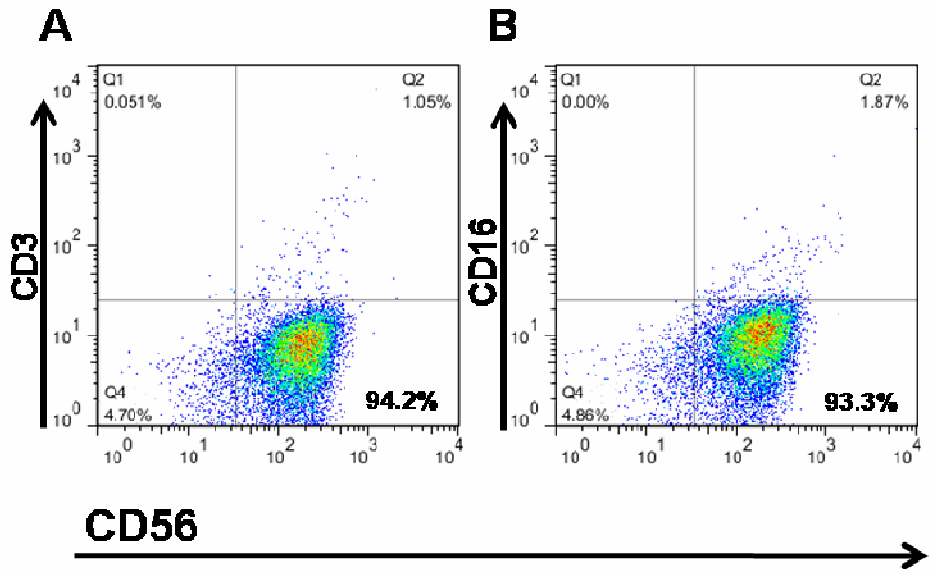

Figure 4.6. Double staining confirms uNK phenotype, CD56+ CD3- CD16 dim. Isolated cells were incubated with two conjugated fluorescent antibody pairs; either CD56-FITC and CD3-PE or CD56-FITC and CD16-PE. The whole ungated population was measured and >93% of the cells were CD56 positive, CD3 negative (A) and >94% of the cells were CD56 positive, CD16 negative (B).

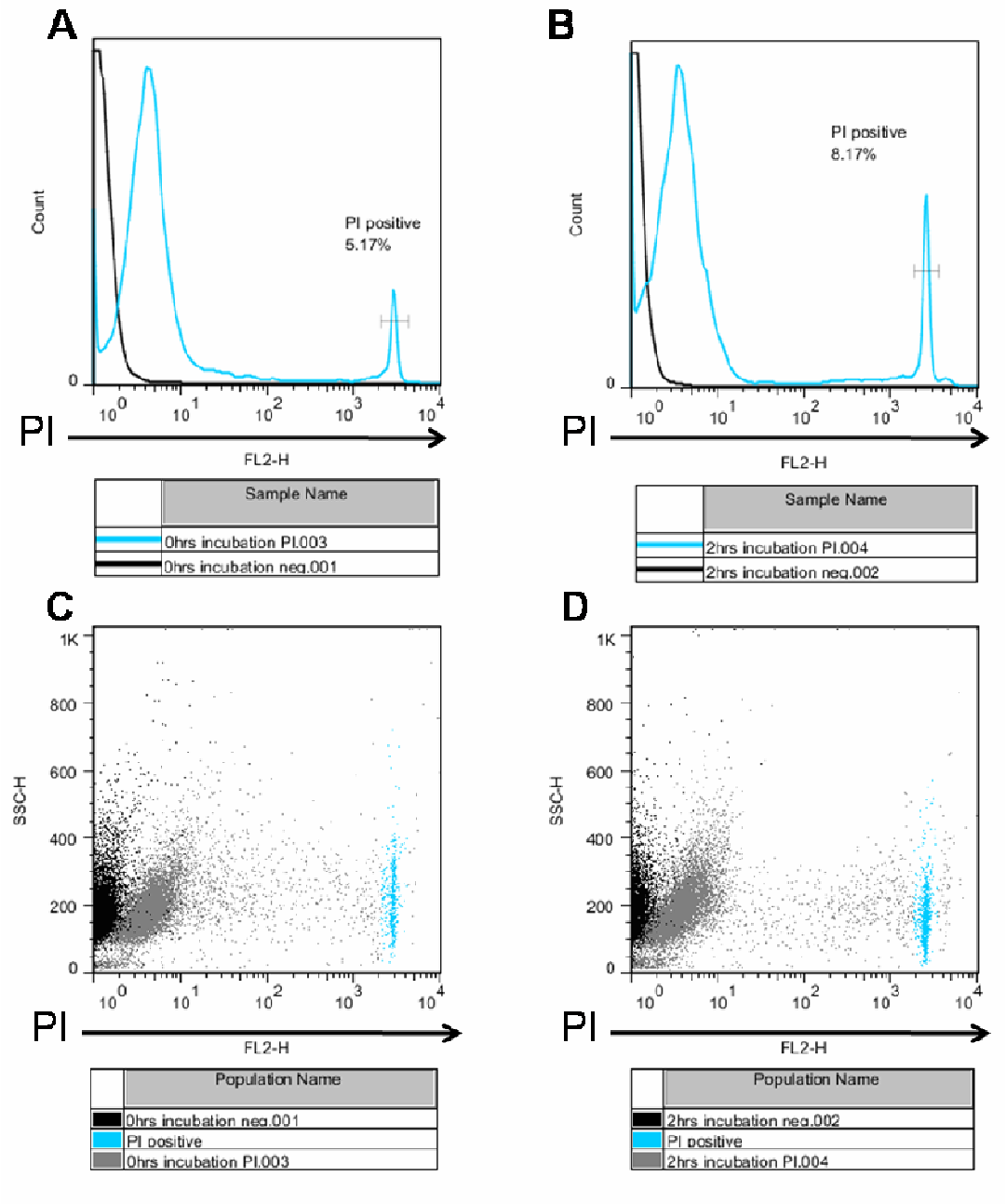

Figure 4.7. Isolated cells are highly viable and remain so following 2 hours incubation. Isolated uNK cells were tested for viability by propidium iodide staining (positive staining shown in blue). At 0 hours (following isolation) the cell population was routinely found to be >94% viable (A, C). Following 2 hours incubation cells retain viability and ungated population was >91% propidium iodide negative (B, D).

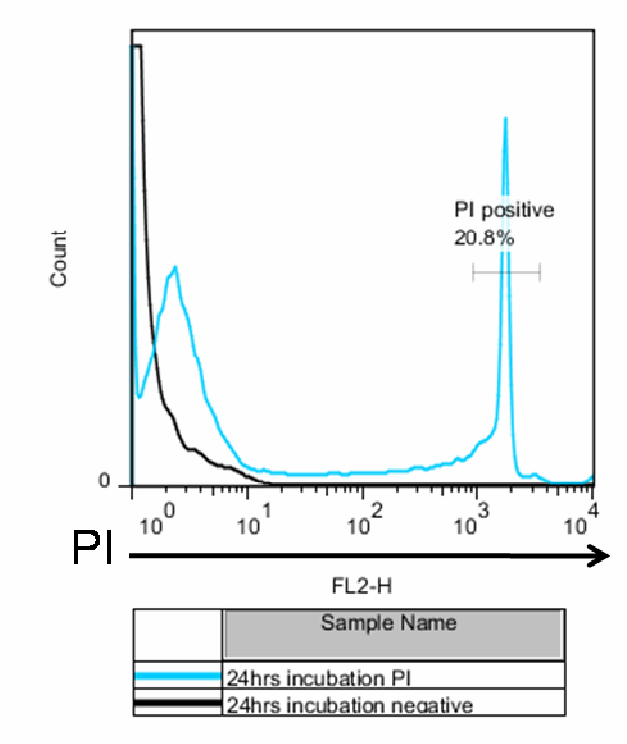

Figure 4.8. Isolated cells are viable following 24 hours incubation. Isolated uNK cells were tested for viability by propidium iodide staining. Following 24 hours incubation cells retain viability and ungated population was >79% propidium iodide negative.

4.4.3. Purified decidual uNK cells have a phenotype identical to that of cells in vivo.

Cytospins of uNK cells purified as above were stained using the same CD56 antibody as had been used on tissue sections and intense immuno-positive staining was detectable in the majority of cells consistent with enrichment of a CD56+ population (Figure 4.9).

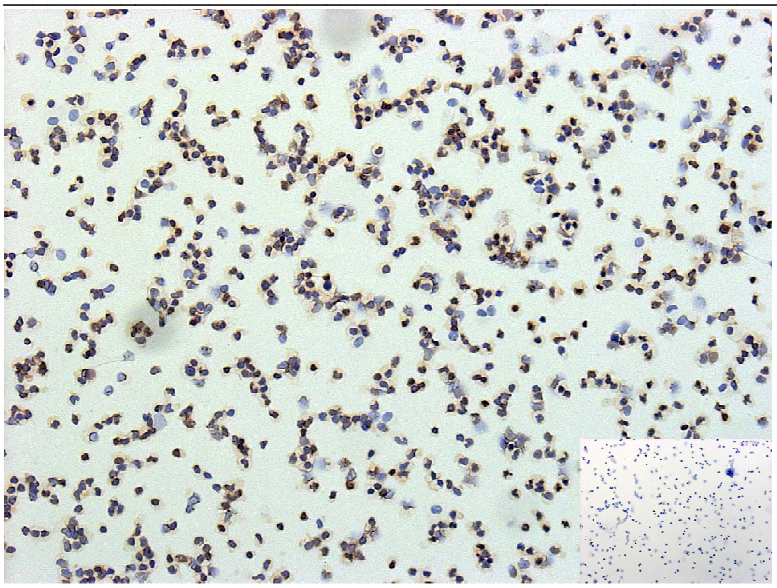

Figure 4.9. Immunolocalisation of CD56 to uNK on cytospins. Cytospins of isolated uNK cells express intense CD56 staining further confirming the phenotype of the isolated cell population and the validity of CD56 immunoexpression as a marker of uNK cells. Insert: negative control incubated in the absence of primary antibody.

To confirm that purified CD56+ uNK cells retained expression of oestrogen receptors qRTPCR was conducted on RNA isolated from 13 samples of uNK cells (Figure 4.10). Concentrations of mRNAs were compared to those in an Ishikawa cell positive control sample (endometrial epithelial adenocarcinoma cell line) as studies within the laboratory (F. Collins unpublished) have demonstrated that the ratio of ERα:ERβ is 1:1 in this cell type. All samples of uNK contained higher concentrations of ERβ mRNA than the control Ishikawa cells whereas ERα mRNA was low/undetectable.

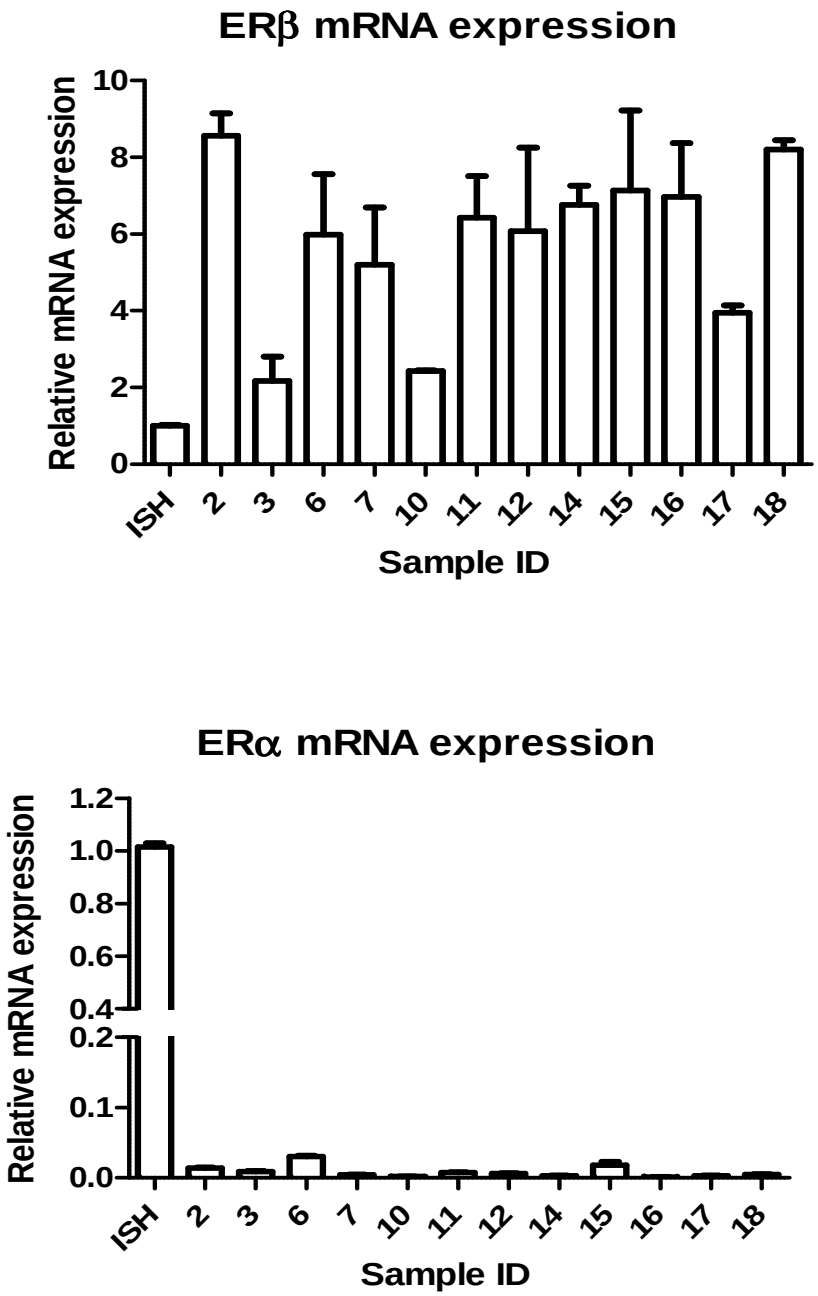

Figure 4.10. Expression of mRNAs encoding oestrogen receptor alpha (ERα) and oestrogen receptor beta (ERβ) in purified decidual uNK cells. Expression of ERβ mRNA (A) is greatly increased compared to Ishikawa (ISH) control RNA. ERα mRNA expression (B) was detected at very low levels in the isolated cells 30 times less than Ishikawa (ISH) control RNA.

4.4.4. **Changes in gene expression in uNK cells in response to oestradiol**

In an experiment undertaken within the laboratory before the start of the current study uNK cells purified from decidua (8-9 weeks, n=4) were incubated +/- E2 for 2h (Saunders, King, Marshall, unpublished). RNA was extracted and hybridized to Illumina's Sentrix® Human-6 v2 Expression Bead Chips at the Finnish DNA Microarray Centre (Turku, Finland). Hybridization was detected with 1μg/ml Cyanine3-streptavidine. Chips were scanned with Illumina Bead Array Reader (Factor=1, PMT=521, Filter=100%) and the numerical results were extracted with Bead Studio v3.2.6. Following the array bioinformatics was undertaken by the Finnish DNA Microarray Centre based on the following criteria: $p<0.005$, fold change (FC) ≥ 1.5, and $\log FC \geq 0.58$, 28 transcripts encoded by known genes were identified. In the present study, nine of these were chosen for validation (Table 4.5).

Gene Symbol	Gene name	Source Reference_ID	P value	FC
CAV2	Caveolin 2	NM_001233.3	**0.003325216**	**-1.65**
SOD2	Superoxide dismutase	NM_001024465.1	**0.004461885**	**-1.63**
PMP22	Peripheral myelin promoter 22	NM_153321.1	**0.003370981**	**-1.58**
TTF3	Tissue Factor	NM_001993.2	**0.004341158**	**-1.52**
IQGAP	IQ motif containing GTPase activating-like protein 1	NM_003870	**0.003721**	**1.32**
ROCK	Rho associated coiled-coil containing protein kinase 1	NM_005406	**0.001398**	**1.45**
ACTR3	Actin related protein 3	NM_005721.3	**0.000371033**	**1.50**
CGA	glycoprotein hormones, alpha polypeptide	NM_000735.2	**0.000637427**	**2.24**
PTMA	Prothymosin alpha	NM_001099285.1	**0.000484506**	**2.26**

Table 4.5. Candidate oestrogen-regulated genes identified by array analysis of uNK cells treated with E2 for 2 hours.

4.4.5. Bioinformatic analysis of interactions between candidate genes and oestrogen receptors.

Metacore (http://www.genego.com/metacore.php) is an integrated knowledge database of manually curated human protein-protein, protein-DNA and protein-compound interactions used in pathway analysis of gene lists. Bioinformatic analysis of interactions between the putative E2-regulated genes revealed potential interactions between ERβ suggesting that whilst regulation of some (PTMA, CAV2) might be altered by direct ERE mediated protein interaction, others (TTF3, SOD2, ROCK1) may be regulated through indirect gene activation via the involvement of ERβ tethered to other transcription factors such as Sp1 (Figure 4.11).

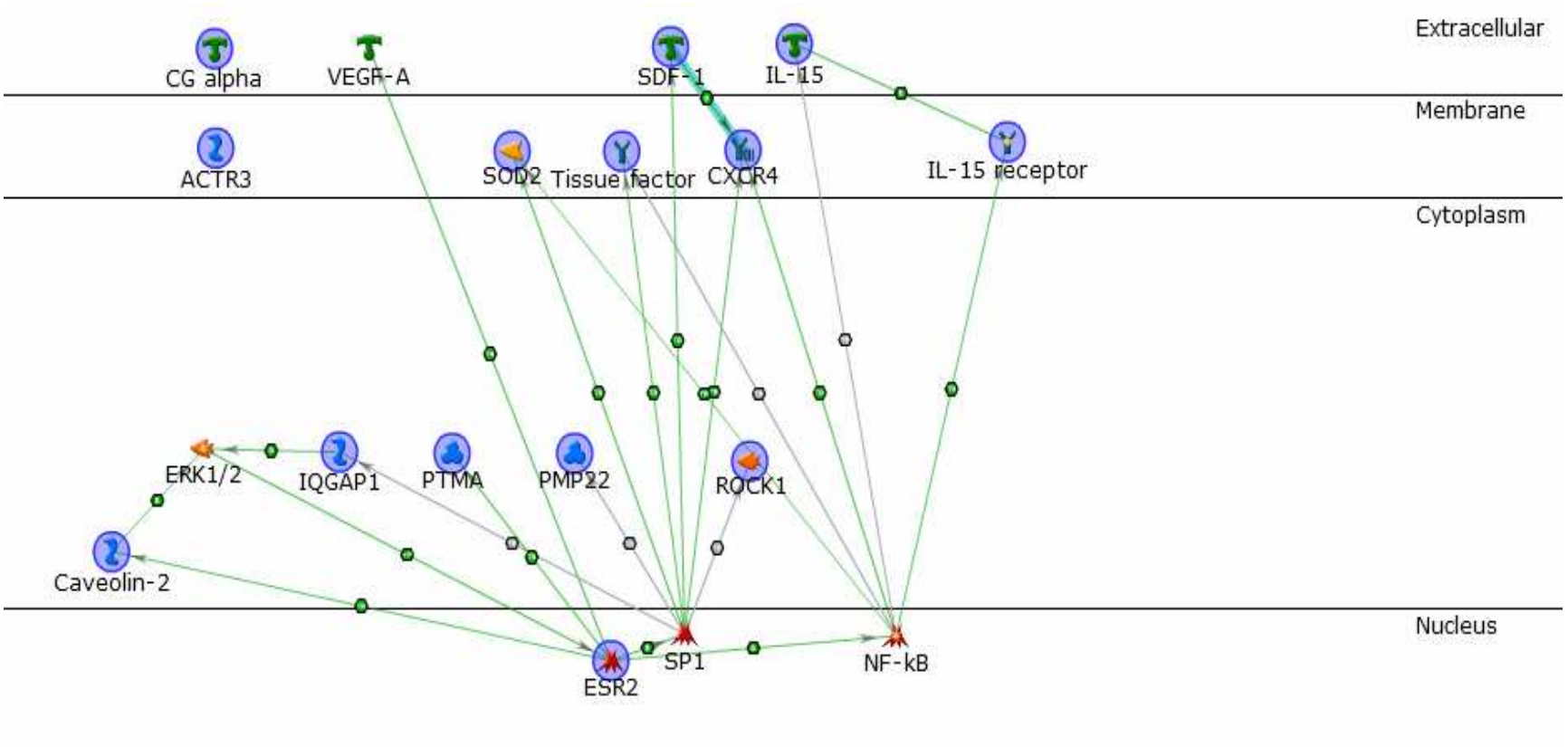

Figure 4.11. Pathway analysis of shortest path interactions with ERβ (ESR2) and candidate genes using Metacore. Interactions between the gene list were expanded to show the shortest path between them. This analysis revealed potential for both direct interaction and involvement of other transcriptional pathways.

4.4.6. Immunodetection of putative E2 regulated genes in human decidual tissue.

In order to investigate protein expression of candidate genes human first trimester decidual tissue sections were stained for CD56 and to antibodies for selected candidate genes. Co-localisation of the uNK cell marker CD56 was observed with SOD2 (Figure 4.12 G). Interestingly, positive immunodetection of SOD2, ACTR3 and PTMA could be also detected in the stromal cells suggesting oestrogens within the local environment might also have an impact on expression of these genes in other cell types.

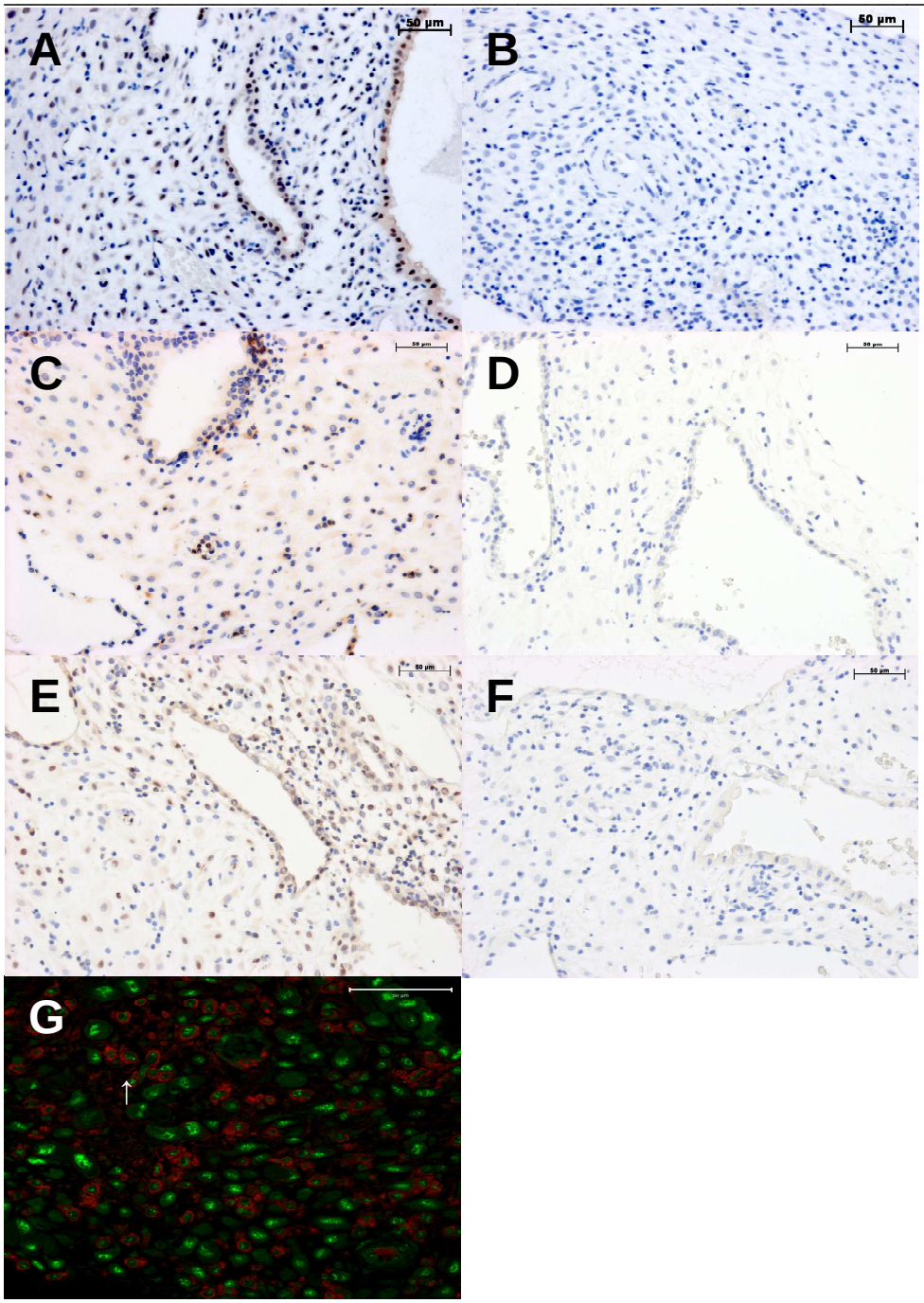

Figure 4.12. Immunolocalisation of selected candidate genes in sections of human first trimester decidua. Positive immunoexpression of SOD2 (A), ACTR3 (C), PTMA (E) could be detected throughout the stromal compartment. Immunohistochemistry shows co-localisation (white arrow, G) of SOD2 (green staining, G) with CD56 (red staining, G). Negative controls were conducted in the absence of primary antibody (B, D and F). Scalebar equivalent to 50µm.

4.4.7. Expression of candidate genes in uNK cells treated +/- E2 is highly variable.

To confirm and extend the results from the array, concentrations of mRNAs encoded by 9 putative E2-regulated genes were measured in a further set of samples (Figure 4.13, n=18 +/- E2 for 2 hours). Changes in gene expression in response to E2 treatment varied between samples and whilst changes in mRNA concentrations in some samples were in agreement with the array findings others were not (Table 4.5). For greater consistency with the samples used for the original array analyses, all of which were matched for gestational age to 8 weeks, a subset of the samples which were from gestations between of 9 to10 weeks (n=6) were grouped together (Figure 4.14). This resulted in more consistency in results with the putatively down-regulated genes showed most agreement with prior findings (Figure 4.15). In this set expression of Caveolin 2 (CAV2) was significantly decreased ($P<0.01$, n=6, 0, A) in agreement with the -1.65 fold change documented in the array data. Tissue factor (TTF3) was also significantly decreased ($P<0.05$, n=6, Figure 4.15, D) in agreement with the -1.52 fold change predicted by the array. Superoxide dismutase 2 (SOD2) mRNA expression appeared unchanged by E2 treatment, while peripheral myelin protein 22 (PMP22) tended to be decreased by E2 treatment ($p=0.0537$, n=6, Figure 4.15, B and C respectively). Putatively up-regulated genes showed less agreement with previous findings and response to E2 was more variable (Figure 4.16). Expression of mRNAs encoding ACTR3 ($p=0.2538$), PTMA ($p=0.3028$) and ROCK ($p=0.1035$) tended to be slightly increased with oestrogen treatment (Figure 4.16, A, B and E respectively) while IQGAP was unchanged (Figure 4.16, D). Paradoxically, CGA appeared to decrease ($p=0.3638$, Figure 4.16, B).

		<9 weeks				<10weeks					<11 weeks				<12 weeks				
Gene	Array Response	Sample # 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
CAV2		-1.34	1.20	1.38	1.03	-1.16	-4.71	-1.24	1.15	1.03	-1.43	-1.06	1.50	1.03	-3.59	1.03	-1.04	1.33	-1.04
SOD2		1.39	1.47	1.58	1.62	1.24	-1.40	-1.07	-1.29	1.62	1.07	-1.12	1.25	1.62	1.72	1.05	-1.04	1.20	-1.22
PMP22		1.40	1.79	1.54	1.34	-1.90	-2.43	1.11		-1.04	-1.23	1.19	1.28	2.66	1.16	1.39	-0.96	1.33	-1.21
TTF3		-1.09	1.11	1.56	-1.39	1.25	-1.92	-1.72	-1.36	-1.53	-1.70	-1.21	1.20	-1.04	-2.01	-1.20	-1.09	1.40	-1.30
ACTR3		-1.42	1.04	1.10	1.47	-1.24	1.17	1.02	1.55	1.48	-1.11	-1.13	1.19	1.06	1.23	-1.25	1.10	1.09	-1.39
IQGAP		-1.06	1.17	1.21	-2.30	-2.02	1.41	-1.14	1.56	0.99	-1.18	-1.18	1.19	-1.11	1.07	-1.04	-1.08	1.09	-1.30
CGA		-1.48	-2.40	1.58	-3.34	-2.27	2.14	-6.16	-2.01	0.17	-1.12	3.61	-4.84	1.42	1.27	1.54	1.84	-1.25	2.44
PTMA		-1.19	1.15	1.05	-1.10	-1.05	-1.03	1.00	1.47	1.35	-1.09	-1.20	1.11	1.51	-1.32	-1.09	1.02	1.19	-1.37
ROCK		1.31	1.12	-1.16	-1.90	1.90	1.11	1.15	1.53	1.32	-1.29	-1.18	1.42	0.60	-1.44	-1.09	1.14	-1.05	-1.26

>-2	>-2<-1.1	>-1.1<1.1	>1.1<1.5	>1.5

Figure 4.13. Gene expression relative to control in E2 treated uNK. Candidate genes were assessed in 18 samples of isolated uNK cells form a range of gestations (8-12 weeks.) Fold regulation values are described and colour coded (see scale). Fold change values less than 1, i.e. down regulated expression, are expressed as the negative inverse e.g. Fold change of 0.5 is equivalent to fold regulation of -2.

Gene	Array Response	Sample # 1	2	3	4	5	6
CAV2		-1.16	-4.71	-1.24	1.15	1.03	-1.43
SOD2		1.24	-1.40	-1.07	-1.29	1.62	1.07
PMP22		-1.90	-2.43	1.11		-1.04	-1.23
TTF3		1.25	-1.92	-1.72	-1.36	-1.53	-1.70
ACTR3		-1.24	1.17	1.02	1.55	1.48	-1.11
IQGAP		-2.02	1.41	-1.14	1.56	0.99	-1.18
CGA		-2.27	2.14	-6.16	-2.01	0.17	-1.12
PTMA		-1.05	-1.03	1.00	1.47	1.35	-1.09
ROCK		1.90	1.11	1.15	1.53	1.32	-1.29

>-2	>-2<-1.1	>-1.1<1.1	>1.1<1.5	>1.5

Figure 4.14. Gene expression relative to control in E2 treated uNK. Candidate genes were assessed in 6 samples of isolated uNK cells form a gestational range of 9 to 10 weeks. Fold regulation values are described and colour coded (see scale). Fold change values less than 1, i.e. down regulated expression, are expressed as the negative inverse e.g. Fold change of 0.5 is equivalent to fold regulation of -2. Samples from this gestational range were more reproducible and responses were in greater agreement with expected values.

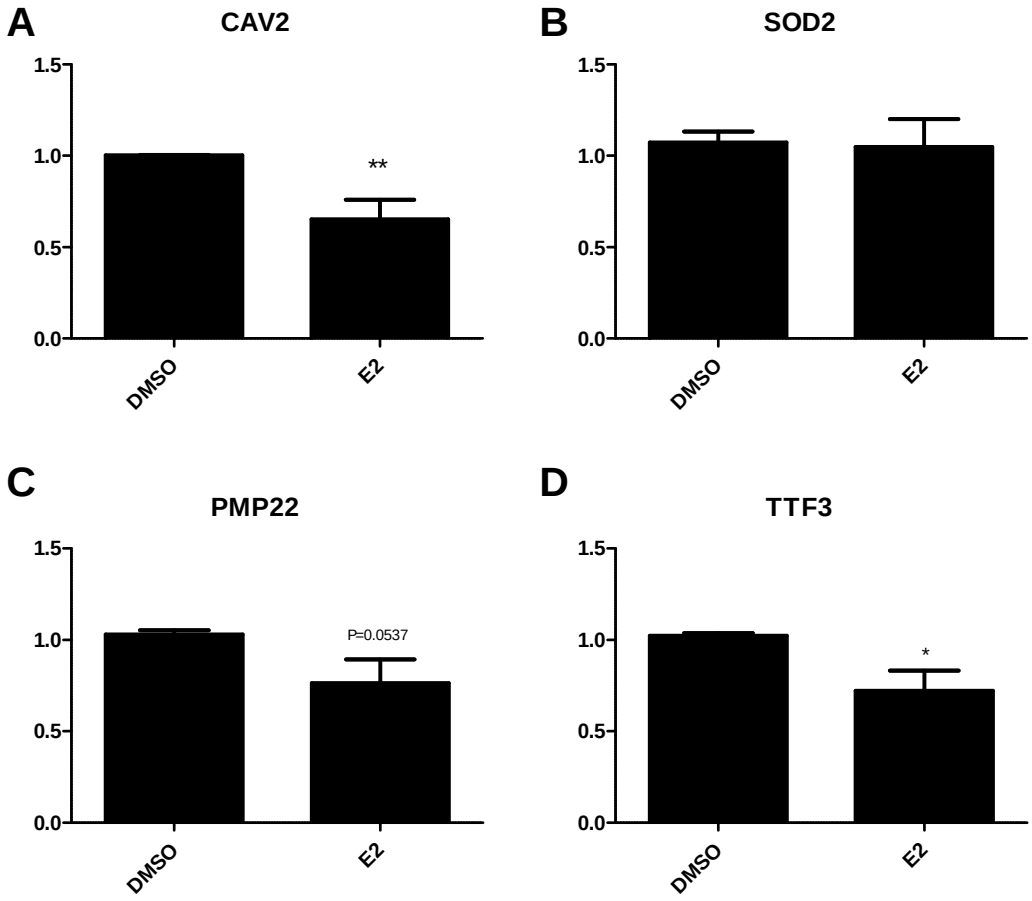

*Figure 4.15. Putatively down-regulated genes showed most agreement with prior array data. The mRNA expression of CAV2 (**A**) was significantly decreased (P<0.01, n=6) which was in agreement with the -1.65 fold change observed in array data. TTF3 (**D**) was also significantly decreased (P<0.05, n=6) in agreement with the -1.52 fold change predicted by the array. SOD2 (**B**) mRNA expression appeared unchanged by E2 treatment, while PMP22 (**C**) tended to decreased by E2 treatment (p=0.0537, n=6).*

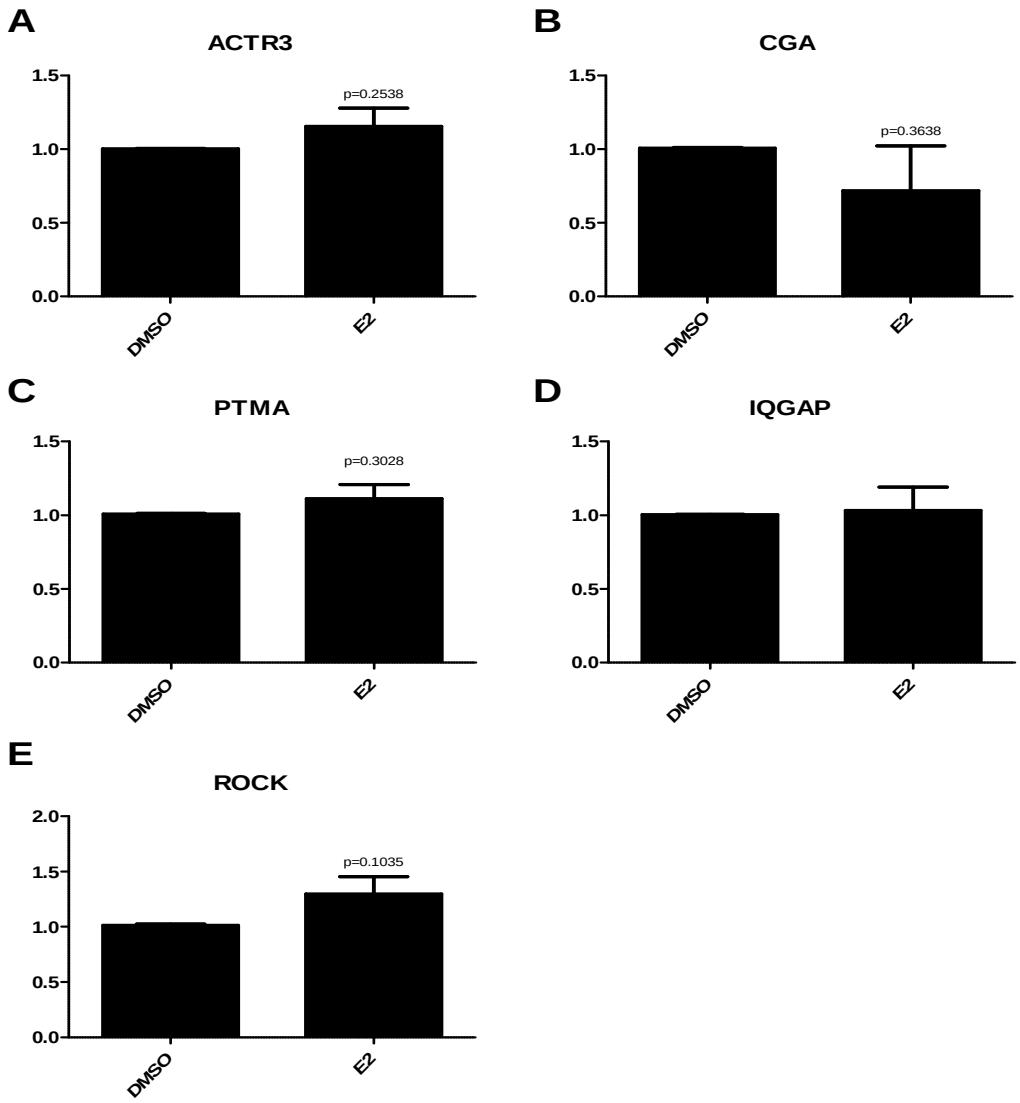

*Figure 4.16. Putatively up-regulated genes showed less agreement with previous array findings and response to E2 was more variable. Expression of mRNAs encoding ACTR3 (**A**, p=0.2536), PTMA (**C**, p=0.3028) and ROCK (**E**, p=0.1035) showed a trend towards increased expression with oestrogen treatment while IQGAP (**D**) was unchanged and in contrast to predicted response CGA appeared to show a non-significant decrease (**B**, p=0.3638).*

4.4.8. The anti-oestrogen ICI 182,780 (ICI) had minimal impact on candidate gene expression in uNK cells.

The expression of array candidate genes were further investigated in 4 additional samples of uNK cells (Figure 4.17) treated +/- ICI for 2 hours to ascertain whether E2 from serum or media could be masking any treatment effect. Interestingly, the expression of PTMA significantly increased (Figure 4.17, A) however this would not be consistent with blocking of E2-mediated increase observed in the array data. IQGAP and SOD2 were unchanged in response to ICI treatment (Figure 4.17, B and C). ACTR3 had a slight non-significant increase following ICI treatment (Figure 4.17, D, p=0.1680) which is not consistent with abrogation of the E2-mediated increase that was observed in the array.

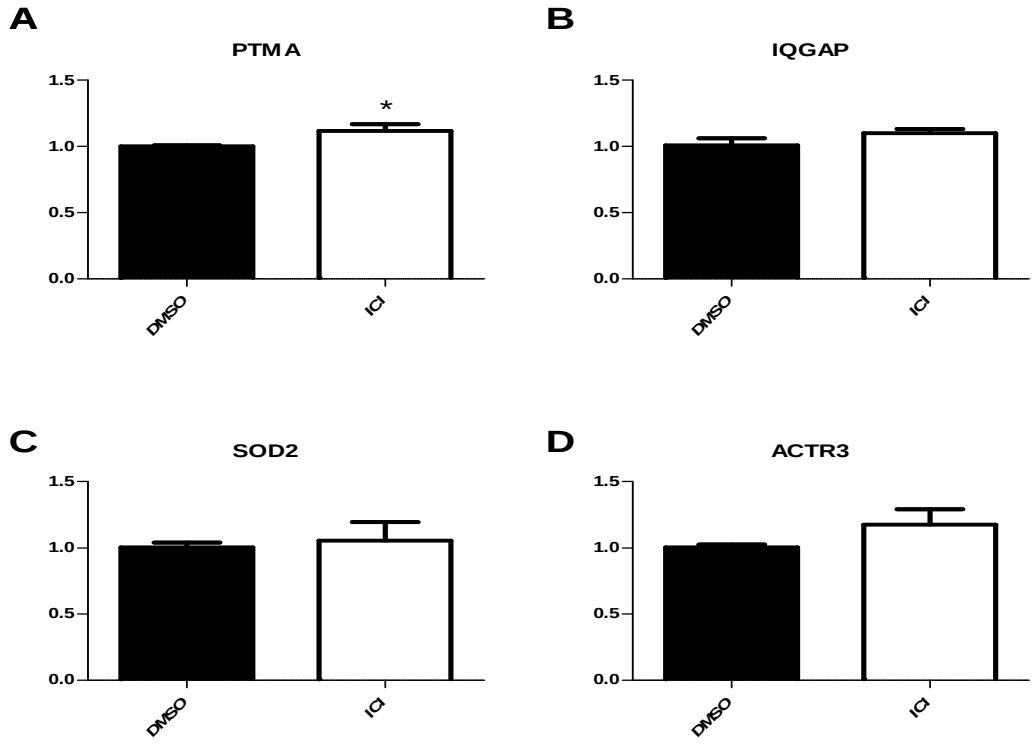

*Figure 4.17. Expression of mRNAs encoding prothymosin alpha (PTMA) are increased in response to ICI treatment. The average response to ICI suggests increased PTMA mRNA expression following treatment (**A**, n=4, p=0.0397). Expression of mRNAs encoding IQGAP (**B**) and SOD2 (**C**) are unchanged in response to ICI treatment (n=4, p=0.0713 and p=0.7380 respectively). The expression of mRNAs encoding ACTR3 (**D**) appear increased in response to ICI treatment (n=4, p=0.1680).*

4.5. Discussion

The present investigation sought to determine the impact of oestrogen receptor-dependent signalling on gene expression in uNK cells purified from 1st trimester decidua. The studies presented herein describe novel regulation of transcription in uNK cells mediated by oestradiol. Our investigation has shown that E2 significantly decreases concentrations of mRNAs encoding CAV2 and TTF3 in uNK cells possibly via ERβ and Sp-1 mediated transcription.

The presence of large numbers of uNK in decidua was confirmed by positive CD56 staining consistent with previous reports (25, 249). First trimester decidual samples ranging from 8 to 12 weeks gestation contained $CD56^+$ $CD16^-$ $CD3^-$ uNK cells, which could be isolated at high purity (> 94% CD56 positive cells in ungated cell population). The isolated population was viable (>94%) and remained viable following *in vitro* treatment for 2 hours, notably ~80% of cells remained viable after 24 hours culture. This was surprising as the cells were cultured in minimally supplemented media in order exclude non-treatment effects. uNK cells are often cultured with additional cytokines such IL-15 (414) to enhance viability however in our hands a highly viable population could be obtained that remained tenable for up to 24 hours without supplementation with cytokines. The uNK cells resident in decidual samples were ERβ+/ERα- and their oestrogen receptor phenotype was therefore identical to uNK cells in non-pregnant endometrium (249) furthermore they retained this phenotype when purified by MACS. To date, transcriptional analysis of uNK cells has been limited to array analysis of subtypes (27) and secretion of cytokines and expression of their receptors (39, 460). However the transcriptional effect of E2 treatment on uNK cells has not been investigated. In the present study having identified and characterised a viable isolated population of uNK from decidua we sought to investigate the impact of oestradiol (E2) treatment on their function. As a starting point for these studies we utilised array analysis results from isolated uNK cells treated +/- E2 for 2 hours from a previous study within our group; Forty-six genes had been identified as being differentially regulated following E2 treatment (6 down-regulated, 40 up-regulated), of which 28 were known genes, from this list 9 candidate genes (4 down-regulated, 5 up-regulated) were selected for follow up validation.

The impact of E2 treatment on expression of candidate genes across 18 samples of isolated uNK was found to be highly variable. However, in the present set of samples, the uNK cells had been isolated from decidua from a wider range of gestational ages (8-12 weeks) than the samples used for the original array analyses all of which were matched as being 8-9 weeks gestation. Interestingly, gross histological analysis suggested there was no difference in uNK

number or distribution with gestation (up to 12 weeks.) However, Lash et al have postulated that uNK cells in decidua at 8-10 weeks and 12-14 weeks are functionally distinct with varied secretion of cytokines and growth factors (41, 43). Furthermore uNK cells may be responsible for mediating different responses at different gestational ages as evidenced by gestation-specific impacts on trophoblast invasion (58). Thus gestational age is likely to be an important factor in uNK responses and was therefore thought to contribute to the variability in oestrogen-dependent gene expression observed across the sample set. To control for this, samples from gestations between 9 and 10 weeks were clustered as this represented the highest number of samples within the narrowest gestational age range from the total sample set. When gene expression was re-analysed using these samples responses were found to be more consistent with those recorded from the array data.

In uNK cells, exposure to E2 for 2 hours resulted in down-regulation of Caveolin-2 (CAV2) mRNA. Caveolins are scaffolding proteins which may be important in mediating non-genomic response to oestrogen (498). CAV2 can regulate caveolin-1-dependent caveolae formation (499) and is associated with tumour progression in breast cancer (500). CAV-1 and CAV-2 have previously been shown to be regulated by ERβ but in that case expression was upregulated in colon cancer cell lines (501). Thus the down-regulation of CAV-2 in uNK cells reported in the current chapter represents novel E2-mediated transcriptional regulation of CAV-2 that is distinct to this cell type.

Tissue factor (TTF3, factor 3, TF) is a cell surface glycoprotein involved in blood coagulation cascades and known to be present in leukocytes. In decidualised endometrial stromal cells expression of tissue factor parallels that of prolactin (157) and it is thus also considered a decidualisation marker. Tissue factor expression is controlled by specificity protein (Sp)-1 which itself may interact with progesterone and/or oestrogen receptors (158). TTF3 expression may be modulated by oestrogen in monocytes (502) and plays a role in mediating cellular migration (503). Progestins increase TTF3 mRNA and protein concentration in human endometrial stromal cells which is synergistically enhanced by co-stimulation with E2, however E2 alone does not increase TF transcription in this cell type (159). In the current investigation we found that TTF3 mRNA expression was significantly decreased following E2 treatment in uNK cells, which may result in modification of cellular migration capacity. This novel finding may be of particular relevance in uNK cells which do not express PR but do express ERβ and thus may be regulated by oestrogens in the endometrium.

Peripheral myelin protein 22 (PMP22) is an integral membrane protein that is a major component of myelin. PMP22 mRNA expression tended to be decreased in uNK cells following treatment with E2.

Actin related protein 3 (ACTR3) encodes a major constituent of the actin related protein (Arp) 2/3 complex. It is essential to cell shape and motility through lamellopodial actin assembly. The Arp2/3 complex initiates formation of actin filament networks in response to intracellular signals and Arp2/3 protein has been co-purified with ligand activated ERα in breast cancer cells suggesting a role for oestrogen in its regulation (504). Interestingly Arp2/3 is required for mediating NK cell cytotoxicity and Arp2/3 knock-down also reduced NK cell adhesion and slowed chemotaxis (505). In the current study E2 treatment tended to increase mRNA expression of ACTR3 in uNK cells. This may represent a mechanism by which oestrogen increases chemotaxis and adhesion of uNK cells and could therefore enhance recruitment to the uterus. However, if oestrogens increase expression of ACTR3 this could also increase cytotoxicity of uNK which may be detrimental in the tissue environment.

IQ motif containing GTPase activating-like protein 1 (IQGAP1) is a scaffold protein that integrates signals for localised actin polymerisation (506). It is important in regulating the cytoskeleton and promoting cell motility (507, 508). Our investigations demonstrated that while array bioinforntics analysis predicted IQGAP expression to be up-regulated by E2, the actual response to treatment in uNK cells was highly variable averaging out to no overall change in expression. IQGAP is known to interact with the Arp 2/3 complex (506) so modulated expression by oestrogen may be influenced by this factor and may explain in part the highly variable response to E2.

Prothymosin alpha (PTMA) is associated with cell proliferation and chromatin remodelling and is known to enhance ER transcriptional activity by sequestering repressors of oestrogen activity and enabling coactivator association with ER. PTMA is itself regulated by oestrogen, the gene promoter contains two half-ERE sites where ER may bind (237). In a study by Martini et al expression of PTMA in ER-positive MCF-7 breast cancer cell line was increased following treatment with E2 (509). Of particular relevance to the current study was that the change in mRNA concentration reported by Martini et al was rapid and observed after only 1-2 hours of treatment, maximal at 4 hours and reduced thereafter (509). In the same study, up-regulation of PTMA was reported to be via ERα or ERβ but was not activated via PR or GR. In the results presented in this chapter, E2 treatment for 2 hours in

uNK cells which are ERβ+ showed a trend towards increased PTMA mRNA expression although a significant difference between treatments could not be found. PTMA was predicted to be increased by the array study (FC 2.26) and mRNA concentrations appeared high with cDNA detected at low Ct values (average Ct 24.5, data not shown). Thus while increased mRNA expression mediated by E2 could not be confirmed, PTMA mRNA expression was detected and this protein could therefore play a role in influencing uNK cell function by modulating responses to E2 and promoting proliferation. The lack of activity with GR described by Martini et al, as well as the lack of ERα and PR expression in uNK also provides strong evidence for steroid hormone responsive transcriptional regulation of this gene by ERβ alone in this cell type.

Rho-associated kinases (ROCKs) are important in many physiological functions of leukocytes including chemotaxis mediated by actin polymerization and cytoskeletal rearrangement (510, 511). Rho associated coiled-coil containing protein kinase 1 (ROCK1) synthesis is essential for macrophage chemotaxis and phagocytosis (512). There is little evidence for E2-regulated transcriptional regulation of ROCK1 although bioinformatics analysis suggests ERβ regulation via a tethered mechanism involving Sp1 could occur (reviewed in (188)). In the current study E2 treatment tended to increase ROCK1 mRNA expression in uNK cells in agreement with predicted array response. Thus regulation of ROCK1 by E2 may be a potential mechanism for mediating recruitment of uNK to decidua.

Immunohistochemistry studies revealed expression of candidate genes in multiple cell types in decidua including the stromal cells. In a pilot study, E2 dependent expression of the candidate genes was examined in a cell line of immortalized human endometrial stromal cells (SHT 290, (465)). Although expression of mRNAs encoding; ACTR3, CAV2, CGA, IQGAP, PTMA, PMP22. TTF3, SOD2 and ROCK, could be detected, treatment of cells with E2 for 2 hours did not alter mRNA concentrations suggesting any stromal contamination of uNK samples would not have accounted for the observed changes in gene expression and highlighting the cell-specific nature of oestrogen-dependent changes in gene expression (data not shown).

Previous studies have documented the influence of proliferative phase stromal cell derived factors (35, 36, 388) on uNK cell function. This, together with the failure to detect progesterone receptor expression in uNK cells had previously led to the assumption that steroid regulation of uNK must be indirect. However detection of ERβ in this cell type (249) raised the possibility that E2-mediated transcriptional regulation is likely. The present study

sought to elucidate such regulation. Seemingly robust array findings suggested a tight network of 46 transcripts which were differentially regulated in uNK cells. However follow-up validation of the array proved to be inconsistent and suggested gestational age might influence transcriptional responses. The genes investigated did yield some significant changes. CAV2 and TTF3 were significantly down regulated and PMP22 showed a strong trend towards decreased expression following E2 treatment. ACTR3, PTMA and ROCK all tended to be increased by E2 treatment while IQGAP appeared unchanged. Bioinformatic analysis of these genes suggested a strong association with genes related to actin cytoskeleton remodelling which may impact on cell motility. Differential expression of ROCK, ACTR3, IQGAP and TTF3 could all influence cell motility while PTMA may influence proliferation of uNK. The response to E2 itself could also be influenced by CAV2 and PTMA consistent with informatics analysis.

uNK cells represent an interesting cell type in which to investigate ER dependent changes in transcription as they only express the ERβ isoform. In the current study, bioinformatic analysis of transcriptional networks associated with candidate genes revealed the potential for ERβ regulation via classical EREs as well as non-classical Sp1 regions. Consistent with this, Liu et al previously reported that chromatin immunoprecipitation (ChIP) analysis performed on MCF7 cells with induced expression of ERβ revealed ERβ-bound regions within the genome had a predominance of classical estrogen response elements (EREs) and GC-rich motifs (i.e. Sp1 regions) (513). Distinct ERβ binding regions in uNK cells may mediate physiological effects which are discrete from other cell types in the stromal compartment. The impact of classical oestrogen agonists, antagonists and selective oestrogen receptor modulators (SERMs) may therefore be unique in this cell population which may allow targeted modulation of uNK by ERβ specific agonists without the pleiotropic endometrial response to classical oestrogens. This may be particularly relevant to disorders of pregnancy such as recurrent pregnancy loss (RPL) where uNK cell numbers appear increased and are associated with greater prevalence of CD16+ NK cells (50, 51).

Taken together these findings suggest a potential role for E2 modulation of uNK cells. However, the functional influence of E2 *in vivo* requires further investigation. Additional bioinformatics analysis of the array dataset (Dr. Elaine Marshall personal communication) suggested E2 impacts on uNK cells might result in alterations in processes associated with cell migration (discussed in chapter 5). In addition to cell migration, angiogenesis might be affected by E2 impacts on uNK cells *in vivo*. uNK cells are known to secrete angiogenic factors such as VEGF-C and angiopoietin-2 (44) and are also known to induce angiogenesis

in human umbilical vein endothelial cells (39). Oestrogen is known to stimulate VEGF and thus may impact expression of angiogenic factors in uNK cells. Increased uNK cell numbers in the human endometrium is concomitant with decidualisation and local angiogenesis. Thus local oestrogen produced by decidualising stromal cells, reported in chapter 3, may influence increases in uNK cell number and angiogenesis during decidualisation.

In summary, in the present study a viable uNK population was isolated from decidua that retained ERβ expression and uNK phenotype. The isolated uNKs were responsive to E2 stimulation, and transcriptional responses suggest modulation of cell motility. The impact of oestrogen on uNK motility and the secretion of angiogenic factors is investigated in Chapter 5.

Chapter 5

5. Modulation of uNK function and angiogenesis during decidualisation: Implications for local oestrogen in cellular cross-talk.

5.1. Introduction

The human endometrium is a complex multicellular organ that undergoes cyclical remodelling in response to fluctuating levels of sex steroid hormones produced by the ovaries (1). The endometrium is bounded by a layer of epithelial cells on its luminal surface with a multicellular stroma containing fibroblasts, blood vessels (lined with endothelial cells), a dynamic immune cell population (including uNK cells and macrophages) as well as glands bounded by secretory epithelium (2). Following menses the functional layer of the endometrium regenerates in response to rising levels of oestrogen produced by ovarian follicles. Oestrogen also induces expression of the progesterone receptor in during the proliferative phase (6-8). Following ovulation circulating levels of progesterone rise (secretory phase) and this, together with regulatory agents that enhance cyclic AMP (cAMP) levels, precipitate changes in endometrial architecture including differentiation of stromal fibroblasts and remodelling of the vasculature, both of which are essential prerequisites for successful implantation of a conceptus and thereafter formation of a functional placenta (9). During the secretory phase there is a marked increase in the population of resident immune cells; leukocytes make up ~5% of uterine cells in the proliferative phase but rise to ~25% in the secretory phase (487). The most prominent population of uterine leukocytes are the uterine natural killer cells (uNK) the number of which further increases to account for 75% of total leukocytes in the decidua of early pregnancy (30). In the human, decidualisation and immune cell invasion occur during every cycle regardless of the presence of a conceptus, however, should implantation occur stromal transformation spreads beyond the perivascular regions and is prolonged. Formation of a mature maternal decidua is considered to play a pivotal role in limiting invasion of the embryonic trophoblast (14).

5.1.1. Immune cell recruitment

The presence of uNK cells in the uterus is essential for successful implantation (514, 515). However, before menarche and after the menopause uNK cell numbers are sparse suggesting

their recruitment and/or proliferation may be steroid regulated. In women who have undergone ovariectomy, administration of exogenous oestrogen and progesterone are required for development of a receptive endometrium which is host to a population of uNK (516). uNK cells are present in small numbers in proliferative and early secretory endometrium but are increased in late secretory endometrium and decidua (25). uNK accumulate around spiral arterioles and are abundant in perivascular sites following decidualisation. The high numbers of uNK cells present in the uterus following decidualisation may be due to increased leukocyte recruitment. There is evidence to suggest this may be mediated by steroid hormones, and in particular oestradiol (E2) (74). For example, adhesion of CD56 positive cells to decidua basalis, mediated via α4 integrin and L-selectin, can be induced in CD56 positive cells from male donors following treatment with E2 (515). There is an increase in α4 integrin in $CD56^{bright}$ pbNK cells at the LH surge a time when oestrogen levels are maximal (517). There are increased numbers of circulating $CD56^{bright}$ NK cells in women of reproductive age compared to males which is notable as this is the population believed to be homed to the uterus (26). Deloia et al. demonstrated that treatment of women with oral oestrogens increased total uterine leukocytes, the majority of which were uNK cells and macrophages while T cell numbers were unaffected (74). Following ovarian stimulation during IVF treatment, supraphysiological levels of sex steroids, including oestrogen, can be detected in the circulation and higher numbers of leukocytes have been detected in the uterus with a shift towards an increased ratio of $CD56^{bright}$ to $CD56^{dim}$ observed. This effect was specific to the uterus as there was no change in peripheral blood NK populations (488). Homing of NK cells to the uterus may be dysregulated in infertility and recurrent pregnancy loss with increased numbers of uNK cells reported in mid-secretory phase non-pregnant endometrium of women with these conditions (50). Taken together, the available data suggest oestrogen may influence the homing of appropriate leukocytes to the uterus.

uNK cells do not express progesterone receptor (PR) or oestrogen receptor (ER) α, but do express ERβ and glucocorticoid receptor (GR) (249) however both PR and ERα/ERβ are abundantly expressed in multiple cell types within the endometrium. Thus regulation of uNK cells by steroid hormones may also be indirect as a result of changes within the uterine environment. Notably, interleukin-15 (IL-15) is one example of a cytokine which can regulate proliferation and activation of NK and T cells. IL-15 is produced by uterine stromal cells under the influence of progesterone and is known to induce proliferation and

recruitment of uNK cells (423). IL-15 can also increase CD56 expression in CD16- pbNK and induce a chemokine receptor repertoire similar to uNK (427).

uNK cells express high levels of the cytokine receptors CXCR3 and CXCR4. Oestrogen induces CXCL10 and CXCL11 expression in the endometrium, with CXCL10 protein being increased in secretory phase endometrial explants treated with oestradiol, and both CXCL10 and CXCL11 can interact with CXCR3. CXCR3 is expressed on uNK cells and may therefore be a further mechanism for uterine control of uNK cells which is stimulated by oestrogen. Indeed, stimulation of CXCR3 by CXL10 is reported to increase the migration of CD16- NK cells *in vitro* (460). CXCL12 (SDF-1) acts via CXCR4 and induces specific migration of CD16- NK cells (390). Oestrogen is known to regulate the CXCL12-CXCR4 pathway (391).

5.1.2. Regulation of the endometrial vascular compartment

Angiogenesis, the formation of new blood vessels from existing vessels, is a feature of pathologies including cancers (102). Functional angiogenesis is rare in normal tissues apart from those of the reproductive system where it is a feature of the dynamic remodelling of the corpus luteum of the ovary (103) and the endometrium (104). In the human uterus endometrial arterioles arise from arcuate arteries in the myometrium which pass into arterioles in the basal endometrium that directly supply the functional layer. Proliferation of endometrial endothelial cells is at a peak in the mid to late proliferative phase and then again in the secretory phase (99). Endometrial arteries are transformed during decidualisation. The thin walled straight arteries characteristic of the proliferative phase become increasingly tortuous with a thicker rim of actin-positive medial cells during the secretory phase, a phenotype which continues in early decidua until remodelling of the vasculature occurs in response to trophoblast invasion (9, 104). Unusually endometrial angiogenesis does not seem to involve vasculogenesis from progenitor cells or capillary sprouting but instead appears to involve elongation and enlargement of existing vessels while still retaining the integral structure of the artery (2).

Many angiogenic factors have been identified in the endometrium which may mediate this process including; VEGFA, VEGFC, PlGF, Ang-1, Ang-2 as well as the receptors VEGF3 and Tie-1 and Tie-2 (44, 104). Despite the presence of angiogenic factors in stroma and epithelium in the endometrium, there may also be a role for uNK cells in mediating this

process. It is notable that uNK cells form aggregates around spiral arteries and glands in the late secretory endometrium and decidua (25). Furthermore, cytokine secretion is an important feature of uNK function and secretion of angiogenic factors VEGFC, PLGF, and Ang-2 has been described (39, 44). In addition, an increased capacity for network formation by HUVEC (Human umbilical vein endothelial cells) is seen following co-culture with uNK but not pbNK cells (39) suggesting a specific role of the uNK cell subset in regulating angiogenesis in the uterus. The functional impact of uNK cells may also be maturation dependent, as VEGFC secretion by uNK cells decreases with gestational age during pregnancy (43). Characterisation of uNK cell cytokine secretion has largely been undertaken in isolated populations and it is interesting that contrary to reports suggesting VEGFC and Ang2 was expressed by uNK cels, laser capture microscopy dissection of uNK cells from sections of decidual tissue resulted in detection of mRNAs encoding VEGFA but not Ang2 in uNK cells *in situ* (518) suggesting cytokine secretion by uNK cells may be modified by the tissue environment. This is further demonstrated by reduced angiogenic growth factor secretion in uNK cells in contact with trophoblast (42) suggesting that the pro-angiogenic function of uNK cells may be modified following implantation. Factors controlling this process are unknown, however, it is notable that uNK cell density is positively correlated with endometrial angiogenesis in women with recurrent reproductive failure (51). Stromal derived factors which may influence uNK cell-mediated angiogenesis include IL-15, which increases VEGFC secretion in uNK cells (44); transcription of VEGFA mRNA can be mediated by oestrogen via either ERα or ERβ in many cell types (104).

5.1.3. Summary

In summary, there is evidence that a range of signalling factors and cytokines produced within endometrial tissue can contribute to recruitment/differentiation of immune cell populations and formation and function of the vascular compartment. Studies detailed in Chapters 3 and 4 of this thesis have reported novel findings demonstrating biosynthesis of oestrogens by human endometrial stromal cells in response to decidualisation and suggested this may in turn provide a previously unrecognised stimulus to alter gene expression in ERβ-positive uNK cells. Experiments described in the current chapter expand on these findings and describe complementary studies on the interplay between oestrogen-dependent interactions between the stromal, immune and vascular cell types that comprise the stromal compartment.

To extend studies reported in Chapter 4, where Ingenuity pathway analysis revealed that treatment of uNK cells with E2 altered expression of genes encoding proteins associated with actin cytoskeleton signalling and motility (Dr Elaine Marshall personal communication), impacts of E2 on migration and chemotaxis of uNK cells were investigated.

5.2. Aims

1. To investigate the impact of oestrogen on migration of uNK cells
2. To investigate the impact of the stromal environment on chemotaxis of uNK cells
3. Investigate the impact of oestrogen treatment on the secretion of angiogenic factors by uNK cells and their impact on human endometrial endothelial cells

5.3. Methods

5.3.1. Isolation of uNK cells

Primary human uterine NK cells were isolated from human first trimester decidua as detailed in Chapter 4 (sections 4.3.1 to 4.3.4). Following their isolation, uNK cells were immune-phenotyped using FACS and both purity and viability was confirmed (see 4.4.2).

	Research #	Tissue #	Lab #	Date	GESTATION
Sample 1[¥§]	7762	554	CD1040D	10/05/11	<8 wks
Sample 2	7644	385	CD774D	21/04/10	8 wks 1 day
Sample 3	7633	380	CD763D	25/03/10	8 wks 4 days
Sample 4[§]	5721	703	CT1205D	12/09/11	8 wks 6 days
Sample 5*	5696	446	CD480D	07/04/11	9 wks
Sample 6*	7648	387	CD778D	22/04/10	9 wks 2 days
Sample 7*	5650	417	CD722D	08/04/10	9 wks 5 days
Sample 8*	7663	397	CD731D	14/06/10	9 wks 6 days
Sample 9*[¥§]	7721	545	CD999D	13/12/10	9 wks 6 days
Sample 10*	7640	383	CD770D	08/04/10	10 wks 1 day
Sample 11	6415	524	CD713D	04/03/10	10 wks 5 days
Sample 12	6418	527	CD716D	08/03/10	10 wks 5 days
Sample 13[¥]	7688	537	CD966D	28/09/10	10 wks 5 days
Sample 14	6413	523	CD798D	01/03/10	11 wks 2 days
Sample 15	7647	386	CD777D	22/01/10	11 wks 2 days
Sample 16	7642	384	CD772D	15/04/10	11 wks 2 days
Sample 17	6417	526	CD715D	08/03/10	11 wks 5 days
Sample 18	7652	390	CD782D	04/05/10	11 wks 5 days

*Table 5.1. Decidual samples from which uNK cells were isolated for treatment. Gestation in weeks (wks) and days based on ultrasound scan and date of last menstrual period. uNK cell samples that were pooled for experiment 5.4.3. are indicated by *. Samples that were used for RNA analysed on motility array (¥, n=3) and angiogenesis array (§, n=3) are also highlighted.*

5.3.2. Primary human endometrial stromal cells

Primary human endometrial stromal cells (hESC) were isolated from biopsies of healthy endometrium as detailed in section 2.3.1. Cells were seeded in 6 well plates at a density of $2x10^5$ cells per well and treated as detailed in Chapter 3, Section 3.3.1.

5.3.3. RNA extraction and Taqman Q-RT PCR

Extraction of RNA from cell extracts was performed using RNAeasy® mini kit (Qiagen, Crawley, UK) as previously described (full method see section 3.3.4). RNA samples were standardised to concentration of 100ng/μL prior to cDNA preparation. Reverse transcription of RNA to form a single strand of complementary DNA (cDNA) was carried out using the SuperScript VILO cDNA synthesis kit (InVitrogen) according to the manufacturer's instructions (Section 3.3.6).

5.3.4. RT^2 PCR profiler arrays

The RT^2 profiler PCR arrays (SABiosciences, Frederick, MD, USA) are designed to analyse a panel of genes related to a specific biological function. This platform combines the performance of real time PCR with the multi gene profiling capabilities of a microarray. Each array contains a panel of 96 primer sets for a set of 84 functionally focussed genes, together with five housekeeping genes and three RNA and PCR quality controls.

RNA samples from uNK cells treated +/- E2 were isolated as previously described (Section 2.3.3) and retrotranscribed using RT^2 single strand cDNA synthesis kit. Samples from 3 sets of treated uNK cells were analysed for differential gene expression following E2 treatment (Table 5.1). RNA samples were converted to first strand cDNA using the RT^2 first strand kit (SABiosciences, Frederick, MD, USA). Briefly, 300ng/ml RNA was mixed with 1x genomic DNA elimination mixture and made up to final volume of 10 μL with nuclease-free H2O per sample. SYBR green dye binds with all types of double stranded DNA, therefore complete removal of genomic DNA is essential for accurate expression data. The contents were mixed and briefly centrifuged before being incubated at 42^{o}C for 5 minutes, then cooled on ice for at least 1 minute. The RT cocktail was prepared as follows for each reaction:

RT Cocktail	x1 rxn	x6 rxn
BC3 (5x RT Buffer 3)	4 µL	24 µL
P2 (Primer & External control Mix)	1 µL	6 µL
RE3 (RT Enzyme Mix 3)	2 µL	12 µL
H2O	3 µL	18 µL
Final Volume	10 µL	60 µL

Table 5.2. RT cocktail mixture for cDNA synthesis

10 µL of RT cocktail was added to each treated RNA sample and mixed by pipette. The samples were then incubated for 15 minutes at 42^{o}C and the reaction was then stopped by heating to 92^{o}C for 5 minutes. 20 µL of cDNA synthesis mix was diluted by addition of 91 µL of H_2O and samples held on ice or stored at -20^{o}C until RT PCR was performed. Frozen samples were thawed on ice immediately prior to use. The RT PCR reaction mixture was as follows:

Reagent	Volume
2X SABiosciences RT^2 qPCR Master Mix	1350 µL
Diluted first strand cDNA synthesis reaction	102 µL
H2O	1248 µL
Total Volume	2700 µL

Table 5.3. RT PCR reaction mixture for each 96 well array plate.

25 µL of the experimental mixture was added to each well of the 96 well array plate using a multi-channel pipette to minimise intra-well variability. Following addition of samples, plates were sealed and held on ice until use. Prior to RT PCR plates were centrifuged for 1 minute at room temperature at 1000 g to remove bubbles. Real time PCR amplification was performed using the Applied Biosystems® 7900HT Fast Real-Time PCR System PCR using RT^2 Real-Timer SyBR Green/ROX PCR Mix kit according to manufacturer's instructions. Using an ABI 7900HT the following two-step cycling program was set up:

Cycles	Duration	Temperature
1	10 minutes	95^{o}C
40	15 seconds	95^{o}C
	1 minute	60^{o}C

Table 5.4. Thermal cycling program for PCR amplification.

SABiosciences RT^2 SYBR Green Master Mix utilises Hot-start Taq DNA polymerase, which prevents polymerase activity before heat activation and thereby reducing amplification of non-specific PCR products. SYBR green fluorescence was detected and recorded during the

annealing step in each cycle and the threshold Ct for each well was calculated. As an additional quality control, a melt curve program was run after every cycling program for each array plate. A first derivative dissociation curve for each well was recorded and checked for single peaks at temperatures greater than 80^{o}C. Wells which did not meet these criteria were excluded from analysis.

PCR array data analysis was performed using the SABiosciences web portal (http://www.SABiosciences.com/pcrarrayanalysis.php). Data analysis was performed using the ΔΔCt method (Section 2.10.3).

5.3.5. Human cell motility array

The Human cell motility RT^2 profiler PCR array (SABiosciences, Frederick, MD, USA) profiles the expression of 84 genes involved in modulating the movement of cells. The array includes growth factors and receptors important for chemotaxis, genes involved in Rho family signalling and adhesion, and genes encoding components of various cellular projections. The gene list is shown below (Table 5.5), responses for each functional grouping for all genes can be found in the appendix and relevant responses are detailed in the appropriate results section.

REF	Symbol	Description
A01	ACTN1	Actinin, alpha 1
A02	ACTN3	Actinin, alpha 3
A03	ACTN4	Actinin, alpha 4
A04	ACTR2	ARP2 actin-related protein 2 homolog (yeast)
A05	ACTR3	ARP3 actin-related protein 3 homolog (yeast)
A06	AKT1	V-akt murine thymoma viral oncogene homolog 1
A07	ARF6	ADP-ribosylation factor 6
A08	ARHGDIA	Rho GDP dissociation inhibitor (GDI) alpha
A09	ARHGEF7	Rho guanine nucleotide exchange factor (GEF) 7
A10	BAIAP2	BAI1-associated protein 2
A11	BCAR1	Breast cancer anti-estrogen resistance 1
A12	CAPN1	Calpain 1, (mu/I) large subunit
B01	CAPN2	Calpain 2, (m/II) large subunit
B02	CAV1	Caveolin 1, caveolae protein, 22kDa
B03	CDC42	Cell division cycle 42 (GTP binding protein, 25kDa)
B04	CFL1	Cofilin 1 (non-muscle)
B05	CRK	V-crk sarcoma virus CT10 oncogene homolog (avian)
B06	CSF1	Colony stimulating factor 1 (macrophage)
B07	CTTN	Cortactin
B08	DIAPH1	Diaphanous homolog 1 (Drosophila)
B09	DPP4	Dipeptidyl-peptidase 4
B10	EGF	Epidermal growth factor
B11	EGFR	Epidermal growth factor receptor
B12	ENAH	Enabled homolog (Drosophila)
C01	EZR	Ezrin
C02	FAP	Fibroblast activation protein, alpha
C03	FGF2	Fibroblast growth factor 2 (basic)
C04	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)
C05	IGF1	Insulin-like growth factor 1 (somatomedin C)
C06	IGF1R	Insulin-like growth factor 1 receptor
C07	ILK	Integrin-linked kinase
C08	ITGA4	Integrin, alpha 4
C09	ITGB1	Integrin, beta 1 (fibronectin receptor, beta polypeptide,)
C10	ITGB2	Integrin, beta 2
C11	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
C12	LIMK1	LIM domain kinase 1
D01	MAPK1	Mitogen-activated protein kinase 1
D02	MET	Met proto-oncogene (hepatocyte growth factor receptor)
D03	MMP14	Matrix metallopeptidase 14 (membrane-inserted)
D04	MMP2	Matrix metallopeptidase 2
D05	MMP9	Matrix metallopeptidase 9
D06	MSN	Moesin
D07	MYH10	Myosin, heavy chain 10, non-muscle
D08	MYH9	Myosin, heavy chain 9, non-muscle

D09	MYL9	Myosin, light chain 9, regulatory
D10	MYLK	Myosin light chain kinase
D11	PAK1	P21 protein (Cdc42/Rac)-activated kinase 1
D12	PAK4	P21 protein (Cdc42/Rac)-activated kinase 4
E01	PFN1	Profilin 1
E02	PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
E03	PLAUR	Plasminogen activator, urokinase receptor
E04	PLCG1	Phospholipase C, gamma 1
E05	PLD1	Phospholipase D1, phosphatidylcholine-specific
E06	PRKCA	Protein kinase C, alpha
E07	PTEN	Phosphatase and tensin homolog
E08	PTK2	PTK2 protein tyrosine kinase 2
E09	PTK2B	PTK2B protein tyrosine kinase 2 beta
E10	PTPN1	Protein tyrosine phosphatase, non-receptor type 1
E11	PXN	Paxillin
E12	RAC1	Ras-related C3 botulinum toxin substrate 1
F01	RAC2	Ras-related C3 botulinum toxin substrate 2
F02	RASA1	RAS p21 protein activator (GTPase activating protein) 1
F03	RDX	Radixin
F04	RHO	Rhodopsin
F05	RHOA	Ras homolog gene family, member A
F06	RHOB	Ras homolog gene family, member B
F07	RHOC	Ras homolog gene family, member C
F08	RND3	Rho family GTPase 3
F09	ROCK1	Rho-associated, coiled-coil containing protein kinase 1
F10	SH3PXD2A	SH3 and PX domains 2A
F11	SRC	V-src sarcoma viral oncogene homolog (avian)
F12	STAT3	Signal transducer and activator of transcription 3
G01	SVIL	Supervillin
G02	TGFB1	Transforming growth factor, beta 1
G03	TIMP2	TIMP metallopeptidase inhibitor 2
G04	TLN1	Talin 1
G05	VASP	Vasodilator-stimulated phosphoprotein
G06	VCL	Vinculin
G07	VEGFA	Vascular endothelial growth factor A
G08	VIM	Vimentin
G09	WASF1	WAS protein family, member 1
G10	WASF2	WAS protein family, member 2
G11	WASL	Wiskott-Aldrich syndrome-like
G12	WIPF1	WAS/WASL interacting protein family, member 1

Table 5.5. Table detailing the 84 genes which were investigated in the cell motility array. The position on the plate (REF) corresponds to reported intensities on the heat map.

5.3.6. Human angiogenesis array

The human angiogenesis RT^2 profiler PCR array (SABiosciences, Frederick, MD, USA) profiles the expression of 84 genes involved in modulating angiogenesis. The array includes growth factors and their receptors, chemokines and cytokines, matrix and adhesion molecules, proteases and their inhibitors and transcription factors, all of which are involved in the development of new blood vessels. The gene list is shown below (Table 5.6), responses for each functional grouping for all genes can be found in the appendix and relevant responses are detailed in the appropriate results section.

REF	Symbol	Description
A01	AKT1	V-akt murine thymoma viral oncogene homolog 1
A02	ANGPT1	Angiopoietin 1
A03	ANGPT2	Angiopoietin 2
A04	ANGPTL3	Angiopoietin-like 3
A05	ANGPTL4	Angiopoietin-like 4
A06	ANPEP	Alanyl (membrane) aminopeptidase
A07	BAI1	Brain-specific angiogenesis inhibitor 1
A08	CCL11	Chemokine (C-C motif) ligand 11
A09	CCL2	Chemokine (C-C motif) ligand 2
A10	CDH5	Cadherin 5, type 2 (vascular endothelium)
A11	COL18A1	Collagen, type XVIII, alpha 1
A12	COL4A3	Collagen, type IV, alpha 3 (Goodpasture antigen)
B01	CXCL1	Chemokine (C-X-C motif) ligand 1
B02	CXCL10	Chemokine (C-X-C motif) ligand 10
B03	CXCL3	Chemokine (C-X-C motif) ligand 3
B04	CXCL5	Chemokine (C-X-C motif) ligand 5
B05	CXCL6	Chemokine (C-X-C motif) ligand 6
B06	CXCL9	Chemokine (C-X-C motif) ligand 9
B07	TYMP	Thymidine phosphorylase
B08	S1PR1	Sphingosine-1-phosphate receptor 1
B09	EFNA1	Ephrin-A1
B10	EFNA3	Ephrin-A3
B11	EFNB2	Ephrin-B2
B12	EGF	Epidermal growth factor
C01	ENG	Endoglin
C02	EPHB4	EPH receptor B4
C03	EREG	Epiregulin
C04	FGF1	Fibroblast growth factor 1 (acidic)
C05	FGF2	Fibroblast growth factor 2 (basic)
C06	FGFR3	Fibroblast growth factor receptor 3
C07	FIGF	C-fos induced growth factor (vascular endothelial growth factor D)
C08	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)
C09	HAND2	Heart and neural crest derivatives expressed 2
C10	HGF	Hepatocyte growth factor (hepapoietin A; scatter factor)
C11	HIF1A	Hypoxia inducible factor 1, alpha subunit
C12	HPSE	Heparanase
D01	ID1	Inhibitor of DNA binding 1
D02	ID3	Inhibitor of DNA binding 3
D03	IFNA1	Interferon, alpha 1
D04	IFNB1	Interferon, beta 1, fibroblast
D05	IFNG	Interferon, gamma
D06	IGF1	Insulin-like growth factor 1 (somatomedin C)
D07	IL1B	Interleukin 1, beta

D08	IL6	Interleukin 6 (interferon, beta 2)
D09	IL8	Interleukin 8
D10	ITGAV	Integrin, alpha V
D11	ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)
D12	JAG1	Jagged 1
E01	KDR	Kinase insert domain receptor
E02	LAMA5	Laminin, alpha 5
E03	LECT1	Leukocyte cell derived chemotaxin 1
E04	LEP	Leptin
E05	MDK	Midkine (neurite growth-promoting factor 2)
E06	MMP2	Matrix metallopeptidase 2
E07	MMP9	Matrix metallopeptidase 9
E08	NOTCH4	Notch 4
E09	NRP1	Neuropilin 1
E10	NRP2	Neuropilin 2
E11	PDGFA	Platelet-derived growth factor alpha polypeptide
E12	PECAM1	Platelet/endothelial cell adhesion molecule
F01	PF4	Platelet factor 4
F02	PGF	Placental growth factor
F03	PLAU	Plasminogen activator, urokinase
F04	PLG	Plasminogen
F05	PLXDC1	Plexin domain containing 1
F06	PROK2	Prokineticin 2
F07	PTGS1	Prostaglandin-endoperoxide synthase 1
F08	SERPINF1	Serpin peptidase inhibitor, clade F
F09	SPHK1	Sphingosine kinase 1
F10	STAB1	Stabilin 1
F11	TEK	TEK tyrosine kinase, endothelial
F12	TGFA	Transforming growth factor, alpha
G01	TGFB1	Transforming growth factor, beta 1
G02	TGFB2	Transforming growth factor, beta 2
G03	TGFBR1	Transforming growth factor, beta receptor 1
G04	THBS1	Thrombospondin 1
G05	THBS2	Thrombospondin 2
G06	TIMP1	TIMP metallopeptidase inhibitor 1
G07	TIMP2	TIMP metallopeptidase inhibitor 2
G08	TIMP3	TIMP metallopeptidase inhibitor 3
G09	TNF	Tumor necrosis factor
G10	TNFAIP2	Tumor necrosis factor, alpha-induced protein 2
G11	VEGFA	Vascular endothelial growth factor A
G12	VEGFC	Vascular endothelial growth factor C

Table 5.6. Table detailing the 84 genes which were investigated in the angiogenesis array. The position on the plate (REF) corresponds to reported intensities on the heat map.

5.3.7. Migration assay

Migration of uNK cells was assessed using modified transwell migration assay. Isolated uNK cells were suspended in culture media; RPMI 1640 phenol red free media (Sigma) supplemented with 10% charcoal stripped foetal calf serum, 1% L-Glutamin and 1% Penicillin/Streptomycin, at a density of $1x10^6$ cells/ml. Cells were treated with vehicle control, 10^{-8}M E2, 10^{-6}M ICI or combination of E2 and ICI for 1 hour prior to assay. Following treatment, cells were pelleted and resuspended in culture media prior to migration assay. A 1.6mg/ml collagen gel was prepared with 3mg/ml bovine collagen I (GIBCO/Invitrogen, A10644-01), 10x MEM (Sigma, M-0275) and 7.5% $NaHCO_3$ (Sigma, S8761) and 200 μL of the gel was pipetted onto the bottom of the well in a 24 well plate and placed in the incubator (37^{o}C, 5% CO2) for 30 minutes to allow for gelation of the collagen matrix. The collagen gel was considered a neutral surface that would allow adherence of uNK cells without acting as a chemoattractant and was prepared as described immediately prior to migration assay. 800μL of serum free RPMI 1640 phenol red free media was added to the well on top of the collagen matrix. Cell culture inserts with 5μm membrane pore (Costar/Corning, 3421, NY, USA) were then placed into the well into which 200μL of uNK suspension was added. Cells were treated in duplicate. Cells were left in the incubator to migrate for 1 hour. Following migration cell culture inserts were removed and cells in lower chamber were counted. Migrated cells were counted by imaging 8 random fields using Axiovert 200 Inverted Fluorescent Microscope (Zeiss) and mean number of migrated cells was calculated for each treatment.

5.3.8. Chemotaxis assay

The chemotaxis of uNK cells was assessed using Ibidi μ-Slide Chemotaxis^{3D} chamber slides (Ibidi, 80326, supplied by Thistle Scientific Ltd, Uddingston, UK). Isolated uNK cells were suspended in a collagen matrix and the response to different chemotaxis stimuli was measured using time lapse microscopy and recorded using Axiovert 200 Inverted Fluorescent Microscope (Zeiss, see Figure 5.1.)

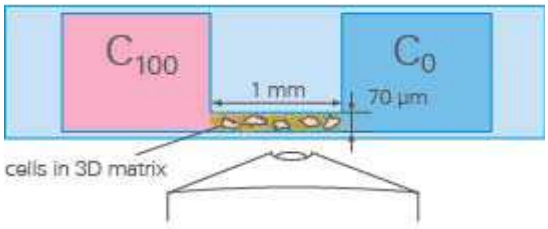

Figure 5.1. Schematic diagram of cells in 3D matrix subject to chemotaxis gradient (+/-). Cells can be viewed using inverted microscope.

Isolated uNK cells were suspended in RPMI 1640 phenol red free media (R7509, Sigma, Dorset, UK) supplemented with 10% charcoal stripped foetal calf serum, 1% L-Glutamin and 1% Penicillin/Streptomycin at a density of $3x10^9$ cells/ml. A 1.6mg/ml collagen gel was prepared with 5 mg/ml bovine collagen I (Gibco, A10644-01, supplied by Invitrogen, Paisley, UK), 10x MEM (M-0275, Sigma, Dorset, UK) and 7.5% $NaHCO_3$ (S8761, Sigma, Dorset, UK) and mixed carefully with the uNK cell suspension avoiding air bubbles during mixing. The collagen gel cell suspension was then filled into the middle chamber of the µ-Slide and placed in the incubator (37°C, 5% CO2) for 30 minutes to allow for gelation of the collagen matrix. Three chambers were seeded and two controls were set up; -/- containing chemoattractant free media in both reservoirs and +/+ containing chemoattractant media in both reservoirs to account for influence of random migration (chemokinesis.) Finally a chamber with chemoattractant free media in one reservoir and chemoattractant media in the other (+/-) was set up which formed a chemotaxis gradient across the chamber. Chemoattractant free media was conditioned media recovered from undecidualised endometrial stromal cells (stromal conditioned media). Chemoattractant media was media recovered from decidualised stromal cells (decidual conditioned media).

Cells were imaged every 5 minutes for 4 hours using Axiovert 200 Inverted Fluorescent Microscope from Zeiss and image stacks analysed using ImageJ (NIH.gov) and the manual tracking plug-in (http://rsbweb.nih.gov/ij/plugins/track/track.html). Results were analysed using Ibidi Chemotaxis and Migration tool software.

5.3.9. Angiogenesis assay

Isolated uNK cells were cultured at a density of $1x10^6$ cells per/ml for cell treatment in phenol red-free RPMI 1640 medium (Sigma, Cat. No. R7509) supplemented with 10% CSFCS, 1% Penicillin/Streptomycin (Sigma, Cat. No. P-4333), 1% L-glutamine (Sigma, Cat.

No. G-7513), and 0.5% fungizone (Invitrogen, Cat. No. 15290-018). Cells were cultured for 24 hours at 37^{o}C under 5% CO_2 in air. Cells were treated with 10^{-8}M E2, 10^{-6}M ICI or combination of E2 and ICI for 24 hours. Following treatment, cells were pelleted and the supernatant, uNK conditioned media (uNK CM), was stored at $^{-}$80^{o}C until assay. uNK cell CM was thawed immediately prior to assay and warmed to 37^{o}C.

Telomerase immortalised human endometrial endothelial cells (HEEC, Gift from Dr. Graciela Krikun, Department of Obstetrics, Gynecology and Reproductive Science, Yale University, New Haven, CT, USA) (466) were plated at a density of 25,000 per transwell insert (inserted into 24 well plates) which had been coated with growth factor reduced matrigel (BD Biosciences, Oxford, UK) and allowed to solidify prior to cell plating. uNK cell CM was added to the bottom chambers and cells incubated overnight at 37ºC with 5% CO_2. Each treatment was set up in duplicate. After 16 hours incubation HEECs were fixed with ice cold methanol for 20 minutes and then stained with hematoxylin. The formation of networks was visualised using Axiovert 200 Inverted Fluorescent Microscope (Carl Zeiss, UK). Three fields of vision were captured at 5x magnification for each well. Network formation was quantified using ImageJ (NIH.gov) and verified by counting branch points.

5.3.10. Statistical Analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Prism version 5.04 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com). The criterion for siginificance for all tests was set at $p<0.05$.

5.4. Results

5.4.1. E2 treatment results in changes in expression of genes associated with cell motility.

Results from studies detailed in chapter 4 suggested that treatment of uNK cells with E2 altered expression of genes encoding proteins associated with actin cytoskeleton signalling and motility. Impacts of E2 on uNK expression of genes associated with cell motility were investigated using PCR arrays.

The expression of 84 genes involved in modulating the movement of cells was investigated in response to E2 in uNK. The array includes growth factors and receptors important for chemotaxis, genes involved in Rho family signalling and adhesion, and genes encoding components of various cellular projections.

E2 treatment appeared to result in down regulation of genes associated with cell motility. The expression of gene candidates was readily detected in uNK cells with >60% of genes having Ct<30. The overall expression response following E2 treatment of all array candidates is summarised in Figure 5.2 and 5.3. Of the 84 genes analysed, only one gene was found not to be expressed (ENAH; Figure 5.2, grey box) and only three genes appeared to be up regulated (Table 5.7; MYH10, ACTN3 and HGF). The sample set of 3 treated pairs of uNK cells that was analysed by the array was small and therefore it is not surprising that although trends were detected only two genes reached significance (Table 5.7; MSN, ITGB1). To provide further insight the data set was narrowed to exclude results that were highly non-significant or exhibited low changes in expression in response to treatment. Fold change in response to E2 from this set is shown in Figure 5.3 and detailed in Table 5.7. Consistent with overall changes, the narrowed data set showed tendency for down-regulation of target genes in response to E2 with only a small number of up-regulated genes. Changes in fold regulation in response to E2 did not appear to be pronounced with only three genes with fold regulation greater than 2 or less than -2 (Table 5.7; EGFR, PIK3CA, HGF). When genes were grouped into functional gene groupings based on information supplied with the array product specification information (see Appendix), E2 appeared to regulate selected genes related to chemotaxis, growth factor receptor expression, Rho signalling, cell-cell adhesion, leukocyte adhesion and rolling, and cellular projections; filopodia and lamellipodia. However, E2 did not appear to have an impact on expression of genes

associated with stress fibers, membrane blebbing, invasive projections, growth cones or cell polarity. Within gene groups of functional processes E2 regulated only selected genes while others appeared to be unchanged. For example, genes associated with cell-cell adhesion were significantly down-regulated by E2 (ITGB1; p=0.04, MSN; p=0.015, n=3, Table 8.16) and non-significant trend suggesting decreased expression of EGFR as a result of E2 treatment (-2.34 fold regulation). However, other genes associated with this process were unchanged; DPP4, EZR, ITGA4 and ITGB2. This was also true of genes associated with lamellipodia; EGFR1, PIK3CA, PLD1 and PXN showed no significant change in expression in response to E2 (despite negative fold change values), while DPP4, FAP, RDX, SVIL, and VCL were unchanged and ENAH was not detected. Interestingly, in contrast to previous findings from our group, actin related genes appeared unchanged by E2 (ACTN1, ACTN4, ACTR2 and ACTR3) with the exception of ACTN3 which was one of only three genes which to display positive fold change values in repsonse to E2 however the change in ACTN3 was not found to be significant.

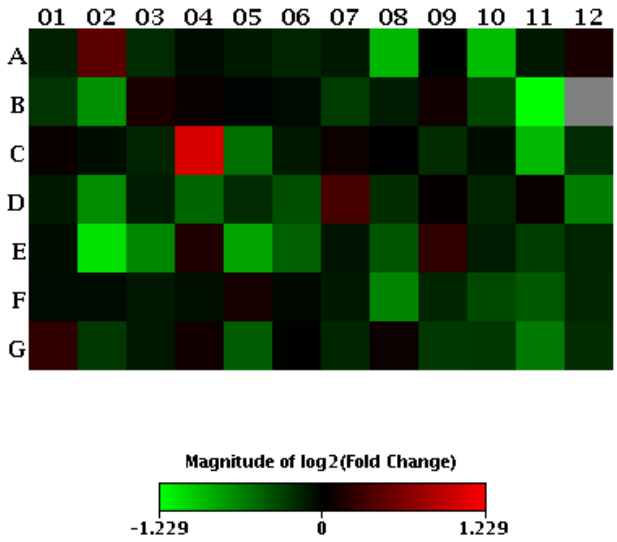

Figure 5.2. Heat map indicating gene expression of cell motility genes following E2 treatment of uNK. The expression of genes associated with cell motility was very high in uNK cells with >60% of genes having Ct<30 and there were low number of no calls. The impact of E2 seemed to decrease expression of most genes in the array.

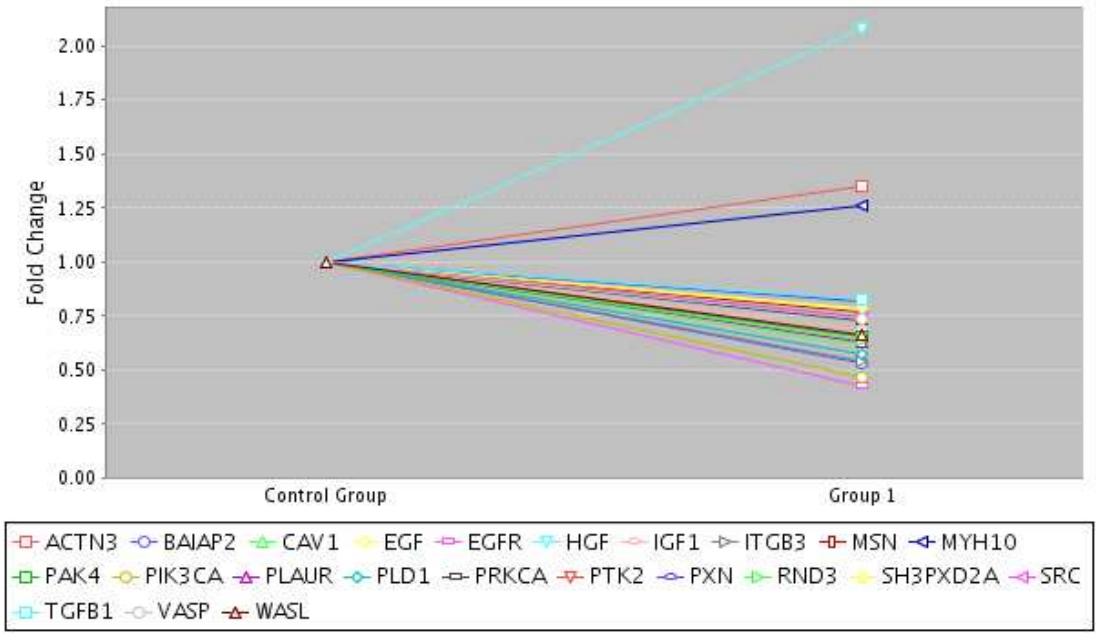

Figure 5.3. Fold change in gene expression of selected cell motility genes following E2 treatment. Genes with low changes in expression or very high "P values" were excluded for simplicity. Most genes were down-regulated with treatment however HGF, MYH10 and ACTN3 were up-regulated. Control group is vehicle treated uNK cells and Group1 is E2 treated uNK.

Symbol	Fold Change	95% CI	P value	Fold Regulation
EGFR	0.4266	(0.00001, 0.90)	0.213332	-2.3442
PIK3CA	0.4694	(0.00001, 1.22)	0.382145	-2.1305
BAIAP2	0.5305	(0.15, 0.91)	0.184226	-1.885
ITGB3	0.5383	(0.08, 0.99)	0.173152	-1.8577
PLD1	0.5736	(0.23, 0.91)	0.133215	-1.7435
CAV1	0.6151	(0.00001, 1.52)	0.600257	-1.6257
PLAUR	0.6339	(0.09, 1.18)	0.368307	-1.5776
RND3	0.6377	(0.00001, 1.82)	0.533238	-1.5681
PAK4	0.6552	(0.16, 1.15)	0.345822	-1.5262
WASL	0.6639	(0.11, 1.22)	0.38761	-1.5063
IGF1	0.6877	(0.01, 1.36)	0.554432	-1.454
MMP2	0.7096	(0.15, 1.27)	0.40729	-1.4092
PRKCA	0.7279	(0.31, 1.15)	0.380198	-1.3739
VASP	0.7341	(0.18, 1.28)	0.431172	-1.3623
SRC	0.7464	(0.17, 1.32)	0.514935	-1.3397
PTK2	0.7481	(0.24, 1.26)	0.440942	-1.3366
MSN	0.7708	(0.67, 0.87)	0.015217	-1.2974
SH3PXD2A	0.7801	(0.50, 1.06)	0.276494	-1.282
EGF	0.7889	(0.00001, 2.25)	0.366281	-1.2676
PXN	0.8146	(0.53, 1.10)	0.341253	-1.2276
TGFB1	0.8269	(0.01, 1.64)	0.686773	-1.2093
ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
MYH10	1.2596	(0.12, 2.40)	0.530781	1.2596
ACTN3	1.3504	(0.20, 2.50)	0.379028	1.3504
HGF	2.0782	(0.00001, 5.34)	0.462532	2.0782

Table 5.7. Table detailing fold change in gene expression of selected cell motility genes following E2 treatment. Most genes were down-regulated with treatment however HGF, MY and ACTN3 were up-regulated. Values shown are differential responses following treatment. 95% CI and P value are also shown. There was low significance in this small sample size.

5.4.2. E2-treated uNK exhibit increased migration.

Overall E2 appears to down regulate expression of genes associated with cell motility in uNK cells. In order to address whether the transcriptional changes observed could have a functional impact we investigated the impact of E2 on migration of uNK cells using a transwell migration assay. Interestingly, when uNK cells were treated with 10^{-8}M E2 for 1 hour an increase in cell migration was observed (Figure 5.5 A, n=4, $p<0.001$). This effect appears to be receptor mediated as incubation of cells with the ER antagonist ICI blocked the E2 effect (Figure 5.5 B n=2, $p<0.05$).

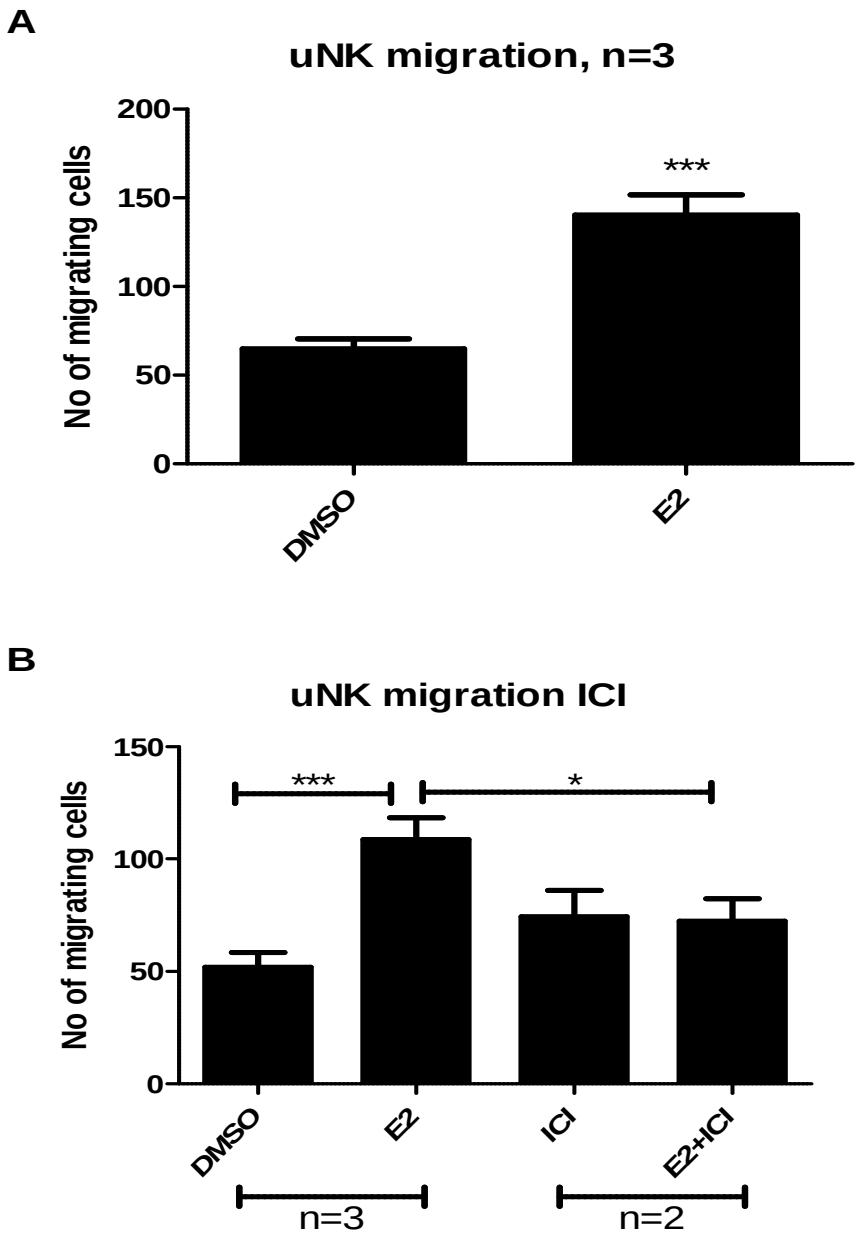

Figure 5.4. Isolated uNK cells pre-treated with E2 have increased migration. uNK cells were treated for 1 hour with 10^{-8}M E2 and then subject to transwell migration assay for 1 hour. E2-treated cells exhibit increased migration (A, n=4, p<0.001). This effect could be blocked by the oestrogen receptor antagonist ICI (B).

5.4.3. Impact of the stromal environment on chemotaxis of uNK cells

5.4.3.1. Primary uNK cells express factors associated with control of chemotaxis

To investigate whether factors from the stromal environment could influence motility of uNK cells and whether E2 could modulate such responses, expression of receptors to known chemotactic stimuli were investigated in uNK cells. Expression of mRNAs encoding receptors for SDF-1 (CXCR4) and IL-15 (IL-15RA) were investigated in uNK cells treated +/- E2 for 2 hours. CXCR4 was detected at low levels (average Ct≈35) in uNK cells however expression tended to be increased by E2 treatment (Figure 5.5 A). IL-15 receptor alpha (IL-15RA) was readily detected (average Ct≈25) however expression was not affected by E2 treatment (Figure 5.5 B). Immunohistochemistry revealed that CXCR4 is expressed in first trimester decidua (Figure 5.6; A, B) and is co-localised to uNK cells confirming CXCR4 protein expression in uNK cells despite low mRNA levels (Figure 5.6 D).

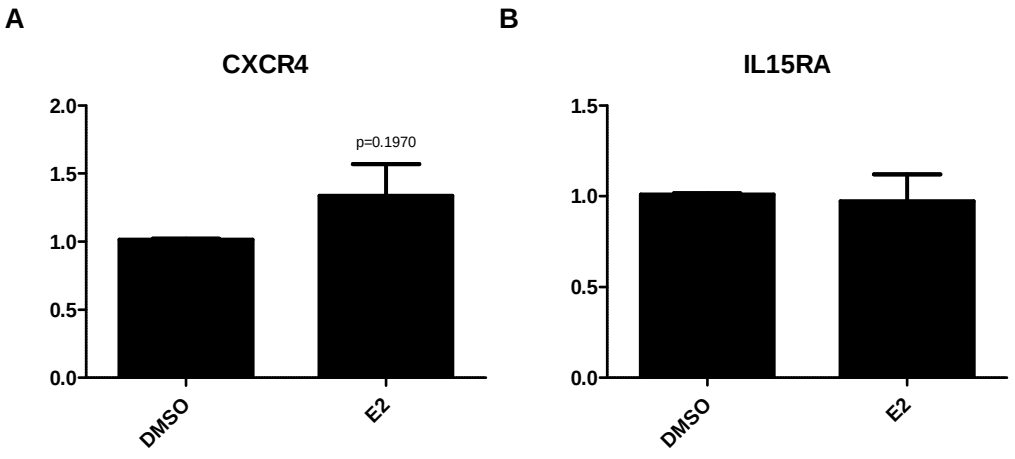

Figure 5.5. The expression of mRNAs encoding the IL-15 receptor α were unchanged (B) while E2 tended to increase mRNA expression of CXCR4 (A, p=0.1970) N=6.

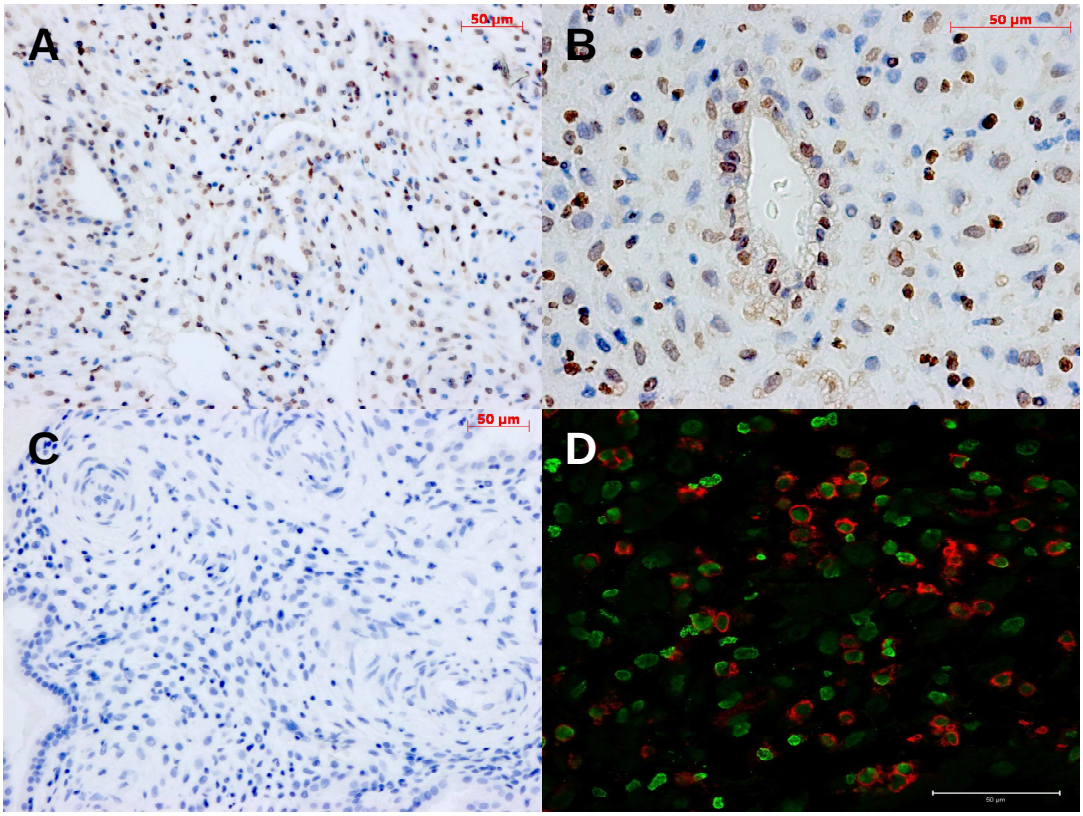

Figure 5.6. CXCR4 is expressed throughout decidua and is colocalised to uNK cells. Positive immunoexpression of CXCR4 was detected in stromal and epithelial cells in fixed sections of first trimester decidua (A, B). Negative controls were conducted in the absence of primary antibody (C). Co-localisation of CXCR4 (D, green staining) with the surface marker CD56 (D, red staining) confirms CXCR4 expression in uNK cells. Scalebar equivalent to 50µm.

5.4.3.2. Expression of factors associated with uNK chemotaxis is increased in decidualised ESC.

Decidualisation is associated with an increase in the number of uNK cells with either migration or proliferation of existing uNK cell populations in the endometrium proposed as a mechanism to explain the increase. SDF-1 is a strong chemoattractant of lymphocytes including uNK cells and this factor may contribute to the recruitment and/or proliferation of uNK cells in the endometrium following decidualisation. IL-15 is known to impact on the proliferation and differentiation of uNK cells and increased expression of IL-15 during decidualisation may also impact on uNK cell populations in the endometrium and contribute to the increased numbers in first trimester decidua. Following decidualisation there is a significant increase in both SDF-1 and IL-15 mRNAs in a time dependent manner (Figure

5.7). SDF-1 expression is increased early in decidualisation at 24 hours (Figure 5.7 A, n=4, $p<0.05$) and most notably at 2 days (Figure 5.7 A, n=4, $P<0.001$). In hESC treated to decidualise, the expression of mRNA encoding IL-15 is increased after 2 days (Figure 5.7 B, n=4, $p<0.001$) and to lesser extent is also increased after 4 and 8 days compared to non-decidualised controls (Figure 5.7 B, n=4, $p<0.001$ and $p<0.05$ respectively).

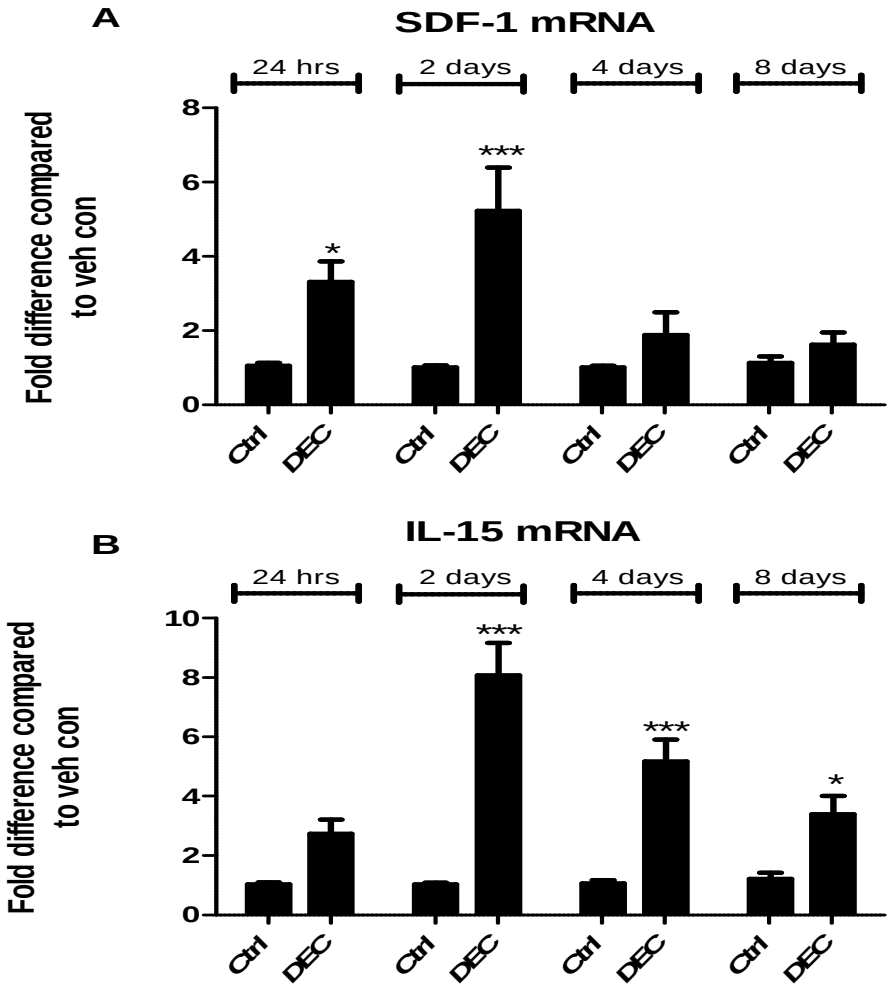

Figure 5.7. Expression of mRNAs encoding SDF-1 (A) and IL-15 (B) are significantly increased in response to decidualisation. SDF-1 expression is increased early in decidualisation at 24 hours (n=4, p<0.05) and most notably at 2 days (n=4, P<0.001). In decidualised ESC IL-15 is greatly increased after 2 days (n=4, p<0.001), with 8 fold increase in expression. IL-15 is also increased after 4 and 8 days decidualisation (n=4, p<0.001 and p<0.05 respectively).

5.4.3.3. uNK cells show positive chemoattraction to decidualised conditioned media.

As expression of SDF-1 and IL-15 by endometrial stromal cells is increased in response to a decidualisation stimulus and uNK cells expressed both receptors for IL15 and SDF-1 (CXCR4) a chemotaxis assay was performed with media recovered from decidualised stromal cells which was used as a chemoattractant in chamber slide chemotaxis assay. Cells showed directed chemotaxis towards the decidualised conditioned media (DCM; Figure 5.8 A) and but not to control media (stromal conditioned media; Figure 5.8 B). The centre of mass of uNK cells was (x, y; -33, -342) for DCM and (x, y; -8, 1) for control indicating directed chemotaxis towards DCM. uNK cells subject to +/- gradient travelled greater distance and at higher velocity than control cells and their movement was directed towards DCM (Figure 5.9).

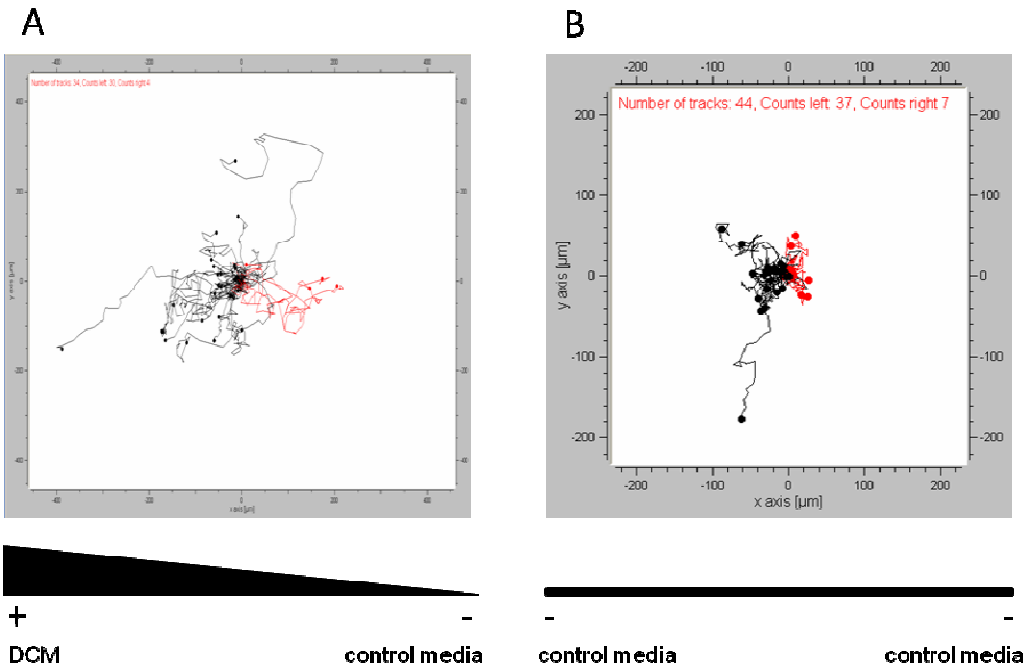

*Figure 5.8. Chemotaxis of uNK cells is directed towards decidualised conditioned media (DCM) but not control (stromal conditioned) media over 4 hours at 37^{o}C 95% CO_2. **A**. Following tracking of cell movement of 34 cells; 30 marked left (DCM) and 4 tracked right (control media). **B**. No directed movement of uNK cells was detected in the absence of DCM. 44 cells were counted of which 37 marked left and 7 marked right in the absence of DCM.*

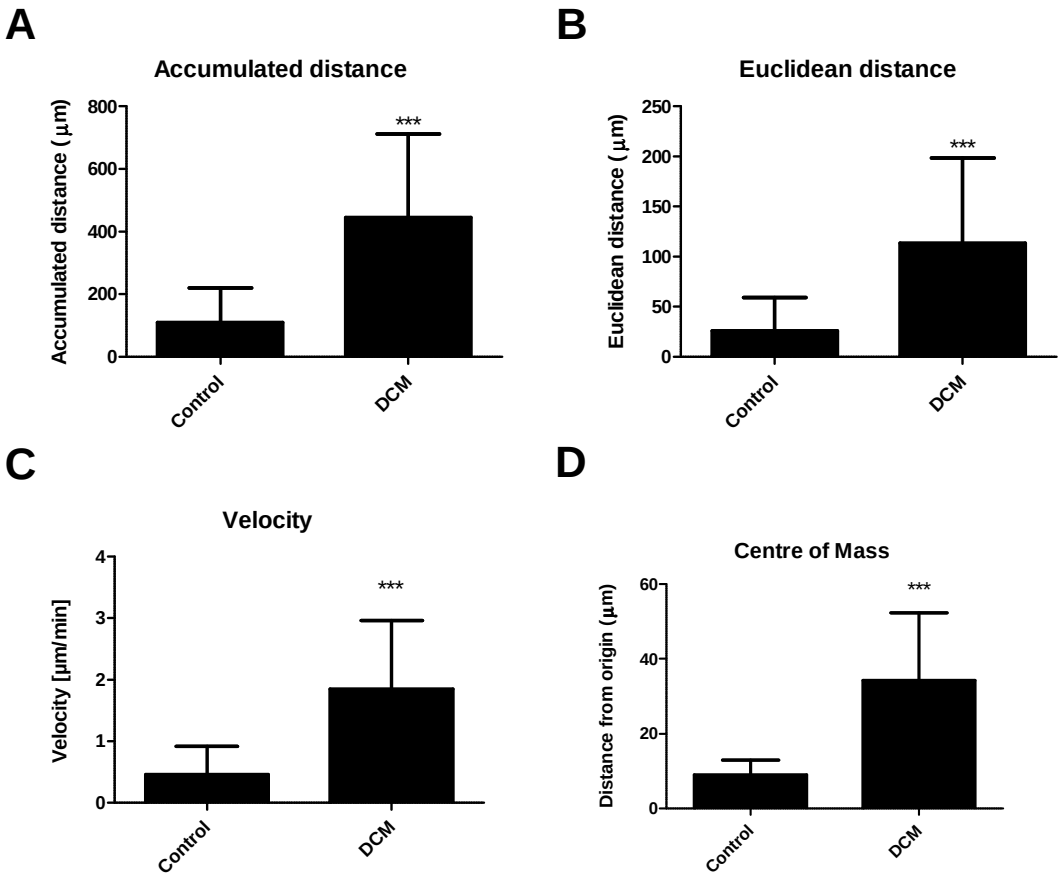

Figure 5.9. Representative values comparing measurements of cell movement in chemotaxis assay for control (-/-) and with a positive gradient to decidualised conditioned media (DCM; +/-). ***A****. The average accumulated distance.* ***B****. The average Euclidean distance (direct distance between point of origin and end point).* ***C****. The average velocity of uNK cells.* ***D****. The distance from origin of the average end point of cells (centre of mass). Each of the measured values for uNK cells under +/- gradient were higher than in control cells indicating uNK cells were directed towards DCM. (Control; n=44 cells, DCM; n=34 cells. *** p<0.001)*

5.4.4. Impact of the stromal environment on angiogenesis

Decidualisation of the endometrium is associated with an increase in angiogenesis. Decidualisation of stromal cells begins in cells proximal to endometrial spiral arteries and thus factors released from stromal cells at this time may influence angiogenesis. Increased expression of angiogenic factors in the endometrium during decidualisation is important for vascular remodelling that takes in preparation for the establishment of pregnancy. Studies in this thesis have revealed that E2 is secreted by stromal cells during decidualisation which

may be important as E2 is known to stimulate VEGF expression; however, it is unknown what effect locally produced E2 may have on angiogenesis. Furthermore, uNK cells accumulate in the endometrial stroma in perivascular sites around the time of decidualisation and are known to secrete angiogenic factors.

To investigate the impact of the stromal environment on angiogenesis, the expression of angiogenic factors in decidualised ESC was investigated and the impact of locally produced E2 as a modulator of uNK cell angiogenic function was assessed.

5.4.4.1. Expression of angiogenic factors is increased in decidualised ESC.

Following decidualisation there was an increase in expression of mRNAs encoding VEGFA (Figure 5.10 A, n=4, $p<0.001$), ADM (Figure 5.10 B, n=4, $p<0.001$) and angiopoeitin 2 (ANGPT2, also Ang-2) (Figure 5.10 C, n=4, $p<0.05$) compared to non-decidualised controls. Angiopoeitin 4 (ANGPT4, also Ang-4) was decreased following decidualisation (Figure 5.10 D, n=4, $p=0.001$).

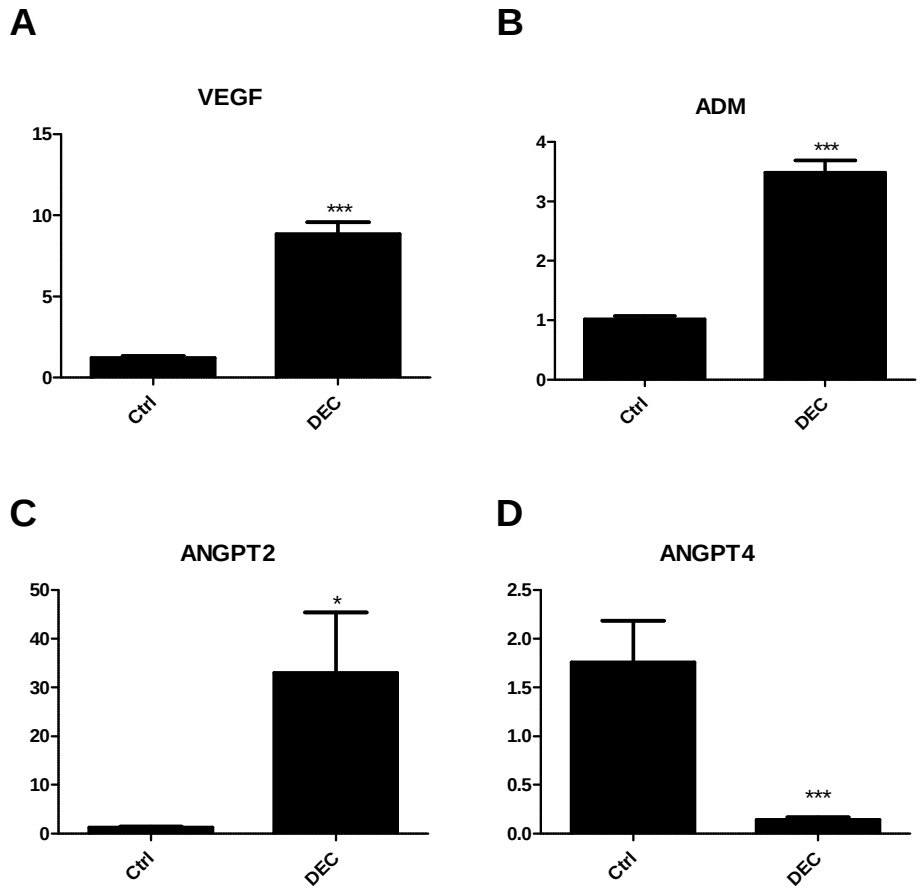

Figure 5.10. Expression of mRNAs encoding VEGF, ADM, ANGPT2 and ANGPT4 in response to decidualisation. VEGF (n=4, p<0.001), ADM (n=4, p<0.001) and ANGPT2 (n=4, p<0.05) are increased after 8 days decidualisation. ANGPT4 is decreased after 8 days decidualisation (n=4, p=0.001).

5.4.4.2. E2 treatment in uNK cells results in changes in expression of genes associated with angiogenesis.

To investigate whether locally produced E2 could impact on the expression of angiogenic factors in uNK cells, the differential response to 2 hours E2 treatment was assessed by PCR array. The expression of 84 genes involved in modulating angiogenesis was investigated. The array includes growth factors and their receptors, chemokines and cytokines, matrix and adhesion molecules, proteases and their inhibitors and transcription factors, all of which have the potential to be involved in the development of new blood vessels. In contrast to the cell motility array analysis (Section 5.4.1) expression of many targets in the angiogenesis array were not detected. Approximately 25% of genes were determined as 'no calls' (Ct>35) and

only ~18% of genes were expressed at Ct<30. However, incubation of cells with E2 seemed to have a wide ranging impact on expression of the genes that were detected. The overall expression response of all array candidates is summarised in Figure 5.12. Of the 84 genes analysed, twenty not detected (Figure 5.11, grey boxes), 18 tended to be up-regulated (Figure 5.11, red boxes), 40 tended to be down-regulated (Figure 5.11, green boxes) and 6 were unchanged (Figure 5.11, black boxes).

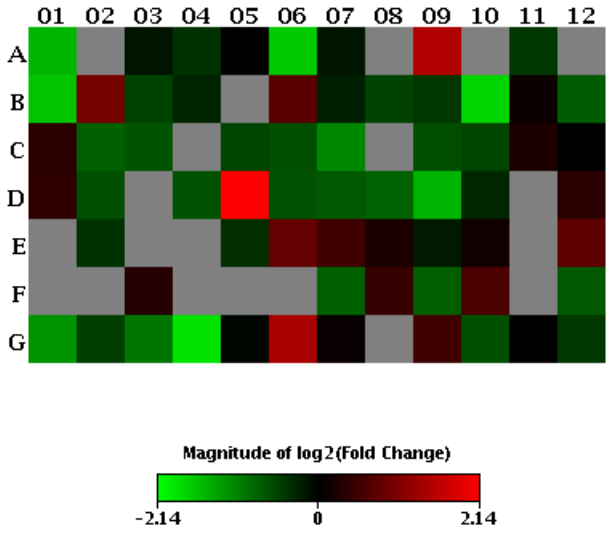

Figure 5.11. Heat map indicating gene expression of angiogenesis genes following E2 treatment of uNK. The expression of genes associated with angiogenesis was appears to be tightly regulated with expression of many targets not detected (grey boxes). The impact of E2 seemed to have wide ranging impact on the genes that were detected.

Despite the small sample size significant decreases in expression following E2 treatment were observed for THBS1, AKT1, TGFB1 and IL1B mRNAs (Table 5.8). There was also a wide dynamic range in changes in expression following E2 treatment (Figure 5.12 and Table 5.8) from -3.8 (THBS1) to 4.4 (IFNG) fold regulation. The data set was narrowed to exclude results from genes that had very high P values or low changes in expression and fold change in response to E2 from this set is shown in Figure 5.12 and detailed in Table 5.8. Incubation of cells with E2 tended to mostly decrease expression of candidate genes however there were several genes which appeared to be up-regulated.

5.4.4.2.1. Gene expression responses in functional groups

When genes were arranged into functional gene groupings based on array product specification information (See Appendix section 8.1), E2 treatment tended to decrease the expression of angiogenic growth factors such as ANPEP, EREG, FIGF. Interestingly, PGF and VEGFC were not detected while VEGFA was unchanged following treatment. Other growth factors such as TGFB1 were significantly decreased ($p=0.028122$, $n=3$) while TFGBR1 and EFNA3 tended to be decreased with treatment. The functional group which showed greatest regulation by E2 was cytokines and chemokines associated with angiogenesis (Table 8.5). E2 significantly decreased the expression of IL1B ($p=0.037542$, $n=3$) and tended to decrease expression of CXCL1, CXCL3, and IL6. However, E2 tended to increase expression of CCL2, CXCL10, CXCL9 and IFNG, while CCL11, CXCL6, IFNA1 and IFNB1 were not detected. Adhesion molecules were largely undetected, but THBS1 was significantly down-regulated in response to E2 ($p=0.020214$, $n=3$) while IL-8, ITGAV and S1PR1 also tended to be decreased with treatment. The expression of ANGPTL3, NAI3, COL4A3, LAMA5, STAB1, CCL11, CDH5 COL18A1, ITGB3, and THBS2 could not be detected. However, CCL2 tended to be increased by E2 and is also associated with adhesion (Table 8.7). The proteases MMP2 and MMP9 tended to be increased by E2 treatment and the expression of the metalloproteinase inhibitor TIMP1 also tended to be increased. LECT1, LEP, PLG, and TIMP3 could not be detected. The transcriptional modulator AKT1 was significantly decreased by E2, and ID3, HAND2 and SPHK1 tended to be decreased by treatment while HIF1A was unchanged. HPSE, ID1, NOTCH4 and PTGS1 were not detected.

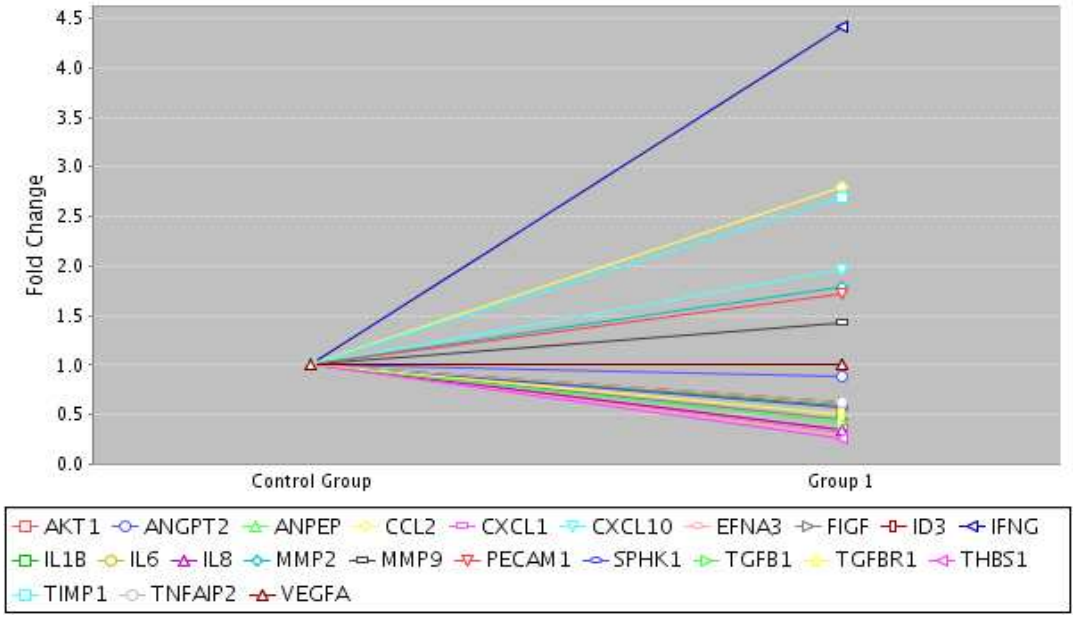

Figure 5.12. Fold change in gene expression of selected angiogenesis genes following E2 treatment. Genes with low changes in expression or very high P values were excluded for simplicity. Differential expression following E2 treatment resulted wide range of responses in target genes. Control group is vehicle treated uNK and Group1 is E2 treated uNK.

	Fold Change	95% CI	P value	Fold Regulation
THBS1	0.2646	(0.00001, 0.60)	0.020214	-3.7791
EFNA3	0.2864	(0.00001, 0.78)	0.12797	-3.4912
ANPEP	0.3043	(0.00001, 0.68)	0.122483	-3.2859
CXCL1	0.3165	(0.00001, 0.76)	0.196089	-3.1599
AKT1	0.3462	(0.21, 0.48)	0.001286	-2.8884
IL8	0.3514	(0.07, 0.64)	0.05618	-2.8458
TGFB1	0.4245	(0.21, 0.64)	0.028122	-2.3557
FIGF	0.4555	(0.00001, 1.48)	0.792319	-2.1955
TGFBR1	0.506	(0.07, 0.94)	0.212099	-1.9761
IL6	0.5666	(0.08, 1.06)	0.270708	-1.7651
SPHK1	0.5803	(0.22, 0.94)	0.10907	-1.7233
IL1B	0.5987	(0.35, 0.84)	0.037542	-1.6704
ID3	0.6329	(0.20, 1.07)	0.262278	-1.58
TNFAIP2	0.6329	(0.34, 0.93)	0.110976	-1.58
ANGPT2	0.8914	(0.42, 1.36)	0.722491	-1.1218
VEGFA	1.007	(0.10, 1.92)	0.756185	1.007
MMP9	1.4272	(0.85, 2.00)	0.150482	1.4272
PECAM1	1.7144	(0.42, 3.01)	0.29493	1.7144
MMP2	1.7916	(0.88, 2.71)	0.066499	1.7916
CXCL10	1.9562	(0.00001, 4.04)	0.446108	1.9562
TIMP1	2.6924	(0.29, 5.10)	0.105164	2.6924
CCL2	2.8044	(0.00001, 5.69)	0.128538	2.8044
IFNG	4.4089	(0.00001, 10.88)	0.108815	4.4089

Table 5.8. Table detailing fold change in gene expression in selected angiogenesis genes following E2 treatment displayed in Figure 5.11. E2 elicited wide ranging changes in expression. Values shown are differential responses following treatment. 95% CI and P value are also shown. There was low statistical significance in this small sample size.

5.4.4.3. Human endometrial endothelial cells show increased network formation following culture with E2-treated uNK conditioned media.

Studies in previous sections have revealed that decidualisation of endometrial stromal cells is associated with production of E2 and increased expression of angiogenic factors such as VEGF, ANGPT2 and ADM (section 5.4.4.1). In order to test whether increased angiogenesis during decidualisation may be mediated by E2-dependent changes in uNK cell function. In uNK cells treated with E2 there are changes in gene expression associated with angiogenesis. The impact of conditioned media from E2 treated uNK cells on angiogenesis was measured using immortalised human endometrial endothelial cells (HEEC) using an angiogenesis assay (see section 2.6. for details.) Conditioned media recovered from uNK cells that had been treated with 10^{-8}M E2 for 24 hours increased network formation in HEEC compared to vehicle treated controls (Figure 5.13, n=4, $p<0.05$), but this effect did not seem to be blocked by the ER antagonist ICI 182, 780 (Faslodex® AstraZeneca).

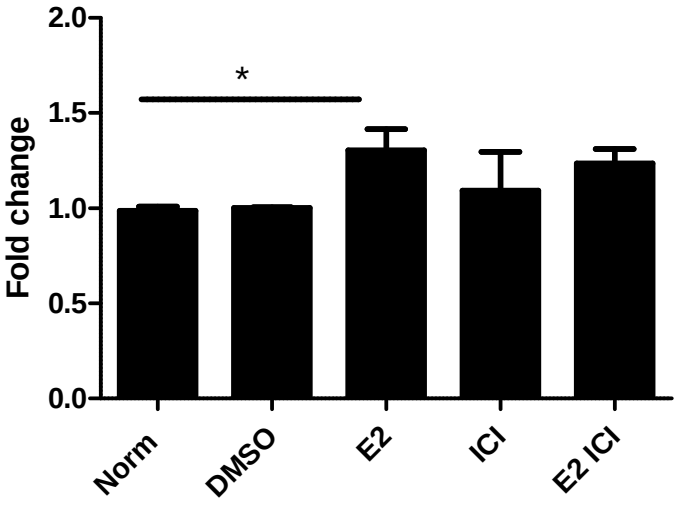

*Figure 5.13. Human endometrial endothelial cells show increased angiogenesis following culture with E2-treated uNK conditioned media. n=4, *p<0.05.*

5.5. Discussion

The functional changes that occur in the endometrium during decidualisation such as angiogenesis and leukocyte recruitment are essential to implantation and establishment of pregnancy but their control is poorly understood. As an extension of studies in Chapters 3 and 4, we investigated the importance of the interplay between E2 and uNK cells in these processes.

The studies presented herein describe a novel mechanism by which oestrogen modulates uNK function. Our investigation has shown for the first time that E2 enhances migration of isolated uNK cells, and that factors secreted from decidualised stromal cells, which may include SDF-1, IL-15 and E1/E2, stimulate directed chemotaxis of uNK cells. We have also shown that E2 stimulates the secretion of soluble factors from uNK cells which enhance angiogenesis of endometrial endothelial cells (HEECs). Gene expression analysis suggests that this may involve VEGFA, CCL2, and PECAM1 but further studies are required to validate these observations. To the best of our knowledge, this study is the first description of a functional impact of modulation of uNK by E2. Together with our previous findings highlighting local production of E2 in the uterus we suggest a novel mechanism for local control of functional processes during differentiation of the endometrium that is mediated by E2 and cellular cross-talk between uNK, stromal and endothelial cells.

It has been reported that E2 increases homing of pbNK cells to the uterus (74) but the impact of E2 on migration of isolated populations of uNK has not previously been investigated. Notably, we report significantly increased numbers of uNK cells that migrated when they were pre-treated with E2 for 1 hour compared with those treated with control media. This effect was blocked by co-treatment with the ER antagonist ICI 182, 780, a finding that could be consistent with E2 having an impact on ERβ-mediated changes in cell function.

In order to investigate which factors may be modulated by E2 to account for the observed increase in migration we analysed an array panel of cell motility genes to investigate differential expression following E2 treatment. These findings were limited by the small sample size (n=3) and previously described variability in gene expression following E2 treatment in uNK (Chapter 4). Expression of mRNAs encoding genes associated with regulation of cell motility were readily detected in uNK. However, in this experiment, only two genes were identified as being significantly regulated by E2; Moesin (MSN) a protein

important in cell-cell recognition and for cell movement and integrin beta 1 (ITGB1) a protein important in mediating cell adhesion. Interestingly, expression of both of these genes was apparently down-regulated following E2 treatment. This small study suggested that expession of myosin heavy chain 10, non-muscle (MYH10) a factor important in cytokinesis, alpha-actin 3 (ACTN3) as well as hepatocyte growth factor (HGF) a paracrine factor important for growth and motility and is also potent angiogenic factor (519), increased in response to E2 although differences between samples meant this was non-significant. Interestingly, in contrast to previous findings from array studies in our group (Chapter 4 and Dr. Elaine Marshall, personal communication), actin related genes appeared unchanged by E2 (ACTN1, ACTN4, ACTR2 and ACTR3) however expression of ACTN3 appeared to show non-significant up-regulation by E2.

PCR-array analysis of expression of cell motility genes following E2 treatment appeared to contradict the observed E2 impact on motility reported in section 5.4.2. Together these findings suggest an apparent paradox in that many genes associated with cell motility were found to be down-regulated in response to E2, yet we described increased migration of uNK cells in response to E2. One possible explanation for this may be that we are not monitoring genes regulated by membrane bound ERs (mERs) expressed in uNK cells. An impact via mERs would be consistent with the rapid response to E2 (1 hour treatment) (520). Propagation of ligand response via membrane ERs could involve membrane-tethered ERβ (521) or G-protein coupled receptors (GPCR) such as GPR30. It has been reported that compounds such as ICI 182780 that act as pure antagonists on ERα and ERβ may have agonist activity for GPR30 (522) adding additional complexity to interpretation of studies. Thus the apparent block of E2-mediated migration by the ER antagonist ICI 182, 780 reported in this chapter may not involve GPR30 interaction in the signalling cascade. Cell surface expression of ERα46 (an isoform of ERα) has been reported in lymphocytes (523) and a membrane impermeable form of E2 has been shown to increase IFN secretion by $CD56^{bright}$ pbNK (523), providing strong evidence that uNK cells may be regulated via a mER. If this is the case a biphasic response to E2 may exist in uNK cells; an increase in cell migration dependent on membrane-ERs that increases homing of uNK to appropriate sites in the uterus in parallel with a transcription dependent down-regulation of cell motility genes in the presence of persistent E2. This may occur in parallel with cytokine dependent changes in cell function as we noted increased expression of CXCR4 mRNA in uNK cells in response to

E2 treatment (Section 5.4.3.1, Figure 5.5). Further experiments are required to investigate these mechanisms.

In the present study, high levels of SDF-1 and IL-15 mRNA were detected in decidualised stromal cells which appeared to be time-dependent. IL-15 is known to be essential for uNK cell homing to implantation sites in mice (428) and is an established regulator of uNK cell functions including proliferation, differentiation and recruitment.

The results presented demonstrate that expression of SDF-1 and IL-15, known to be chemoattractant to uNK cells (35, 57), is increased during decidualisation of endometrial stromal cells which *in vivo* is concomitant with uNK cell accumulation in the endometrium.

Thus, decidual conditioned media (DCM), which may contain secreted IL-15 and SDF-1, was tested to determine whether it can act as a chemoattractant to uNK cells. Using a chamber slide chemotaxis assay we were able to report novel data demonstrating directed movement of uNK towards DCM but not control media. Previous studies have reported that co-culture of uNK cells with decidual stromal cells protects uNK cells from apoptosis (524) and conditioned media from ESC from secretory phase endometrium and early pregnancy decidua promotes uNK cell proliferation (38). However to the best of our knowledge this is the first data demonstrating an effect of conditioned media from decidualised ESC on chemotaxis of isolated uNK cells. SDF-1 and IL-15 secreted by decidual stromal cells may be acting as a chemoattractant, however an ELISA for IL-15 could not detect the protein in DCM (data not shown).

The receptor-mediated increase in human uNK cell migration stimulated by E2 reported herein, is in agreement with previous reports that suggest E2 enhances homing of pbNK to the uterus (74). However, it is distinct in that it describes a local mechanism of control for uNK cells, which may augment pbNK cell homing. For example, while an increase in circulating E2 is correlated with pbNK cell homing to the uterus, results presented in Chapter3 of this thesis have demonstrated that oestrogen is produced by decidualising cells, and that E2 increases migration of tissue derived uNK cells which are distinct from pbNK cells (27). Thus, local E2 may mediate tissue migration of uNK cells within the uterus to decidualising cells and consequently to spiral arterioles. It is interesting that uNK cells may retain this migratory potential once homed to the uterus and it is conceivable that active gradients of steroids and cytokines derived from the stromal environment may modulate and regulate uNK cells *in situ*. Furthermore, transcriptional down-regulation of cell motility

genes observed in response to E2 may enhance this effect as a reduction in migration in an E2-rich tissue environment would encourage retention of uNK cells in the tissue. Consistent with the proposed temporal E2 regulation of uNK cell recruitment, expression of IL-15 and SDF-1 by endometrial stromal cells was transiently up-regulated early in decidualisation and decreased thereafter. The results described may represent a novel mechanism of uNK cell regulation during a brief window in which the cells within the decidualising stroma signal the recruitment of uNK cells.

As an aside to the current investigations, it was noted that one uNK cell sample which was outwith the gestational range of this study (>12 weeks) did not respond to E2 treatment and had significantly reduced overall migration (data not shown). This may be anomalous; however, it is possible that the migratory response of uNK cells to E2 may diminish with gestational age, as has been described for other functions of uNK cells (43). Alternatively, as the uNK cells have been derived from a later gestation and thus have been resident in the tissue for longer, persistent signals from the local environment, such as E2, may have led to down-regulation of cell motility genes. The contribution of specific motility factors and their regulation by E2 in uNK cells requires further investigation however results presented herein have described novel evidence that E2 increases migration of uNK cells and that stromal derived factors in DCM, which may include oestrogen and cytokines such as SDF-1 and IL-15, can directly influence uNK cell motility.

Decidualisation is associated with an increase in angiogenesis of the spiral arteries of the endometrium (104). VEGF is a prime regulator of angiogenesis during decidualisation (525) and it has been reported that oestrogen stimulates VEGF expression in human endometrial stromal and epithelial cells (114) providing a link between our novel findings of local E1/E2 biosynthesis in decidualised cells and angiogenesis, consistent with results reported in the mouse (128). Angiopoietins (Ang) act with VEGF to promote angiogenesis (111).

In the current investigation an increase in expression of VEGFA, ADM and Ang-2 mRNAs was detected following decidualisation of endometrial stromal cells. This is consistent with previous reports that suggest VEGFA is up-regulated following decidualisation and in the secretory phase (112, 526) but is in contradiction to previous studies which suggest Ang-2 mRNA expression is decreased in secretory phase endometrium (527). ADM expression has only previously been reported in human endometrial epithelial and endothelial cells (528, 529) and these data therefore extend the range of endometrial cell types capable of producing

this factor. Ang-4 mRNA was decreased following decidualisation, to our knowledge there is no previous evidence of Ang-4 regulation during decidualisation in human endometrial stromal cells, however the aromatase inhibitor letrozole has been shown to decrease Ang-4 expression during *in vitro* decidualisation of mice uterine stromal cells (128) suggesting a link with oestrogen biosynthesis. Data presented in the current chapter desrcibe increased expression of VEGFA, Ang-2 and ADM in decidualised stromal cells which would appear to be consistent with a role in stimulation of angiogenesis and may be important for vascular remodelling that takes place during modification of the implantation site. In addition to its stimulatory affect on VEGF, E2 itself is also reported to directly stimulate proliferation and migration of endothelial cells (530) and uterine angiogenesis is impaired in ERαKO mice (115). Thus local E2 acting in a paracrine manner in the uterus may be pro-angiogenic. Our new studies would be consistent with changes which correlate with increased angiogenesis being influenced by local oestrogen produced in decidualised stromal cells.

In addition to decidualised stromal cells, increased numbers of uNK cells accumulate close to spiral arteries in the endometrium. uNK cells have been reported to secrete angiogenic factors such as VEGFC, PLGF, and Ang-2 (39, 44, 531). It was hypothesised that local oestrogen may also impact on the capacity of uNK cells to secrete angiogenic factors.

The impact of uNK cell conditioned media (uNK CM) on HEECs was analysed using an angiogenesis assay. HEECs were chosen for the assays as this endothelial cell is more representative of the endometrial vascular bed than the commonly used HUVECs ((532) and Dr. Erin Greaves personal communication). Notably, supernatants from E2 treated uNK cells significantly increased network formation in HEECs compared to unconditioned media or media recovered from control uNKs suggesting E2 might enhance the ability of uNK cells to influence the stromal environment by increased secretion of angiogenic factors. The secretion of angiogenic factors by uNK cells has been shown to be gestation dependent (43) and for this reason only the response to uNK CM from gestations of <10 weeks were analysed in this study. It is notable that in uNK CM from one sample that was out with the gestational range of this study, E2 uNK CM did not increase angiogenesis network formation in HEECs (data not shown) consistent with gestation dependent response described by Lash et al (43). Thus response to E2 as a modulator of angiogenic secretion by uNK cells may also be dependent on gestation.

To investigate what factors may be modulated by E2 the expression of an array panel of angiogenesis genes in uNK cells treated +/- E2 were investigated. The expression of genes associated with angiogenesis in uNK appeared to be tightly regulated, with several genes not detected. Despite the small sample size, expression of four genes were significantly down-regulated by E2 treatment; Thrombspondin (THBS1), Rac-alpha serine/threonine-protein kinase (AKT1), Transforming growth factor 1 (TGFB1), and interleukin β1 (IL-1β).

THBS1 is inhibitor of angiogenesis and suppresses the expression of the angiogenic factor PECAM1 (533, 534). Thus decreased expression of THBS1 in uNK cell mediated by E2 may be indirectly pro-angiogenic. AKT1 is serine/threonine protein kinase that is activated by a number of different growth factors and cytokines. AKT signalling is critical to vascular homeostasis and mediating VEGF signalling (535) and AKT1 -/- mice have impaired VEGF-induced angiogenesis (536). Thus down-regulation of AKT1 by E2 in uNK may impair VEGF signalling. TGFB1 is an evolutionarily conserved cytokine which is involved in a variety of cellular functions (537). TGFB1 can be both a stimulator and inhibitor of angiogenesis depending on experimental conditions (538) and is reported to disrupt angiogenesis in microvascular cells in the bovine corpus luteum (539). As E2 treated uNK cell conditioned media was pro-angiogenic, decreased expression of TGFB1 in response to E2 may suggest TGFB1 is acting as an inhibitor of angiogenesis in this instance. Interleukin 1β (IL-1β, also IL-1B) is a pleiotropic cytokine with effects in many cell types. While IL-1β is reported to be pro-angiogenic (540), IL-1β inhibits decidualisation (396, 397) and thus decreased secretion of IL1β by uNK cells as a result of E2 stimulation may be essential in maintaining the uterine environment during decidualisation.

A further 11 genes exhibited large fold changes (greater than 2 or less than -2) in response to treatment (8 down, 3 up; Table 5.8) however due to the small sample size analysed, many of these responses were non-significant. Interestingly, mRNAs encoding expression of VEGF-C and placental growth factor (PLGF) were not detected and VEGFA expression was unaffected by E2 treatment a finding at odds with previous reports (43, 114). In the current experiment, expression of growth factors associated with angiogenesis such as ANGPT2, ANPEP and FIGF, showed a non-significant decrease in expression as a result of E2 treatment, while others were not detected (ANGPT1, FGF1 and PGF). These results suggest that any impact of uNK cells on angiogenesis may not be mediated by classical growth factor secretion.

Although some pro-angiogenic cytokines were expressed by uNK cells there was a non-significant decrease in the expression of IL6 and CXCL1, and a significant decrease in IL1β as a result of E2 treatment. However, a non-significant trend in increased expression of CCL2 (2.8 fold up-regulation, p=0.13, Table 5.8) by E2 was detected. CCL2 is known to induce angiogenesis directly (541) and the gene expression studies reported in the current study suggests E2 may enhance its expression. Furthermore, CCL2 combined with E2 has been shown to increase VEGF in stromal cells (531). Thus, combined secretion of CCL2 and E2 by stromal cells and uNK cells respectively may increase VEGF in the stromal environment and influence angiogenesis.

At the start of this study it was proposed that locally produced E2 might influence the ability of uNK cells to produce angiogenic factors. In the results presented herein novel findings are reported which demonstrate, using uNK cell conditioned media, that E2 stimulates the secretion of soluble factors from uNK cells which enhance angiogenesis of HEECs. To the best of our knowledge this is the first evidence of regulation of HEECs by uNK cells and of an oestrogen-mediated regulation of angiogenic cytokine secretion by uNK cells. Transcriptional analysis of genes which may be regulated by E2 revealed that many of the classical growth factors did not appear to be expressed in our samples. In addition, a mixture of changes in expression of mRNAs encoding both pro- and anti-angiogenic factors was detected in cells treated with E2 including; AKT1, reported to be a modulator of VEGF signalling and was significantly down regulated by E2, and inhibitors of angiogenesis such as THBS1 and TGFB1 which were also significantly down-regulated by E2. However, CCL2 and PECAM1, which may be pro-angiogenic, appeared to be increased in response to E2. The changes in expression of individual genes do not point to a single factor being responsible for the observed impacts on angiogenesis but rather it is likely that the sum of these changes results in a ‘cocktail’ of factors that have an impact on endothelial cells when they are exposed to uNK cell conditioned media for several hours.

5.5.1. Summary

In summary, the findings described in the current chapter contribute further evidence to support a role for intra-uterine biosynthesis of oestrogen in mediating the functional changes occurring in the stromal compartment during decidualisation of the endometrium. Novel evidence is reported that suggests oestrogen enhances the motility of uNK cells that may ‘home’ uNK cells to decidualising cells and spiral arteries in the endometrium *in vivo*.

Furthermore, we have shown for the first time that E2 increases the angiogenic capacity of uNK cells and that uNK secreted factors have a direct, positive impact on network formation of cells derived from the endometrial vascular bed. The precise factors involved and the transcriptional impact of E2 on uNK cell migration and uNK cell-mediated angiogenesis remain to be fully elucidated. However, these findings are consistent with a functional interplay between uNK cells, endothelial cells and decidualised stromal cells that is mediated by oestrogen, which may promote an appropriate environment for establishment and maintenance of pregnancy.

Chapter 6

6. Final discussion

6.1. Introduction

The human endometrium is a complex multi-cellular tissue that occupies the luminal compartment of the uterus. The luminal surface is defined by a layer of epithelial cells supported on a multi-cellular stroma containing fibroblasts, glands (lined by a secretory epithelium), blood vessels (lined with endothelial cells) and several populations of immune cells; the latter includes a unique population of natural killer cells (uNK). In women of reproductive age, the endometrium undergoes dynamic remodelling in response to fluctuating levels of sex steroids secreted by ovarian cells: phases of endometrial function include an oestrogen-dominated proliferative phase, a progesterone-dominated secretory phase and menses (endometrial shedding precipitated by falling levels of progesterone). A key feature of the secretory phase is differentiation (decidualisation) of stromal fibroblasts (ESC) an event characterized by transformation of cell shape, secretion of growth factors/cytokines, accompanied by angiogenesis/vascular remodelling and an increase in the numbers of resident immune cells by migration/proliferation. Decidualisation ensures an appropriate nutritional and hormonal environment exists during the establishment of pregnancy. Although mice do not menstruate, regulation of uterine function essentially mirrors that in woman with cell proliferation during an oestrogen dominated phase (proestrous) and functional maturation during a progesterone-dominated phase leading to development of a “window of uterine receptivity” stimulated by the action of oestrogen on the progesterone primed uterus (169, 174). Notably, studies in mice treated with an aromatase inhibitor suggest that *de novo* biosynthesis of oestrogen within the uterus may play an essential role in regulation of decidualisation but no data exist for human. Studies from this laboratory have previously reported that human endometrial endothelial and uNK cells both express oestrogen receptor beta (248, 249) but the impact of oestrogens on their function had not been explored prior to the studies described in this thesis.

6.2. Key findings

In the current study three questions were addressed:

1. Is oestrogen biosynthesis a feature of endometrial stromal cell decidualisation in women?
2. What is the impact of oestrogen on uNK cell function?

3. What role (if any) does oestrogen play in the interplay between decidual, immune and vascular cells within the endometrial stroma?

It was hypothesized that, consistent with evidence from studies in mice, decidualisation of human endometrial stromal cells results in alterations in biosynthesis of oestrogens and that the local (intra-uterine) biosynthesis of oestrogens could have a functional impact on cell populations in the endometrial stroma, including those that are ERβ positive such as the abundant uterine natural killer cells, and endothelial cells of the endometrial vasculature. During the secretory phase of the cycle decidualising endometrial stromal cells and uNK cells are in close proximity to the endometrial spiral arteries which are lined by endothelial cells. It was postulated that oestrogen produced by cells within the stromal compartment alter cell function in either a paracrine or an autocrine manner and thus impact on the functional interplay between cells within the endometrial stroma.

6.2.1. Human endometrial stromal cells demonstrate the capacity for *de novo* oestrogen biosynthesis

In Chapter 3 results are presented that confirm our primary hypothesis that decidualisation of human ESC results in alterations in their capacity for biosynthesis of oestrogens. Notably, it was found that *in vitro* decidualisation of primary cells was associated with expression of aromatase protein and time-dependent changes in expression of genes encoding proteins important in steroid biosynthesis including transient up-regulation of *StAR* and *CYP11A1*. A change in enzyme activity in decidualised cells was also detected characterized by increased aromatase activity and an apparent reduction in 17βHSD2 activity. Secretion of both E1 and E2 by primary ESC was confirmed using ELISA, with increasing production of E2 associated with progressive decidualisation of cells. Secretion of E1 and E2 was partially ameliorated by inhibitors of aromatase and STS. There was evidence to suggest that treatment of cells with STX64 (an STS inhibitor) resulted in reduced biosynthesis of E1 as well as expression of the decidualisation marker protein IGFBP1, which may indicate that E1 reinforces the process of decidualisation in women. The production of E1 exceeded that of E2. Consistent with this, incubation with the aromatase inhibitor anastrozole had a greater impact on concentrations of E1 than E2 in culture the media suggesting aromatase-mediated conversion from androstenedione (A4) to E1 is the more active reaction in decidualised ESC.

The results obtained provide the first evidence of *de novo* biosynthesis of oestrogens during decidualisation of human ESC. Aromatase is essential in the biosynthesis of all oestrogens. Aromatase activity in human ESC has previously been reported (345), however, a subsequent study failed to detect either aromatase enzyme activity or mRNA expression in

normal ESC (311). In the studies reported in Chapter 3 of this thesis, both aromatase protein expression and increased aromatase activity were reported and although aromatase mRNA concentrations were low, an increase in aromatase mRNA concentrations was detected following decidualisation of hESC *in vitro* for 8 days. In support of induction of aromatase expression and activity during decidualisation, concentrations of E1 and E2 were reduced by addition of an aromatase inhibitor.

In humans, expression of the aromatase gene is regulated by tissue-specific promoters (327, 542). For example, in ovarian endometriosis, gene expression is stimulated by cAMP and PGE2 acting via promoter II (378). Bulkulmez et al have reported that in proliferative phase endometrial explants aromatase mRNA transcripts containing exon IIa at their 5' ends were increased in response to stimulation with A4 (349). The authors reported that the ER antagonist ICI 182,780 (Faslodex®, AstraZeneca), and the aromatase inhibitor fadrozole (543) suppressed A4-mediated induction of aromatase mRNA suggesting the impact of A4 was in fact a reflection of its conversion to oestrogens. Consistent with this, they also reported that the non-aromatisable androgen dihydrotestosterone did not induce aromatase mRNA expression. It is likely that E1, as the direct aromatized product of A4, may be of key importance in mediating increases in aromatase expression both in the studies reported by Bulkulmez and those reported in this thesis during decidualisation of ESC.

High levels of E1 were reported in response to decidualisation which may therefore impact on induction of aromatase expression. We speculate that an intracrine/autocrine positive feedback loop may also contribute to the increased aromatase expression observed as a result of persistent high levels of E1 detected in culture media from decidualised ESC in our study. Aromatase may also be regulated by progesterone. A study by Tseng et al. reported that aromatase activity in ESC was increased by progestins but not androgens or glucocorticoids (346). The authors reported that oestrogen alone had no impact on aromatase activity but potentiated progestin-mediated increases. In the current study aromatase gene expression appeared to be increased when ESC were exposed to a steroid environment of high progesterone and high secreted oestrogens, conditions which have been shown by Tseng et al to induce aromatase expression in ESC (345, 346).

Previous studies from our laboratory group have revealed an important role for the orphan nuclear receptor, oestrogen-related receptor α (ERRα) in decidualisation of human ESC. ERRα is reported to be increased in decidualisation and inhibition of ERRα reduces expression of IGFBP1 in ESC (305) a result that mirrors our findings following inhibition of STS. Miao et al reported that in prostatic stromal cells, ERRα up-regulates the expression of

aromatase via the I.3/II promoter region and that prostaglandin E2 (PGE2) enhanced ERRα recruitment to the same promoter (544). Interestingly, Miao et al also reported that stimulation of the PKA pathway by PGE2, which increases intracellular cAMP, also increased ERRα expression in prostatic stromal cells (544). During decidualisation of ESC, the increased expression of ERRα may therefore also act to up-regulate aromatase expression via a similar mechanism to that reported in prostatic stromal cells. Further studies are required to confirm which promoter element is the most important in regulation of the *CYP19A1* gene in endometrial stromal cells.

The effects of ERRα are potentiated by the ERRα co-activator peroxisome proliferator-activated receptor gamma co-activator-1 alpha (PGC-1α). Interestingly, PGC-1α together with ERRα increases the expression of the steroidogenic enzymes, CYP11A1 and CYP17A1 in hepatic cells (545). Furthermore, in the same study, the authors identified ERR response elements (ERRE) in the promoters of both *CYP11A1* and *CYP17A1* genes and ERRα was reported to bind these elements by chromatin immunoprecipitation (ChIP) analysis. An increase in CYP11A1 mRNA expression was detected in decidualised ESC which may also be influenced by ERRα-mediated transcriptional regulation. In addition, it has been reported that ERRs have the capacity for transcriptional cross-talk with ER regulated genes such as osteopontin (reviewed in (298)) which may have additional impacts on the transcriptional response to oestrogens produced during decidualisation. Taken together these studies suggest that ERRα may act as a transcriptional regulator during decidualisation which may account for some of the changes in expression in steroidogenic enzymes reported in this thesis.

Aghajanova et al compared the regulation of steroid metabolizing enzymes in response to cAMP treatment in normal endometrial stromal cells and stromal cells isolated from eutopic endometrium from women with endometriosis (313). The authors reported increased immunoexpression of 17βHSD2 protein in ESC treated with cAMP for 96 hours that was absent in ESC isolated from women with endometriosis. Furthermore, there was no impact on expression of 17βHSD1 regardless of whether the women had endometriosis or not. The main focus of the study by Aghajanova et al was a comparison of steroidogenic enzymes in women with or without endometriosis and their treatment regime differed from the studies presented in chapter 3 as their cells were treated with cAMP or P4 alone and results only presented for a single time point (96 hours.) In contrast, the impact of cAMP alone or in combination with P4, over multiple time points was investigated in the current study. Additionally, in the study by Aghajanova et al enzyme expression was expressed as fold change to ‘no endometriosis’ controls so only limited information can be extrapolated for comparison to the results presented in chapter 3. In addition, the findings that expression of

mRNA encoding 17βHSD2 is up-regulated whilst 17βHSD1 is unchanged in response to decidualising stimuli are in agreement with the results reported by Aghajanova et al (313). However, novel time-dependent increases in expression of mRNAs encoding StAR, CYP11A1, SRD5A1 and STS in response to cAMP and P4 treatment were also reported in the present study. Additionally, the results presented herein demonstrated decreased 17βHSD2 activity was detected, as measured by TLC, a result apparently at odds with observed changes in mRNA concentrations emphasizing the need to expand studies beyond that of measurement of mRNA alone. Taken together these results suggest a novel description of the regulation of steroidogenic enzymes and metabolism during decidualisation and shed new light on regulation of fertility in women.

6.2.2. A role for oestrone?

In addition to secreted E2, significantly high levels of E1 secreted by decidualised hESC were detected across all time points investigated. The level of E1 was consistently high from the earliest time point. In contrast, levels of E2 increased with progression of decidualisation leading to a near equivalent ratio of E1/E2 by 8 days decidualisation. This may in part account for the decrease in 17βHSD2 activity (decreased conversion of E2 to E1) detected in decidualised stromal cells as the comparative excess of E1 compared to E2 during decidualisation may impact on the dynamics of their respective metabolism. This may also impact on the capacity to detect the conversion of E1 to E2 by TLC.

High concentrations of E1 may also have an impact on ER mediated transcription. Classically E1 is considered to be the 'weaker' oestrogen compared to that of E2. This perception has been based on affinity studies for the oestrogen receptor. Oestrogens are lipophilic and thus are able to diffuse in and out of cells. Oestrogens are retained within cells with high affinity following intranuclear binding to the ER protein and subsequently are able to modulate gene expression (546). The dissociation rate of an oestrogen from the activated ER gives an indication of the effectiveness of an oestrogenic ligand in maintaining the receptor in an activated state (547). In 1980, Weichmann et al (548) reported that in calf uterus, E2-ER complexes had greater retention than E1-ER complexes; this publication predates the identification of ERβ. The authors suggested that the greater interaction and retention of E2 with ER may make possible long term uterine growth responses to E2 (548). However, in a more recent paper E1 has been reported to induce uterine growth in the rat (549). Kuiper et al (550) investigated saturation binding of *in vitro* synthesised human ERα and rat ERβ protein. The authors reported relative binding affinity (RBA) of a range of oestrogenic ligands, expressing them as a proportion of E2 binding, and found that E1 had

60% RBA for the human ERα protein and 37% RBA for the ERβ protein (rat) (550). While the reported affinity of E1 was lower than that of E2 for both receptor isoforms, in this study E1 had greater affinity than tamoxifen, genistein and bisphenol A for both ERs (550). The capacity for E1 to induce expression of an ERE-reporter construct in T47D human breast cancer cells was investigated by Legler et al (551). Legler et al reported that E2 was ten times more potent than E1 at inducing ERE luciferase activity for either human ERα or ERβ protein demonstrated by the concentration of oestrogen required to elicit EC_{50} (nominal concentration at which 50% maximum response is reached) which was around ten times higher for E1 than E2 (551). In Chapter three it was reported that the ratio of E1/E2 in hESC following two days decidualisation was approximately 4:1. An excess in the relative concentrations of E1 compared to E2 could favour E1 stimulation of ER-mediated transcription and if higher concentrations of E1 than E2 are present then this may compensate for the lower affinity of E1 for ERα and ERβ proteins. Selective oestrogen receptor modulators (SERMs) are reported to have tissue-specific agonist and antagonist actions which are influenced by the expression of co-activators and co-repressors and the relative expression of different ER isoforms in a given tissue context (552). The specific capacity for E1-ER complexes to recruit cofactors and the influence of different ER isoforms on the impact of E1-mediated transcription requires further investigation to fully appreciate the impact, if any, E1 could have on transcription in the context of different tissue environments. The impact of E1 on non-classical ER signalling such as via indirect tethering to transcription factors (Sp1, AP-1) or via membrane localised ERs is also poorly understood. The molecular response to E1 in the stromal environment may result in tissue-specific agonist profiles that are distinct from E2, however this requires further investigation. In this regard, temporal changes in the E1 to E2 ratio during decidualisation may account for some of the temporal changes in mRNA expression reported in Chapter 3.

Baird et al (553) reported that plasma concentrations of E1 were highest in women during the proliferative phase but that E1 was proportionally lower than E2 in the secretory phase. In contrast, it is reported in the current study that during decidualisation the ratio of locally produced oestrogens favours E1 initially but is equivalent by 8 days decidualisation. Baird et al also reported that in postmenopausal women the plasma E1 to E2 ratio was approximately 7 to 1 (553). In postmenopausal women the majority of circulating oestrogen is produced from peripheral conversion which is influenced by obesity (554); conversion of A4 to E1 has been reported in human female fat tissue (555) and adipose is thought to be the major source of oestrogens in postmenopausal women (556). Furthermore, Hemsell et al (557) have reported that with advancing age there is a progressive increase in the efficiency with which

A4 is converted to E1 in plasma. Despite the reported high circulating levels of E1 in postmenopausal women this does not appear to impact on uterine growth as postmenopausal uteri are atrophic. However, ERs from postmenopausal uteri may be less active than those pre-menopause as Strathy et al (558) reported that ER from postmenopausal uteri have minimal capacity to bind nuclear acceptor sites in a cell-free assay. The efficacy of ERs in the postmenopausal uterus may also be influenced by the relative abundance/availability of co-regulatory proteins. The expression/activity of steroid metabolising enzymes in postmenopausal uteri may give an indication of the potential for high circulating E1 to be converted locally in target cells. As E1 is reported to have lower affinity than E2 for ER, E1 may require conversion to the more potent E2 in order to elicit effects at ERs in the postmenopausal uterus.

The presence of high concentrations of local oestrogen may also influence receptor turnover. E2 is known to promote down-regulation of ERα concomitant with the receptor's transcriptional activation (559) while SERMs such as tamoxifen and raloxifene are reported to increase steady state levels of ERα (560, 561). Degradation of ERα is mediated by the ubiquitin-proteasome degradation system and is promoted by E2-mediated recruitment of co-regulatory proteins. When bound to the receptor complex, these proteins contribute to receptor down-regulation, as mutations in co-activator binding residues in ERα are reported to stabilise the receptor and abolishes E2-mediated receptor degradation (559).

In Chapter 3, it was reported that the expression of mRNAs encoding ERα and ERβ were decreased and increased respectively following decidualisation for 8 days. The observed decrease in ERα mRNA may parallel degradation of the ERα protein although this was not measured in the current study. If this is the case, the local biosynthesis of E2 by hESC during decidualisation may mediate ERα turnover in the endometrial stroma which could be critically important as down-regulation of ERα is essential to implantation (479, 562). Interestingly, Tateishi et al (563) reported that there is an ubiquitin-proteasome pathway for ERβ that is not coupled to transcription. The authors indentified two regions within ERβ that are essential for ubiquitination and degradation; an N-terminal region essential for recruitment of ubiquitin ligase and a C terminal F domain which protects ligand unbound ERβ from degradation, however the conformation of the F domain was proposed to change following oestrogen binding which would permit recruitment of the 26 proteasome and degradation of the receptor (563). Whether E1 influences receptor turnover is not known but high levels of E1 and/ or E2 may differentially affect ERβ protein expression/turnover as in contrast to ERα, increased concentrations of ERβ mRNA were detected in response to decidualisation in the current study.

6.2.3. Oestrogen alters gene expression in uNK cells

Studies presented in Chapter 4 demonstrated that a viable uNK population could be isolated from first trimester decidua that retained ERβ expression and had a phenotype apparently identical to uNK from non-pregnant endometrium (249). The isolated uNKs were responsive to E2 stimulation, and bioinformatic analysis of transcriptional responses suggested steroid exposure could modulate cell motility. Although these results provided preliminary evidence that E2 can modulate the function of uNK it was notable that stratification of results according to the gestational age of the decidua from which the cells were recovered suggested that functional responses may be subject to 'conditioning' by the local environment. It has been reported that tissue-resident uNK cells have a phenotype that is distinct from those of peripheral blood NK subsets (27).

The current study utilized human first trimester decidua as an abundant source of primary uNK cells (75). Descriptions of uNK from non-pregnant endometrium are limited but they are reported to be phenotypically similar to those from decidua being $CD56^{+}$, $CD16^{-}$, with a granular appearance and lacking cytotoxicity. However, uNK from non-pregnant endometrium have recently been shown by Manaster et al to differ from decidual uNK because of lack of NKp30 expression (also natural cytotoxicity receptor, NCR3, an NK cell activating receptor (564)) and fail to secrete a full range of cytokines (565). Interestingly, in the same study, treatment of uNK with IL-15 restored NKp30 expression and increased their ability to secrete cytokines resulting in a shift to a phenotype more closely resembling that of decidua-derived uNK (565) which suggests reported differences in phenotype of uNK from non-pregnant endometrium may be a reflection of an 'inactivated' state. IL-15 expression is high in the secretory phase endometrium (36) and this cytokine may therefore activate uNK at this time.

A recent transcriptional analysis by Kopcow et al has suggested that uNK cells derived from first trimester decidua are distinct from those in the cycling endometrium (566) however in our hands decidua-derived uNK appear phenotypically similar to uNK being both ERβ+ and $CD56^{bright}$. In the study by Kopcow et al (566), several differences in methodological practices could account for the transcriptional differences observed between uNK from decidua and non-pregnant endometrium. Firstly, the isolation methods utilized to recover the two populations were different; endometrial NK cells were isolated based on a selection of $CD45^{+}$, $CD56^{+}$ $CD3^{-}$ phenotype while decidual NK cells were isolated as a selected $CD56^{+}$ $CD16^{-}$ $CD3^{-}$ phenotype. Thus, the cells isolated from the endometrium may contain both $CD56^{+}$ $CD16^{+}$ and $CD56^{+}$ $CD16^{-}$ populations of NK cells; notably, while the latter is

considered to be a mature uNK cell phenotype, the former may represent an undifferentiated precursor of uNK, that is homologous to the pbNK $CD56^{bright}$ population and could thus be functionally different from 'mature' uNK. This makes it likely that whilst a homogenous uNK population was derived from decidua, a heterologous NK population was derived from the non-pregnant endometrium. In addition, uNK derived from decidua in the study by Kopcow were from a wide range of gestational ages (6-12 weeks) and as revealed by the studies presented in Chapter 4 it is likely that those cells may vary significantly in their transcriptional responses dependent upon the gestation and age of the source tissue. As reported in Chapter 4 and by others (43, 58), the present study has described gestational impacts on uNK function and further studies on decidual uNK from early gestations and those recovered at greater than 12 weeks gestation are required to explore whether they have functionally distinct transcriptional networks in response to E2 stimulation.

It seems likely that uNK from non-pregnant endometrium and decidua may have different functional properties as a result of variations in their tissue micro-environment. This would be consistent with the large body of data showing that the levels of hormones and cytokines change throughout the menstrual cycle and that the decidua of early pregnancy also has a distinct steroid/cytokine profile. To determine whether a distinct 'subpopulation' of uNK really exists transcriptional responses to ligands such as IL-15 could be compared. Interestingly, NK cells in secondary lymphoid tissues such as spleen, lymph nodes and tonsils, all express different phenotypes. For example, splenic NK are $CD56^{dim}$ $CD16^{+}$ and express perforin, natural cytotoxicity receptors (NCRs) and Killer-cell immunoglobulin-like receptors (KIRs), whereas NK cells in lymph nodes and tonsils are $CD56^{+}$ $CD16^{-}$, lack perforin and NCR expression (567). However, stimulation of lymph and tonsil NK cells with IL-2 activates cytolytic activity, increases perforin and expression of CD16 and KIRS (567). IL-15 is structurally similar to IL-2 and they share common binding properties (568).

Crucial to these studies is the fact that ERβ mRNA and protein are both expressed in tissue-resident uNK in both non-pregnant endometrium and decidua. The studies presented complement and extend existing information which suggests that uNK are a functionally dynamic population (43, 58), furthermore ERβ-dependent transcriptional targets have been identified which may further modulate uNK function in oestrogen-rich tissues. In light of these novel findings, we suggest E2 (or E1) may regulate functional processes of uNK particularly in late-secretory endometrium (following stromal cell decidualisation) and in first trimester decidua, at which point uNK cell populations are likely to have reached a similar 'mature'/'activated' phenotype due to the increased expression IL-15 reported at these times in the endometrium.

6.2.4. Oestrogens enhance the functional interplay between endometrial stromal cells (uNK, stromal fibroblasts and endothelial cells)

Our investigations have shown for the first time that exposure to E2 enhances migration of uNK cells; these results complement those suggesting that factors secreted from decidualised stromal cells, which may include SDF-1 and IL-15 stimulate directed chemotaxis of uNK cells (see Figure 6.1). Notably, expression of SDF-1 by human ESC is reported to be increased by E2 (386) and increased expression of SDF-1 and IL-15 occurs during stromal cell decidualisation with both factors able to stimulate NK recruitment to the uterus (57, 390, 425). It has been reported that treatment of premenopausal women with E2 increases the number of uNK cells and macrophages found in the uterus an effect postulated to include homing of pbNK (74). A direct impact of E2 on migration of purified populations of uNK has not previously been studied. In the current study, using an *in vitro* assay, we detected a significant increase in uNK cell migration as a result of treatment with E2, an effect that was blocked by co-treatment with the ER antagonist ICI. As uNK cells are ERβ+/ERα- this provides the first evidence that E2 can increase migration in an ERβ dependent manner. Changes in expression of known cell motility genes in response to E2 did not reveal alterations in the expression of any factors which might account for the observed increase in migration in response to E2. However, we speculate stimulation of membrane bound ERs might account for the rapid response to E2 (1 hour treatment) (520) and that a biphasic response to E2 may exist in uNK; an increase in cell migration dependent on membrane-ERs that increases homing of uNK to appropriate sites in the uterus, which is augmented by a transcription-dependent down-regulation of cell motility genes in the presence of persistent E2. Further experiments are required to investigate these mechanisms and to determine the balance between the direct impact of E2 on uNK cells and the indirect impacts mediated by stromal cell derived factors (Figure 6.1).

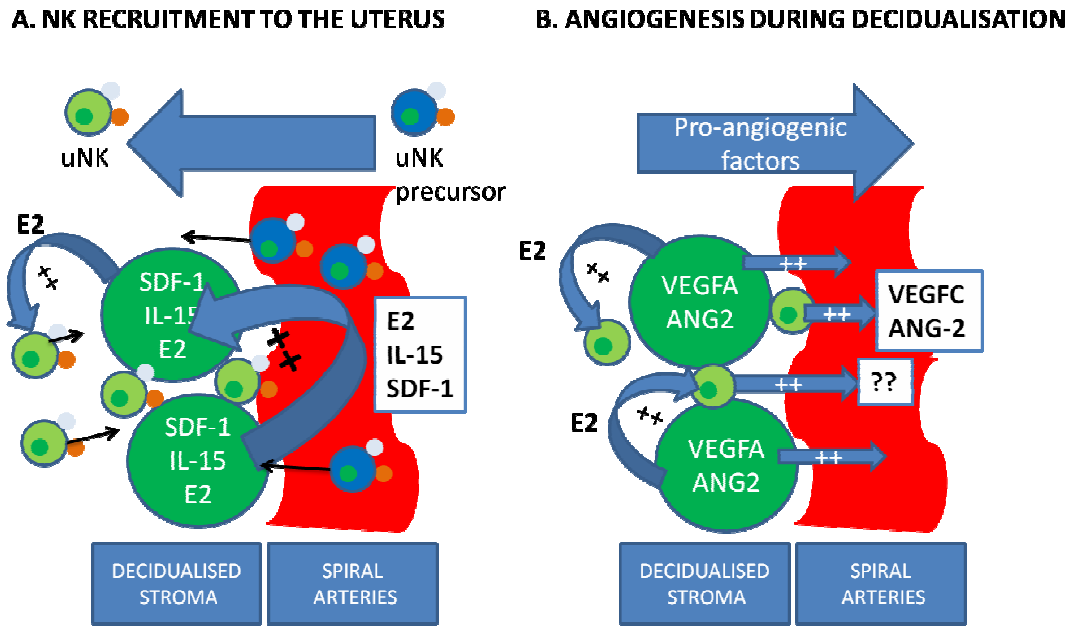

Figure 6.1. Summary diagram depicting the proposed role of oestrogen and stromal-derived factors in regulating uNK cell functions during decidualisation. ***A****. NK recruitment to the uterus. Decidualisation results in the increased expression of SDF-1 and IL-15 in decidualised ESC, factors which are known to enhance recruitment of NK cells to the uterus. In addition, local production of E2 enhances migration of uNK cells which may home uNK cells to peri-vascular sites during decidualisation. White circle; CXCR4 (SDF-1 receptor), orange circle; IL-15Rα (IL-15 receptor.)* ***B****. Angiogenesis during decidualisation. Decidualisation results in increased expression of the pro-angiogenic factors VEGFA and ANG2 in decidualised ESC. Locally produced E2 enhances the secretion of factors by uNK cells which increase 'network formation' in endometrial endothelial cells consistent with a role for local E2 in enhancing the angiogenic capacity of uNK cells. In addition, uNK cells are reported to induce angiogenesis by secretion of VEGFC and Ang-2.*

Our results have also revealed for the first time that incubation of uNK with E2 stimulates the secretion of soluble factors which enhance 'network formation' (an *in vitro* angiogenesis assay) of endometrial endothelial cells (HEECs) which is indicative of an increase in angiogenesis. Previous studies have reported that uNK cells induce 'network formation' of HUVECs (human umbilical vein endothelial cells) (39, 43) however the data reported in Chapter 5 represent the first evidence of modulation of HEECs by uNK and a role for E2 in this process. This is particularly pertinent to our studies elaborating on the interplay between endometrial cell populations as studies in our own (Dr Erin Greaves, personal communication) and other laboratories (532) suggest that HEECs provide more representative model of the vascular bed in the endometrium than do HUVECs. To gain insight into those factors responsible for this E2-mediated cell to cell dialogue we used targeted gene array expression analysis. Preliminary results suggested that this may involve E2-dependent suppression of the angiogenesis inhibitor THBS1 and modulation of AKT1

signalling. In addition, expression of the pro-angiogenic factors VEGFA, CCL2, and PECAM1 by uNK cells may also be increased by exposure to E2 but further studies are required to validate this. To the best of our knowledge, this study is the first description of a functional impact of modulation of uNK by E2 on a different cell type.

Studies in mice using an aromatase inhibitor have revealed uterine biosynthesis of oestrogens play a critical role in neovascularisation during decidualisation (128). The current studies that have detected oestrogen secretion by decidualised ESC would be consistent with direct and indirect impacts on angiogenesis due to locally increased concentrations of oestrogens as well as factors released by decidualised stromal cells or uNK cells in response to E1 or E2 (see Figure 6.1). For example, an increase in production of angiogenic factors such as VEGFA, Ang-2 and ADM by decidualised ESC would be consistent with the development of a pro-angiogenic environment during decidualisation. While individual factors from the different cellular compartments may all contribute to increased angiogenesis in the endometrium their production could be orchestrated by local oestrogen biosynthesis which might augment the cross-talk between stromal, uNK and vascular cells. This might also involve the secretion of cytokines by tissue-resident uNK cells, for example, we have demonstrated that E2 can stimulate increased CCL2 expression by uNK cells. This factor has been shown by others to combine with E2 to increase stromal VEGF expression (531) which in turn may further stimulate angiogenesis. Our results also suggest that E2 may increase secretion of interferon γ (IFNγ) by uNK cells; this factor is reported to induce production of IL-15 expression by stromal cells. IL-15 also plays a key role in regulation of uNK function, survival and recruitment consistent with a dynamic interplay between the stromal and immune compartments in the endometrium.

A number of studies have previously shown functional interactions between ESC and uNK some of which may also be modulated by local oestrogen biosynthesis although this possibility was not considered in the original papers. For example, co-culture of uNK cells with decidual stromal cells protects uNK cells from apoptosis (524) and conditioned media from ESC from secretory phase endometrium and early pregnancy decidua promotes uNK cell proliferation (38). In addition, it has been reported that conditioned media from uterine leukocytes (which were predominantly uNK) substantially altered ESC gene expression (414); increased expression of cytokines IL-8, CCL8, and CXCL1 and also increased stromal expression of IL-15 and IL-15Rα was reported. Interestingly, IL-8 is expressed in perivascular cells in late-secretory phase endometrium (410) and mRNA expression is reported to be highest in late-secretory and mid-proliferative phase in total endometrial extracts (411). Additionally, IL-8 secretion by uNK cells is reported to increase the

invasiveness of extra-villous trophoblast cells (59). Our studies presented in Chapter 5 suggested that E2 may decrease expression of IL-8 mRNA in uNK cells and thus expression of IL-8 may be another example of a factor that is regulated by local E2 and participates in stromal-uNK cell cross-talk.

If local production of E2 by decidual cells also persists in pregnancy, E2 may further potentiate the role of uNK cells in spiral artery remodelling and trophoblast invasion through stimulation of factors such as IFNγ. There is evidence to suggest uNK cells have a functional role in tissue vascular remodelling and placental development and that dysregulation of uNK may be a component of the aetiology of pre-eclampsia (9). In pregnancy, transformation of spiral arteries is generally complete by 10-12 weeks gestation (45) and expression of angiogenic growth factors by uNK is reported to be increased at 8-10 weeks gestation compared with 12-14 weeks gestation (43) suggesting uNK may specifically influence vascular remodeling at the earliest stages of gestation. Notably, uNK cells are reported to accumulate within the vascular wall of first trimester placental-decidual co-cultures and this appears to be associated with loss of vascular smooth muscle cells and widening of the vessel lumens (569). Evidence from animal models also suggests uNK cells could be essential to vascular remodelling during pregnancy. In NK-deficient mice spiral artery remodelling is impaired; however administration of IFNγ rescues this phenotype (570). Thus, IFNγ from decidual uNK cells may be essential in spiral artery remodelling in mice. In addition, IFNγ has also been shown to control migration of cytotrophoblast cells in human co-cultures (446). In the preliminary studies presented in Chapter 5, we report that E2-treated uNK exhibit a non-significant increase in the expression of IFNγ mRNA as a result of E2 treatment. IL-8 and CXCL10 have also been shown to regulate trophoblast invasion (39, 571), factors which were both differentially regulated by E2 in uNK cells in this study. Regulation of cytokine secretion from uNK cells by E2 in the local uterine environment in early pregnancy may therefore also regulate vascular remodelling and trophoblast invasion *in vivo*.

Taken together these data suggest that E2 may modulate many of the factors which are key to functional roles of uNK, firstly by modulating angiogenesis during decidualisation and then in pregnancy influencing trophoblast invasion and spiral artery remodelling. This would be consistent with dynamic functions of uNK in response to changing tissue environments. Whether local oestrogen production persists in decidua and whether responsiveness of uNK to E2 is retained as pregnancy progresses requires further investigation, however we have reported novel data that support the capacity for E2 to modulate uNK function.

6.3. Clinical implications

Oestrogen action is an essential requisite for implantation. In mice, although oestrogens are a critical determinant of the window of receptivity, their concentration/action needs to be tightly regulated as excessive concentrations of oestrogens are associated with aberrant expression of genes normally conducive to implantation, while suboptimal levels do not promote progression from the pre-receptive state (174). Several oestrogen-regulated factors, such as leukemia inhibitory factor (LIF), are locally modulated within different cellular compartments of the endometrium allowing the transition to a receptive state which is permissive to implantation. Dysregulation of local steroid metabolism in the endometrial stroma may contribute to production of factors which result in an 'out of phase' endometrium precipitating implantation failure.

Furthermore, supraphysiological levels of sex steroids as a result of controlled ovarian hyperstimulation (COH) during *in vitro* fertilization (IVF) treatment regimes can imbalance local steroid metabolism and this might also affect uterine receptivity. Indeed it has been reported that 'high responders' to COH i.e. women with significantly elevated serum E2 and P4, have low implantation rates which may be as a result of an altered steroid milieu (572).

We have also shown that oestrogens can alter the function of uNK cells and therefore any imbalances in local hormone levels could further impact on reproductive function. These findings may be relevant to subfertility associated with obesity as oestrogen biosynthesis takes place in adipose tissue and also in disorders associated with alterations in the androgen:oestrogen balance such as polycystic ovarian syndrome (PCOS). PCOS is an endocrine disorder affecting women of reproductive age and is associated with menstrual cycle disturbances, hyperandrogenism and infertility (362). Disruptions in local steroid metabolism in the endometrium as a result of hyperandrogenism in PCOS may account for some of the associated infertility due to altered gene expression in ER positive cells including the uNK cells. Disorders of pregnancy such as recurrent pregnancy loss (RPL) have been associated with dysregulation of uNK cells but the mechanism is unclear (50). uNK numbers appear increased in RPL and are associated with greater prevalence of CD16+ NK cells (50, 51). This may also be as a result of disturbances in inter-cellular dialogue within the stromal compartment resulting in inappropriate homing of uNK cells to the uterus, dysregulation of uNK function by an altered steroid environment, or dysregulation of oestrogen mediated cross-talk with the vasculature. Factors which could be involved include IL-15, which is responsible for modulating the functional phenotype of uNK cells, but also

oestrogens which we have suggested may be important in regulating angiogenesis, spiral artery remodelling and trophoblast invasion associated with the regulation of uNK cell function. Impaired decidualisation associated with decreased prolactin production is also associated with RPL and dysregulation of local steroid metabolism may impact on this process (484). The proposed roles for uNK in regulating vascular function may also be important in heavy menstrual bleeding (HMB). For example, oestrogens may enhance vascular stability through stimulating VEGF production from cells within the stromal environment, however, whether impaired local oestrogen biosynthesis is associated with HMB is unknown and merits further investigation.

Endometriosis is classified as an oestrogen-dependent inflammatory disease that is associated with pain and the formation of lesions within the peritoneal cavity (373). The novel findings reported in this thesis which describe a capacity for normal endometrial stromal cells to differentiate into cells capable of oestrogen biosynthesis may help in part to explain one of the paradoxes of Sampson's theory of endometriosis (573). Sampson proposed that retrograde menstruation of endometrial material into the peritoneal cavity was responsible for lesion formation in various ectopic sites. However retrograde menstruation occurs in 90% of women, yet the incidence of endometriosis is much lower (373). It is conceivable that the efflux of endometrial stromal cells with the capacity for oestrogen biosynthesis (decidualised ESC) may increase the prevalence of lesion formation as endometriosis is an oestrogen-dependent disease and these cells may be more likely than non-oestrogen producing cells to flourish outside the uterine cavity as a result of stimulation of proliferation or neoangiogenesis. If this is the case, the state of differentiation of stromal cells during menses may be important and thus duration of menses may influence whether lesions become established. Menses is triggered by cessation of ovarian steroid support following demise of the corpus luteum and progesterone withdrawal. In our study progesterone was required for oestrogen biosynthesis in ESC *in vitro*. As a result of progesterone withdrawal during menses, oestrogen biosynthesis may cease in ESC *in vivo*, in parallel with processes that occur during normal menstrual breakdown. If this is the case, the time it takes for oestrogen biosynthesis to cease following progesterone withdrawal may affect the likelihood of lesion formation. Shorter menstrual cycles are associated with increased risk of endometriosis (574) and perhaps efflux of stromal cells that are still capable of oestrogen biosynthesis during menses may play a previously unrecognised role in the aetiology of this condition, however further studies are required.

Another interesting distinction between the decidualised stromal cells described in this study, and eutopic stromal cells from women with endometriosis, is the differential expression of 17βHSD1 and 17βHSD2. Following decidualisation we reported an increase in expression of 17βHSD2 mRNAs and no change in expression of 17βHSD1. It is reported that decidualisation of ESC from eutopic endometrium from women with endometriosis by cAMP promoted an expression profile of increased 17βHSD1 and decreased 17βHSD2 indicative of altered gene regulation in response to decidualising stimuli (313). This could be a consequence of epigenetic modification of 17βHSD1 or 17βHSD2 in the eutopic endometrium of women with endometriosis. An increase in the survival of extra-uterine endometrial tissue could be linked to prior modification of gene expression or dysregulation as a result of an aberrant decidualisation response. Thus, an increased understanding of the precise temporal expression of steroid metabolizing enzymes in the healthy endometrium, throughout the menstrual cycle and during decidualisation may lead to the discovery of factors which may indicate an increased risk of endometriosis in advance of lesion formation. Such an approach may be particularly beneficial in this condition as early diagnosis is difficult, and there is often a delay of several years between onset of symptoms and a definitive diagnosis (575).

6.4. Future Studies

Future studies should focus on elaborating the steroid metabolome of normal cycling endometrium, and aim to investigate not only the expression of mRNAs encoding steroid metabolising enzymes, but also the capacity for metabolism of steroids within the different cellular compartments of the endometrium.

The function of uNK cells appears to be dynamic and characterisation of functional responses to hormones/cytokines by uNK cells isolated from different phases of the normal cycle as well as decidua derived from different gestational ages will be essential in order to unravel the complex role of this cell type in regulating endometrial function.

Thus, understanding the hormonal milieu that is present in the stromal micro-environment of the endometrium across dynamic changes in cycling endometrium and decidua of pregnancy, would allow better understanding of cellular cross-talk mediated by steroid hormones and the control of functional processes in reproductive function in health and disease.

6.5. Conclusion

The data presented in this thesis provide evidence that local biosynthesis of oestrogens within the endometrial stroma may play a previously unrecognised role in regulating the

function of uNK cells and endometrial endothelial cells in women. These results have implications for treatment of disorders such as infertility, heavy menstrual bleeding and endometriosis.

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8. Appendix

The RT^2 profiler PCR arrays (SABiosciences, Frederick, MD, USA) are designed to analyse a panel of genes related to a specific biological function. Each array contains a panel of 96 primer sets for a set of 84 functionally focussed genes, together with five housekeeping genes and three RNA and PCR quality controls.

The responses for each functional grouping for functional genes in the angiogenesis and cell motility array can be found in the following appendix. Differential gene expression in response to treatment with E2 in uNK cells is shown. Gene tables are grouped according to broad functional groupings as denoted in the product specification literature provided by the manufacturer (SABiosciences, Frederick, MD, USA). There is a degree of overlap between genes in functional groups and as such the responses to certain genes appear in more than table.

8.1. Angiogenesis array

8.1.1.1. Angiogenic factors

	Symbol	FC	95% CI	P value	Fold Regulation
A02	ANGPT1				
A03	ANGPT2	0.8914	(0.42, 1.36)	0.722491	-1.1218
A06	ANPEP	0.3043	(0.00001, 0.68)	0.122483	-3.2859
B07	TYMP	0.8425	(0.25, 1.43)	0.797764	-1.1869
C03	EREG	0.6153	(0.18, 1.05)	0.236852	-1.6252
C04	FGF1				
C05	FGF2	0.6766	(0.00001, 1.49)	0.723398	-1.4781
C07	FIGF	0.4555	(0.00001, 1.48)	0.792319	-2.1955
C08	FLT1				
D12	JAG1	1.2803	(0.06, 2.50)	0.916332	1.2803
E01	KDR				
E02	LAMA5				
E09	NRP1	0.8631	(0.00001, 1.88)	0.861361	-1.1586
E10	NRP2	1.1008	(0.03, 2.18)	0.941099	1.1008
F02	PGF				
F05	PLXDC1				
F10	STAB1				
G11	VEGFA	1.007	(0.10, 1.92)	0.756185	1.007
G12	VEGFC				

Table 8.1. Growth factors and their receptors

	Symbol	FC	95% CI	P value	Fold Regulation
A04	ANGPTL3				
A07	BAI1				
A12	COL4A3				
D09	IL8	0.3514	(0.07, 0.64)	0.05618	-2.8458
E02	LAMA5				
E09	NRP1	0.8631	(0.00001, 1.88)	0.861361	-1.1586
E10	NRP2	1.1008	(0.03, 2.18)	0.941099	1.1008
F10	STAB1				

Table 8.2. adhesion molecules

	Symbol	FC	95% CI	P value	Fold Regulation
A05	ANGPTL4				
E12	PECAM1	1.7144	(0.42, 3.01)	0.29493	1.7144
F01	PF4				
F06	PROK2				
F08	SERPINF1	1.3725	(0.44, 2.30)	0.341365	1.3725
G10	TNFAIP2	0.6329	(0.34, 0.93)	0.110976	-1.58

Table 8.3. Proteases, inhibitors other matrix proteins

	Symbol	FC	95% CI	P value	Fold Regulation
C09	HAND2	0.6335	(0.00001, 1.56)	0.768935	-1.5786
F09	SPHK1	0.5803	(0.22, 0.94)	0.10907	-1.7233

Table 8.4. Transcription factors

8.1.1.2. Other factors involved in angiogenesis

	Symbol	FC	95% CI	P value	Fold Regulation
A08	CCL11				
A09	CCL2	2.8044	(0.00001, 5.69)	0.128538	2.8044
B01	CXCL1	0.3165	(0.00001, 0.76)	0.196089	-3.1599
B02	CXCL10	1.9562	(0.00001, 4.04)	0.446108	1.9562
B03	CXCL3	0.6848	(0.00001, 1.47)	0.482861	-1.4602
B04	CXCL5	0.8119	(0.00001, 2.22)	0.840271	-1.2317
B05	CXCL6				
B06	CXCL9	1.6734	(0.00001, 6.99)	0.816324	1.6734
D03	IFNA1				
D04	IFNB1				
D05	IFNG	4.4089	(0.00001, 10.88)	0.108815	4.4089
D07	IL1B	0.5987	(0.35, 0.84)	0.037542	-1.6704
D08	IL6	0.5666	(0.08, 1.06)	0.270708	-1.7651
E05	MDK	0.7609	(0.13, 1.39)	0.688258	-1.3142

Table 8.5. Cytokines and chemokines.

	Symbol	FC	95% CI	P value	Fold Regulation
B08	S1PR1	0.68	(0.00001, 1.75)	0.406441	-1.4706
B09	EFNA1	0.7325	(0.00001, 1.70)	0.751892	-1.3652
B10	EFNA3	0.2864	(0.00001, 0.78)	0.12797	-3.4912
B11	EFNB2				
B12	EGF	0.5841	(0.00001, 1.67)	0.871683	-1.7119
C02	EPHB4				
C06	FGFR3				
C10	HGF				
D06	IGF1	0.6174	(0.00001, 2.00)	0.827903	-1.6197
D11	ITGB3				
E11	PDGFA				
F11	TEK				
F12	TGFA	0.6012	(0.00001, 1.71)	0.84366	-1.6634
G01	TGFB1	0.4245	(0.21, 0.64)	0.028122	-2.3557
G02	TGFB2	0.6986	(0.00001, 1.91)	0.935521	-1.4315
G03	TGFBR1	0.506	(0.07, 0.94)	0.212099	-1.9761

Table 8.6. Other growth factors and their receptors

	Symbol	FC	95% CI	P value	Fold Regulation
A08	CCL11				
A09	CCL2	2.8044	(0.00001, 5.69)	0.128538	2.8044
A10	CDH5				
A11	COL18A1				
B08	S1PR1	0.68	(0.00001, 1.75)	0.406441	-1.4706
C01	ENG	1.2795	(0.33, 2.23)	0.621572	1.2795
D10	ITGAV	0.7992	(0.10, 1.50)	0.485518	-1.2512
D11	ITGB3				
G04	THBS1	0.2646	(0.00001, 0.60)	0.020214	-3.7791
G05	THBS2				

Table 8.7. adhesion

	Symbol	FC	95% CI	P value	Fold Regulation
E03	LECT1				
E04	LEP				
E06	MMP2	1.7916	(0.88, 2.71)	0.066499	1.7916
E07	MMP9	1.4272	(0.85, 2.00)	0.150482	1.4272
F03	PLAU	1.229	(0.69, 1.77)	0.382705	1.229
F04	PLG				
G06	TIMP1	2.6924	(0.29, 5.10)	0.105164	2.6924
G07	TIMP2	1.0518	(0.00001, 2.26)	0.973573	1.0518
G08	TIMP3				

Table 8.8. Proteases, inhibitors and other matrix proteins

	Symbol	FC	95% CI	P value	Fold Regulation
A01	AKT1	0.3462	(0.21, 0.48)	0.001286	-2.8884
C11	HIF1A	1.1817	(0.45, 1.92)	0.774817	1.1817
C12	HPSE				
D01	ID1				
D02	ID3	0.6329	(0.20, 1.07)	0.262278	-1.58
E08	NOTCH4				
F07	PTGS1				

Table 8.9. Transcription factors

8.1.2. Cell motility array

8.1.2.1. Chemotaxis

	Symbol	FC	95% CI	P value	Fold Regulation
C03	FGF2	0.8883	(0.15, 1.63)	0.722328	-1.1258
C10	ITGB2	0.9545	(0.00001, 2.33)	0.93106	-1.0477
D01	MAPK1	0.9179	(0.78, 1.05)	0.327079	-1.0894
D07	MYH10	1.2596	(0.12, 2.40)	0.530781	1.2596
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
E03	PLAUR	0.6339	(0.09, 1.18)	0.368307	-1.5776
E05	PLD1	0.5736	(0.23, 0.91)	0.133215	-1.7435
E06	PRKCA	0.7279	(0.31, 1.15)	0.380198	-1.3739
F01	RAC2	0.9649	(0.83, 1.10)	0.688026	-1.0363
G02	TGFB1	0.8269	(0.01, 1.64)	0.686773	-1.2093
G07	VEGFA	0.881	(0.33, 1.43)	0.627208	-1.135
G10	WASF2	0.8361	(0.69, 0.99)	0.113536	-1.1961
G12	WIPF1	0.8624	(0.55, 1.17)	0.515482	-1.1596

Table 8.10. Chemotaxis

8.1.2.2. Receptors

	Symbol	FC	95% CI	P value	Fold Regulation
B11	EGFR	0.4266	(0.00001, 0.90)	0.213332	-2.3442
C06	IGF1R	0.9245	(0.57, 1.28)	0.777562	-1.0817
C08	ITGA4	0.9985	(0.53, 1.46)	0.907358	-1.0015
C09	ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
C10	ITGB2	0.9545	(0.00001, 2.33)	0.93106	-1.0477
C11	ITGB3	0.5383	(0.08, 0.99)	0.173152	-1.8577
D02	MET	0.6275	(0.00001, 1.64)	0.766728	-1.5936
E03	PLAUR	0.6339	(0.09, 1.18)	0.368307	-1.5776
F04	RHO	0.9448	(0.70, 1.19)	0.735465	-1.0584

Table 8.11. Receptors

8.1.2.3. Growth factors

	Symbol	FC	95% CI	P value	Fold Regulation
B06	CSF1	0.9664	(0.41, 1.52)	0.826921	-1.0348
B10	EGF	0.7889	(0.00001, 2.25)	0.366281	-1.2676
C03	FGF2	0.8883	(0.15, 1.63)	0.722328	-1.1258
C04	HGF	2.0782	(0.00001, 5.34)	0.462532	2.0782
C05	IGF1	0.6877	(0.01, 1.36)	0.554432	-1.454
G02	TGFB1	0.8269	(0.01, 1.64)	0.686773	-1.2093
G07	VEGFA	0.881	(0.33, 1.43)	0.627208	-1.135

Table 8.12. Growth factors

8.1.2.4. Rho family GTPases

	Symbol	FC	95% CI	P value	Fold Regulation
A04	ACTR2	0.9592	(0.70, 1.22)	0.74566	-1.0425
A05	ACTR3	0.9183	(0.79, 1.05)	0.307675	-1.089
A08	ARHGDIA	0.5423	(0.00001, 1.15)	0.338822	-1.844
C12	LIMK1	0.8705	(0.70, 1.04)	0.227567	-1.1488
D06	MSN	0.7708	(0.67, 0.87)	0.015217	-1.2974
D09	MYL9	1.0166	(0.35, 1.68)	0.925491	1.0166
E04	PLCG1	1.1095	(0.00001, 3.12)	0.91189	1.1095
E05	PLD1	0.5736	(0.23, 0.91)	0.133215	-1.7435
E06	PRKCA	0.7279	(0.31, 1.15)	0.380198	-1.3739
E07	PTEN	0.9432	(0.73, 1.16)	0.700471	-1.0602
E10	PTPN1	0.9102	(0.71, 1.11)	0.438296	-1.0987
F04	RHO	0.9448	(0.70, 1.19)	0.735465	-1.0584
F05	RHOA	1.0739	(0.91, 1.24)	0.407981	1.0739
F06	RHOB	0.9768	(0.50, 1.45)	0.877817	-1.0238
F07	RHOC	0.9184	(0.66, 1.17)	0.649835	-1.0888
F08	RND3	0.6377	(0.00001, 1.82)	0.533238	-1.5681
F09	ROCK1	0.8819	(0.31, 1.46)	0.662993	-1.1339
G08	VIM	1.0401	(0.24, 1.84)	0.90575	1.0401

Table 8.13. Rho signaling

	Symbol	FC	95% CI	P value	Fold Regulation
A04	ACTR2	0.9592	(0.70, 1.22)	0.74566	-1.0425
A05	ACTR3	0.9183	(0.79, 1.05)	0.307675	-1.089
A10	BAIAP2	0.5305	(0.15, 0.91)	0.184226	-1.885
B04	CFL1	1.0366	(0.81, 1.27)	0.762844	1.0366
B05	CRK	0.9844	(0.74, 1.23)	0.886593	-1.0159
D11	PAK1	1.0309	(0.04, 2.02)	0.832824	1.0309
D12	PAK4	0.6552	(0.16, 1.15)	0.345822	-1.5262
E05	PLD1	0.5736	(0.23, 0.91)	0.133215	-1.7435
E06	PRKCA	0.7279	(0.31, 1.15)	0.380198	-1.3739
E12	RAC1	0.8847	(0.58, 1.19)	0.551169	-1.1303
F01	RAC2	0.9649	(0.83, 1.10)	0.688026	-1.0363
F12	STAT3	0.881	(0.19, 1.57)	0.779725	-1.1351
G09	WASF1	0.8235	(0.32, 1.32)	0.774144	-1.2143
G10	WASF2	0.8361	(0.69, 0.99)	0.113536	-1.1961
G11	WASL	0.6639	(0.11, 1.22)	0.38761	-1.5063

Table 8.14. Rac signalling

	Symbol	FC	95% CI	P value	Fold Regulation
A04	ACTR2	0.9592	(0.70, 1.22)	0.74566	-1.0425
A05	ACTR3	0.9183	(0.79, 1.05)	0.307675	-1.089
B03	CDC42	1.0976	(0.82, 1.37)	0.558195	1.0976
E01	PFN1	0.9556	(0.77, 1.14)	0.65679	-1.0465
G09	WASF1	0.8235	(0.32, 1.32)	0.774144	-1.2143
G10	WASF2	0.8361	(0.69, 0.99)	0.113536	-1.1961
G11	WASL	0.6639	(0.11, 1.22)	0.38761	-1.5063

Table 8.15. Cdc42 signalling

8.1.2.5. Adhesion

	Symbol	FC	95% CI	P value	Fold Regulation
B09	DPP4	1.0593	(0.00001, 2.43)	0.924135	1.0593
B11	EGFR	0.4266	(0.00001, 0.90)	0.213332	-2.3442
C01	EZR	1.0373	(0.02, 2.06)	0.998311	1.0373
C08	ITGA4	0.9985	(0.53, 1.46)	0.907358	-1.0015
C09	ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
C10	ITGB2	0.9545	(0.00001, 2.33)	0.93106	-1.0477
D06	MSN	0.7708	(0.67, 0.87)	0.015217	-1.2974
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
F09	ROCK1	0.8819	(0.31, 1.46)	0.662993	-1.1339
G02	TGFB1	0.8269	(0.01, 1.64)	0.686773	-1.2093

Table 8.16. cell-cell adhesion

	Symbol	FC	95% CI	P value	Fold Regulation
A01	ACTN1	0.8957	(0.52, 1.27)	0.536094	-1.1165
A02	ACTN3	1.3504	(0.20, 2.50)	0.379028	1.3504
B06	CSF1	0.9664	(0.41, 1.52)	0.826921	-1.0348
C07	ILK	1.0427	(0.83, 1.26)	0.738315	1.0427
C09	ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
C10	ITGB2	0.9545	(0.00001, 2.33)	0.93106	-1.0477
C11	ITGB3	0.5383	(0.08, 0.99)	0.173152	-1.8577
D03	MMP14	0.9089	(0.00001, 2.38)	0.820498	-1.1003
E07	PTEN	0.9432	(0.73, 1.16)	0.700471	-1.0602
E09	PTK2B	1.1644	(0.24, 2.09)	0.724516	1.1644
E11	PXN	0.8146	(0.53, 1.10)	0.341253	-1.2276
F02	RASA1	0.9695	(0.70, 1.24)	0.838768	-1.0315
F05	RHOA	1.0739	(0.91, 1.24)	0.407981	1.0739

Table 8.17. cell-matrix adhesion

	Symbol	FC	95% CI	P value	Fold Regulation
A01	ACTN1	0.8957	(0.52, 1.27)	0.536094	-1.1165
A02	ACTN3	1.3504	(0.20, 2.50)	0.379028	1.3504
A09	ARHGEF7	0.9903	(0.33, 1.65)	0.93552	-1.0098
A11	BCAR1	0.9217	(0.00001, 1.93)	0.846037	-1.0849
A12	CAPN1	1.0813	(0.17, 2.00)	0.555978	1.0813
B01	CAPN2	0.8394	(0.38, 1.30)	0.526571	-1.1913
B02	CAV1	0.6151	(0.00001, 1.52)	0.600257	-1.6257
B12	ENAH	1.0781	(0.92, 1.23)	0.369607	1.0781
C07	ILK	1.0427	(0.83, 1.26)	0.738315	1.0427
C09	ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
D09	MYL9	1.0166	(0.35, 1.68)	0.925491	1.0166
E08	PTK2	0.7481	(0.24, 1.26)	0.440942	-1.3366
E09	PTK2B	1.1644	(0.24, 2.09)	0.724516	1.1644
E11	PXN	0.8146	(0.53, 1.10)	0.341253	-1.2276
G04	TLN1	1.0593	(0.34, 1.78)	0.954997	1.0593
G05	VASP	0.7341	(0.18, 1.28)	0.431172	-1.3623
G06	VCL	0.9932	(0.51, 1.48)	0.986411	-1.0068

Table 8.18. focal adhesion

	Symbol	FC	95% CI	P value	Fold Regulation
C01	EZR	1.0373	(0.02, 2.06)	0.998311	1.0373
C08	ITGA4	0.9985	(0.53, 1.46)	0.907358	-1.0015
C09	ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
C10	ITGB2	0.9545	(0.00001, 2.33)	0.93106	-1.0477
D06	MSN	0.7708	(0.67, 0.87)	0.015217	-1.2974
F09	ROCK1	0.8819	(0.31, 1.46)	0.662993	-1.1339

Table 8.19. leukocyte adhesion and rolling

8.1.2.6. Integrin mediated signalling

	Symbol	FC	95% CI	P value	Fold Regulation
A11	BCAR1	0.9217	(0.00001, 1.93)	0.846037	-1.0849
C07	ILK	1.0427	(0.83, 1.26)	0.738315	1.0427
C08	ITGA4	0.9985	(0.53, 1.46)	0.907358	-1.0015
C09	ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
C10	ITGB2	0.9545	(0.00001, 2.33)	0.93106	-1.0477
C11	ITGB3	0.5383	(0.08, 0.99)	0.173152	-1.8577
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
E08	PTK2	0.7481	(0.24, 1.26)	0.440942	-1.3366

Table 8.20. Integrin mediated signalling

8.1.2.7. Cellular Projections

	Symbol	FC	95% CI	P value	Fold Regulation
A10	BAIAP2	0.5305	(0.15, 0.91)	0.184226	-1.885
B03	CDC42	1.0976	(0.82, 1.37)	0.558195	1.0976
B08	DIAPH1	0.9094	(0.52, 1.30)	0.645942	-1.0996
B11	EGFR	0.4266	(0.00001, 0.90)	0.213332	-2.3442
B12	ENAH	1.0781	(0.92, 1.23)	0.369607	1.0781
C01	EZR	1.0373	(0.02, 2.06)	0.998311	1.0373
D06	MSN	0.7708	(0.67, 0.87)	0.015217	-1.2974
F03	RDX	0.9253	(0.80, 1.05)	0.320237	-1.0808
G01	SVIL	1.1654	(0.33, 2.01)	0.904226	1.1654
G05	VASP	0.7341	(0.18, 1.28)	0.431172	-1.3623

Table 8.21. Filopodia

	Symbol	FC	95% CI	P value	Fold Regulation
B07	CTTN	0.8164	(0.17, 1.47)	0.873956	-1.2249
B09	DPP4	1.0593	(0.00001, 2.43)	0.924135	1.0593
B11	EGFR	0.4266	(0.00001, 0.90)	0.213332	-2.3442
B12	ENAH	1.0781	(0.92, 1.23)	0.369607	1.0781
C02	FAP	0.956	(0.24, 1.67)	0.802964	-1.046
E02	PIK3CA	0.4694	(0.00001, 1.22)	0.382145	-2.1305
E05	PLD1	0.5736	(0.23, 0.91)	0.133215	-1.7435
E08	PTK2	0.7481	(0.24, 1.26)	0.440942	-1.3366
E11	PXN	0.8146	(0.53, 1.10)	0.341253	-1.2276
F03	RDX	0.9253	(0.80, 1.05)	0.320237	-1.0808
G01	SVIL	1.1654	(0.33, 2.01)	0.904226	1.1654
G05	VASP	0.7341	(0.18, 1.28)	0.431172	-1.3623
G06	VCL	0.9932	(0.51, 1.48)	0.986411	-1.0068

Table 8.22. lamellipodia

	Symbol	FC	95% CI	P value	Fold Regulation
A03	ACTN4	0.8693	(0.51, 1.23)	0.565807	-1.1504
B08	DIAPH1	0.9094	(0.52, 1.30)	0.645942	-1.0996
D07	MYH10	1.2596	(0.12, 2.40)	0.530781	1.2596
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
D10	MYLK	0.8879	(0.38, 1.39)	0.675214	-1.1263
F05	RHOA	1.0739	(0.91, 1.24)	0.407981	1.0739
F06	RHOB	0.9768	(0.50, 1.45)	0.877817	-1.0238
F07	RHOC	0.9184	(0.66, 1.17)	0.649835	-1.0888

Table 8.23. stress fibers

	Symbol	FC	95% CI	P value	Fold Regulation
A01	ACTN1	0.8957	(0.52, 1.27)	0.536094	-1.1165
A02	ACTN3	1.3504	(0.20, 2.50)	0.379028	1.3504
A03	ACTN4	0.8693	(0.51, 1.23)	0.565807	-1.1504
C01	EZR	1.0373	(0.02, 2.06)	0.998311	1.0373
D07	MYH10	1.2596	(0.12, 2.40)	0.530781	1.2596
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
D10	MYLK	0.8879	(0.38, 1.39)	0.675214	-1.1263
F08	RND3	0.6377	(0.00001, 1.82)	0.533238	-1.5681
F09	ROCK1	0.8819	(0.31, 1.46)	0.662993	-1.1339

Table 8.24.membrane bleb

	Symbol	FC	95% CI	P value	Fold Regulation
A04	ACTR2	0.9592	(0.70, 1.22)	0.74566	-1.0425
A05	ACTR3	0.9183	(0.79, 1.05)	0.307675	-1.089
A07	ARF6	0.92	(0.51, 1.33)	0.707735	-1.087
B03	CDC42	1.0976	(0.82, 1.37)	0.558195	1.0976
B04	CFL1	1.0366	(0.81, 1.27)	0.762844	1.0366
B07	CTTN	0.8164	(0.17, 1.47)	0.873956	-1.2249
B09	DPP4	1.0593	(0.00001, 2.43)	0.924135	1.0593
B10	EGF	0.7889	(0.00001, 2.25)	0.366281	-1.2676
C01	EZR	1.0373	(0.02, 2.06)	0.998311	1.0373
C02	FAP	0.956	(0.24, 1.67)	0.802964	-1.046
D03	MMP14	0.9089	(0.00001, 2.38)	0.820498	-1.1003
D04	MMP2	0.7096	(0.15, 1.27)	0.40729	-1.4092
D05	MMP9	0.8688	(0.00001, 1.89)	0.870163	-1.151
D06	MSN	0.7708	(0.67, 0.87)	0.015217	-1.2974
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
E03	PLAUR	0.6339	(0.09, 1.18)	0.368307	-1.5776
F01	RAC2	0.9649	(0.83, 1.10)	0.688026	-1.0363
F10	SH3PXD2A	0.7801	(0.50, 1.06)	0.276494	-1.282
F11	SRC	0.7464	(0.17, 1.32)	0.514935	-1.3397
G01	SVIL	1.1654	(0.33, 2.01)	0.904226	1.1654
G02	TGFB1	0.8269	(0.01, 1.64)	0.686773	-1.2093
G07	VEGFA	0.881	(0.33, 1.43)	0.627208	-1.135
G11	WASL	0.6639	(0.11, 1.22)	0.38761	-1.5063
G12	WIPF1	0.8624	(0.55, 1.17)	0.515482	-1.1596

Table 8.25. invasive projections

	Symbol	FC	95% CI	P value	Fold Regulation
A09	ARHGEF7	0.9903	(0.33, 1.65)	0.93552	-1.0098
B03	CDC42	1.0976	(0.82, 1.37)	0.558195	1.0976
B04	CFL1	1.0366	(0.81, 1.27)	0.762844	1.0366
E09	PTK2B	1.1644	(0.24, 2.09)	0.724516	1.1644

Table 8.26. growth cones

	Symbol	FC	95% CI	P value	Fold Regulation
A04	ACTR2	0.9592	(0.70, 1.22)	0.74566	-1.0425
A05	ACTR3	0.9183	(0.79, 1.05)	0.307675	-1.089
A07	ARF6	0.92	(0.51, 1.33)	0.707735	-1.087
A10	BAIAP2	0.5305	(0.15, 0.91)	0.184226	-1.885
A11	BCAR1	0.9217	(0.00001, 1.93)	0.846037	-1.0849
B07	CTTN	0.8164	(0.17, 1.47)	0.873956	-1.2249
B08	DIAPH1	0.9094	(0.52, 1.30)	0.645942	-1.0996
C01	EZR	1.0373	(0.02, 2.06)	0.998311	1.0373
C09	ITGB1	0.8645	(0.78, 0.95)	0.042202	-1.1567
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
E12	RAC1	0.8847	(0.58, 1.19)	0.551169	-1.1303
F01	RAC2	0.9649	(0.83, 1.10)	0.688026	-1.0363
F02	RASA1	0.9695	(0.70, 1.24)	0.838768	-1.0315
F03	RDX	0.9253	(0.80, 1.05)	0.320237	-1.0808
F05	RHOA	1.0739	(0.91, 1.24)	0.407981	1.0739
G04	TLN1	1.0593	(0.34, 1.78)	0.954997	1.0593
G10	WASF2	0.8361	(0.69, 0.99)	0.113536	-1.1961

Table 8.27. membrane ruffles

8.1.2.8. Cell polarity

	Symbol	FC	95% CI	P value	Fold Regulation
B03	CDC42	1.0976	(0.82, 1.37)	0.558195	1.0976
B04	CFL1	1.0366	(0.81, 1.27)	0.762844	1.0366
C01	EZR	1.0373	(0.02, 2.06)	0.998311	1.0373
C06	IGF1R	0.9245	(0.57, 1.28)	0.777562	-1.0817
C07	ILK	1.0427	(0.83, 1.26)	0.738315	1.0427
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646

Table 8.28. Cell polarity

8.1.2.9. proteolysis

	Symbol	FC	95% CI	P value	Fold Regulation
A06	AKT1	0.8816	(0.51, 1.25)	0.591216	-1.1343
A12	CAPN1	1.0813	(0.17, 2.00)	0.555978	1.0813
B01	CAPN2	0.8394	(0.38, 1.30)	0.526571	-1.1913
B09	DPP4	1.0593	(0.00001, 2.43)	0.924135	1.0593
C02	FAP	0.956	(0.24, 1.67)	0.802964	-1.046
C04	HGF	2.0782	(0.00001, 5.34)	0.462532	2.0782
D03	MMP14	0.9089	(0.00001, 2.38)	0.820498	-1.1003
D04	MMP2	0.7096	(0.15, 1.27)	0.40729	-1.4092
D05	MMP9	0.8688	(0.00001, 1.89)	0.870163	-1.151
D08	MYH9	0.8587	(0.45, 1.26)	0.535791	-1.1646
E03	PLAUR	0.6339	(0.09, 1.18)	0.368307	-1.5776
G03	TIMP2	0.9211	(0.13, 1.71)	0.871882	-1.0857

Table 8.29. Proteolysis