The molecular basis of chemokine action in human endometrium

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Abbreviations

2-ME	2-Mercaptoethanol
A	Adenine
ACTB	Beta actin
ADORA3	Adenosine A3 receptor
ADP	Adenosine diphosphate
AIP4	E3 ubiqutin ligase
Akt	V-akt murine thymoma viral oncogene homolog
AMV-RT	Avian myeloblastosis virus reverse transcriptase
ANOVA	Analysis of variation
AP-1	adaptor-related protein complex 1
APS	Ammonium persulphate
Asn	Asparagine
B2M	Beta-2-microglobulin
bFGF	Basic fibroblast growth factor
BL	Blastocyst
bp	Base pairs
C	Cytosine
CAM	Cellular adhesion molecule
CC	Cytotrophoblast cell column
CCD	Charge coupled device
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	Cluster of differentiation
CDK2	Cyclin-dependent kinase 2
CDK2AP1	Cyclin-dependent kinase 2-associated protein 1
cDMEM	Complete Dulbecco's modified eagle's medium
cDNA	Complementary deoxyribonucleic acid
CG	Chorionic gonadotropin
cRNA	Complementary ribonucleic acid
C_t	Cycle threshold
CT	Cytotrophoblast
CTP	Cytosine triphosphate
CX3CL	CX3C chemokine ligand
CX3CR	CX3C chemokine receptor
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
Cy3	Cyanine 3
Cy5	Cyanine 5
DEFA6	Defensin alpha 6
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSCs	Decidual stromal cells
dsDNA	Double stranded deoxyribonucleic acid
DTT	Dithiothreitol
EBI3	Homo sapiens Epstein-Barr virus induced gene 3

ECM	Extracellular matrix
ED_{50}	Effective dose 50
EDTA	Ethylenediaminetetraacetic acid
EEC	Epithelial endometrial cell line
EGF	Epidermal growth factor
EMBL	European molecular biology laboratory
EP	Early proliferative
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinase
ES	Early secretory
Ets1	E26 transformation-specific 1
EVT	Extravillous trophoblast
FAM	Fluorecein
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
G	Guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GE	Gene expression
GE	Glandular epithelium
GH	Growth hormone
GHSR	Growth hormone secretagogue receptor
GM-CSF	Granulocyte macrophage-colony stimulating factor
gp	Glycoprotein
GPCR	G protein-coupled receptor
GR	Glucocorticoid receptor
GRK	G protein-coupled receptor kinase
hCG	Human chorionic gonadotropin
HESC	Human endometrial stromal cell line
HI RPM	High Revolutions per minute
HIV	Human immunodeficiency virus
HK	Housekeeping gene
HPRT1	Hypoxanthine phosphoribosyl-transferase 1
HRT	Hormone replacement therapy
iEVT	Interstitial extravillous trophoblast
IFN	Interferon
IGFBP1	Insulin-like growth factor binding protein 1
IL	Interleukin
ISP1	Implantation serine protease 1
ISP2	Implantation serine protease 2
IVF	*In vitro* fertilisation
JAK	Janus kinase
KCNMA1	Calcium-activated pottassium channel
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
LP	Late proliferative
LS	Late secretory
MAP1A	Microtubule associated protein 1A
MAPK	Mitogen-activated protein kinase

MCP	Monocyte chemoattractant protein
MDC	Macrophage derived chemokine
MGB	Minor groove binder
$MgCl_2$	Magnesium chloride
MHC	Major histo-compatibility
microRNA	Micro ribonucleic acid
MIP	Macrophage inhibitory protein
MMLV-RT	Moloney murine leukaemia virus reverse transcriptase
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Mid secretory
MT-MMP	Membrane-type matrix metalloproteinase
NCBI	National Center for Biotechnology Information
NFκB	Nuclear factor kappa B
NRF-1	Nuclear respiratory factor-1
NTP	Nucleoside triphosphate
Oligo-dT	oligodeoxythymidylic acid
P	Pinopode
P	Probability
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCD4	Programmed cell death factor 4
PEA-3	Polyomavirus enhancer activator-3
PEG	Polyethylene glycol
PI3 kinase	Phosphatidylinositol 3-kinase
PL	Placental lactogen
PLC-β	Phospholipase C-β
PMT	Photon multiplier tube
PR	Progesterone receptor
PTX	Pertussis toxin
RANTES	Regulated upon activation, normally T-expressed and secreted[2]
RDC1	Chemokine orphan receptor 1
RIF	Recurrent implantation failure
RM	Recurrent miscarriage
RNA	Ribonucleic acid
ROX	6-carboxyl-X-rhodamine
RPL13A	Ribosomal protein L13a
rRNA	Ribsomal ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SDF-1	Stromal cell derived factor-1
SDHA	Succinate dehydrogenase complex, subunit A
ST	Syncytiotrophoblast
STAT	Signal transducer and activator of transcription
SYBR	Synergy brands
T	Thymine
TBE	Tris/borate/EDTA
TEMED	Tetramethylethylenediamine

TGF	Transforming growth factor
Th	T helper
TIMP	Tissue inhibitors of matrix metalloproteinases
TNF	Tumour necrosis factor
TRIS	Tris (hydroxymethyl)-aminomethane
Tyr	Tyrosine
UBC	Ubiquitin C
uNK cell	Uterine natural killer cell
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
vEVT	Vascular extravillous trophoblast
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide
YY1	Ying Yang 1
ZP	Zona pellucida

Abstract

The function of the human endometrium is to accept the implanting embryo and provide an environment for its subsequent growth and development by forming the maternal side of the placenta. The endometrium undergoes rapid cyclic changes of cell proliferation, differentiation and renewal and briefly becomes receptive to the embryo allowing its attachment and subsequent invasion during the implantation window. These processes are controlled systemically by steroid hormones which are thought to activate and control local molecular effectors including chemotatic cytokines known as chemokines. Chemokines are known to recruit leukocytes by chemotaxis, but also have a wider multifunctional in the control of cell function.

Limited studies have shown expression of the chemokine ligand CXCL12 and its receptor CXCR4 in the human endometrium. However, little is known about the precise role of these molecules in endometrial function. To investigate this further, real-time RT-PCR was used to measure the expression of CXCL12 and CXCR4 mRNA in endometrial biopsies throughout the menstrual cycle. Both CXCR4 and CXCL12 were expressed by the endometrium at all stages of the cycle. Expression of CXCL12 mRNA was relatively low and did not change throughout the menstrual cycle. However, CXCR4 mRNA significantly increased during the early proliferative phase of the cycle, with a second slight increase during the mid secretory phase.

Expression of CXCL12 and CXCR4 in cultured human endometrial epithelial and stromal cells was investigated using real-time RT-PCR used to measure levels of CXCL12 and CXCR4 mRNA expression. CXCL12 expression was found to be higher in stromal cells compared to epithelial cells, while expression of CXCR4 was highest in the endometrial epithelial cells. Expression of CXCR4 mRNA was also investigated in Ishikawa and HEC-1-B cell lines. Both cell lines were found to express CXCR4 mRNA, though levels were higher in Ishikawa cells compared to HEC-1-B cells, which was more comparable to their primary epithelial cell counterparts.

Although the functions of CXCL12 in the endometrium remain unknown, CXCL12 has been shown to increase the expression of interleukin 6 (IL6), interleukin8 (IL8), matrix metalloproteinase 2 (MMP2), matrix metalloproteinase 9 (MMP9) and vascular endothelial growth factor A (VEGFA) in various non-endometrial cell types. As all of these factors have previously been shown to be of potential importance to endometrial function, their basal mRNA expression was quantified in primary epithelial and stromal endometrial cells and Ishikawa and HEC-1-B cell lines using real-time RT-PCR. To investigate effects of CXCL12 on these endometrial factors, all four endometrial cell types were incubated with CXCL12 and the expression of IL6, IL8, MMP2, MMP9 and VEGFA was measured using real-time RT-PCR. Basal levels of IL8 and MMP9 mRNA were found to be significantly higher in endometrial epithelial cells in comparison to stromal cells, while VEGFA was significantly higher in stromal cells in comparison to epithelial cells. Expression of IL6, IL8, MMP2 and MMP9 was several orders of magnitude lower in the Ishikawa and HEC-1-B cell lines in comparison to their primary epithelial counterparts suggesting that these cell lines are not good models to investigate endometrial function. Incubation with CXCL12 had no significant effect on the expression of any of these factors, in primary epithelial, stromal, Ishikawa or HEC-1-B cells, which suggests CXCL12 may not regulate their expression in the endometrium.

To investigate the potential wider effects of CXCL12 on the expression of endometrial factors, a genome-wide microarray expression analysis was carried out in Ishikawa cells. Incubation of these cells with CXCL12 resulted in the identification of fifty one mRNA transcripts that were shown to be significantly and reproducibly up-regulated by CXCL12. Fourteen of these transcripts were produced by unknown genes, but thirty seven transcripts could be identified and potential functions attributed to them. Several of these genes have previously been shown to be of potential importance to endometrial function or to be known components of the CXCR4 signaling pathways.

Overall, the results of this study have increased our knowledge of the expression and cellular distribution of CXCL12 and CXCR4 in the endometrium. This study has also identified several potential roles for CXCL12 and CXCR4 in endometrial function which would warrant further investigation.

Chapter 1
General Introduction

1.1 The human female reproductive tract

The functions of the human female reproductive tract are to facilitate fertilisation of the oocyte by spermatozoa and to provide a suitable environment to allow implantation and the subsequent development of the resulting embryo (Johnson and Everitt, 1995, Chapter 5). The morphology of the human female reproductive tract is shown in Figure 1.1 and can be divided into distinct components. The ovaries are responsible for the cyclic release of oestrogen and progesterone which control female reproductive function and are the sites of episodic oocyte maturation and release. The function of the fallopian tubes (oviducts) is to transport released oocytes from the ovaries to the uterus. They consist of the fimbria, which are adjacent to the ovaries followed by the ampullary and isthmus regions of the tube which connects to the uterus. The ampullary region is the site of fertilisation where spermatozoa travel to meet the oocyte following their deposition in the vagina. The fallopian tube is also the site of early embryonic development that occurs prior to implantation.

The processes of implantation and placentation take place in the uterus. It is the site of embryonic and fetal growth and development (Johnson and Everitt, 1995, Chapter 5). The upper portion of the uterus is termed the body and the fundus, which is the part of the uterine body that extends above the junctions of the fallopian tubes. The lower portion of the uterus is termed the cervix. The uterus is covered with a connective tissue layer called the peritoneum which extends laterally to form the broad ligaments from which the ovaries are suspended. The wall of the uterus is composed of a thick smooth muscle layer termed the myometrium and a vascular mucosal layer called the endometrium which varies in thickness through the phases of the menstrual cycle and is the first point of maternal contact for the implanting embryo. Adjacent to the cervix is the vagina, which are the sites of spermatozoa deposition and capacitation but also allows the delivery of the fully developed infant.

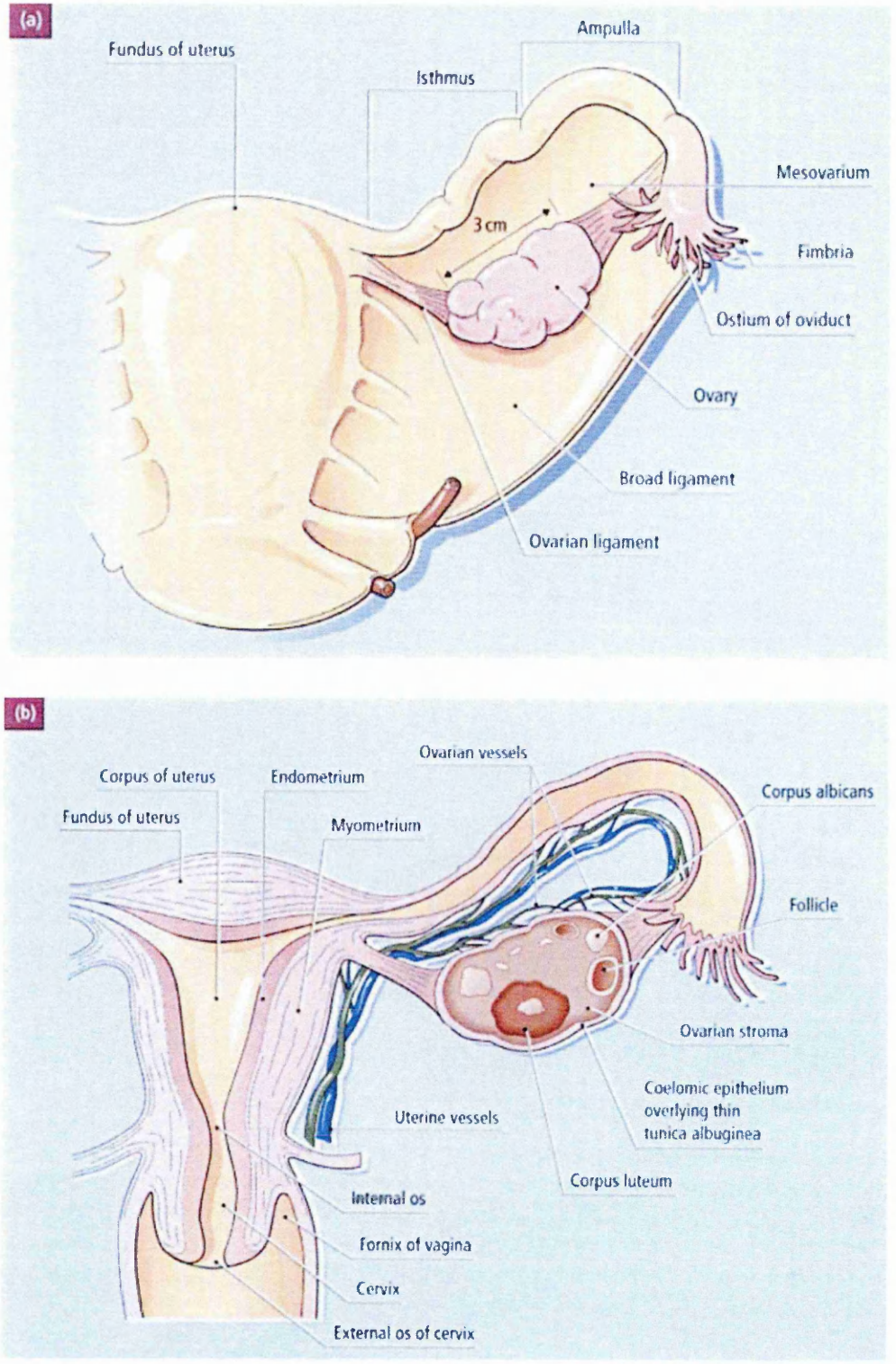


Figure 1.1: Anatomy of the female reproductive tract (Taken from Johnson and Everitt, 1995, Chapter 5)

The major components of the female reproductive tract showing; the ovaries, fallopian tubes, uterus, endometrium and cervix.

1.1.1 The ovaries and oocyte maturation and release

The adult human ovary contains an inner vascular medulla containing blood vessels which branch off into smaller blood vessels that penetrate the surrounding outer cortex (Sadow, 1980). The cortex is composed of stromal cells and contains the primordial follicles. The follicles are the site of oocyte maturation and are considered the fundamental functional unit of the ovary. The primordial follicle consists of flattened mesenchymal cells known as granulosa cells, condensed around a primordial germ cell which becomes the primary oocyte.

At birth each ovary contains approximately $2x10^6$ primary oocytes, the majority of which undergo atresia during infancy (Jonhson and Everitt, 1995, Chapter 5). By the onset of puberty approximately only 40,000 oocytes remain. Following the onset of puberty, a small number of follicles are recruited daily into further growth resulting in a process of continuous follicular growth and maturation. Between the onset of puberty and menopause the ovarian cycle allows the release of one mature oocyte from the ovarian follicle approximately every 28 days, a process that will occur approximately 400 times in a human females reproductive life (Chabbert-Buffet and Bouchard, 2002). Figure 1.2 illustrates the process of follicular development, which occurs during the ovarian cycle. The first half of this cycle is termed the follicular phase and is the period during which ovarian follicles mature leading to ovulation. The latter half of the cycle is termed the luteal phase in which the corpus luteum forms.

When a primordial follicle is selected to recommence growth, it passes through three developmental phases prior to ovulation (Johnson and Everitt, 1995, Chapter 5). It first becomes a primary preantral follicle before developing into a secondary antral follicle and finally becomes a mature tertiary follicle just prior to ovulation itself. The duration of these phases varies with the preantral phase being the longest and the preovulatory phase the shortest. The preantral phase is characterised by an increase in the diameter of the primordial follicle. The primary oocyte is responsible for much of this growth as it reaches its final size. The oocyte remains in a state of meiotic prophase but synthesises large amounts of mRNA and rRNA to facilitate protein production, which is vital for the later stages of

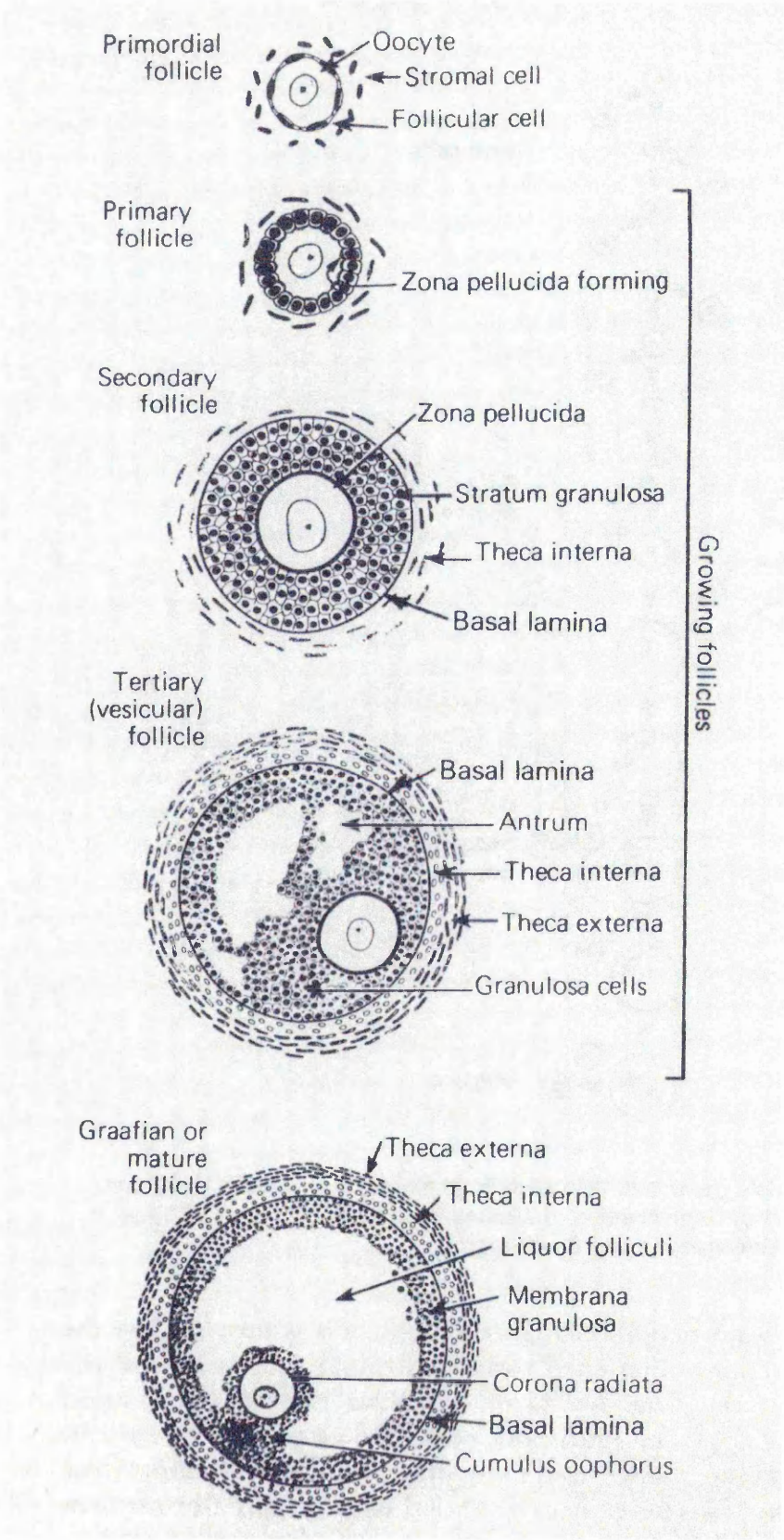


Figure 1.2: Maturation of human ovarian follicles (Taken from Findlay, 1984)

Shows the transition from the primordial follicle to the mature follicle prior to oocyte release.

oocyte maturation and the first days of development of the fertilised oocyte. In the preantral to antral transition, granulosa cells continue to proliferate resulting in a further increase in follicular size. This is accompanied by the appearance of follicular fluid between the granulosa cells. This is comprised partly of granulosa cell secretions and partly of serum transudate. The fluid coalesces to form a single follicular antrum marking the onset of the antral phase.

When the maturing antral follicle is ready to enter the preovulatory phase, the pituitary hormones, luteinising hormone (LH) and follicle-stimulating hormone (FSH) are required to complete the development of the maturing oocyte (Johnson and Everitt, 1995, Chapter 5). Follicular receptors for LH and FSH first appear on cells in the late preantral and early antral phases. In the early antral phase, only cells of the theca interna bind LH while the granulosa cells bind FSH. The antral follicles produce increasing amounts of steroids as they grow under the influence of the gonadotrophins LH and FSH. The main oestrogens produced by the antral follicles are oestradiol 17β and oestrone. The antral follicles also account for the production of 30-70% of the androgens, androstenedione and testosterone that circulate in human females, with the remaining androgen production coming from the adrenal gland. Thecal cells are thought to synthesise androgens and oestrogens from acetate and cholesterol and LH is known to stimulate this process. Granulosa cells are capable of the aromatization of androgens to produce oestrogens, a process stimulated by FSH. The oestrogens, progestagens and androgens can all be detected in the follicular fluid as well as systemically in the blood and are thought to have intrafollicular roles as well as implementing their wider effects. A positive feedback mechanism in which oestrogens produced in the follicle bind receptors on granulosa cells causing their proliferation and leading to further conversion of androgens to oestrogens, leads to further granulosa cell proliferation. This process culminates towards the end of antral expansion in a surge of circulating oestrogens produced by the most advanced follicles. This surge in circulating oestrogens causes a rapid increase in FSH levels by acting on the pituitary and hypothalamus by a positive feedback mechanism. The FSH surge occurs on approximately day 14 of the menstrual cycle with levels peaking approximately 18 hours before ovulation.

Just prior to the onset of the preovulatory phase, receptors for LH appear on the outer layer granulosa cells in response to increased levels of oestrogen and FSH. Approximately 18 hours prior to oocyte release there is a rapid surge in LH levels that is responsible for causing ovulation. LH binds LH receptors on both the granulosa cells and the thecal cells, causing the antral follicle to enter the preovulatory phase. If this does not happen the antral follicle dies.

The LH surge causes a rapid preovulatory growth phase in the oocyte and its subsequent release into the fallopian tube at ovulation, which leaves the follicle to become a corpus luteum once the oocyte is released at ovulation (Johnson and Everitt, 1995, Chapter 5). As the length of the follicular phase can vary and ovulation does not always occur precisely on day 14 of the menstrual cycle, a more precise means of describing the day of ovulation is to refer to it as LH+0 (day of the LH surge) with subsequent days numbered accordingly (e.g. day 20 of the 'ideal' menstrual cycle would be termed LH+6) (Li *et al.*, 1988).

Corpus luteum formation occurs during the luteal phase of the ovarian cycle (Jones and Lopez, 2006). The post-ovulatory follicle becomes a collapsed sac. The initial post-ovulatory structure is termed the corpus haemorrhagicum due to blood clot formation on its surface. The luteinised granulosa cells within the collapsed follicle begin to divide and enter the antral cavity to form the corpus luteum. This is penetrated by blood vessels from the thecal layer which grows into the central luteal cell mass. LH is essential for corpus luteum function in human females and cells of the corpus luteum secrete high amounts of progesterone and moderate amounts of estradiol. The corpus luteum begins to degenerate just prior to menstruation but if pregnancy occurs it does not die and continues to function in the first trimester of pregnancy.

1.1.2 The human uterus

The uterus is an inverted pear-shaped organ situated in the pelvic cavity of the human female (Jones and Lopez, 2006). The uterus is supported by bands of connective tissue known as the uterine ligaments, which attaches the uterus to the pelvic wall on each side. Paired uterosacral ligaments attach the lower end of the uterus to the sacrum. Lateral cervical ligaments connect the cervix and vagina to the pelvic wall and round ligaments attach near the entrance of the oviducts to the lower pelvic wall. These

ligaments carry blood vessels and nerves to the uterus and serve to support the uterus and other pelvic organs in their normal position. The walls of the uterine cavity are composed of three layers of tissue. The external surface of the uterus is covered by a thin membrane called the perimetrium, a continuous layer, which covers the oviducts and other peritoneal organs. Inside the perimetrium is a thick layer of smooth muscle known as the myometrium. The myometrium is capable of undergoing strong contractions during labour and also exhibits contractile patterns throughout the menstrual cycle. The final innermost layer of tissue within the uterus lines the myometrium and is known as the endometrium.

1.1.3 The human endometrium

The human endometrium is composed of two layers which form the innermost walls of the uterine cavity. It is composed of two major cell types: epithelial cells and stromal cells (Jones and Lopez, 2006). The epithelial layer is composed of a mixture of ciliated and secretory simple columnar epithelial cells on the endometrium's inner surface which fold inwards to form the luminal epithelium and is the first point of contact for the implanting blastocyst. Glandular epithelium is formed by the budding of luminal epithelium into distinct structures. The endometrial glands secrete a wide range of proteins and other factors that are thought to contribute to the function of the endometrium and provide nutrition to the embryo in the earliest stages of pregnancy (Gray *et al.*, 2001). Stromal fibroblasts are found beneath the luminal epithelial layer and surround the glandular epithelium to form the main body of the endometrium (Sadow, 1980). A population of endometrial leukocytes is present in the endometrial stroma. The upper layer of endometrium is known as the stratum functionalis and acts as the functional layer of the endometrium. It is this layer of the endometrium that is shed during menstruation. It contains characteristic spiral arteries which provide blood supply. The stratum basale is the basal layer of the endometrium and is connected to the uterine smooth muscle layer known as the myometrium. This layer of the endometrium is not shed during menstruation but provides the base for endometrial proliferation following menses at the beginning of a new menstrual cycle. Endometrial blood supply is provided by the uterine arteries. Basal arteries provide the stratum basale with a continuous supply of blood while spiral arteries permeate

the stratum functionalis and are shed with the rest of this layer during menstruation. Steroid hormones produced within the ovaries tightly regulate the proliferation and cellular activity of the endometrium to facilitate its function, which is to allow the implantation of the embryo during the implantation window (Figure 1.3).

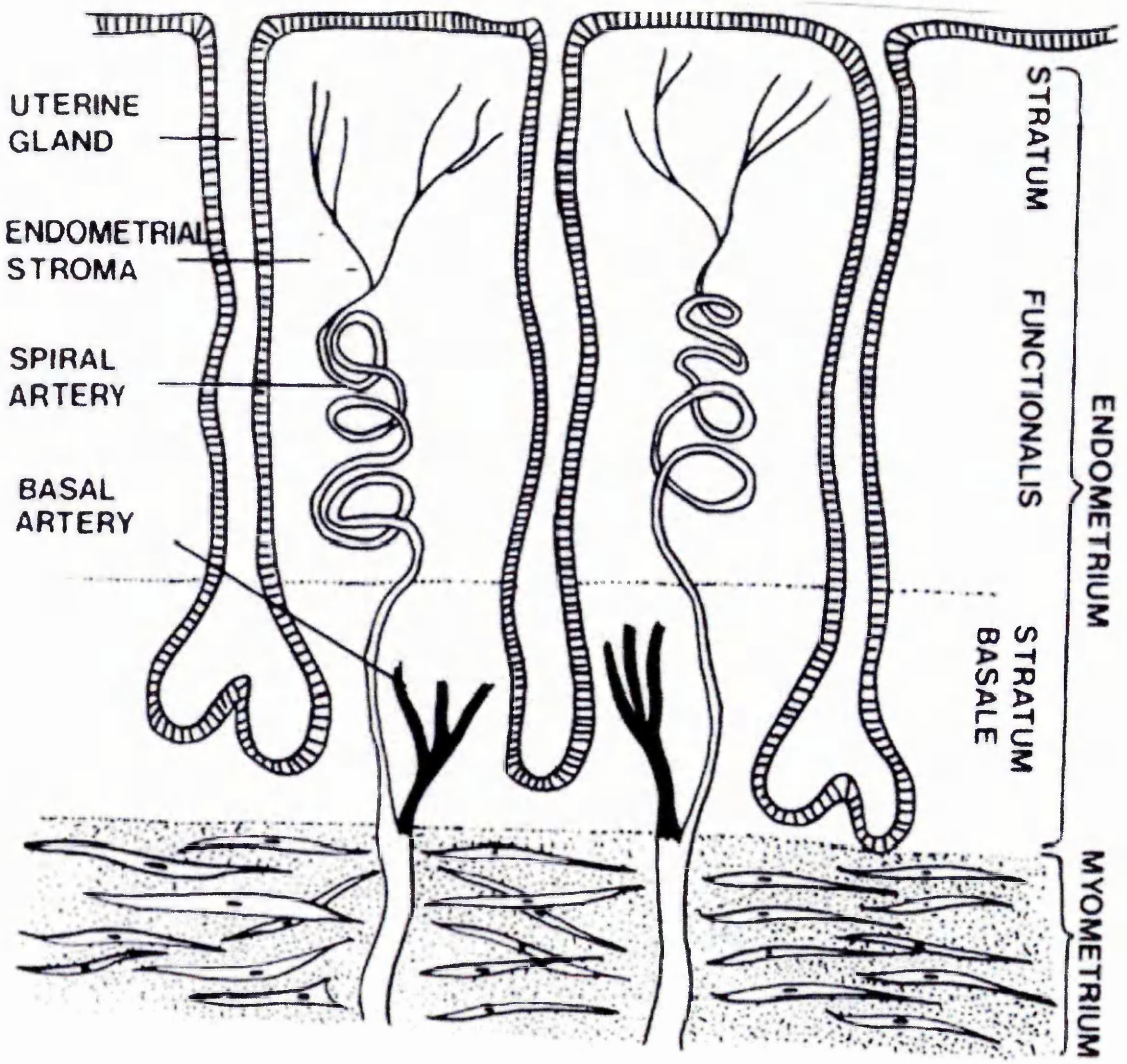


Figure 1.3: The structure of the human endometrium (Taken from Sadow, 1980)

Showing the layers of the endometrium, spiral and basal arteries and the smooth muscle myometrium.

1.1.4 The Menstrual cycle

The human endometrium is dynamic. Endometrial morphology and function undergo characteristic changes with each menstrual cycle (Chabbert-Buffet and Bouchard, 2002; Jabbour *et al.,* 2006 and Johnson and Everitt, 1995, Chapter 5). The human female menstrual cycle lasts for approximately 28 days with a range of 25 to 35 days considered to be normal. Changes in ovarian oestrogen and progesterone levels are responsible for the regulation of a cascade of locally acting factors within the endometrium which controls both endometrial thickness and its receptivity to implantation by the embryo. Endometrial thickening occurs in response to the oestrogen surge in the latter half of the follicular phase of the ovarian cycle. Oestrogens bind receptors found throughout the endometrium and causes endometrial thickening due to a proliferation of stromal cells. This is often termed the proliferative phase of the menstrual cycle (days 0-14). Endometrial thickening must occur to provide an adequate environment for the implanting embryo with a suitable blood and nutrient supply for further development (Figure 1.4).

One of the molecular effects of the oestrogen surge is to increase the expression of endometrial progesterone receptors in the endometrium, which bind progesterone during the luteal phase of the ovarian cycle (Chabbert-Buffet and Bouchard, 2002; Jabbour *et al.,* 2006 and Johnson and Everitt, 1995, Chapter 5). Progesterone acts on the endometrium to cause cellular differentiation and initiates the release of endometrial mucosal secretions by the epithelial cells in the endometrial glands. These secretions are rich in glycoproteins, sugars and amino acids. Progesterone also acts to increase the proliferation and size of the stromal cells and stimulates full development of the spiral arteries. This latter phase of the menstrual cycle is often termed the secretory phase (days 15-28) and acts to allow the endometrium to receive the implanting blastocyst. If pregnancy does not occur and under the influence of falling progesterone levels the death of endometrial tissue occurs. Shedding of the secretory epithelium and stratum functionalis occurs along with blood from ruptured spiral arteries via the cervix and vagina in the process termed menstruation. Menstruation marks the beginning of a new menstrual cycle following a brief rise in FSH which is thought to initiate the new ovarian and therefore endometrial cycles.

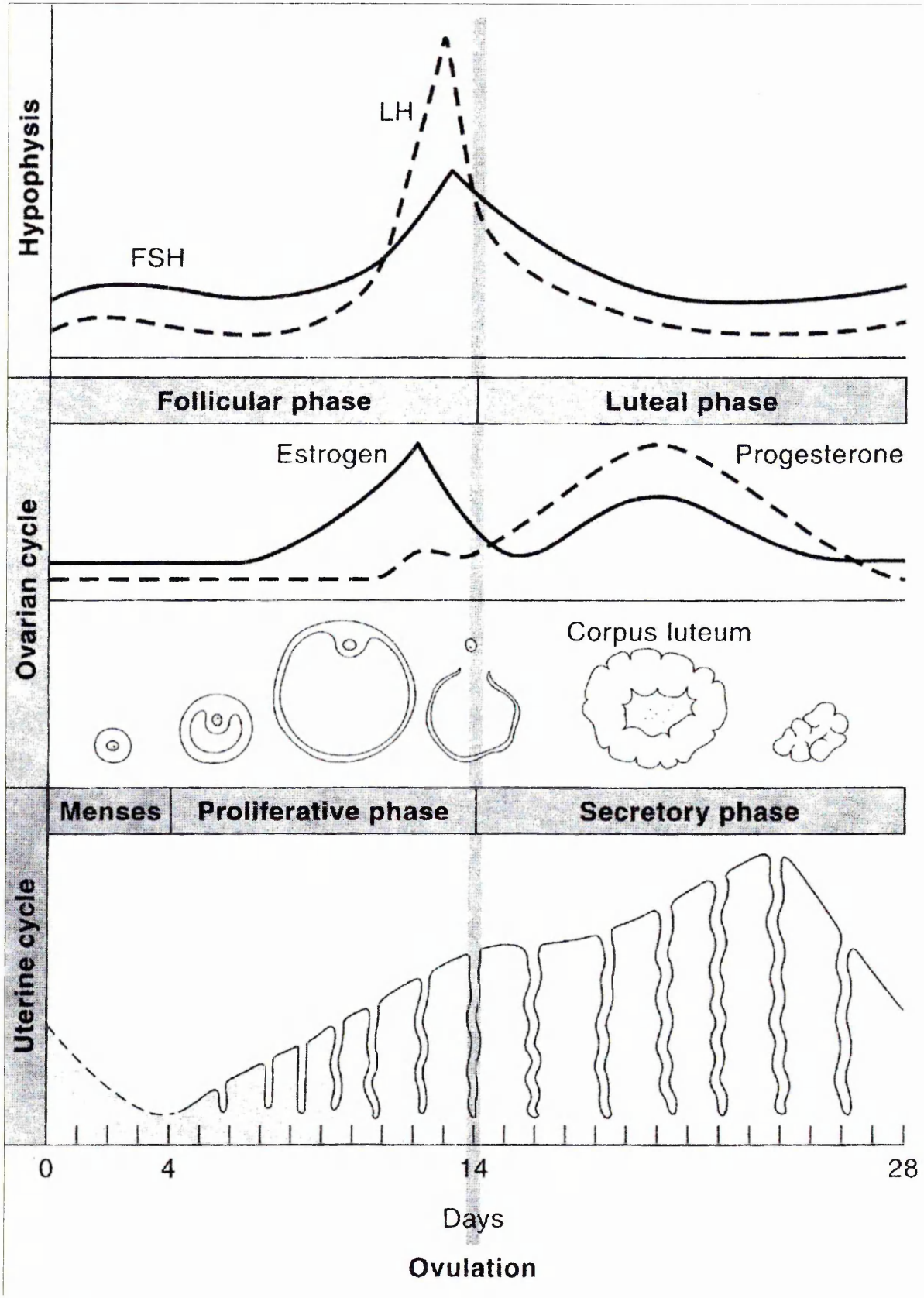


Figure 1.4: Effects of cyclic hormonal changes on the menstrual and ovarian cycles (Taken from Jones and Lopez, 1984)

Effective human female reproductive function relies on coordinated hormone release to control the progression of the ovarian and uterine cycles.

1.1.5 Morphological changes in the endometrium throughout the cycle

Microscopy has revealed that the cellular structure of the endometrium changes throughout the menstrual cycle in a characteristic manner (Noyes *et al.*, 1950). During the early proliferative phase of the menstrual cycle (days 4-7), the epithelial surface is observed as very thin prior to its regeneration following menstruation. The epithelial glands are generally short, straight and narrow and the surrounding stromal cells appear as compact and spindle shaped with large nuclei. As the endometrium develops into the mid-proliferative phase (days 8-10) the surface epithelium becomes columnar and the endometrial glands become longer, there is also evidence of stromal oedema. The late proliferative phase (days 11-14) is characterised by active growth and pseudostratification of the epithelium and an increase in stromal density.

The secretory epithelial cells are either elongated or polygonal and vary in size. The microvilli are short but develop as the endometrium moves through the proliferative phase (Nikas *et al.*, 1999a). By day 16 the epithelial glands have increased in size and tortuosity and by day 17 the microvilli are long, thick and upright. The appearance of subnuclear vacuoles by the nucleus of the glandular epithelium is also important for dating endometrial samples at this time (see B and C, Figure 1.5). By day 18 the microvilli appear to be swollen and by day 19 they begin to fuse and disappear and are replaced by smooth membrane projections which fold in on themselves to form pinopodes which appear on day 20 (Nikas *et al.*, 1999b). Pinopodes are progesterone dependent organelles which are visible between days 20 and 21 of the menstrual cycle. The functional significance of pinopodes appearing during the implantation window still remains unclear but they are thought to possibly play a role in the adhesion of the blastocyst to the endometrial epithelium. Following their appearance for 24-48 hours the pinopodes are replaced by reappearing microvilli (Cavanga and Mantese, 2003). It is at this time in the menstrual cycle that intraluminal secretory material peaks and there is the onset of massive stromal oedema. On day 23, the spiral arteries have become more prominent which indicates the predecidualisation of the stroma and by day 24 there are collections of predecidual cells around the arterioles with continued stromal proliferation. Differentiation of the predecidua continues into day 26 by which time the cells have developed into decidual cells. If by day 24, pregnancy has not occurred, the glandular secretions cease and the epithelium

undergoes involution while the glands dilate. There is a characteristic increase in the abundance of polymorphonuclear leukocytes by day 27 (Bulmer *et al.*, 1991) and areas of focal necrosis and haemorrhage become apparent a few hours prior to the onset of menses (Noyes *et al.*, 1950).

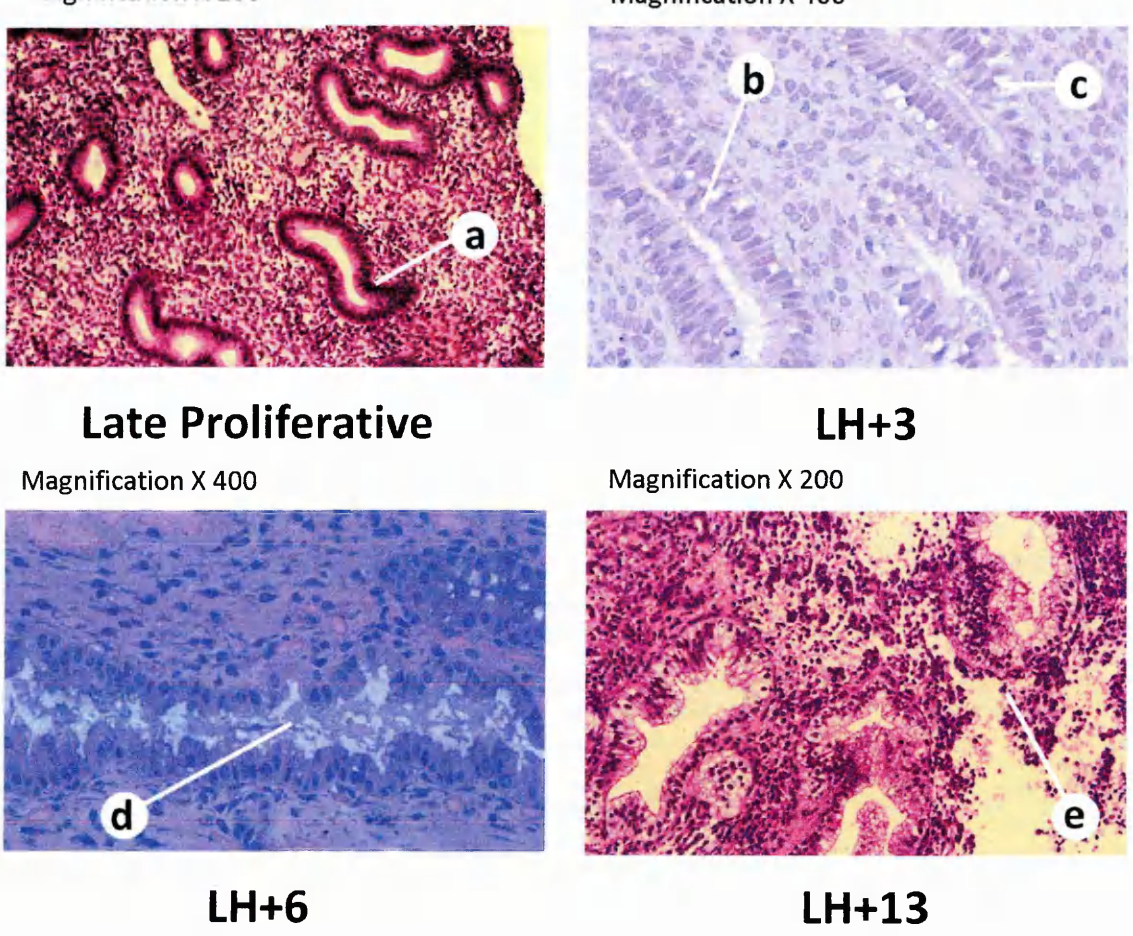


Figure 1.5: Morphological changes of the endometrium through the menstrual cycle (Images courtesy of Dr Susan Laird)

Characteristic changes in endometrial cell morphology can be observed through the menstrual cycle. (a) During the proliferative phase of the cycle the endometrial glands are compact with the majority of the endometrial tissue composed of stromal cells. (b) Following the LH surge and under the influence of progesterone the endometrial glands become visibly larger occupying more of the endometrial tissue space. (c) By LH+3 the presence of secretary vesicles within the glandular epithelial cells can be noticed. (d) By LH+6 secretory material is released from the glandular epithelial cells and accumulates within the lumen of the endometrial glands. (e) Following the end of the implantation window and providing pregnancy does not occur; oedema occurs in the stromal compartment. The epithelial cell layer collapses and along with cells of the stratum functionalis start to die as menses begins.

1.2 Implantation

Human embryo implantation is a complex process and requires synchronous development of a receptive endometrium and activated blastocyst (Hannan *et al.*, 2006).

1.2.1 Gamete transport to the site of fertilisation

Following coitus and spermatozoa deposition in the vagina, spermatozoa travel into the cervix and uterus under their own propulsion and aided by currents of fluid created by the action of uterine cilia (Johnson and Everitt, 1995, Chapter 10). Spermatozoa migrate to the isthmus of the oviduct and have been identified in the oviduct as soon as 2-7 hours post-coitus. The number of living spermatozoa found in the oviduct rarely exceeds several hundred. Although it is not known how the flow of spermatozoa to the oviduct is regulated it is thought the cervical crypts may act as a reservoir to control sperm release into the uterus and that the uterotubal junction may regulate entry into the oviduct by action as an intermittent sphincter. Upon reaching the isthmus of the oviduct, spermatozoa become immotile and linger only re-acquiring motility at ovulation where they swim to the ampullary-ishthmic junction, the site of potential fertilisation.

While spermatozoa are moving towards the oviducal ampulla, the ovulated oocyte is picked up from the ovary surface in the peritoneal cavity by the fimbriated ostium of the oviduct and swept by oviducal cilia along the ampulla towards the junction of the isthmus.

It is only when spermatozoa reach the uterus and oviduct that they reach their full capacity to fertilise the oocyte (Johnson and Everitt, 1995, Chapter 10). This process is called capacitation and involves the stripping of glycoprotein molecules from the spermatozoa surface. Providing spermatozoa are present when the oocyte reaches the ampullary-ishthmic junction, a capacitated spermatozoon can fertilise the occyte causing maternal and paternal chromosomes to come together to form the zygote.

1.2.2 Blastocyst Development

Upon zygote formation cell cleavage occurs to form a two-cell stage embryo which remains in the oviduct for 3-4 days where each of the two cells undergoes a series of divisions (Johnson and Everitt, 1995, Chapter 10). Once the 8-16 cell stage is reached the embryo undergoes compaction resulting in the formation of a morula, which is complete by the time the developing embryo reaches the uterine cavity. The morula becomes polarised following the minimising of intracellular contacts and two cell types are formed. This initial cellular differentiation allows the formation of the inner cell mass and the trophoectoderm cells. This process occurs at the 32-64 cell stage and it is at this stage that the embryo is termed the blastocyst.

The trophoectoderm forms an outer rim of cells. This is the first extra-embryonic tissue and it is termed the trophoblast. The cells that form the trophoblast are responsible for the initial contact at the feto-maternal interface which continues to develop to become the fetal component of the placenta. The trophoblast cells surround a blastocoelic cavity which contains blastocoelic fluid and the inner cell mass, the inner cell mass moves to reside within the trophoblastic wall. It is this part of the trophoblast that makes contact with the endometrium at the luminal epithelium.

The blastocyst enters the uterine cavity approximately 3-4 days after ovulation. It then floats freely within the uterine cavity for a further 3-4 days before implanting (Johnson and Everitt, 1995, Chapter 10). Nutritional support for the blastocyst is provided by secretions from the glandular epithelium of the endometrium promoted by progesterone during the secretory phase of the menstrual cycle. Approximately 6 days post-ovulation (LH+6), the zona pellucida of the blastocyst comes away and exposes the trophoblast allowing the inner cell mass of the embryo to implant into the endometrial epithelium (Figure 1.6).

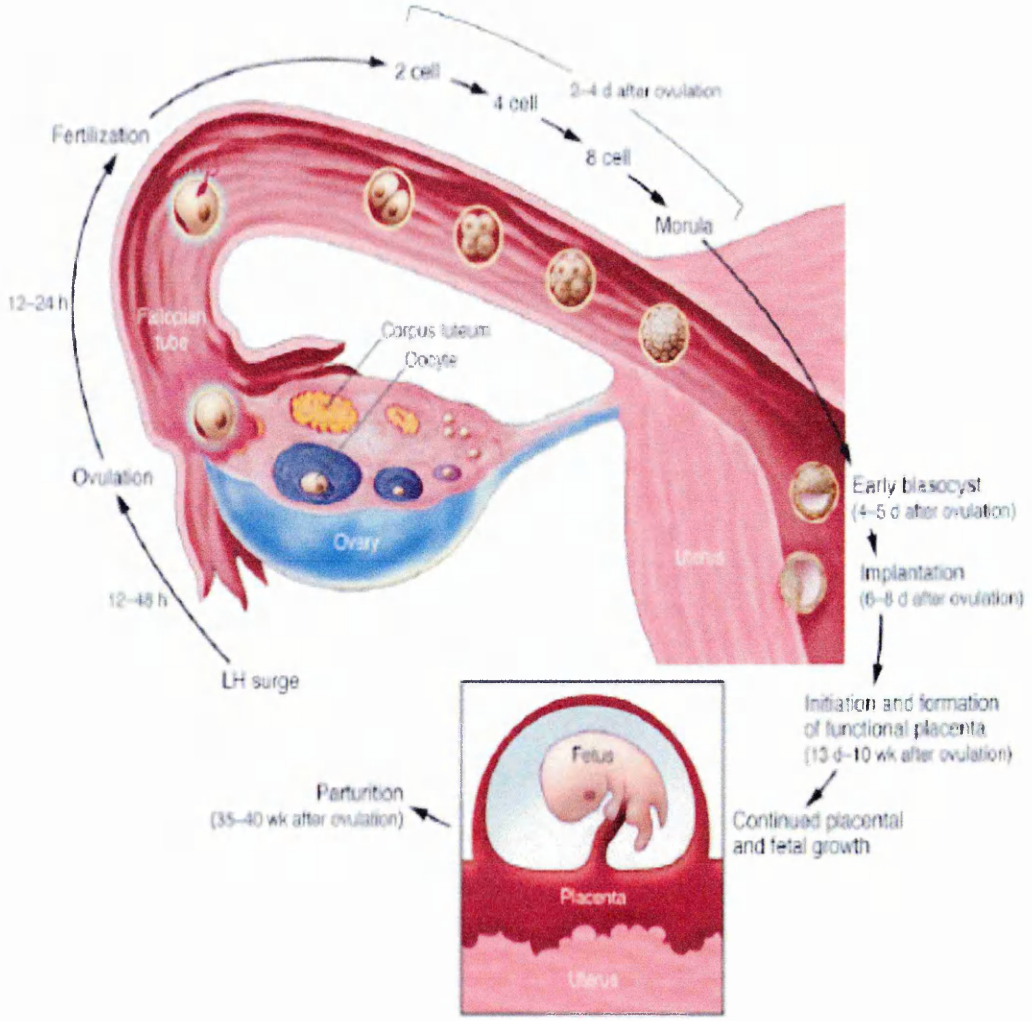


Figure 1.6: Diagram showing the development of the embryo prior to endometrial implantation (Taken from Dey et al., 2010)

Showing oocyte release following the LH surge, fertilisation in the fallopian tube and subsequent development prior to implantation in the receptive endometrium leading to placental formation and subsequent parturition.

1.2.3 Endometrial receptivity to implantation

For successful human embryo implantation to take place, the endometrium must be receptive to the apposition, adhesion and invasion of the blastocyst (Achache and Revel, 2006 and Cavanga and Mantese, 2003). The endometrium is only receptive to these processes for a short period of time which is termed the 'implantation window'. In humans, the implantation window is approximately four days long and occurs between days 20 and 24 of the menstrual cycle (LH+7 to LH+10). The preparation of the endometrial tissues to facilitate this process occurs under the influence of the steroid hormones as the endometrium develops through the proliferative phase under the influence of oestrogens and enters the secretory phase under the influence of progesterone. Steroid hormones bring about their effects in the endometrium by affecting the expression of many cytokines and growth factors that finely control the cyclic cellular processes of proliferation, differentiation and apoptosis within the endometrium. This is thought to be achieved using autocrine, paracrine and endocrine mechanisms. These factors must act together during the implantation window to facilitate a synchronised cross-talk between the functional blastocyst and receptive endometrium to allow successful blastocyst attachment to the endometrium's mucosal epithelium and subsequent invasion into the stroma.

1.2.4 The implantation process

The process of implantation consists of three distinct phases. The first phase is the apposition, in which the blastocyst correctly orientates itself towards the endometrial epithelium so that the inner cell mass faces the epithelial surface (Johnson and Everitt, 1995, Chapter 10). Following this, the zona pellucida (ZP) of the blastocyst is shed exposing the trophoblast cells. The process of ZP shedding is thought to be aided by two serine proteases named implantation serine protease 1 (ISP1) and ISP2. ISP1 is produced by the blastocyst while ISP2 is secreted from the glands of the endometrium. The blasotcyst itself undergoes metabolic activation at this stage, increasing mRNA production and preparing it for implantation and subsequent development (O'Sullivan *et al.*, 2001). As no direct contact between the blastocyst and endometrial epithelial surface occurs during the process of apposition, crosstalk must occur between the two

via soluble mediators including growth factors and cytokines such as LIF and IL11 (Hannan *et al.*, 2006) (Figure 1.7).

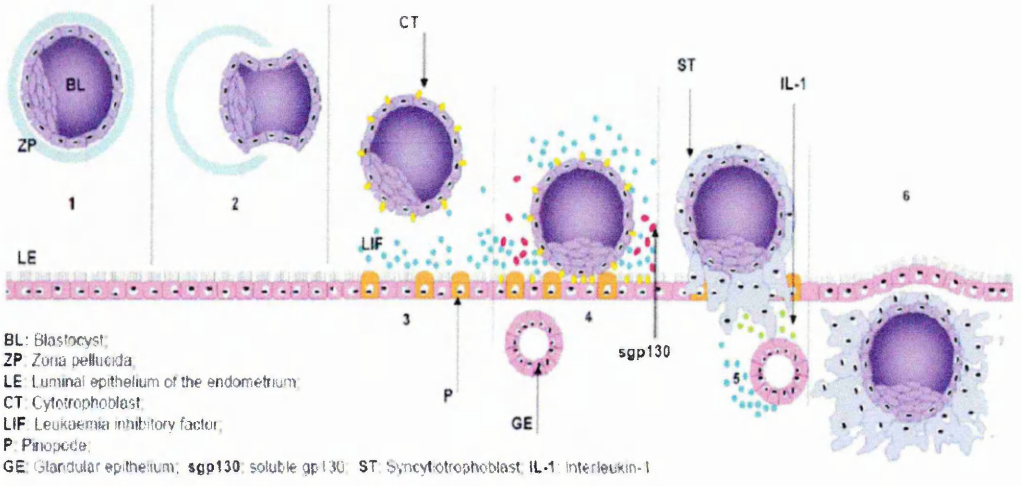


Figure 1.7: The implantation process (taken from Fitzgerald et al., 2008)

The figure shows a proposed scheme of blastocyst attachment and stromal invasion during the implantation window including implicated morphological and biochemical markers: (1) Representation of the free floating blastocyst (BL) surrounded by the zona pellucida (ZP). (2) Blastocyst hatching from the zona pellucida. (3) Gradual apposition of blastocyst to the endometrium during the onset of the implantation window accompanied by the presence of two biomarkers of implantation; Pinopode (P) formation and maximal endometrial expression of LIF (blue) which binds to its receptor on the surface of the blastocyst (green). (4) The blastocyst adheres to the endometrium. The endometrium itself secretes gp130 (red) and expresses the LIF receptor. (5) The syncytiotrophoblast (ST) invades the luminal epithelium of the endometrium and secretes cytokines such as IL1 which in turn stimulates LIF expression in the glandular epithelium (GE). (6) The implantation window is complete. The blastocyst has invaded the decidualising stroma and the epithelium of the endometrium has been restored (Fitzgerald *et al.*, 2008).

The second phase of implantation is the adhesion of the trophoblast cells of the blastocyst to luminal epithelial surface of the endometrium. It is at this time that the characteristic morphological markers of implantation, such as the presence of pinopodes and biochemical markers, including cellular adhesion molecules (CAMs), integrins and selectins are thought to be of greatest importance (Diedrich *et al.*, 2007) (see section 1.2.6). These adhesion molecules allow the trophectoderm to make and maintain contact with the endometrial epithelium, while cytokines such as Leukemia inhibitory factor (LIF), interleukin 1 (IL1) and interleukin 11 (IL11) are thought to maintain crosstalk between the trophoblast and decidualising stromal cells.

The final phase of the implantation process is invasion. Within a few hours following the attachment of the blastocyst to the endometrial epithelium, the epithelial cells that lie beneath the trophoblast cells become eroded allowing trophoblast invasion to occur (Johnson and Everitt, 1995). To facilitate the invading trophoblast and support subsequent development of the embryo the stromal fibroblasts undergo transformation to decidual cells (Fazleabas and Strakova, 2002). Morphologically the fibroblast-like stromal cells become polygonal and begin to express decidual proteins. This process is thought to be central to controlling the invasion of the trophoblast, providing the necessary nutrional support and protecting the embryo from maternal immune rejection.

Trophoblast is an embryonic tissue which plays a crucial role in the processes of implantation and placentation (Hannan *et al.*, 2006 and Lunghi *et al.*, 2007). The implanting embryo begins to form floating and anchoring villi which expands as pregnancy develops. The villi are composed of specialised trophoblast cell types named the syncytiotrophoblast (ST). The initial function of the ST is lytic and mediates early invasion. Later the ST acts as a barrier through which nutrient exchange can take place. Other trophoblast cells retain their phenotype and line the chorionic villi. These are known as cytotrophoblast (CT) cells and act as stem cells for the production of further ST cells. ST fuse cells together to form an external layer of non-proliferative multinucleated ST, which grows due to the steady incorporation of new cells provided by the CT and occurs as EVTs invade the maternal arteries. The ST functions to mediate the exchange of nutrients, gas and waste products between the fetal and maternal blood.

The ST possesses endocrine activity and releases hormones required for successful pregnancy including chorionic gonadotrophin (CG) and placental lactogen (PL). CG is known to directly contribute to corpus luteum rescue and also modulates the uterine environment (Cameo *et al.*, 2004 and Lunghi *et al.*, 2007). At approximately 14 days post implantation, CT cells penetrate through the syncytium, some cells remain at the tips of the villi as a solid core and are known as the cytotrophoblast cell column (CC). This anchors the placental villi to the decidua, establishing a physical connection between the fetus and mother. These columns spread laterally and form the CT shell from which, invading extravillous cytotrophoblast (EVT) cells originate (Hannan *et al.*, 2006 and Lunghi *et al.*, 2007).

CT cells break through the ST layer and differentiate into extravillous trophoblast (EVT) cells which begin to invade the uterine stroma as trophoblastic cell columns. At the distal portion of the cell column, cytotrophoblast cells differentiate into an invasive phenotype and then either invade the maternal blood vessels and become vascular EVTs (vEVTs) or migrate through the decidua as interstitial EVTs (iEVTs). The iEVTs invade the maternal spiral arteries where they contribute critically, to extensive remodeling. The vEVTs invade the maternal arteries at the point where the shell contacts the opening of the maternal vessels and aggregate to form plugs (Hannan *et al.*, 2006 and Lunghi *et al.*, 2007).

1.2.5 Decidualisation and placentation

Evidence found in rodents shows, within a few hours following the attachment of the blastocyst to the endometrial epithelium, the immediately underlying stroma undergoes an increase in vascular permeability, displays oedema and changes in extracellular matrix structure and stromal cell morphology (Johnson and Everitt, 1995, Chapter 10). This is accompanied by a progressive sprouting and ingrowth of capillaries. These stromal cell changes in response to blastocyst attachment are collectively called the primary decidualisation reaction. In the next 2-3 days following the initiation of the primary decidualisation reaction, further secondary decidualisation spreads throughout the endometrial stroma.

The process of decidualisation involves functional and morphological changes in endometrial stromal cells, uterine glands and vessels as well as endometrial leukocyte

populations (Lunghi *et al.*, 2007). In humans, decidualisation is independent of the blastocyst's presence in the uterine cavity and begins in the late secretory phase of the menstrual cycle and is thought to be evoked by progesterone.

Decidualisation continues during early pregnancy and is thought to regulate subsequent trophoblast invasion and formation of the placenta by altering expression of regulatory factors including; matrix metalloproteinases (MMPs), cytokines, surface integrins and major histo-compatibility (MHC) molecules (Hess *et al.*, 2006). The trophoblast releases paracrine signals in response to this, including cytokines, chemokines and angiogenic growth factors, which further modulates decidual stromal cell gene expression. Decidual stromal cell morphology changes to show ultrastructural similarities with myofibroblast and epithelial cells (King, 2000). They are known to release several factors including; prolactin, relaxin, rennin, insulin-like growth factor binding protein (IGFBP1) and specific extracellular matrix proteins such as laminin and fibronectin. The decidualised stromal cells themselves produce various growth factors and cytokines such as IL6 and IL8 and MMPs, which regulate trophoblast invasion under the general control of progesterone (Brar *et al.*, 2001 and Hess *et al.*, 2006). *In vitro* studies have demonstrated that this event correlates to changes in steroid hormone receptor expression and steroid metabolism, remodeling of the extracellular matrix (ECM) and cytoskeleton, altered expression of enzymes, growth factors and cytokines and also induction of apoptosis modulators and specific transcription factors.

Elongation of the maternal spiral arteries occurs through endometrial angiogenesis (Lunghi *et al.*, 2007). The decidual tissue that surrounds the invading trophoblast acts as a nutrient reservoir for the embryo which survives from using maternal secretions provived by the glandular epithelial glands of the decidualising endometrium (Burton *et al.*, 2002.) Maternal blood flow to the developing placenta is not fully established until the third month of gestation. This strongly suggests that initial fetal nutrition is histiotrophic in nature. The maternal uterine glands are present and remain active until at least week 10 of pregnancy during which time, their secretions are freely delivered freely into the placental intervillous space. Initial organogenesis of the fetus therefore occurs when metabolism is essentially anaerobic. However, this process of

histiotrophic nutrition soon becomes inadequate to support the growing embryo (Johnson and Everitt, 1995, Chapter 10).

To supply the developing fetus with the nutrients it requires as it grows, the decidualised endometrium develops a region of highly specialised and vascularised tissue (Johnson and Everitt, 1995, Chapter 10). The embryo also develops a corresponding vasculature and this zone of contact between the mother and the embryo is called the placenta. The endometrial epithelium becomes incorporated into the developing placenta which provides haemotrophic nutrition to the embryo by allowing the rapid exchange of materials between two distinct maternal and embryonic vasculatures. Placental formation is characterised by extensive proliferation of embryonic tissue to give a large surface area for material exchange, the presence of a highly developed vasculature between the fetal and maternal components and an intimately composed, but physically separate fetal and maternal blood flows.

1.2.6 Role of adhesion molecules in the implantation process

The endometrium's capacity to be receptive to the implantation of the blastocyst during the implantation window is associated with the expression of genes encoding proteins which function to aid the implantation process and can therefore act as biomarkers for the normal functioning of the receptive endometrium and the onset of the implantation window (Achache and Revel, 2006).

During blastocyst apposition, trophoblast cells adhere to the receptive endometrial epithelium. This process is thought to be facilitated by the presence of cellular adhesion molecules (CAMs) on the surfaces of the epithelial and trophoblast cells and provides an obvious target for the identification of implantation markers.

The CAM family consists of four subtypes of adhesion molecule; the integrins, selectins, cadherins and the immunoglobulin-like receptors. The classical function of these cell surface ligands includes; maintenance of tissue integration, wound healing and cellular migration. Both the integrins and selectins are thought to be of direct importance to the implantation process, while cadherins and immunoglobulin-like receptors are known to be expressed in the endometrium throughout the cycle and at

the implantation window yet their functional significance is still not fully understood in process of implantation itself.

Integrins are a family of transmembrane glycoproteins formed by the association of two non-covalently linked α and β subunits. Approximately 18 α chains and 8 β chains have been identified to date and can form 24 distinct integrin heterodimers which differ in function. The extracellular domain of integrins acts as a receptor to extracellular matrix (ECM) components, complement and other cells. The intracellular domain of integrins are able to interact with the cytoskeleton (Achache and Revel, 2006). A variety of integrins have been described within the luminal and glandular epithelium of the endometrium and the majority of them have been shown to be constitutively expressed throughout the menstrual cycle (Lessey *et al.*, 1992). However, the integrins α1β1 and α4β1 have been shown to be elevated during the implantation window and the integrin αVβ3 showed increased β3 subunit mRNA expression following day 19 and was undetectable beforehand. Due to its increased expression at the implantation window and its cellular location on the endometrial luminal epithelial surface, integrin αVβ3 has been proposed as a potential receptor for the attachment of the blastocyst (Apparao *et al.*, 2001 and Lessey, 2003).

Selectins are a family of glycoproteins that include P-selectin, L-selectin and E-selectin. Human L-selectin has been demonstrated to be of importance in the implantation process. It consists of a large highly glycosylated extracellular domain, a single transmembrane domain and a small cytoplasmic tail. L-Selectins are expressed on leukocytes and are known to interact with carbohydrate ligands expressed on inflamed vascular endothelium allowing the rolling of leukocytes over the endothelium before firm adhesion and transmigration. A parallel between this phenomena and blastocyst apposition can be made. Strong L-selectin staining over the embryo surface has been observed at the time of implantation (Genbacev *et al.*, 2003) and maternal expression of L-selectin ligands such as MECA-79 or HECA-452 is upregulated during the implantation window allowing the trophoblast cells to contact the endometrial epithelial surface. This process may constitute the initial step of the implantation process (Genbacev *et al.*, 2003 and Lai *et al.*, 2005).

1.2.7 Cytokines and growth factors involved in implantation

Cytokines and growth factors are cell derived polypeptides and proteins that have the capacity to bind cell surface receptors specifically and have the potential to act as intracellular signals that regulate the function of endometrial cells (Achache and Revel, 2006). They regulate cell proliferation, differentiation and apoptosis by autocrine, paracrine and endocrine mechanisms. Many cytokines are produced by the uterine mucosa and the embryo and may play a role in the maternal-embryonic interactions, enhancing endometrial receptivity by controlling the expression of adhesion molecules.

Classically cytokines have been classified according to the immune cell type from which they are produced and the effects their expression brings about (Laird *et al.*, 2003). CD4+ T cells are the major class of immune cells responsible for cytokine production and can be divided into three functional subsets based on their cytokine production. T helper 1 (Th1) cells produce interferon gamma (IFNϒ) and tumour necrosis factor beta (TNFβ) and contribute to the control of the cell-mediated response. Th2 cells produce IL4, IL5, IL6, and IL10 and contribute to the control the antibody mediated response. Th0 cells are precursor cells that can transform to either Th1 or Th2 cells and produce both types of cytokines as well as tumour necrosis factor alpha (TNFα) and granulocyte-macrophage colony stimulating factor (GM-CSF). Additional to the Th1/Th2 cytokines, the proinflammatory cytokines constitute another cytokine group which includes IL1, TNFα, IL6 and leukaemia inhibitory factor (LIF). They are produced by macrophages and are important in mediating responses to tissue damage and repair.

It has become clear that all these cytokines can be produced by non-immune cell types including epithelial and stromal cells of the endometrium and also decidual and cytotrophoblast cells (Laird *et al.*, 2003). As cytokines act locally and as T helper cells are a minor immune-cell population within the first trimester placental tissues, this suggests they are not the main source of cytokines present within the feto-placental unit. It is thought the Th2 type response is the predominant human maternal immune response during pregnancy (Wegmann *et al.*, 1993).

The cytokines IL1, LIF and IL11 have all been shown to be of importance in the process of implantation (Achache and Revel, 2006). LIF (Stewart *et al.* 1992) and IL11 (Robb *et al.*, 1998) are thought to be of particular importance as they have been shown to be the only two cytokines essential for successful implantation in mouse models. In humans, LIF and IL11 are thought to be involved in the control of early blastocyst interactions with the luminal epithelial surface of the endometrium and in the processes of decidualisation and the invasion of EVT (Achache and Revel, 2006). LIF (Arici *et al.*, 1995; Charnock-Jones *et al.*, 1994; Kojima *et al.*, 1994 and Sharkey *et al.*, 1995) and IL11 (Cork *et al.*, 2001 and Dimitriadis *et al.*, 2000) are maximally expressed in the glandular and luminal epithelium during the mid secretory phase of the menstrual cycle. IL11 (Cork *et al.,* 2001) and LIF (Kojima *et al.,* 1994) are also expressed in the stroma. *In-vitro* studies have identified a role for LIF and IL11 in the adhesion of the blastocyst to the endometrial epithelium by differentially affecting the expression of integrins (Achache and Revel, 2006). LIF and IL11 are also thought to play roles in the regulation of EVT invasion and decidualisation under the general control of progesterone potentially by modulating matrix metalloproteinase activity. LIF has also been shown to be produced by uterine natural killer cells (uNK) cells and may facilitate EVT trophoblast invasion via LIF receptor expression present on the cell surface of EVTs (Saito *et al.,* 1993).

The growth factors heparin binding-epidermal growth factor, colony-stimulating factor-1, insulin-like growth factor binding protein-1 and keratinocyte growth factor have all been shown to be of potential importance to the implantation process (Achache and Revel, 2006). Many other additional factors such as the mucins and prostaglandins are thought to be of importance in controlling endometrial receptivity at the time of implantation.

1.3 The endometrial leukocyte population

Effective ovarian and uterine function relies on a complex interplay between the endocrine and immune systems (Lea and Sandra, 2007). Ovarian leukocyte release has been shown to be central to ovarian follicle development, ovulation and corpus luteum formation. In the uterus, sex steroids cyclically regulate the number and distribution of endometrial and decidual immune cells and other immune factors. It is generally

accepted that oestrogens have a typical pro-inflammatory immune-reproductive effect while progesterone initiates an anti-inflammatory response (Wegmann *et al.,* 1993). The uterine mucosa is unique; it must tolerate the presence of commensal organisms, sperm and the allogenic blastocyst but not compromise immune surveillance against potential pathogens (Lea and Sandra, 2007).

The leukocyte population that exists within the endometrium and decidua is distinctly different to that of the peripheral blood (Laird *et al.*, 2003). Within the population there are essentially no B cells and very few neutrophils (Bulmer *et al.*, 1991; Johnson *et al.,* 1999 and Salamonsen and Lathbury, 2000). Instead, the endometrial and decidual leukocyte population consists of T cells, macrophages and uterine natural killer (uNK) cells (large granular lymphocytes). Figure 1.8 summarises the process of leukocyte migration.

The most predominant leukocyte in the endometrium and decidua are uNK cells. They are phenotypically different to those found in the peripheral blood (Bulmer *et al.,* 1991 and Laird *et al.*, 2003). They express CD56 and CD38 cell markers but not the classical T cell or NK cell markers; CD3, CD4, CD8, CD16 and CD57. However, a small proportion of uNK cells show similarity to peripheral blood NK cells and express minimal levels of CD56 and CD16. These cells are referred to as $CD56^{dim}$ uNK cells while the majority of the uNK cell population is $CD56^{bright}$.

The numbers and proportions of the endometrial and decidual leukocytes population varies throughout the menstrual cycle and in early pregnancy (Bulmer *et al.*, 1991). T cells make up approximately 45% of leukocytes found in the proliferative endometrium. Their numbers remain relatively constant throughout the menstrual cycle and in early pregnancy but their relative numbers decrease in comparison to the uNK cells as the cycle progresses. Macrophages make up 15-20% of endometrial leukocytes. Their numbers increase slightly during the secretory phase of the menstrual cycle and early pregnancy comprising 20% of the leukocytes found in the placental bed (Laird *et al.,* 2003).

It is the uNK cells that show the most dramatic changes in numbers through the menstrual cycle (Bulmer *et al.*, 1991 and Jabbour *et al.*, 2006). During the proliferative phase, their numbers are approximately equal to the T cell population but by the mid

secretory phase, they comprise 70% of the endometrial leukocyte population and are found in close contact with endometrial glands and spiral arteries. Numbers continue to increase further into early pregnancy.

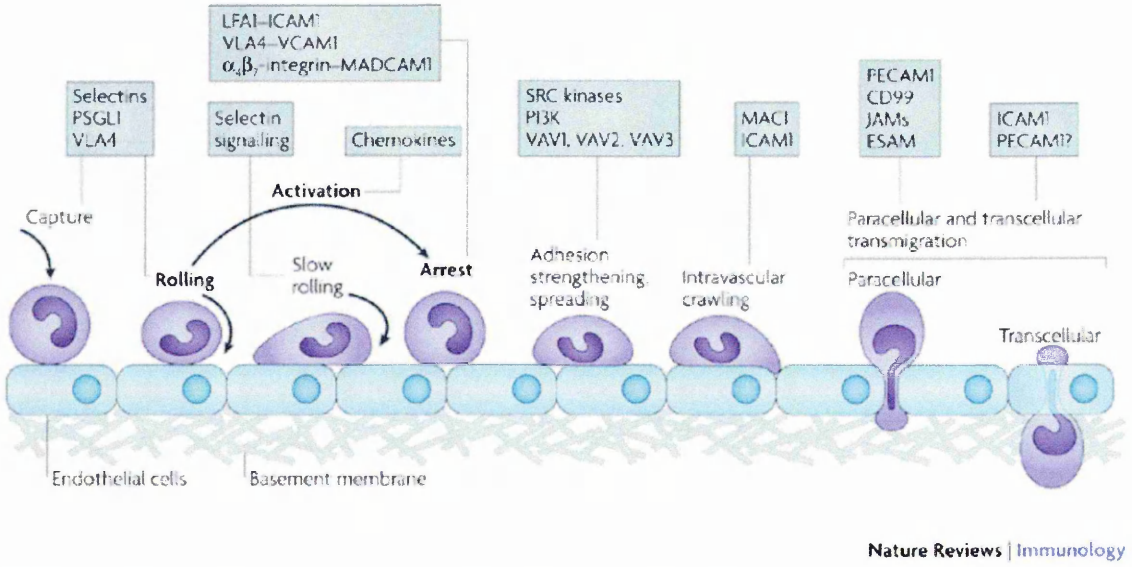


Figure 1.8: Summary of the process of leukocyte migration (Taken from Ley et al., 2007)

The process of leukocyte migration, including the stages of leukocyte capture, rolling and extravasation through the endothelial cells of blood vessels such as the spiral arteries of the endometrium.

1.3.1 uNK cell proliferation and trafficking

It has not yet been determined whether the increase in uNK cell number is solely due to *in situ* proliferation or due to *de novo* migration from the peripheral circulation. In mice there strong evidence that self-renewing proginators of uNK cells are derived from trafficking precursors which are found in peripheral lymphoid sites such as the spleen and lymph nodes (Chantakru *et al.*, 2002). In humans there is evidence for both the trafficking of uNK cells from the peripheral circulation and *in situ* differentiation and proliferation (Bulmer *et al.*, 2010). It has been shown that in late secretory phase endometrium and early pregnancy decidua, that uNK cells are found predominantly in the stratum functionalis and often form aggregates around spiral arteries and glands (Bulmer *et al.*, 1991). This perivascular distribution of uNK cells has been considered to be evidence of the trafficking of uNK cells or their precursors from the peripheral circulation (Trundley and Moffett, 2004). Some studies have focused on whether factors produced by the trophoblast may play a role in the trafficking of uNK cells. Molecules such as macrophage inhibitory protein 1α (MIP1α) (Drake *et al.*, 2001), MIP1β (Kitaya *et al.*, 2003) and CXCL12 (Hanna *et al.*, 2003), which are all expressed by trophoblast have all been shown to potentially fulfill this role. There are also arguments against the trophoblast playing a role in recruiting the uNK cells to the endometrium. uNK cells are known to be prominent in the endometrium in the absence of trophoblast in pre-menstrual endometrium and in intrauterine decidua in ectopic pregnancy. This suggests trophoblast does not recruit uNK cells as they are not found at the site of the tubal pregnancy (Von Rango *et al.*, 2001). uNK cells are most prominent in the endometrium in the progesterone dependent secretory phase of the menstrual cycle and are likely to respond to exogenous progesterone. Further to this, if the trophoblast played a role in the recruitment of uNK cells into the endometrium, it would be expected that uNK cell numbers would differ consistently between the decidua basalis in the presence of extravillous trophoblast and the decidua parietalis which lacks trophoblast (Bulmer *et al.*, 2010). This has not been observed to be the case.

If uNK cells are trafficked from the peripheral blood, it is possible and likely that the endometrium releases factors to control their recruitment. Several studies have shown that cytokines and chemokines released by the endometrium may be

responsible for the recruitment of uNK cells to the endometrium including macrophage derived chemokine (MDC), monocyte chemoattractant protein (MCP)3, fractalkine, 6Ckine, MIP1β (Jones *et al.*, 2004), CXCL10, CXCL11 (Sentman, 2004) and IL15 (Kitaya *et al.*, 2005). In some cases, studies have shown cyclical changes in expression suggesting potential regulation by the steroid hormones. While the endometrium secretes many factors which may cause uNK cell recruitment, endometrial uNK cells themselves have been shown to lack some of the chemokine receptors which would allow uNK cells to respond via chemotaxsis (Manaster *et al.*, 2008).

Recruitment of cells from the peripheral blood into the endometrial tissues does not exclude the possibility of local differentiation of precursor cells which could first be recruited into the endometrium before differentiating to display their uNK phenotype in response to local stimuli (Bulmer *et al.*, 2010). Evidence for this occurring has mainly been observed in the mouse (Bilinski *et al.*, 2008 and Lynch *et al.*, 2007). This hypothesis is supported in the human by the identification of the presence of CD34+ CD56+ leukocyte stem cells in the secretory phase endometrium and the local proliferation of CD56+ uNK cells in secretory phase endometrium and early pregnancy decidua (Pace *et al.*, 1989). While it is possible that uNK cells may differentiate from precursor cells found in the peripheral blood, it is also possible that they may differentiate into their uterine specific phenotype from typical peripheral blood NK cells. uNK cells are phenotypically distinct and to date, similar cells have not been described in substantial numbers in other organs. It is also the case that no typical peripheral blood NK cells have been identified in the endometrium in significant numbers at any stage of the cycle or in early pregnancy (Bulmer *et al.*, 2010). Incubation of peripheral blood NK cells with transforming growth factor (TGF)β1 has been shown to lead to the conversion of these cells to a uNK phenotype (Keskin *et al.*, 2007). However, if this was the case *in vivo*, it would be expected at some stage of the menstrual cycle or early pregnancy that peripheral type NK cells could be indentified in the endometrium (Bulmer *et al.*, 2010).

1.3.2 uNK cell function

uNK cells are the major endometrial leukocyte population during the onset of the implantation window and early pregnancy. For this reason, the study of endometrial leukocyte function has focused on this cell type. The identification of this cell population as a NK cell type has led studies to investigate their cytotoxic nature in relation to their endometrial function (Bulmer *et* al., 2010 and Bulmer and Lash, 2005). CD56+ cells isolated from non-pregnant endometrium during various stages of the menstrual cycle (Jones *et al.*, 1997) and in early pregnancy decidua (King *et al.*, 1989) have been shown to exhibit cytotoxic activity against the NK cell target K562. However, this cytotoxic activity has been demonstrated to be lower than peripheral blood NK cells (Kopcow *et al.*, 2005). It has been suggested that uNK cells cytotoxic potential may play a role *in vivo* in controlling the invasion of trophoblast into the decidua but extravillous trophoblast has been shown to be protected from uNK cell cytotoxicity. This may be due interactions between inhibitory receptors on the NK cells and MHC molecules present on the trophoblast cells (Manaster and Mandelboim, 2008). Abnormal expression of these molecules has been associated with preeclampsia and recurrent miscarriage (Hiby *et al.*, 2004 and Hiby *et al.*, 2008). uNK cells are a rich source of many different cytokines and It has been suggested that they may mediate the action of uNK cells in trophoblast invasion. The cytokines TNFα, TGFβ1 and IFNϒ have all been shown to inhibit trophoblast invasion in placental explants lending support to this idea (Lash *et al.*, 2006a and Otun *et al.*, 2003).

It has also been shown that uNK cells are a major source of angiogenic growth factors in early pregnancy (Hanna *et al.*, 2006 and Lash 2006b). A role for uNK cells in trophoblast independent spiral artery remodeling has been proposed (Pijnenborg *et al.*, 2006), though it has been demonstrated that complete remodeling of the uterine spiral arteries requires a contribution from the trophoblast and uNK cells alone may only be involved in the early stages (Kam *et al.*, 1999). A study which investigated infiltration of leukocytes into decidual arteries showed infiltration of uNK cells and macrophages into smooth muscle of the deciduas basalis via endothelial cells (Smith *et al.*, 2009). Early vascular changes were shown to be independent of vascular trophoblast strongly suggesting a role for the uNK cells and macrophages found to be present at this time. Most recently, a study which used a placental-decidual co-culture

model to investigate remodeling showed that presence of placenta was required to intiate spiral artery remodeling but uNK cells and macropahges were shown to mediate this process at a cellular level during the early stages of this process (Hazan *et al.*, 2010).

While the presence of uNK cells aggregates around the spiral arteries has been suggested to be evidence of uNK cell trafficking from the peripheral blood, it may be the case that this occurs because uNK cells play an important role in vascular remodeling during pregnancy (Bulmer *et al.*, 2010).

Quantitative real-time RT-PCR and protein studies show an absence of the oestrogen receptor ERα and the progesterone receptor (PR) in uNK cells but the oestrogen receptor ERβ isoform and glucocorticoid receptor (GR) mRNA and protein has been identified, raising the possibility that oestrogens and glucocorticoids can act directly on uNK cells to affect cell and endometrial function (Henderson *et al.*, 2003). Indirectly, oestrogens and progesterone may also exert effects on uNK cells via stimulation of cytokines such as IL-15 and prolactin and other soluble factors by endometrial cells. Progesterone is thought to be central to these effects, due to the location of uNK cells within the endometrial stroma where most of these factors are released and the retention of the PR expression in stromal cells during the secretory phase of the menstrual cycle (Jabbour *et al.*, 2006 and Lea and Sandra, 2007).

1.3.3 uNK cells and reproductive failure

While the precise functions of uNK cells remains unknown, their importance in endometrial and placental function is suggested by the presence of abnormal numbers or populations in women with reproductive failure such as recurrent miscarriage (RM) or implantation failure after *in vitro* fertilisation (IVF) (Laird *et al.*, 2003).

There have been several studies of uNK and peripheral NK cells in the decidua and peripheral blood in women with recurrent and sporadic miscarriage (Laird *et al.*, 2003). Differences in the numbers of uNK cells and their state of activation have been implicated in the aetiology of RM. Several studies have showed increased numbers of CD56+ uNK cells in the peripheral blood of women with RM prior to and during pregnancy compared to healthy controls (Aoki *et al.*, 1995 and Kwak *et al.*, 1995).

CD56+ uNK cell activity has been shown to decrease in the first trimester of pregnancy in normal women but the activity of these cells in women with RM remains high (Higuchi *et al.*, 1995). It has also been shown that CD56+ uNK cell number and activity is only seen in RM women with chromosomally normal fetuses suggesting they are a cause of RM rather than an immunological response to chromosomally abnormal fetuses (Coulam *et al.*, 1995 and Yamada *et al.*, 2001).

Other studies however have contradicted these findings. While an increased number of CD56+ NK cells have been observed in the peripheral blood, a decreased number of decidual CD56+ uNK cells have been reported in the placental tissue from women who underwent spontaneous miscarriage in comparison to those who requested terminations (Quack *et al.*, 2001 and Yamamoto *et al.*, 1999b). Several reports have also shown increased numbers of uNK cells in secretory phase endometrium of both women with unexplained RM and recurrent implantation failure using immunohistochemistry (Clifford *et al.*, 1999; Laird *et al.*, 2005; Quenby *et al.*, 1999 and Tuckerman *et al.*, 2007).

Differences in the activation state of uNK cells may be important in their function or potential reproductive pathogenesis (Laird *et al.*, 2003). The activation status of both T cells and CD56+ NK cells has been investigated by measurement of CD25 (IL2Rα) (T cell activation marker) and CD69 (NK cell activation marker). Increased expression of CD69 on peripheral CD56+ (both $CD56^{bright}$ and $CD56^{dim}$) NK cells has been observed in women with unexplained recurrent miscarriage in contrast to normal controls and cells from women with RM showed significantly higher CD69 expression when co-cultured with trophoblast cell lines compared to cells from normal controls (Ntrivalas *et al.*, 2001).

1.4 Endometrial receptivity and female reproductive failure

The endometrium plays an important role in successful pregnancy outcome and abnormalities in endometrial function lead to reproductive failure, which can manifest itself as a failure to conceive (infertility), repeated failure to establish implantation in women undergoing IVF and recurrent miscarriage in women who successfully conceive (Laird *et al.*, 2006).

1.4.1 Endometrial receptivity and repeated implantation failure (RIF)

The pregnancy rate following one cycle of IVF and embryo transfer can be as high as 60%. Embryonic reasons for RIF include; genetic abnormalities of the male or female, sperm defects, embryonic aneuploidy or zona hardening. Various uterine pathologies such as hyperplasia, polyps, endometritis, synechiae and leiomyomata have been associated with RIF in up to approximately 30% of cases (Demirol and Gurgan, 2004). This still leaves a significant proportion of cases in which the cause of RIF is undefined and no morphological pathology of the endometrium can be observed despite the transfer of 'good quality' embryos. The altered expression of specific endometrial factors may be responsible for altering endometrial function and negatively affecting endometrial receptivity.

Endometrial dysregulation of numerous factors has been implicated in RIF including the abnormal levels of cytokines; IL12, IL15 and IL18 (Lédéé-Bataille *et al.,* 2005). Increased IL1β and decreased IFNY and IL10 expression in uterine fluid has also shown to be of potential importance in the aetiology of RIF along with increased levels of the MMPs 2 and 9 (Inagaki *et al.*, 2003). Adhesion molecules found to be expressed on the epithelium of the endometrium at the time of implantation are an obvious target for investigation in RIF. Absence of the integrin-αVβ3 during the implantation window in cases of RIF has been shown and may suggest a potential mechanism in which the blastocyst is unable to attach to the endometrial epithelial surface (Inagaki *et al.,* 2003). Mucins have also been shown to be potentially involved in RIF and the increased expression of MUC1 glycans has been demonstrated using immunohistochemistry (Horne *et al.*, 2005).

1.4.2 Endometrial receptivity and recurrent miscarriage

Recurrent miscarriage (RM) affects 1% of women and is defined as three or more consecutive first trimester losses of pregnancy. RM has both maternal and embryonic aetiologies, with known probable causes including; parental and foetal chromosome abnormalities, thrombophilia, metabolic disorders and anatomical causes. Despite this knowledge, around 50% of RM cases remain unexplained. Successful implantation requires concerted actions between the blastocyst, leukocyte populations and the endometrial epithelia and stroma. The expression of key molecules by the

endometrium at the time of implantation implies its importance in allowing the blastocyst to attach to the endometrial surface and invade into the decidual stroma Laird *et al.*, 2006).

Previous work has suggested that abnormal endometrial development may be associated with recurrent miscarriage (Li *et al.*, 2002). This may be identified by morphological analysis, which has shown that the endometrium of a sub-group of women with recurrent miscarriage at day LH+7 is retarded and would be dated at least 2 days earlier using the Noyes criteria. Immunohistochemical studies have also shown decreased expression of a number of endometrial proteins, such as MUC1 (Hey *et al.*, 1995), IL6 and LIF (Cork *et al.*, 1999) and αvβ3 integrin expression (Lessey *et al.*, 1996) in the peri-implantation period. Levels of the endometrial marker protein placental protein 14 are also reduced in women with recurrent miscarriage (Dalton *et al.*, 1995).

It is thought a proportion of unexplained RM may be due to abnormal maternal immunological function leading to destruction of the foetus. While it is clear that leukocyte populations such as uNK cells may play pivotal roles in the pathology of unexplained RM, the identification of the expression of 'immune' and other factors by the endometrium itself may be centrally important (Laird *et al.*, 2006).

1.4.3 Role of cytokines in successful pregnancy outcome

It has been shown in murine models that rejection of pregnancy is mediated by Th1 cytokines such as IL2 and IFNγ, while successful pregnancy depends on the presence of Th2 cytokines such as IL4, IL6 and IL10 (Wegmann *et al.*, 1993). While cytokines are expressed by CD4+ T helper cells in the endometrium and have traditionally been divided into such families based on the immunological effects they bring about it, seems clear that the endometrial epithelium is a major source of cytokines in the non-pregnant endometrium. Stromal and decidual cells have also been shown to produce cytokines (Linjawi *et al.*, 2004 and Tuckerman *et al.*, 2004).

It has been shown that women with RM have higher levels of Th1 cytokines (IFNγ, IL2, IL12 and TNFβ) and lower levels of Th2 cytokines such as IL6 in comparison to the endometrium and blood of normal controls (Lim *et al.*, 2000). Another study showed reduced expression of IL6 and LIF in RM endometrium in comparison to normal

controls (Cork *et al.*, 1999). The chemotatic cytokine (chemokine) IL8 (CXCL8) has been shown to be elevated in samples from the cervical mucus from RM patients (Hattori *et al.*, 2007). However, this study also showed increased levels of IL6 which is in disagreement with the study by Lim *et al.* (2000), which suggested decreased expression of IL6.

While the importance of LIF and IL11 in normal pregnancy is well established (see section 1.2.5), there is also evidence for abnormal expression of these cytokines playing a role in pregnancy failure due to RIF or RM (Laird *et al.*, 2006). Some studies have shown decreased production of LIF in endometrial samples obtained from women with unexplained infertility (Delage *et al.*, 1995 and Laird *et al.*, 1997), but other studies have shown no observable difference compared to normal controls (Sherwin *et al.*, 2002). Decreased secretion of LIF in women with multiple IVF failures has been shown (Hambartsoumian, 1998) and high levels of LIF expression at times in the menstrual cycle when it would be expected to be decreasing in women with multiple IVF failure has also been shown (Lédéé-Bataille *et al.*, 2002). Decreased expression of LIF in peri-implantation endometrium of non-pregnant women with RM has been demonstrated suggesting the importance of LIF in preventing pregnancy loss (Cork *et al.*, 1999).

Investigations into the expression of IL11 in the endometrium of women with implantation and pregnancy failure is limited, but it has been shown that IL11 is reduced in stromal and epithelial cells from women with infertility in comparison to normal controls (Karpovich *et al.*, 2005 and Linjawi *et al.*, 2004).

1.4.4 Trophoblast invasion and preeclampsia

Failure of the trophoblast to invade the endometrium successfully and remodel the maternal environment, including the spiral arteries is thought to be an aetiological factor for preeclampsia. This leads to restricted blood flow to the developing foetus. The disorder occurs in approximately 3-5% of pregnancies and is a cause of both foetal and maternal morbidity and mortality (Weiss *et al.*, 2007). Preeclampsia is characterised by hypertension and proteinuria in previously normal subjects, which can progress to eclampsia, which is characterised by convulsions. The superficial trophoblast invasion seen with preeclampsia has been associated with abnormal

genetic polymorphism, vascular endothelial cell activation, immune responses by the maternal immune system and exaggeration of a systemic imflammatory response (Schiesssl, 2007). Immuno-competent cells such as neutrophils, monocytes, NK cells, T and B cells have been found to activated in preeclampsia and hypoxic conditions, excessive inflammation or oxidative stress induces necrosis or aponecrosis of trophoblasts (Weiss *et al.*, 2009). Syncytiotrophoblast apoptotic debris, which is shed into the maternal circulation in increased amounts in preeclampsia, may be the stimulus for inducing the observed inflammatory response.

While failure of the trophoblast to correctly invade and remodel the maternal environment could occur due to the trophoblast's inability to correctly implant, it is possible that pathological endometrial receptivity or function may contribute. Recent studies have shown abnormally increased expression of endometrial factors including IL11 and MMP9 in cases of preeclampsia in decidualised endometrial cells (Basar *et al.*, 2010 and Lockwood *et al.*, 2008). This adds support to this idea and again highlights the importance of the tightly regulated crosstalk that must occur between the endometrium and blastocyst to achieve a successful pregnancy.

1.5 Chemokines

Chemoattractant cytokines (chemokines) are a family of small proteins (8-14 kDa) that are traditionally known to be involved in the recruitment of leukocytes during the inflammatory process acting on neutrophils, monocytes, lymphocytes and eosinophils. It has become clear however that chemokines and their receptors are central to the activity of many biological processes and affect the function of non-immune cell types (Taub and Oppenheim, 1994; Wells *et al*, 1998 and Zlotnik and Yoshie, 2000). Advances in cloning and bioinformatic research strategies have led to the identification of a large number of chemokines and their G protein coupled receptors. Chemokines can be divided into four subfamilies based on the presence or absence of amino acid residues between the first two of four conserved cysteines at the N-terminus of the protein (Figure 1.9). At least twenty eight CC, seventeen CXC, two C chemokines and a CX_3C chemokine have been identified (Wells *et al*, 1998 and Zlotnik *et al.*, 2006). Table 1.1 summarises the known chemokines and their receptors according to their subtypes and systematic name.

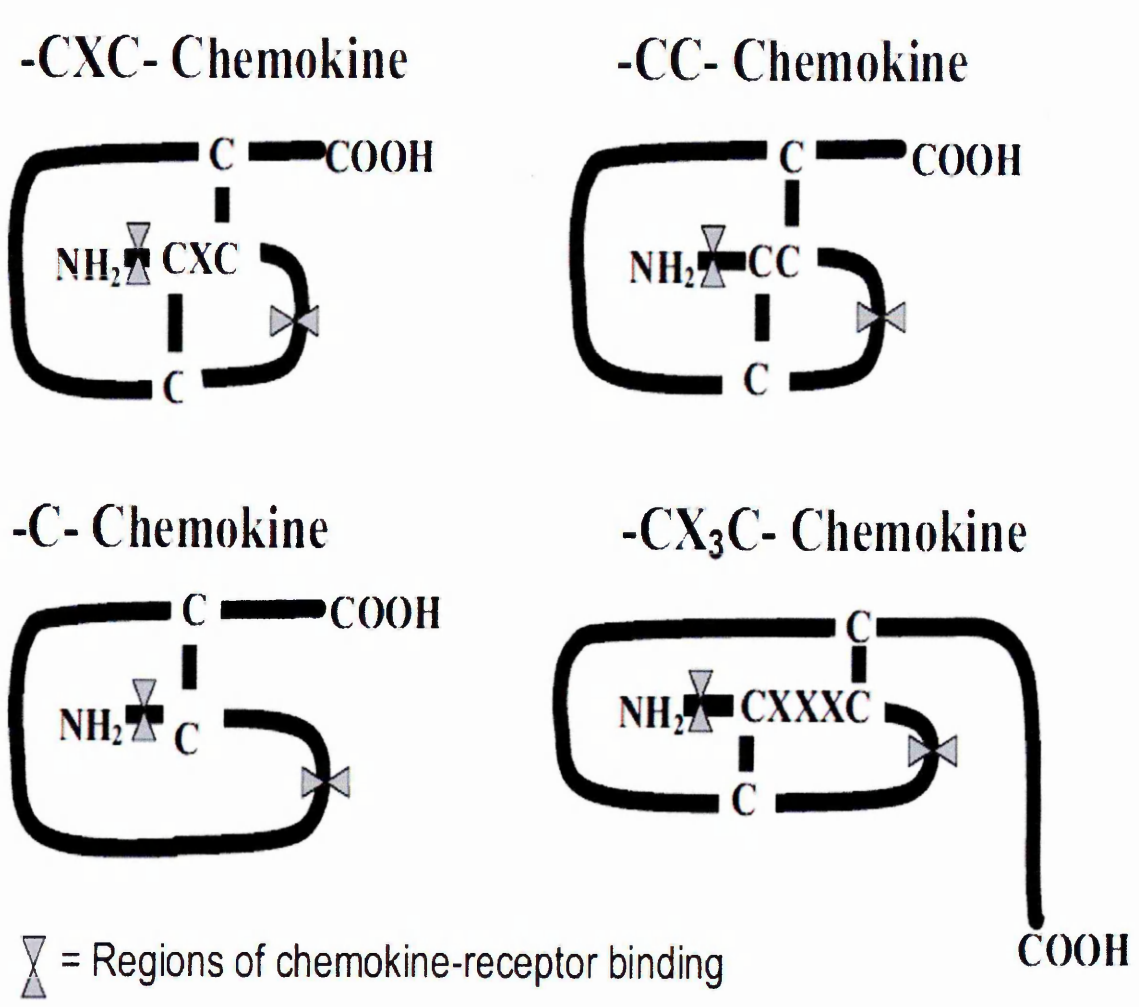


Figure 1.9: Structural differences of chemokine ligands (Taken from Townson and Liptak, 2003).

Shows the structural differences between the chemokine ligand subtypes and their regions of ligand/receptor binding.

Family	Other names	Chromosome	Function	Receptor
CXC family				
CXCL1	Melanoma growth stimulating activity alpha (Groα)	4q13.3	I	CXCR2, CXCR1
CXCL2	Melanoma growth stimulating activity beta (Groβ)	4q13.3	I	CXCR2
CXCL3	Melanoma growth stimulating activity gamma (GroΥ)	4q13.3	I	CXCR2
CXCL4	Platelet factor 4 (PF4)	4q13.3	U	CXCR3B
CXCLV1		4q13.3	U	Unknown
CXCL5	Epithelial-derived neutrophil-activating peptide-78 (ENA-78)	4q13.3	I	CXCR2
CXCL6	Granulocyte chemotactic protein 2 (GCP-2)	4q13.3	I	CXCR1,CXCR2
CXCL7	Pro-platelet basic protein	4q13.3	I	Unknown
CXCL8	Interleukin 8 (IL-8)	4q13.3	I	CXCR1,CXCR2
CXCL9	Mig receptor (MIGR)	4q21.1	I	CXCR3, CXCR3β
CXCL10	Interferon gamma-produced protein (IP-10)	4q21.1	I	CXCR3, CXCR3β
CXCL11	Interferon-inducible T-cell alpha chemoattractant (I-TAC)	4q21.1	I	CXCR3, CXCR3β, CXCR7‡
CXCL12	Stromal cell-derived factor (SDF-1α/β)	10q11.21	H	CXCR4, CXCR7‡
CXCL13	B lymphocyte chemoattractant (BLC)	4q21.1	H	CXCR5
CXCL14	Chemokine breast and kidney	5q31.1	I	Unknown

	(BRAK), Bolekine			
CXCL16		1713.2	I	CXCR6
CXCL17	Dendritic cell and monocyte chemokine-like protein (DMC)	19q13.2	U	Unknown
CC family				
CCL1		17q11.2	I	CCR8
CCL2	Monocyte chemoattractant protein-1 (MCP-1)	17q11.2	I	CCR2
CCL3	Macropahge inflammatory protein-1 alpha (MIP-1α)	17q11.2	I	CCR1,CCR5
CCL3L1		17q12	I	Unknown
CCL3L3		17q12	I	Unknown
CCL4	Macrophage inflammatory protein 1 beta (MIP-1β)	17q12	I	CCR5
CCL4L1	Lymphocyte activation gene 1 protein (LAG-1)	17q12	I	Unknown
CCL4L2	Lymphocyte activation gene 1 protein (LAG-1)	17q12	I	Unknown
CCL5	Regulated on activation normal T expressed and secreted (RANTES)	17q12	I	CCR1,CCR3,CCR5
CCL7	Monocyte chemoattractant protein 3 (MCP-3)	17q11.2	I	CCR1,CCR2,CCR3
CCL8	Monocyte chemoattractant protein 2 (MCP-2)	17q11.2	I	CCR1,CCR2,CCR3 ,CCR5
CCL11	Eotaxin	17q11.2	I	CCR3
CCL13	Monocyte chemoattractant protein-4 (MCP-4)	17q11.2	I	CCR1,CCR2,CCR3
CCL14	Haemofiltrate CC chemokine-1 (HCC-1)	17q12	H	CCR1

CCL15	Haemofiltrate CC chemokine-2 (HCC-2)	17q12	H	CCR1,CCR3
CCL15	Haemofiltrate CC chemokine-2 (HCC-2)	17q12	H	CCR1,CCR3
CCL16	Haemofiltrate CC chemokine-4 (HCC-4)	17q12	H	CCR1,CCR2,CCR5 ,HRH4
CCL17	Thymus and activation-regulated chemokine (TARC)	16q13	D	CCR4
CCL18	Pulmonary and activation-regulated chemokine (PARC)	17q12	H	Unknown
CCL19	Macrophage inflammatory protein 3-beta (MIP3β)	9p13.3	H	CCR7
CCL20	Macrophage inflammatory protein 3-alpha (MIP3α)	2q36.3	D	CCR6
CCL21	Secondary lymphoid-tissue chemokine (SCL)	9p13.3	D	CCR7
CCL22	Macrophage derived chemokine (MDC)	16q13	D	CCR4
CCL23	Myeloid progenitor inhibitory factor-1 (MPIF-1)	17q12	I	CCR1, FPRL-1
CCL24	Eotaxin 2	7q11.23	I	CCR3
CCL25	Thymus expressed chemokine (TECK)	19p13.2	H	CCR9
CCL26	Eotaxin 3	7q11.23	I	CCR3
CCL27	Cutaneous T-cell attracting chemokine (CTACK)	9p13.3	H	CCR10
CCL28	Mucosae-associated epithelial chemokine (MEC)	5p12	U	CCR10, CCR3
Other classes				
XCL1	Lymphotactin	1q24.2	D	XCR1
XCL2	Small inducible cytokine subfamily (SCM-1β)	1q24.2	D	Unknown

CX3CL1	Fractalkine	16q13	I	CX3CR1

Table 1.1: The known human chemokines and receptors (Adapted from Zlotnik et al., 2006

Systematic symbols for chemokine ligands are derived by the position of conserved N-terminus cystiene residues. For example, CXCL1 is: Chemokine (C-X-C motif) ligand 1. Chemokine receptors use 'R' to denote receptor status, e.g. CXCR1 is: Chemokine (C-X-C motif) receptor 1.

Functions: I, inflammatory; H, homeostatic; D, dual (homeostatic and inflammatory); U, unknown. The lists of alternative names are not fully comprehensive. ǂ Binding has been reported but signaling is still controversial.

1.5.1 Chemokine ligands and receptor

Chemokines act as ligands for chemokine receptors. Chemokine receptors belong to the G protein-coupled receptor (GPCR) super-family which contains receptors for a wide range of ligands including hormones, neurotransmitters and inflammatory mediators (Murdoch and Finn, 2000 and Zlotnik and Yoshie, 2000). They all structurally consist of seven trans-membrane protein domains with an extracellular N-terminus and an intracellular C-terminus (Figure 1.10).

Secreted chemokine ligands bind the extracellular N-terminus region of its target chemokine receptor causing activation of an intracellular signaling cascade potentially in both a G protein dependent and independent manner causing a cellular response (Busillo and Benovic, 2007). Chemokine ligands and receptors exhibit promiscuity with many of the chemokine receptors binding multiple ligands (see Table 1.1) (Zlotnik *et al.*, 2006), which in one part is due to their similar tertiary protein structures and binding properties (Figure 1.9) (Townson and Liptak, 2003). Expression of chemokines and their receptors are subject to transcriptional control with the identification of mRNA and protein splice variants and other forms of control such as DNA hypermethylation and regulation by microRNAs also playing roles (Asirvatham *et al.*, 2008 and Busillo and Benovic, 2007).

The effects of chemokines within a wide range of human tissues has been observed. Chemokines were initially studied for their ability to act as molecular attractants, recruiting leukocytes to sites of inflammation via chemotaxis. Since these findings, it has become clear that chemokines have the ability to carry out a wide range of functions (Onuffer and Horuk, 2002 and Shao *et al.*, 2008). These functions are thought to include roles in leukocyte, dendritic cell and stem cell migration and function, mediation of the Th1 and Th2 responses, organogenesis and control of developmental processes, cell adhesion, angiogenesis and angiostasis.

Chemokine function has been implicated in a number of human diseases in a range of different cell types, tissues and systems (Busillo and Benovic, 2007). The two most studied diseases in which chemokines have been implicated, HIV and numerous types of cancer metastases, both involve the chemokine receptor CXCR4 and its ligand CXCL12.

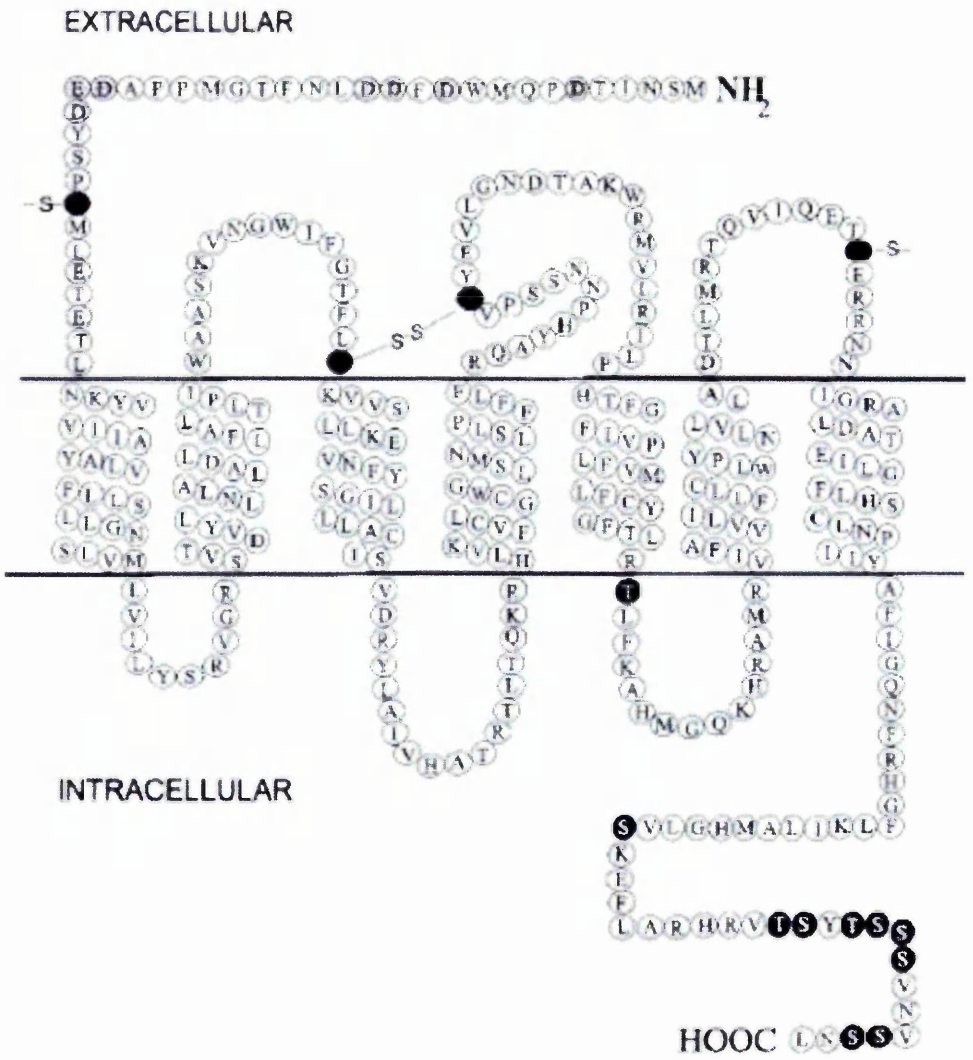


Figure 1.10: Representation of the chemokine receptor CXCR1 situated in the cell membrane (Taken from Murdoch and Finn, 2000).

Showing the seven transmembrane domains, the extracellular N-terminus and intracellular C-terminus characteristic of G protein-coupled receptors.

1.5.2 Evidence for the role of chemokines in endometrial function

Chemokines have been implicated in playing a role in the control of female reproductive function including in the endometrium and ovaries. Recruitment and control of leukocyte populations within the ovaries and endometrium is thought to be controlled by chemokine chemotaxis. It has been shown that several chemokines and their receptors are expressed in the endometrium and are potentially involved in the recruitment of endometrial leukocytes including uNK cells. Expression of a number of different chemokine mRNAs was generally up-regulated during the implantation window. Protein expression of these chemokines was found to be localised to endometrial epithelial cells leading up to implantation, while differentiated stromal cells were found to be a major source of chemokines following decidualisation (Jones *et al.*, 2004). Another study demonstrated concordance between the expression of chemokines ligands in the uterus and expression of their receptors on decidual leukocytes suggesting a possible mechanism for leukocyte recruitment during pregnancy (Red-Horse *et al.*, 2001).

Beyond acting as a simple chemoattractant, there is evidence that chemokine ligands act as signal molecules between cells and tissues where receptor activation leads to changes in intracellular signaling and gene expression. This is particularly relevant in the endometrium where there is potentially a complex and rapidly changing crosstalk between the endometrial epithelium and stroma, implanting blastocysts and leukocytes such as uNK cells all of which may be involved in the control of implantation, decidualisation, placentation and menses (Dominguez *et al.*, 2003 and Garcia-Valesco and Arici, 1999). The identification of mRNA expression of chemokine transcripts CX3CL1 and CCL7, along with their receptors CX3CR1, CCR1, CCR2, CCR3 and CCR5 has been demonstrated in human endometrium and first-trimester placenta. Immuno-reactive CCR1, CCR3 and CX3CR1 was also shown in human first-trimester implantation sites, lending support to the involvement of endometrial chemokines in the crosstalk that occurs at the feto-maternal interface (Hannan *et al.*, 2006).

There is evidence that chemokine production is under the influence of steroid hormone control within the endometrium, where it is thought they may play centrally important roles in many processes of this highly dynamic tissue throughout the menstrual cycle (Dominguez *et al.*, 2003 and Garcia-Valesco and Arici, 1999). The

chemokine ligands IL8, MCP-1 and RANTES (CXCL8, CCL2 and CCL5) and the chemokine receptors CXCR1, CXCR4, CCR5 and CCR2B have been identified in human endometrium and their expression has been shown to change throughout the normal menstrual cycle and in a steroid hormone dependent manner in women undergoing hormone replacement therapy (HRT) (Dominguez *et al.*, 2003). IL8 mRNA expression has been found to peak in the late secretory and early proliferative human endometrium, where IL8 expression was found predominantly in the surface epithelium and glands of the endometrium (Arici *et al.*, 1998 and Dominguez *et al.*, 2003). MCP-1 has also been shown to be localised to the epithelium and glands of the endometrium and expression would appear to increase during the onset of the implantation window. RANTES expression has been shown to be localised to the endometrial stroma and perivascular cells during the implantation window (Arici *et al.*, 1995; Arici *et al.*, 1998; Dominguez *et al.*, 2003a).

Several studies have demonstrated that the expression of some chemokines can be induced by steroid hormones. Estradiol and progesterone treatment has been shown to increase CXCL10 and CXCL11 protein expression in cultured human endometrium and $CD56^{bright}$ uNK cells have been shown to express the receptor CXCR3, the receptor for these chemokine ligands (Sentman *et al.*, 2004).

17 β-estradiol has been shown to increase CXCL12 and CXCR4 mRNA transcript expression in oestrogen receptor positive Ishikawa adenocarcinoma cell lines (Kubarek and Jagodzinski, 2007) and oestrogen induced CXCL12 expression has been shown in the uterus of rats (Glace *et al.*, 2009). In primary cell cultures of decidual stromal cells (DSCs); oestrogen, progesterone and hCG was shown to increase the level of CCL2 (He *et al.*, 2007).

Analysis of the chemokine receptors CXCR1, CXCR4, CCR2B and CCR5 mRNA transcript levels in endometrial samples obtained from normal fertile women throughout the menstrual cycle showed hormone dependent changes in expression (Dominguez *et al.*, 2003). CXCR1 and CCR5 mRNA was shown to be highest during the late secretory phase of the menstrual cycle while CXCR4 mRNA was apparently increased during the implantation window. Immunohistochemical staining for CXCR4, CCR2B and CCR5 was shown to be located within the epithelium and glands of the endometrium but was

weak in HRT patients receiving oestrogen suggesting a suppressive effect. CXCR1 staining was shown to increase in the glands of the endometrium approaching and during the implantation window. An *in vitro* model of blastocyst apposition, showed an increase in endometrial cell expression of CXCR1, CXCR4 and CCR5 in the presence of a blastocyst in comparison to its absence. Tables 1.2 and 1.3 summarise the known expression of chemokine ligands and receptors in the endometrium.

Chemokine ligand (systematic name)	Endometrial expression	Proposed functions and regulation
CXCL1	Stroma/decidua, increased during secretory phase (Nasu *et al.*, 2001)	Regulated by thrombin via MAP kinases (Kawano *et al.*, 2011)
CXCL2	First trimester decidua (Huang *et al.*, 2006)	Increased expression in response to IL1β (Huang *et al.*, 2006)
CXCL3	First trimester decidua (Huang *et al.*, 2006)	Increased expression in response to IL1β (Huang *et al.*, 2006)
CXCL5	Stroma (Nasu *et al.*, 2001)	Regulated by progesterone and pro-inflammatory mediators (Nasu *et al.*, 2001)
CXCL6	Cultured stromal cells (Mine *et al.*, 2003)	Regulated by inflammatory cytokines (Mine *et al.*, 2003)
CXCL8	Highest in epithelium, present in stroma (Caballero-Campo *et al.*, 2002), Peaks during the secretory phase (Arici *et al.*,, 1998a)	Regulated by steroid hormones (Kelly *et al.*, 1994) and cytokines (Arici *et al.*, 1993). Potential functions in neutrophil migration prior to menses (Kelly *et al.*, 1994) and angiogenesis (Arici *et al.*, 1998a)
CXCL10	Epithelium (Paiva *et al.*, 2011)	Regulated by hCG, involved in blastocyst migration and adhesion (Paiva *et al.*, 2011)
CXCL11	Epithelium (Hirota *et al.*, 2006)	Regulated by pro inflammatory cytokines (Hirota *et al.*, 2006)
CXCL12	Epithelium and stroma throughout the menstrual cycle (Laird *et al.*, 2011)	Potential regulation by steroid hormones (Tsutsumi *et al.*, 2011), potential function in uNK cell recruitment (Kitaya *et al.*, 2004)
CXCL14	Glandular epithelium, increased during mid secretory phase (Mokhtar *et al.*, 2011)	Potential implantation role (Mokhtar *et al.*, 2011)
CCL2	Stroma (Huang *et al.*, 2006)	Regulated by IL1β (Huang *et al.*, 2006)
CCL3	Epithelium (Akiyama *et al.*, 1999)	Potentially involved in monocyte chemotaxis (Akiyama *et al.*, 1999)

CCL4	Epithelium and stroma (Hannan *et al.*, 2006)	Promotes trophoblast migration at feto-maternal interface (Hannan *et al.*, 2006)
CCL5	Stroma and endothelial cells (Caballero-Campo *et al.*, 2002)	Potentially involved in implantation, regulated by IL1β via NF kappa B (Caballero-Campo *et al.*, 2002)
CCL8	Epithelium, no differential expression through cycle (Hampton *et al.*, 2001)	Unlikely to be involved in macrophage recruitment (Hampton *et al.*, 2001)
CCL14	Epithelium, decidua and vasculature (Hannan *et al.*, 2008)	Promotes trophoblast migration (Hannan *et al.*, 2008)
CCL16	Epithelium (Chand *et al.*, 2007)	Regulated by inflammatory cytokines (Chand *et al.*, 2007)
CCL19	Epithelium and stroma throughout the menstrual cycle (Daikoku *et al.*, 2004)	Not involved in chemotaxis (Daikoku *et al.*, 2004)
CCL20	Endometrial Cell lines (Sun *et al.*, 2002)	Potential function in regulating/trafficking dendritic and T memory cells (Sun *et al.*, 2002)
CCL21	Epithelium and perivascular stroma (Nakayama *et al.*, 2003)	Increased during the mid secretory phase, may be involved in uNK cell chemotaxis (Nakayama *et al.*, 2003)

Table 1.2: Summary of endometrial chemokine expression, regulation and function

The known chemokine ligand expression in the human endomtrium, including potential regulation and function.

Chemokine receptor (systematic name)	Endometrial expression	Proposed functions and regulation
CXCR1	Epithelium and stroma, peak expression during mid secretory (Mulayim *et al.*, 2003)	Potential steroid hormone regulation (Dominguez *et al.*, 2003)
CXCR2	Epithelium and stroma (Mulayim *et al.*, 2003)	-
CXCR3	Epithelial and stromal cell lines (Hirota *et al.*, 2006)	MAP kinase signalling (Hirota *et al.*, 2006)
CXCR4	Throughout the endometrium, highest in epithelium during proliferative phase (Laird *et al.*, 2011).	Potential role during proliferative phase (Laird *et al.*, 2011) and in implantation during the MS phase (Dominguez *et al.*, 2003)
CCR1	Found at first trimester implantation sites (Hannan *et al.*, 2006)	Potential role in implantation (Hannan *et al.*, 2006)
CCR2	Decidual stromal cells (He *et al.*, 2007)	Roles in the maternal-fetal interface (He *et al.*, 2007)
CCR3	Epithelial cells (Zhang *et al.*, 2000)	Role in leukocyte recruitment/implantation (Zhang *et al.*, 2000)
CCR7	Stromal cell cultures (Shi *et al.*, 2007)	-
CCR8	Stromal cell cultures (Shi *et al.*, 2007)	Potential regulation by oestrogen (Shi *et al.*, 2007)

Table 1.3: Summary of endometrial chemokine receptor expression, regulation and function

The known chemokine ligand expression in the human endomtrium, including potential regulation and function.

1.5.3 CXCL12

CXCL12 is an 8 kDa chemokine which was named stromal cell-derived factor 1 (SDF-1) after identification of its constitutive expression in bone-marrow derived stromal cells. It exhibits several features which distinguish it from other chemokines (Holmes *et al.*, 2001). Genes encoding the CXC and CC chemokines are generally found on chromosomes 4 and 17 respectively. However, the CXCL12 gene is found on chromosome 10. CXCL12 is expressed in a broad range of tissues while other chemokines appear to be more restricted in where they are expressed. The nucleotide and amino acid sequences for CXCL12 are highly conserved between humans and murine models indicating it is of core importance for mammalian cell function. Until recently it was believed that CXCL12 exclusively bound the chemokine receptor CXCR4. However, it has been shown that CXCL12 can bind to what was originally thought to be an orphan receptor named RDC1 (Thelen and Thelen, 2008). This receptor has since been renamed CXCR7 and while signal transduction via the receptor remains controversial, strong CXCL12 binding has been shown and a possible scavenging role proposed. Interestingly, CXCR7 has been shown not to play roles in leukocyte trafficking.

CXCL2 was chosen as the focus for this study due to its unique attributes, such as the location of its gene on chromosome 10 and not chromosomes 4 and 17, its widespread expression in human tissues (Holmes *et al.*, 2001) and limited evidence for its presence and potential functional importance in the endometrium (Kitaya *et al.*, 2004). Complementary protein studies in our laboratory also showed the presence of CXC12 in non-pregnant endometrium. CXCL12's receptor CXCR4 has also been shown to be expressed in the endometrium and its expression characterised throughout the menstrual cycle (Dominguez *et al.*, 2003). As CXCR7 was only very recently discovered as a potential receptor to CXCL12 with potentially significant functioning, the expression of this new receptor could not be investigated in this study.

Two alternatively spliced isoforms of CXCL12 were originally isolated and termed SDF-1α and SDF-1β. SDF-1α is composed of 89 amino acids and is the predominantly expressed isoform. SDF-1β contains a four amino acid extension at the carboxyl terminus. Both isoforms bind CXCR4 with comparable affinity. More recently, an additional four splice variants have been identified which vary in length at the carboxyl

terminus. While these isoforms are functional and have differential tissue distributions, their functional significance remains unknown (Busillo and Benovic, 2007 and Yu *et al.*, 2006).

1.5.4 CXCR4

CXCR4 is a 352 amino acid rhodopsin-like GPCR and selectively binds the CXC chemokine CXCL12 (Busillo and Benovic, 2007). Mice lacking either CXCR4 or CXCL12 exhibit an almost identical phenotype of late gestational lethality and defects in B cell lymphopoesis, bone marrow colonisation and cardiac septum formation. These findings and other studies have confirmed CXCR4 is essential for development, hematopoiesis, organogenesis and vascularisation as well as functioning as a classical chemokine receptor in the adult. CXCR4 has been shown to be expressed in a number of tissues in addition to the leukocytes from which it was originally cloned.

1.5.5 Transcriptional control of CXCR4

The CXCR4 promoter region contains a number of predicted regulatory consensus sequences and is thought to be positively regulated by Nuclear Respiratory Factor-1 (NRF-1) (Moriuchi *et al.*, 1997 and Wegner *et al.*, 1998), with a potential negative upstream element that may be mediated by Ying Yang 1 (YY1). Roles for other transcription factors have also been suggested (Moriuchi *et al.*, 1999). Transcriptional expression of CXCR4 is thought to be affected by a number of signaling molecules. An increase in CXCR4 expression has been observed in response to intracellular second messengers such as calcium and cyclic AMP in addition to a number of cytokines such as IL2, IL4, IL7, IL8, IL10, IL15 and TGF-1β (Busillo and Benovic, 2007). Simultaneous CD3 and CD28 engagement (Moriuchi *et al.*, 1997) as well exposure to growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) (Salcedo *et al.*, 1999) and epidermal growth factor (EGF) (Philips *et al.*, 2005) have also been shown to increase CXCR4 transcription. Pro-inflammatory cytokines such as tumour necrosis factor α (TNFα), interferon ϒ (IFNϒ) and IL1β have been shown to decrease the expression of CXCR4 (Gupta *et al.*, 1998 and Han *et al.*, 2001).

1.5.6 Translational control of CXCR4

A number of translational modifications have been shown to contribute to the expression of CXCR4 at the protein level (Busillo and Benovic, 2007). The extracellular domain of CXCR4 contains two potential N-linked glycosylation sites (Asn11 and Asn176) though only Asn11 appears to be glycosylated in mammalian cells and has been shown to inhibit CXCL12 binding (Chabot *et al.*, 2000). CXCR4 can also undergo extracellular tyrosine sulfation at Tyr7, Tyr12 and Tyr21 and this has been shown to affect CXCL12 ligand binding (Farzan *et al.*, 2002). CXCR4 is thought to be able to homodimerise, which may occur in the absence of CXCL12 (Babcock *et al.*, 2003). It is thought that CXCR4 can also heterodimerise with CCR2 and CD4. While little is known about the functionality of dimerisation, it is potentially a further explanation for the functional heterogeneity of CXCR4 signaling in various cell types and tissues (Issafras *et al.*, 2002 and Basmaciogullari *et al.*, 2006).

1.5.7 Binding of CXCL12 to its receptor

The binding of CXCL12 to its receptor CXCR4 is proposed to be a two-step process. Initially, an interaction between residues 12 to 17 of CXCL12 and residues 2 to 36 of CXCR4 are thought to cause a conformational change in the receptor allowing the first eight amino acids of CXCL12 to access a binding pocket in CXCR4's extracellular loop structures (Brelot *et al.*, 2000 and Huang *et al.*, 2003). During an inflammatory response, proteases have been shown to inhibit CXCL12/CXCR4 binding by cleaving N-terminus residues of both the ligand and receptor (Delgado *et al.*, 2001 and Valenzuela-Fernandez *et al.*, 2002). CXCL12 is able to interact with glycosaminoglycans such as heparin sulphate which may immobilise CXCL12 *in vivo* aiding gradient formation. This association may also allow CXCL12 to oligomerise, a phenomenon observed at high CXCL12 concentrations. Recent evidence suggests that inflammatory response products may 'prime' the CXCL12 response and enhance hematopoietic stem/progenitor cell migration at lower CXCL12 concentrations. This may be due to changing membrane localisation of CXCR4 through incorporation into lipid rafts. It has been argued that lipid raft localisation is required to allow the proper functioning of CXCR4 and it has recently been shown that CXCL12 promotes the incorporation of Src tyrosine kinases, focal adhesion kinases, PI3 kinase and the G protein Rac into lipid

rafts. This ligand promoted clustering of receptor and effectors may ensure activation of the appropriate/correct signal pathways (Majka *et al.*, 2000 and Wysoczynski *et al.*, 2005).

1.5.8 CXCR4 and G protein Signaling

Upon CXCL12 ligand binding to CXCR4, a number of signaling pathways can be activated leading to a variety of biological responses. CXCR4 couples to the G_i family of proteins (Busillo and Benovic, 2007). This interaction can be disrupted by pertussis toxin (PTX) which inhibits GPCR/G_i association by ADP-ribosylation. This interaction has been used as a tool to investigate CXCR4 signaling and has demonstrated the capability of CXCR4 signaling to occur in both a G protein dependent and independent manner. G protein dependent signaling relies on activated G_i protein causing inhibition of adenylyl cyclase and activating the Src family of tyrosine kinases. Liberated GβΥ activates phospholipase C-β (PLC-β) and phosphoinositide-3 (PI3K). These events ultimately lead to regulation of processes such as gene transcription, cell migration and cell adhesion (Kucia *et al.*, 2004). G protein independent signaling has been proposed to take place through activation of the JAK/STAT pathway in which the transient association of JAK2 and JAK4 with CXCR4 causes the activation and nuclear translocation of a number of STAT proteins (Vila-Coro *et al.*, 1999). CXCR4 dependent signal transduction has also been shown to occur through the activation of the MAPK/ERK1/2 and PI3K/Akt pathways leading to the activation of transcription factors such as nuclear-factor kappa B (NF_KB) and Ets1 (Chinni *et al.*, 2006; Huang *et al.*, 2009 and Tang *et al.*, 2008).

1.5.9 Regulation of CXCR4 signaling

GPCRs are regulated by the processes of desensitisation, internalisation and degradation. Homologous desensitisation (decreased receptor activity in response to continuous stimuli) occurs due to phosphorylation of serine/threonine residues present within the cytoplasmic tail domain of the receptor by G protein-coupled receptor kinase (GRK). This phosphorylation event allows subsequent binding of arrestin-2 and/or arrestin-3 which uncouples the receptor from further G protein activation and potentially targets it for internalisation (Krupnick *et al.*, 1998). Upon

CXCL12 binding, CXCR4 is rapidly phosphorylated and internalised. Upon internalisation, GPCRs can be recycled back to the plasma membrane but this process occurs poorly for CXCR4. CXCR4 has been shown to be ubiquinated, sorted to the lysosome and degraded in a process mediated by the E3 ubiqutin ligase AIP4. It is thought the phoshorylation of specific residues within CXCR4 may dictate the receptors fate following its internalisation (Haribabu *et al.*, 1997).

1.6 Evidence for the role of CXCL12 and CXCR4 in the function of the endometrium

Although expression of CXCL12 in monkey and bovine endometrium has been reported (Ace and Okuliez, 2004 and Mansouri-Attia *et al.*, 2009), studies of CXCL12 and CXCR4 in human endometrial epithelial and stromal cells are limited. One immunohistochemical study showed that CXCL12 could be detected during the proliferative phase of the menstrual cycle only (Kitaya *et al.*, 2004). A study which investigated the expression of CXCL12 and other chemokines in the human endometrium through the menstrual cycle using gene arrays did not detect the presence of CXCL12 (Jones *et al.*, 2004). Unpublished data from our laboratory has suggested that CXCL12 protein is detectable in the endometrium and is localised predominantly to the glandular and luminal epithelium (see Chapter 3, Figure 3.1). A study by Dominguez *et al.* (2003) quantified the expression of CXCR4 mRNA through stages of the menstrual cycle from endometrial biopsies. This study found CXCR4 to be expressed throughout the menstrual cycle with an apparent decrease in CXCR4 mRNA expression through the proliferative phase and a significant increase at the time of implantation.

Several studies of CXCL12 and CXCR4 in uNK cells, trophoblast and the blastocyst have demonstrated a potential role for the chemokine ligand and its receptor in the processes of implantation and uNK cell trafficking. It has been demonstrated that decidual uNK cells isolated from elective terminations expressed CXCR4 mRNA as did the decidual tissue and placenta. Positive immunohistochemical staining for CXCL12 was also shown in the placenta and it was demonstrated that CXCL12 (but not CXCL9 or CXCL10) attracted CD56+ CD16- NK cells. The same group also showed that CXCL12

protein is expressed *in vivo* on invasive trophoblast and trophoblast cells lining maternal blood vessels (Hanna *et al.,* 2003).

Another study also demonstrated (using RT-PCR and immunohistochemistry) that trophoblasts and decidual stromal cells express both CXCL12 and CXCR4. CXCL12 was found to induce an apparent increase in the invasiveness of the trophoblast and upregulated MMP2 and MMP9 activity in both trophoblast and stromal decidua. It was proposed that CXCL12 secreted by human trophoblasts enhances the coordination between the trophoblast and the decidual stromal cells via MMP2 and MMP9 (Zhou *et al.,* 2008). It has been shown that CXCR4 and CXCR3 were highly expressed in $CD56^{bright}$ $CD16^{-}$ first trimester decidual NK cells (Wu *et al.,* 2005). The same study demonstrated that trophoblast cells spontaneously secrete CXCL12 and showed that both recombinant CXCL12 and trophoblast cell supernatents caused the chemotaxsis of $CD56^{bright}$ CD16- natural killer cells.

Endometrial biopsies taken from patients undergoing hormone replacement therapy showed that when patients were treated solely with estradiol, weak staining for CXCR4 was seen to be localised to the luminal and glandular epithelium of the endometrium. It was also shown in an *in vitro* apposition model of implantation that in the presence of a blastocyst, endometrial cells showed an increase in staining for CXCR4. Staining for CXCR4 on the blastocyst was negative (Valles and Dominguez, 2006).

Endometrial and peripheral T lymphocytes taken from RM and fertile women showed that endometrial lymphocytes expressed CXCR4 and that its expression was decreased by CCL5 (Ramhorst *et al.,* 2004). CCL5 was also shown to be expressed in the endometrium, which was shown to be sensitive to progesterone.

Finally, in an investigation into genome wide mRNA transcript levels between three fertility groups subjected to exogenous estradiol and progesterone, microarray analysis showed reduced expression of CXCR4 in a group of women who showed repeated implantation failure during IVF treatments in comparison to women who underwent successful IVF or fertile controls (Tapia *et al.,* 2008). Although expression of CXCR4 was decreased in comparison to both the successful IVF treatment and fertile control groups, the expression of CXCR4 mRNA was highest in women who had undergone successful IVF treatment and not fertile controls.

1.7 Evidence for the effects of CXCL12 on the expression of other factors

Outside of the endometrium, there is evidence that CXCL12 acts not just as a chemotactic agent but also functions to modulate the expression of a number of genes and subsequent protein production (Busillo and Benovic, 2007). A number of genes that have been shown to be of potential importance in endometrial function have been shown to be up-regulated by CXCL12 in non-endometrial cell types. However, the effects of CXCL12 on these genes expression levels remains unstudied in human endometrial cells.

CXCL12 increases IL6 expression in human microglia, basal carcinoma cells and prostate cancer cell lines. It is proposed that is achieved by activation of the PI3K/Akt, ERK and NF Kappa B signal transduction pathways (Chu *et al.*, 2009; Lu *et al.*, 2009 and Wang *et al.*, 2005). CXCL12 increases IL8 expression in prostate cancer and mast cell lines (Lin *et al.*, 2000 and Wang *et al.*, 2005) and expression of IL8 has been shown to be inhibited following treatment with a CXCR4 antagonist in malignant human glioma cells. This was also demonstrated for vascular endothelial growth factor (VEGF) expression in the same study (Ping *et al.*, 2007). VEGF expression has been shown to be increased by CXCL12 in human breast carcinoma cells via the Akt signaling pathway (Liang *et al.*, 2007) and in human prostate cancer cell lines (Wang *et al.*, 2005). Matrix metalloproteinases MMP2 and MMP9 have been shown to be increased by CXCL12 treatment in prostate cancer cell lines (Singh *et al.*, 2004), bone marrow proginator cells (Shao *et al.*, 2008) and implicated in transendothelial haematopoietic stem cell migration, where CXCL12 increases MMP2 and MMP9 expression (Lapidot *et al.*, 2005).

1.8 Evidence for the functional importance of IL6, IL8, MMP2, MMP9 and VEGF in the human endometrium

Various studies have implicated IL6 (Laird *et al.*, 1993; Laird *et al.*, 1994; Margni and Zenclussen, 2001; Robertson *et al.*, 2000; Tabibzadeh *et al.*, 1995; Vandermolen and Gu, 1996 and Zenclussen *et al.*, 2003), IL8 (Arici *et al.*, 1998a; Arici *et al.*, 1998b; Caballero-Campo *et al.*, 2002; Garcia-Valesco and Arici, 1999 and Koch *et al.*, 1992), MMP2 (Fernandez *et al.*, 1992; Jeziorska *et al.*, 1996 and Salamonsen and Woolley, 1996), MMP9 (Lockwood *et al.*, 2008 and Salamonsen *et al.*, 1997) and VEGF (Ferrara, 2004; Ferrara and Davis-Smyth, 1997; Girling and Rogers, 2005 and Risau, 1997) in a

range of female reproductive and endometrial functions throughout the menstrual cycle and during implantation.

1.8.1 Interleukin 6 (IL6)

Interleukin 6 (IL6) is a multifunctional secreted cytokine that regulates various aspects of the immune response, acute phase reaction and haematopoiesis. IL6 deficient mice have reduced fertility and a decrease in viable implantation sites (Robertson *et al.*, 2000). A report by Tabibzadeh (1989) showed the production of IL6 by human stromal endometrial cells which was decreased by incubation with oestradiol, showing that IL6 production can be modulated by a major controller of endometrial function. Further immunolocalisation studies of IL6 expression in the human endometrium showed that it was weakly expressed during the proliferative phase of the menstrual cycle but highly expressed during the mid secretory phase. The expression was found mainly to be in the endometrial glands and to a lesser extent in the stroma (Tabibzadeh *et al.*, 1995 and Vandermolen and Gu, 1996). IL6 was also found to be expressed in cultured human endometrial cells. Endometrial epithelial cells were shown to produce significantly higher levels of IL6 protein than stromal cells and addition of progesterone and oestradiol increased IL6 production in these cells (Laird *et al.*, 1993). Addition of IL1 to cultured endometrial epithelial cells increased IL6 production (Laird *et al.*, 1994). Inhibition of IL6 production in the Ishikawa cell line following treatment with 17β-oestradiol potentially through NF_KB signaling has also been demonstrated (Ray *et al.*, 1997). Some studies have shown differences in serum IL6 expression between normal fertile females and those that undergo spontaneous abortion in both humans and murine models, however uterine levels were not measured (Margni and Zenclussen, 2001; Zenclussen *et al.*, 2003). The localisation of IL6 mainly to the glandular and luminal epithelium of the endometrium, with expression peaking in the mid-secretory phase combined with evidence that IL6 levels differ between fertile and infertile individuals suggests a plausible role for IL6 in the implantation process. However, a further study which measured levels of IL6 expression in human endometrial biopsies obtained between days LH+6 and LH+13 from fertile and infertile human women, showed no difference between the two groups of women, suggesting that IL6 does not play a direct role in uterine receptivity (Sherwin *et al.*, 2002).

IL6 expression has also been identified in the blastocyst (Sharkey *et al.*, 1995) and in the trophoblast. Investigations into the role of IL6 in trophoblast growth and placental development have shown its capacity to affect the expression of placental hormones such as hCG (Nishino *et al.*, 1990). The trophoblast has also been shown to express the interleukin 6 receptor (IL6R) and its associated signal transducer, gp130. It has also been shown in an *in vitro* study that cytotrophoblast cells produce higher levels of IL6 mRNA than syncytiotrophoblast cells (Stephanou *et al.*, 1995). The IL6R and its associated gp130 signal protein has also been localised to within the trophoblast (Nishino *et al*, 1990). IL6 has been shown to induce the activation of trophoblast MMPs 2 and 9 and also to increase trophoblast integrin expression (Das *et al.*, 2002 and Meisser *et al.*, 1999).

Tissue culture experiments have found that first trimester placenta released IL6 into the supernatant, while histochemical studies showed the presence of IL6 in trophoblast cells with greater staining intensity found in the syncytiotrophoblast rather than the cytotrophoblast (Kameda *et al.*, 1990). Other tissue culture experiments found IL6 to be expressed in both the trophoblast and villous core compartments of the placenta, with the trophoblast producing significantly higher levels of IL6. It was suggested that the regulation of IL6 was post-transcriptional as mRNA levels in the villous core and trophoblast did not correlate with the observed differential production of IL6 (Kauma *et al.*, 1993). Another study found IL6 to be expressed by both the syncytiotrophoblast and extravillous trophoblast and suggested that IL6 produced by the trophoblast may account for observed IL6 in coelomic and amniotic fluids. It has been suggested that IL6 may play a role in tissue remodeling associated with placentation and during the first trimester of pregnancy in the haematopoeitic function of the secondary yolk sac and in the generation of new vessels in placental villous tissue (Jauniaux *et al.*, 1996).

1.8.2 Interleukin 8 (IL8) (CXCL8)

IL8 is a secreted chemokine that is known to recruit leukocytes by chemotaxsis. It has also shown to be involved in non-immune cell function both outside and within the endometrium (Arici *et al.*, 1998b and Garcia-Valesco and Arici, 1999). IL8 has been shown to be expressed in the human endometrium, where it has been immunolocalised mainly to the glandular and luminal epithelium and vascular

endothelial cells (Caballero-Campo *et al.*, 2002 and Milne *et al.*, 1999). It has been suggested that the function of IL8 in the endometrium is to act as a regulator for immune cell recruitment, a process which is known to change throughout the menstrual cycle (Garcia -Velasco and Arici, 1999). IL8 is expressed in cultured epithelial and stromal endometrial cells and addition of the pro-inflammatory cytokines IL1 and TNFα increased its expression (Arici *et al.*, 1993). Endometrial IL8 protein levels change during the menstrual cycle, with peak levels of expression during the early to mid proliferative and late secretory phases (Arici *et al.*, 1998a). It was suggested that the increased expression of IL8 in the late secretory phase may regulate the increased recruitment of neutrophils into the endometrium prior to menstruation (Milne *et al.*, 1999), where they are thought to be involved in the degradation and scavenging of endometrial tissue. The increased expression of IL8 in the latter early to mid proliferative phase of the menstrual cycle may suggest a role in neo-vascularisation of growing endometrium, which is a known effect of IL8 (Arici *et al.*, 1998a and Koch *et al.*, 1992). Progesterone has been shown to increase IL8 mRNA expression and inhibit protein production in cultured endometrial cells and this might account for the lower IL8 expression seen in the early luteal phase *in vivo*. The decrease in progesterone during the late luteal phase would allow IL8 expression to increase allowing increased neutrophil recruitment into the endometrium (Kelly *et al.*, 1994). This is supported by the observation that increased immuno-reactivity to IL8 occurs in premenstrual decidua and perivascular cells, which is consistent with IL8 functioning to recruit leukocytes from the vasculature at this time (Jones *et al.*, 1997).

IL8 has not only been shown to act as a chemoattractant and angiogenic factor but also can stimulate cell growth. It has been shown that IL8 stimulates the proliferation of various cells types including endothelial cells (Koch *et al.*, 1992), epidermal cells (Tuschil *et al.*, 1992), and smooth muscle cells (Yue *et al.*, 1994). The ability of IL8 to act as a growth factor in stromal endometrial cells has been demonstrated previously (Arici *et al.*, 1998b).

1.8.3 Matrix metalloproteinase 2 (MMP2) and 9 (MMP9)

Matrix metalloproteinases (MMPs) are enzymes which degrade components of the extracellular matrix (ECM). The MMPs are thought to critically control the degradation and remodeling of ECM and collectively can degrade virtually all components of ECM including both interstitial matrix and basement membranes (Birkedal-Hansen *et al.*, 1993; Powell and Matrisan, 1996 and Salamonsen and Woolley, 1999). The MMPs can be divided into subfamilies; the collagenases, gelatinases, stromelysins and membrane-types. Most MMPs are secreted extracellulary as latent zymogens, which have been shown to be activated by a number of proteases such as plasmin, tryptase and elastin as well as by other MMPs which can form MMP activation cascades. The activation of pro-MMP2 is unusual as it activated at the cell surface by membrane-type MMPs (MT-MMPs) (Salamonsen, 1999 and Salamonsen and Woolley, 1996). MMPs are inhibited by tissue inhibitors of matrix metalloproteinases (TIMPs) which block the activity of the MMPs by the formation of 1:1 complexes. Growth factors, cytokines, steroid hormones (including progesterone and androgens) differentially regulate the expression of MMPs. This gives the MMPs the ability to act differentially in both a tissue and cell specific manner independent of the system to which they belong (Salamonsen, 1999 and Salamonsen *et al.*, 1997). Both transcriptional and post-transcriptional mechanisms regulate MMP and TIMP production. The promoter regions of the MMP and TIMP genes contain an AP-1 and PEA-3 biding site, which binds fos and jun and Ets respectively. The fos, jun and Ets proteins have been shown to be present in the endometrium (Edwards *et al.*, 1996). The mechanisms by which MMP gene transcription is regulated in the endometrium and trophoblast remains unknown (Salamonsen, 1999 and Salamonsen and Woolley, 1999).

The expression pattern of MMPs in the normally functioning endometrium during the menstrual cycle is consistent with a role in the process of normal menstruation. Studies have demonstrated that progesterone has the ability to act as a negative regulator of transcription for MMPs 1, 3 and 9 and it has been suggested that falling levels of progesterone towards the end of the menstrual cycle may be responsible for allowing an increased expression of MMPs at the time of menstruation (Salamonsen *et al.*, 1997). However, it has been suggested that this may not reflect the true *in vivo* situation. For example, some cytokines such as IL1 and TNFα, which increase MMP

production, have been shown to have the ability to override the action of progesterone on MMP expression suggesting that transcriptional control of MMP expression may be carried out at the local level (Salamonsen *et al.*, 1997; Singer *et al.*, 1997 and Zhang *et al.*, 1998). MMP1, 2 and 3 are expressed by the human endometrial stroma at menstruation (Salamonsen and Woolley, 1996).

Studies that have immunolocalised the expression of MMP2 in the human female reproductive tract have found it to be expressed in a variety of cell types including decidual cells and the trophoblast as well as a variety of leukocytes (Fernandez *et al.*, 1992). During the secretory phase of the menstrual cycle, MMP9 protein has not been detected in the endometrial stroma using immunohistochemistry but MMP9 has been shown to be expressed within the luminal and glandular epithelium during the postovulatory period. It has been observed that MMP9 protein moves to the apical surface of the epithelium and are released into the uterine lumen during the period of endometrial receptivity (Jeziorska *et al.*, 1996 and Salamonsen and Woolley, 1996). The expression of MMP9 has been shown in subsets of endometrial eosinophils, macrophages and neutrophils, while CD4+ T lymphocytes have been shown to express MMP2 and 9 during menstruation (Shi *et al.*, 1995).

MMP2 and 9 are thought to play role in controlling the invasion of the trophoblast into the endometrial stroma (Ioannidis *et al.*, 2010 and Jovanovic *et al.*, 2010). The cytokine LIF, which is known to be involved in the control of implantation has been shown to increase the secretion of TIMP1 and 2 in primary first trimester human extravillous trophoblast (EVT), but did not affect the expression of MMP2 and 9 (Tapia *et al.*, 2008). In preeclampsia, there is shallow EVT invasion and impaired spiral artery and arteriole remodeling to reduce uteroplacental blood flow. Studies have shown increased expression of MMP9 in decidual cells and adjacent interstitial trophoblasts in placental sections taken from preeclamptic women versus normal controls demonstrating MMP9's potential importance in successful normal implantation (Lockwood *et al.*, 2008).

Proteolytic degradation of the ECM by MMPs and the control of ECM degradation by TIMPs appears to be the most obvious reason for the expression of MMPs in the endometrium, especially given its highly dynamic nature. However, alternative

functions of the MMPs have been described for these molecules which may be important when considering endometrial function (Salamonsen, 1999). For example, several MMPs degrade regulatory molecules including IL1β and insulin-like growth factor binding protein (Fowlkes *et al.*, 1994 and Ho *et al.*, 1996). The TIMPs also have been shown to have alternative functional activities. For example, TIMP1 and 2 have shown to have growth factor activity (Hayakawa *et al.*, 1994).

1.8.4 Vascular endothelial growth factor A (VEGFA)

Angiogenesis is the formation of new blood vessels from pre-existing vasculature and is a fundamental process of the human menstrual cycle. The endometrium is recognised as one of the few adult human tissues where significant angiogenesis occurs on a routine physiological basis due to the endometrium's dynamic cycles of tissue growth and breakdown. Abnormal endometrial angiogenesis may contribute to several endometrial pathologies including; endometrial cancer, endometriosis, menorrhagia and breakthrough bleeding (Girling and Rogers, 2005).

Endometrial growth and differentiation occurs under the control of oestrogen and progesterone, but the sex steroids exert the majority of their effects indirectly via a variety of growth factors (Girling and Rogers 2005). This includes the process of angiogenesis, in which vascular endometrial growth factor (VEGF) is known to act as a potent endothelial cell mitogen. It also has the ability to increase vascular permeability and plays a central role in inflammation and other pathologies (Ferrara, 2004; Ferrara and Davis-Smyth, 1997 and Risau, 1997).

The human VEGF gene is organized in eight exons on chromosome 6p21.3 and is known to produce four alternatively spliced isoforms (VEGFA-D) which vary in amino acid length, protein structure and potential functioning. VEGF acts through tyrosine kinase receptors VEGFR1 and VEGFR2 to exert its cellular effects (Ferrara and Davis-Smyth, 1997).

There is evidence that oestrogen stimulates angiogenesis by acting directly on endometrial endothelial cells, which is supported by the identification of the oestrogen receptors in the endometrial vascular smooth muscle and in endothelial cells both *in vitro* and *in vivo* (Girling and Rogers, 2005). Studies which have investigated the effects of oestrogen on endometrial epithelial and stromal cells have produced some

conflicting results. For example, a study which investigated the effects of oestrogen on cultured endometrial stromal cells found it to increase the mRNA expression of VEGF isoforms (Huang *et al.*, 1998), yet a more recent study which used immunolocalisation to demonstrate expression of VEGF in both the endometrial epithelium and stroma found that expression of VEGF was decreased under the influence of oestrogen (Punyadeera *et al.*, 2006).

While evidence for the direct action of oestrogen on the endometrium and its vasculature in relation to angiogenesis and VEGF expression has been identified, it is also thought that angiogenesis in the endometrium must also occur indirectly via other endometrial cell types. For example, following hormonal withdrawal at the end of the secretory phase, the angiogenesis required to repair the vascular bed occurs in the absence of steroid hormones (Girling and Rogers, 2005). This is supported by observations using human co-cultures of endometrial cells and microvascular endothelial cells, which showed that neither oestrogen nor endometrial stromal cells promoted angiogenesis in endothelial cells but instead demonstrated increased angiogenesis by endothelial cells in the presence of glandular epithelial cells. This led to the proposal that oestrogen regulates endometrial angiogenesis by regulating the expression and secretion of angiogenic factors such as VEGF by glandular epithelial cells in the human endometrium. Expression of VEGF by glandular epithelial cells has also been demonstrated in non-human primates (Albrecht *et al.*, 2003 and Albrecht and Pepe, 2003).

1.9 Aims and Objectives of the study

This introduction has highlighted that successful reproduction in the human female involves the concerted action between different organs, tissues and cell types. The endometrium plays a particularly important role in the process as it is the site of embryo implantation and develops to form the maternal side of the placenta during pregnancy. The endometrium contains many different cell types, including a unique population of immune cells and undergoes dynamic changes through the menstrual cycle. These highly dynamic processes are systemically regulated by the action of the steroid hormones. However, the diverse cellular functions that occur throughout the menstrual cycle and during implantation requires fine cellular control. Chemokines

have the potential to fill many of these roles using their ability to form chemotatic gradients and potentially exert wide effects on the expression of many genes and the proteins they encode.

The endometrium undergoes rapid changes in tissue development and organisation, vascularisation and cell function. Chemokines have been implicated in all of these roles and may play vital roles in the paracrine control of endometrial function (Busillo and Benovic, 2007). The chemokine ligand CXCL12 and its receptor CXCR4 are unique in terms of their genetic locations in respect to other chemokines and receptors, their widespread expression and secretion in human tissues and are essential to achieve fetal development. The ligand and receptor pair are known to be involved in leukocyte chemotaxis and other functions including controlling cell proliferation and angiogenesis. All of these effects could be of potential importance in the endometrium. While CXCL12 and CXCR4 have been heavily studied in pathological roles such as cancer metastases and HIV, their potentially diverse normal functions in human tissues have received less attention. Within the endometrium the expression of CXCL12 and CXCR4 has been characterised but the expression patterns through the menstrual cycle, cellular distribution and functions remain unknown but may potentially be of crucial importance to the general functions of the endomtrium (Dominguez *et al.,* 2003 and Kitaya *et al.,* 2004).

1.9.1 Hypotheses

It is our hypotheses that CXCL12 and CXCR4 may be differentially expressed in the endometrium throughout the menstrual cycle and between endometrial cell types, which may affect function. Furthermore, CXCL12 may regulate the expression of factors important to successful endometrial function such as IL6, IL8, MMP2, MMP9 and VEGFA, which have all been shown to be regulated by CXCL12 in non-endometrial human tissues. CXCL12 may act via CXCR4 to exert completely unknown functional changes on endometrial cells.

1.9.2 Aims and objectives

Therefore, it is our aim to quantify CXCL12 and CXCR4 mRNA expression throughout the menstrual cycle using endometrial samples obtained from healthy volunteers using

real-time RT-PCR to identify differential expression throughout the cycle. To investigate any potential differences in CXCL12 and CXCR4 mRNA expression between different endometrial cell types, we aimed to use real-time RT-PCR to quantify levels of CXCL12 and CXCR4 mRNA in primary endometrial cell cultures. To investigate any potential regulatory effects of CXCL12 on IL6, IL8, MMP2, MMP9 and VEGFA in the endometrium, primary cultures of endometrial cells would be incubated with CXCL12 and any effects measured using real-time RT-PCR after establishing basal expression. As CXCL12 may have effects on endometrial gene expression that are completely unknown, we aim to use genome-wide microarray analysis in endometrial cell lines incubated with CXCL12 to identify gene transcripts which may respond to the ligands presence. The suitability of using cell lines to investigate endometrial cell function will also be considered by comparing the gene expression of Ishikawa and HEC-1-B cells to primary epithelial counterparts.

Chapter 2
Materials and methods

2.1 Human subjects

Endometrial biopsies were obtained from women attending the Jessop Wing, Sheffield Teaching Hospitals, to investigate the expression of CXCL12 and CXCR4 through the menstrual cycle and for preparation of primary cell cultures. All samples were collected with written informed consent from the patient and ethical committee approval was obtained for this study (ethics REC reference number: 04/Q2305/23, Sheffield Research Ethics Committee). The total number of samples used was 37 and all samples were obtained from normal fertile women undergoing surgery for non-endometrial pathology. All of the women were aged between 20 and 40 and had menstrual cycles of between 25 and 35 days, had normal uterine anatomy and had no steroid treatment for at least 2 months prior to the study. The samples were dated according to the day of the last menstrual period and confirmed by histological staging.

2.2 Cell culture

All cell culture reagents, media and supplements were obtained from Gibco™, Invitrogen UK unless otherwise stated. All cell culture methods were carried out under sterile conditions in a class II laminar flow cabinet. Ishikawa endometrial adenocarcinoma cells were obtained from the European Collection of Cell Cultures (ECACC) through Sigma-Aldrich, UK. HEC-1-B endometrial adenocarcinoma cells were obtained from the American Type Culture Collection (ATCC) through LGC Standards, UK.

2.2.1 Cell line stocks

Ishikawa and HEC-1-B cells were stored in cryovials immersed in liquid nitrogen until required for use. Cells were preserved in 2 ml of fetal bovine serum (FBS) containing 10% (v/v) dimethyl sulfoxide (Sigma-Aldrich, UK).

2.2.2 Ishikawa cell line culture

Thawed stocks of adherent Ishikawa adenocarcinoma cells were routinely grown in a cell culture incubator at 37°C and 5% CO_2 in 25 cm^2 cell culture flasks bathed in 20 ml of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS, 2% (v/v) L-glutamine (200 mM) and 2% penicillin-streptomycin to make complete DMEM (cDMEM). When cell monolayers were determined to be near-confluent (approximately 80%), cells were passaged to new 25 cm^2 flasks by removal of culture medium followed by washing with 10 ml of 1X phosphate buffered saline (PBS) solution. The cells were detached from the flask by incubating at 37°C for 5 minutes in 5 ml of trypsin/EDTA solution (containing 0.25% trypsin) followed by gentle tapping of the flask. The action of the trypsin/EDTA solution was stopped by addition of 5 ml of cDMEM. The resulting 10 ml cell solutions were placed into 50 ml Falcon tubes and centrifuged for 5 minutes at 300 x g and the supernatant discarded. Cell pellets were washed by re-suspension in 10 ml of PBS to remove any remaining trypsin/EDTA. Centrifugation was repeated. The supernatant was discarded and the pellet was either immediately used for RNA extraction (section 2.2) or re-suspended in 8 ml of cDMEM and passaged at a 1:8 ratio into new 25 cm^2 flasks by addition of 1 ml of cell/DMEM solution to 19 ml of cDMEM. Flasks were placed back into the incubator to allow cells to adhere and grow.

2.2.3 HEC-1-B cell line culture

The same procedure as was used for Ishikawa cells was used to culture HEC-1-B cells, except that HEC-1-B cells were grown in McCoy's 5A medium (Sigma-Aldrich, UK) which was supplemented with 10% FBS, 2% L-glutamine and 2% penicillin-streptomycin solution.

2.2.4 Primary cell culture

Primary endometrial epithelial and stromal cell cultures were prepared using an established method (Cork *et al.*, 2002; Laird *et al.*, 1993 and Tuckerman *et al.*, 2000). Human endometrial tissue was collected and placed in 2.5 ml Hanks balanced salt solution (Sigma-Aldrich, UK) containing 100 µl penicillin-streptomycin solution. 20 mg of collagenase 1A (Sigma-Alrich, UK) was placed into a sterile Falcon tube and 10 ml of

cDMEM containing 0.005% (v/v) sodium pyruvate Solution (Sigma-Aldrich, UK) was added to the tube and syringe filtered into two 5 ml aliquots in 50 ml Falcon tubes and stored at 4°C. Using sterile scissors and forceps, endometrial tissue was removed from the Hanks solution and cut into fine pieces in one of the tubes containing the collagenase/cDMEM solution.

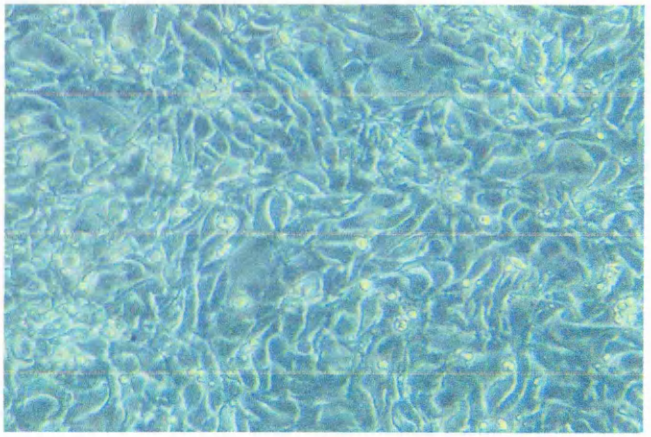
The sample was incubated at 37°C for 1 hour to allow the collagenase 1A to act on the endometrial tissue resulting in separation of epithelial cell glands and stromal cells from the tissue. This process was aided by gentle pipetting at 30 minutes and 1 hour during the incubation to aid dispersion. After 1 hour, the dispersed cell suspension was centrifuged at 100 x g for 5 minutes, the resulting supernatant containing stromal cells was removed and centrifuged at 300 x g for 5 minutes to pellet the cells and allow removal of collagenase/cDMEM solution. The supernatant was discarded and the stromal cell pellet was re-suspended in 5 ml of cDMEM and stored at 37°C. The remaining epithelial cell pellet was re-suspended in the second aliquot of collagenase/cDMEM and incubated at 37°C and pipetted to disperse cells as previously stated. Centrifugation at 100 x g produced a pellet of epithelial cells in glandular clumps and the supernatant was placed into the previously removed and stored stromal cell suspension which was centrifuged at 300 x g to produce a pellet.

Density gradient separation was performed to further purify epithelial and stromal cell cultures. Two 15 ml sterile Falcon tubes were labeled epithelial gradient and stromal gradient. 8 ml of cDMEM was placed into the epithelial gradient tube and 8 ml of 50% cDMEM and 50% Nutrient Mixture F-12 Ham medium (F-12) supplemented in the same manner as cDMEM (section 2.2.2). 2 ml of cDMEM was used to re-suspend the epithelial cells and was layered over its gradient. 2 ml of cDMEM/F-12 was used to re-suspend the stromal cells, which were also layered over its respective gradient. Both gradients were left for 30 minutes to allow separation. The larger epithelial cell gland fragments fall to the bottom of the tube, the top 8 ml of the epithelial tube was discarded and the remaining 2 ml was retained for cell counting. The smaller stromal cells remain in the upper 8 ml of the tube which was removed and retained. The lower 2 ml of the media was discarded.

Previous immunocytochemical analysis of vimentin (a stromal cell marker), cytokeratin (an epithelial cell marker) and CD45 (leukocyte marker) of cells prepared and grown this way have shown that the epithelial and stromal cells are essentially pure and free of leukocyte contamination after 48 hours in culture (Tuckerman, *et al.*, 2000).

Cells were counted using a haemocytometer by counting the number of cells in 5 of the large squares (outer four corners and the central square) which was multiplied by 50, 000 to give the number cells per ml. Approximately $4x10^5$ cells in 5 ml of appropriate medium (cDMEM for epithelial cells and cDMEM/F-12 for stromal cells) was added to 12.5 cm^2 flasks which were pre-coated with 500 µl of FBS to aid cell adhesion. Cells were incubated at 37°C and 5% CO_2 with medium changed every 48 hours while growing. Cells were not passaged but underwent addition of CXCL12, 24 hours prior to expected confluence (section 2.2.5). Figure 2.1 shows images of primary epithelial and stromal cells cultured using this method. A total of six samples were used for cell culture obtained throughout the menstrual cycle, three of known date (day 8, 25 and 29) and three of unknown date.

A.



B.

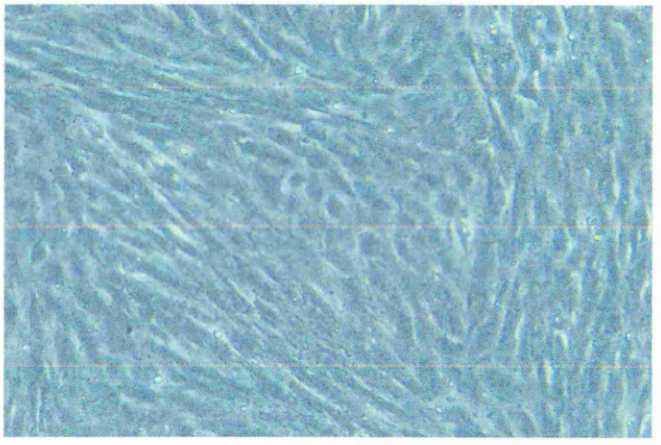


Figure 2.1: Images of cultured primary endometrial epithelial and stromal cells

The figure shows confluent primary epithelial (A) and stromal (B) endometrial cells. No visible contamination is apparent in either culture model.

2.2.5 Addition of CXCL12α/SDF-1α to endometrial cell cultures

A recombinant polypeptide sequence representing amino acid residues 22-89 of human CXCL12α (NCBI sequence ID: 1352728) with an ED_{50} of 3-9 ng/ml for cultured T lymphocytes was obtained from R&D Systems, UK. 10 µg of lyophilised CXCL12 was reconstituted to a recommended concentration of 100 µg/ml by addition of 100 µl of sterile PBS to create a stock solution. This was separated into 10 µl aliquots designed for single use to minimise damage due to freeze/thawing. Aliquots were stored at -80°C.

For use in microarray analysis, Ishikawa cells were incubated 24 hours prior to expected confluence with 0 and 5 ng/ml of recombinant CXCL12. This concentration was chosen to be relatively low as the sensitivity of microarray analysis should detect any changes in any mRNA transcript expression. For real-time RT-PCR experiments Ishikawa and HEC-1-B cells were incubated with 0, 10 and 100 ng/ml of CXCL12 for 24 hours prior to expected confluence. Primary epithelial and stromal cell cultures also incubated with 0, 10 and 100 ng/ml of CXCL12 24 hours before reaching confluence. 24 hours after addition of CXCL12, Ishikawa and HEC-1-B cells were removed from flasks by trypsinisation as described in sections 2.2.2 and 2.2.3. Primary epithelial and stromal cells were detached from the flask by addition of 1 ml of trypsin/EDTA solution followed by incubation at 37°C for 5 minutes followed by gentle tapping and use of a cell scraper if necessary to remove cells. The action of trypsin/EDTA was stopped by addition of 1 ml of cDMEM. Cells were pelleted by centrifugation at 300 x g for 5 minutes. The supernatant was removed and discarded and cells were washed by addition of 10 ml of PBS. Centrifugation was repeated and the supernatant was discarded. Pellets were kept on ice until RNA was extracted.

2.3 RNA extraction

RNA was extracted from all cells cultures using the Genelute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, UK) which uses spin columns to extract RNA from cell cultures or tissue. RNA in human endometrial tissue biopsy samples was extracted using TRI REAGENT™ (Sigma-Aldrich, UK).

2.3.1 Genelute™ Mammalian Total RNA Miniprep Kit

RNA extraction from Ishikawa and HEC-1-B cells and primary endometrial epithelial and stromal cells was carried out according to the manufacturer's protocol:

Cell lysis solution was prepared by addition of 10 µl of 2-Mercaptoethanol (2-ME) per ml of provided lysis solution. 500 µl of 2-ME/lysis solution was added to each cell pellet and was vortexed thoroughly to lyse the cells and inactivate RNases. The lysate was then added to the Genelute™ filtration column and receiving tube and centrifuged at 16,000 x g for 2 minutes, to remove cellular debris and to shear genomic DNA.

The filtration column was discarded and the filtered lysate was prepared for RNA binding by adding an equal volume (500 µl) of 70% ethanol solution. The lysate/ethanol solution was bound to a Genelute™ RNA binding column by a two-step addition of 500 µl of lysate/ethanol solution followed by centrifugation at 16,000 x g for 15 seconds. The flow-through was discarded and the binding insert containing the RNA was retained.

Bound RNA was washed by addition of 500 µl of wash solution 1 to the RNA binding column which was placed into a collection tube, followed by centrifugation at 16,000 x g for 15 seconds. The binding column was then placed into a fresh collection tube and 500 µl of wash solution 2 was run through the column by further centrifugation at 16,000 x g for 15 seconds. This was repeated with a third column wash which was again centrifuged for 2 minutes to dry the column. As the column must be free of any ethanol before eluting the RNA a further drying step was carried out by centrifugation at 16,000 x g for 1 minute.

RNA was eluted from the column by adding 50 µl of elution solution to the RNA binding column followed by centrifugation at 16,000 x g for 1 minute. The resulting purified RNA in the eluate was stored at -80°C.

2.3.2 Extraction of total RNA from human endometrial tissue samples using TRI REAGENT™

RNA was directly extracted from endometrial tissue using TRI REAGENT™ according to manufacturer's instructions (Sigma-Aldrich Technical Bulletin MB-205): Human

endometrial tissue samples were collected into 1.5 ml of RNA*later*® tissue collection RNA stabilisation solution (Applied Biosystems, UK) which rapidly permeates tissue to preserve and protect samples from RNase degradation. Samples were stored at -80°C. When required, samples were thawed at room temperature and weighed to ascertain the amount of TRI REAGENT™ required to successfully extract the RNA. Samples were then finely chopped with surgical scissors and homogenised by hand in a plunging glass homogeniser with 1 ml of TRI-REAGENT™ added per 100 mg of endometrial tissue.

The homogenised tissue/TRI REAGENT™ mixture was placed into 1.5 ml micro-centrifuge tubes and centrifuged at 12,000 x g for 10 minutes at 4°C to remove insoluble materials such as extracellular membranes, polysaccharides and high molecular weight DNA. The resulting supernatant was transferred to a fresh micro-centrifuge tube and allowed to stand at room temperature for 5 minutes to ensure dissociation of nucleoprotein complexes. Addition of 0.2 ml of chloroform (Sigma-Aldrich, UK) per ml of TRI REAGENT™ to each sample was followed by vigorous shaking and each sample was left to stand for 15 minutes at room temperature. The mixture was centrifuged at 12,000 x g for 15 minutes at 4°C. This procedure resulted in the separation of each sample into three phases. A red organic phase containing protein, an inter phase containing DNA and the colourless upper phase that contained RNA.

To isolate and precipitate RNA, the upper aqueous phase was removed to a fresh micro-centrifuge tube to which, 0.5 ml of isopropanol (Fisher Scientific, UK) per ml of TRI REAGENT™ (first used in sample homogenisation) was added and thoroughly mixed. The sample was allowed to stand for 10 minutes at room temperature. Centrifugation at 12,000 x g for 10 minutes at 4°C led to the formation of an RNA pellet on the bottom of the tube. The pellet was washed by removing the supernatant and adding 1 ml of 75% ethanol per ml of TRI REAGENT™ followed by vortexing and centrifugation at 7,500 x g for 5 minutes at 4°C. The supernatant was removed and the pellet left to air dry ensuring it did not do so completely. 50 µl of nuclease free water (Sigma-Aldrich, UK) was added to the pellet which was re-suspended by repeated pipetting of the sample. Samples were stored until required at -80°C.

2.4 Assessing RNA quantity and integrity

2.4.1 Quantification of RNA samples using NanoDrop ND-1000 UV-VIS Spectrophotometer

RNA quantity was accurately measured in using the NanoDrop ND-1000 UV-VIS Spectrophotometer in the RNA40 nucleic acid mode. The spectrophotometer was initialised using nuclease-free water and then blanked using the appropriate solution (nuclease-free water or elution solution from the Genelute™ Mammalian Total RNA Miniprep Kit). 1 µl of each RNA sample was used to obtain the RNA concentration. Generally, 500 ng aliquots of RNA in 10 µl of nuclease-free water were prepared and stored at -80°C for use in cDNA synthesis. Samples considered to have a low abundance of total RNA were adjusted to 250 ng.

2.4.2 Assessing RNA integrity using Experion™ LabChip microfluidic technology

Assessment of RNA integrity by automated electrophoresis was carried out using the Experion™ LabChip microfluidic technology in conjunction with the Experion™ RNA Standard Sensitivity Analysis Kit (Biorad, UK) and was carried out according to the manufacturer's instructions.

Cleaning of the electrodes on the electrophoresis station was carried out before each run by filling the provided cleaning chip with 800 µl of Experion™ electrode cleaning solution and placing in the instrument with the lid closed for 2 minutes, immersing the electrodes into the solution within the cleaning chip. A separate cleaning chip containing 800 µl of nuclease-free water was then placed in the instrument for 5 minutes followed by replacement of the water and further electrode immersion for 1 minute. The lid was then left open to allow evaporation of any remaining water from the electrodes.

Experion™ RNA Standard Sensitivity Analysis Kit reagents were removed from storage at 4°C and left at room temperature for 15 minutes to equilibrate while the provided RNA ladder was removed from storage at -80°C and placed on ice. Stocks of filtered gel were prepared by pipetting 600 µl of RNA gel into a provided spin filter tube followed by centrifugation for 10 minutes at 1,500 x g. The filter insert was discarded

and the resulting filtered gel was viable for 4 weeks after this procedure when stored at 4°C.

65 µl of the filtered gel was pipetted into an RNase free micro-centrifuge tube and 1 µl of gel stain (foil wrapped to protect it from light damage) was added and briefly vortexed to obtain the appropriate ratio of gel to gel stain. This solution was prepared on the day of use.

2 µl of the RNA ladder and 2 µl of each RNA sample to be analysed (maximum 12 per chip) were placed into RNase free micro-centrifuge tubes. The ladder and RNA samples were denatured at 70°C in a heating block for 2 minutes to remove secondary RNA structures and cooled by immediately placing on ice for a minimum of 5 minutes.

An Experion™ RNA Standard Sensitivity Chip was removed from its packaging and placed on the platform of the instrument's priming station. 9 µl of the filtered gel solution was placed into the gel priming well labelled GS on the RNA chip. Care was taken to avoid bubble formation when applying reagents to the RNA chip wells and was fundamental to achieving successful analysis. The lid of the priming station was closed and the chip primed for 30 seconds at time setting 1 and pressure B. The chip was removed from the priming station and placed on a clean surface.

9 µl of filtered gel-stain solution was placed into the second GS well on the RNA chip. 9 µl of the filtered gel was placed into the well labeled G. 5 µl of provided loading buffer was placed into each sample well (1 to 12) and the ladder well labeled L. 1 µl of denatured RNA ladder was pipetted into the well labeled L and a maximum of 12 denatured RNA samples were removed from ice and placed into the sample wells. 1 µl of nuclease-free treated water was pipetted into any unused wells.

The loaded chip was clipped into the instrument's vortex station which operates to mix the reagents and samples for 60 seconds and automatically shuts down. The chip was removed from the vortex station and placed immediately into the instrument's electrophoresis station. Each run was performed using the RNA StdSens Eukaryotic protocol which was selected via the provided computer software.

2.4.3 Assessing mRNA integrity from endometrial cell extracts

Successful and accurate real-time RT-PCR and microarray analysis depends upon good quality mRNA for reverse transcription to cDNA. To assess the integrity of mRNA obtained from Ishikawa, HEC-1-B, epithelial and stromal endometrial cells Experion™ LabChip microfluidic technology was used. Figure 2.2 shows an example of the results obtained from this automated electrophoresis procedure in which peaks at 18S and 28S rRNA show the presence of intact RNA which has not been degraded by the extraction procedures. The results displayed in Figure 2.2 are for RNA extracted from primary endometrial epithelial and stromal cells but RNA extracted from all cell types were tested in this way before their use in real-time RT-PCR and microarray analysis and similar peaks for 18S and 28S rRNA were seen in all samples tested showing that all RNA was of good integrity.

Total RNA extracted from endometrial biopsy tissue generally showed poorer levels of RNA integrity when investigated by the same method (Figure 2.3). This was expected due to unavoidable degradation that occurs in obtaining the sample prior to its stabilisation in RNA*later*® solution. All tissue samples used for calculating the *in-vivo* expression of CXCL12 and CXCR4 were consistently and immediately placed into RNA later solution once removed from the volunteer to minimise this effect.

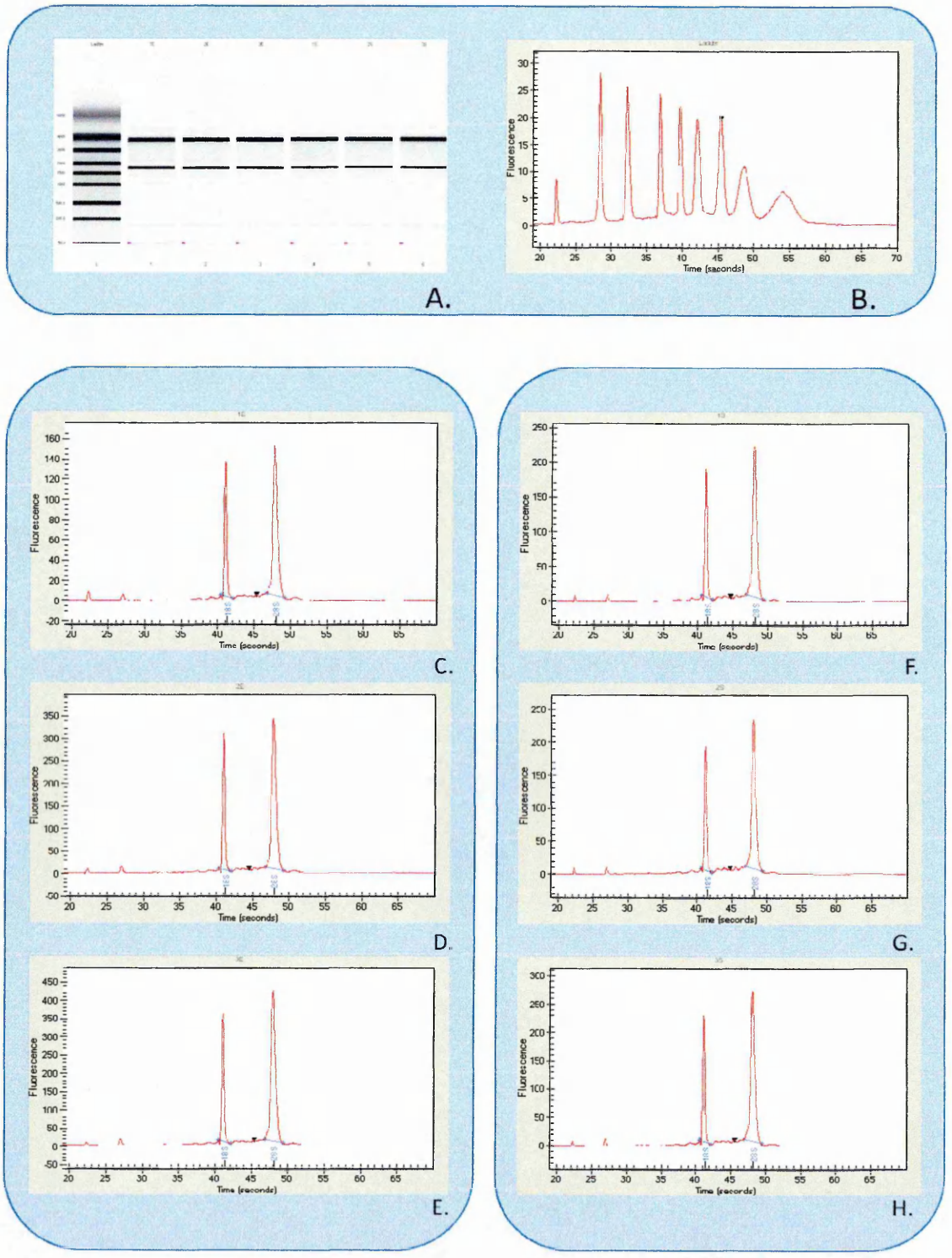


Figure 2.2: Example of results obtained from analysis of RNA by Experion™ LabChip microfluidic technology in primary endometrial cells

A. Shows a digital gel produced after analysis of primary epithelial and stromal cell RNA extracts. Lane 1 shows the RNA ladder which allows identification of the 18S and 28S rRNA subunits in the experimental samples. Lanes 2-5 show RNA from epithelial cells incubated with 0, 10 and 100 ng/ml of CXCL12, while lanes 5-7 show stromal cells incubated in the same manner. B. Shows a plot of recorded fluorescence displayed as a

function of time for the RNA ladder used by Experion™ to identify the 18S and 28S rRNA subunits. C, D and E shows the results obtained for epithelial cells while F, G and H show stromal cells. The samples all show tight peaks for 18S and 28S rRNA and demonstrates that the extracted RNA is intact RNA and has not been subjected to degradation during the extraction process.

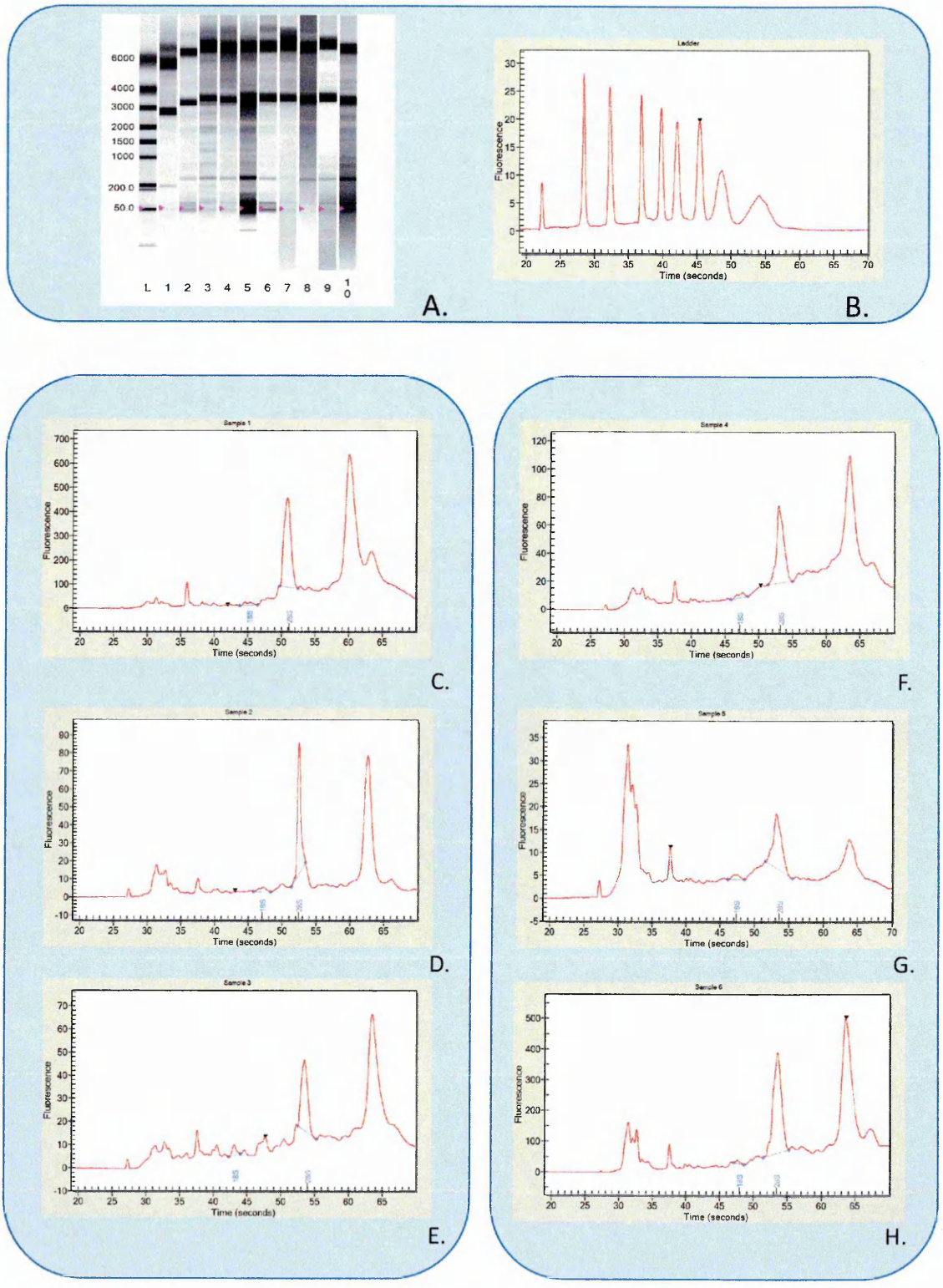


Figure 2.3: Example of results obtained from analysis of RNA by Experion™ LabChip microfluidic technology in endometrial biopsies

A. Shows a digital gel produced after analysis of endometrial biopsy RNA extracts. Lane 1 shows the RNA ladder which allows identification of the 18S and 28S rRNA subunits in the experimental samples.

B. Shows a plot of recorded fluorescence displayed as a function of time for the RNA ladder used by Experion™ to identify the 18S and 28S rRNA subunits. C-H shows the results obtained from six endometrial biopsy RNA extracts.

2.5 Real-time RT-PCR assay preparation and validation

2.5.1 Complementary DNA synthesis from RNA samples for use in real-time RT-PCR

First strand complementary DNA (cDNA) synthesis was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, UK).

10 µl aliquots of extracted total RNA previously adjusted (section 2.4.1) to 500 ng or 250 ng in 0.2ml micro-centrifuge tubes were reversed transcribed to cDNA by addition of 10 µl of cDNA master mix which contained 2.0 µl 10X reverse transcriptase (RT) buffer, 0.8 µl 25X dNTP mix (100 mM), 2 µl 10X RT random primers, 1 µl MultiScribe™ Reverse Transcriptase and 4.2 µl of nuclease-free water per required reaction to a total volume of 20 µl. Mixing was carried out using repeated pipetting and brief centrifugation to drive down tube contents and remove air bubbles.

Reaction tubes were placed in a PCR thermocycler at 25°C for 10 minutes, 37°C for 120 minutes and 80°C for 5 seconds respectively. Samples were held at 4°C until stored at -20°C.

Negative controls for the cDNA synthesis reaction were prepared in the same manner but with 1 µl of nuclease-free water instead the Multiscribe™ Reverse Transcriptase so no cDNA could be produced.

Master mixes were made by multiplying the amounts of each component in the cDNA reaction by the number of samples to be prepared. One additional reaction for both the experimental and control mixes was added to account for evaporation and pipetting error.

2.5.2 Custom primer design of oligonucleotide primers for use in real-time RT-PCR

Oligonucleotide PCR Primers were synthesised by Invitrogen, UK. Housekeeping genes sequences were selected from established primers reported by Vandesompele *et al.* (2002) designed for use as reference transcripts to normalise real-time RT-PCR data (Table 2.1).

Primers for chemokine ligand CXCL12 and receptor CXCR4 were designed after obtaining mRNA sequences from NCBI. The selected portion of the mRNA sequence was pasted into Primer3 software and sequences were generated. A G:C content of approximately 50% was considered acceptable for use (Table 2.2).

Sequences for primers to be used in real-time RT-PCR for IL6, IL8, MMP2 and MMP9 were obtained from a report by Wang *et al.* (2006), while sequences for primers for VEGFA was obtained from Thijssen *et al.* (2004) (Table 2.3).

symbol	Number		Reverse sequence	Product Size (base pairs)
ACTB	NM_001101.2	**Beta actin** Cytoskeletal structural protein	CTGGAACGGTGAAGGTGACA AAGGGACTTCCTGTAACAATGCA	141
B2M	NM_004048.2	**Beta-2-microglobulin** Beta-chain of major histocompatibility complex class I molecules	TGCTGTCTCCATGTTTGATGTATCT TCTCTGCTCCCCACCTCTAAGT	86
GAPDH	NM_002046.2	**Glyceraldehyde-3-phosphate dehydrogenase** Oxidoreductase in glycolysis and gluconeogenesis	TGCACCACCAACTGCTTAGC GGCATGGACTGTGGTCATGAG	125
HPRT1	NM_000194.1	**Hypoxanthine phosphoribosyl-transferase I** Purine synthesis in salvage pathway	TGACACTGGCAAAACAATGCA GGTCCTTTTCACCAGCAAGCT	98
RPL13A	NM_012423.2	**Ribosomal protein L13a** Structural component of the large 60S ribosomal subunit	CCTGGAGGAGAAGAGGAAAGAGA TTGAGGACCTCTGTGTATTTGTCAA	126
SDHA	NM_004168.1	**Succinate dehydrogenase complex, subunit A** Electron transporter in the TCA cycle and respiratory chain	TGGGAACAAGAGGGCATCTG CCACCACTGCATCAAATTCATG	86
UBC	M26880.1	**Ubiquitin C** Protein degradation	ATTTGGGTCGCGGTTCTTG TGCCTTGACATTCTCGATGGT	90
YWHAZ	NM_003406.2	**Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide** Signal transduction by binding to phosphorylated serine residues on a variety of signalling molecules	ACTTTTTGGTACATTGTGGCTTCA CCGCCAGGACAAACCAGTAT	94

Table 2.1: House-keeping genes analysed for stability in different cell types using real-time RT-PCR

Eight established housekeeping genes (Vandesomple *et al.,* 2002) were assessed for their stability across different endometrial cell types under different conditions. The above table lists the housekeeping genes assessed, their NCBI sequence ID, gene function, forward and reverse probe sequences and the expected amplicon product size.

Chemokine/ chemokine receptor name	Accession number	Forward sequence Reverse sequence	Product size (base pairs)
CXCR4	NM_001008540.1	CATTTTTCAGATATAAAAGACTGAC GGTCCTGCCTAGACACACATC	151
CXCL12	NM_199168.2	TCAGCCTGAGCTACAGATGC CTTTAGCTTCGGGTCAATGC	121

Table 2.2: Custom designed primers used in real-time RT-PCR

Primer3 software (http://primer3.sourceforge.net/) was used to create oligonucleotide real-time RT-PCR primers for chemokine ligand CXCL12 and receptor CXCR4. The table shows their mRNA sequence ID from NCBI and the resulting forward and reverse primer sequences created along with the expected product size for each transcript of interest.

Gene name	Accession number	Forward sequence / Reverse sequence	Product size (Base pairs)
IL6	NM_000600	GACAGCCACTCACCTCTTCA	211
		TTCACCAGGCAAGTCTCCTC	
IL8	NM_000584	CTGCGCCAACACAGAAATTATTGTA	170
		TTCACTGGCATCTTCACGTATTCTT	
MMP2	NM_002801	TGCTGGAGACAAATTCTGGA	200
		ACTTCACGCTCTTCAGACTTTGG	
MMP9	NM_004994	TGCCCGGACCAAGGATACAG	182
		TCAGGGCGAGGACCATAGAG	
VEGFA	NM_001025366	AAGGAGGAGGGCAGAATCAT	100
		CCAGGCCCTCGTCATTG	

Table 2.3: Previously published real-time RT-PCR primers for five genes of interest

Established real-time RT-PCR primers were used to investigate mRNA expression for five transcripts of interest. Oligonucleotide probes for IL6, IL8, MMP2 and MMP9 first published by Wang *et al.* (2006) and VEGFA (Thijssen *et al.*, 2004) are shown along with their transcript ID obtained from NCBI, forward and reverse probe sequences and the expected amplicon size.

2.5.3 Real-time RT-PCR primer preparation

A stock solution of concentration 100 µM was prepared from the lyophilised oligonucleotide primers by addition of nuclease-free water and thorough vortexing. Working solutions for each primer were prepared by adding 12.5 µl of stock primer solution to 87.5 µl of nuclease-free water to a total volume of 100 µl and concentration of 12.5 µM. 1 µl of forward and 1µl of reverse primer solution was added to each 25 µl PCR reaction giving a final primer concentration of 0.5 µM.

2.5.4 Components of a SYBR® Green real-time RT-PCR reaction

Each individual real-time RT-PCR reaction consisted of 12.5 µl Absolute QPCR SYBR® Green Mix (ABgene, UK), which contains all the components necessary to perform real-time RT-PCR except for oligonucleotide primers and cDNA. This includes a Thermo-Start™ DNA polymerase which requires activation by heating at 95°C for 15 minutes and prevents non-specific amplification during the reaction set-up, proprietary reaction buffer optimised to $MgCl_2$ which was at a final concentration of 3 mM, dNTPs and SYBR® Green I dye which fluoresces upon binding double stranded DNA. The overall fluorescence increases proportionally to the concentration of double stranded DNA present. Inclusion of a fluorescein reference dye allowed normalisation of reactions in the PCR plate (referred to as the well plate factor) to account for the well position and inherent pipetting error.

Each reaction also contained 1 µl of both the forward and reverse of oligonucleotide primer for the gene of interest from its 12.5 µM working solution giving a final concentration of 0.5 µM per reaction (section 2.4.3). 4.5 µl of nuclease-free water and 2 µl of cDNA from a reverse transcribed RNA sample were also added. The total reaction volume was 25 µl.

A negative control reaction was included which contained 2 µl of nuclease-free water in place of 2 µl of cDNA and a minus reverse transcriptase (-RT) reaction was included which contained 2 µl of –RT control in place of cDNA. Master mixes were made by multiplying the amount of each component required by the number of reactions required plus one to account for possible evaporation and pipetting error. The cDNA was individually pipetted into wells of an iCycler iQ™ 96-well PCR plate (Bio-Rad, UK)

which was sealed using Microseal® B Adhesive Seals (Bio-Rad, UK) before placing into the Bio-Rad iCycler iQ™ Multicolour Real-Time Dectection System. All SYBR® Green real-time RT-PCR reactions were subjected to the same thermal protocol shown in Figure 2.4.

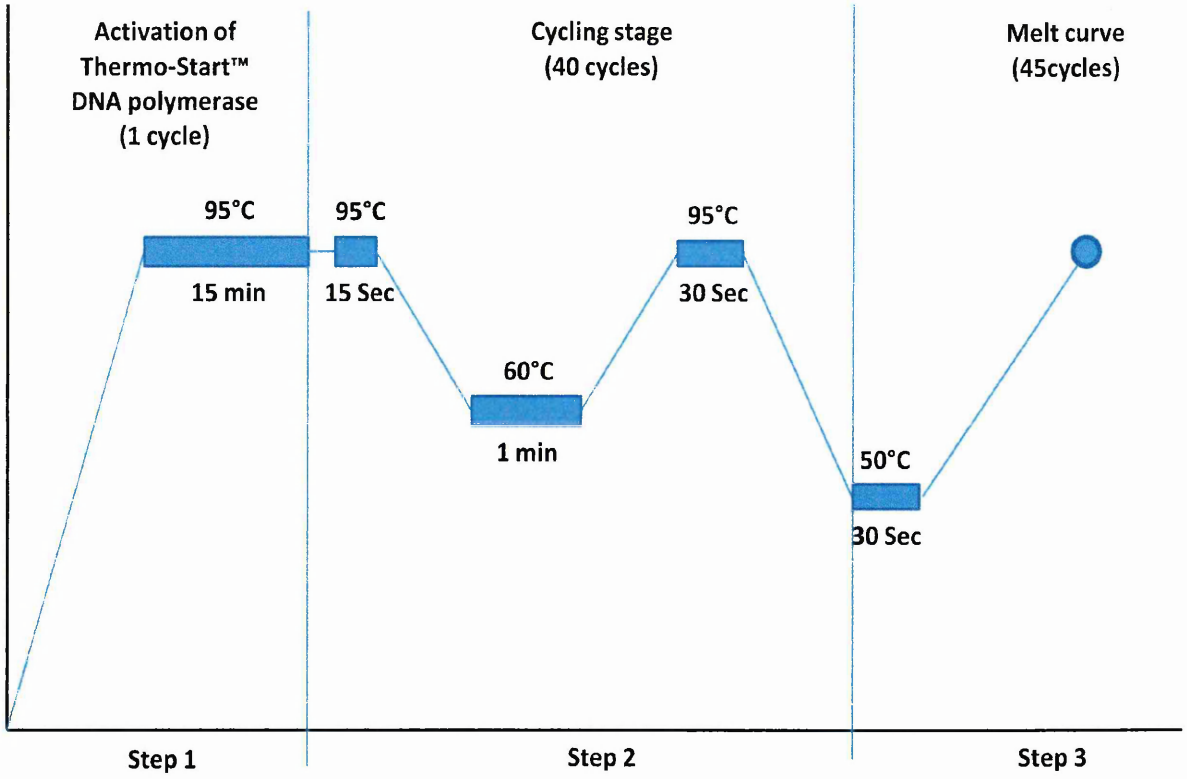


Figure 2.4: Bio-Rad iCycler iQ™ Multicolour Real-Time Detection System thermocycler protocol

All SYBR® Green real-time RT-PCR was subjected to the thermal protocol shown above. Step 1 activated the Termo-Start™ DNA polymerase. Step 2 allowed amplification of the target of interest, while Step 3 consisted of 45, 10 second cycles starting at 50°C (initially for 30 seconds) with an increase of 1°C per cycle enabling melt curve data collection to distinguish the PCR product from possible non-specific DNA amplification.

2.5.5 Assessing reference gene transcript stability

Reference gene transcript stability between Ishikawa and HEC-1-B cell lines was compared. Individual 25 µl SYBR Green real-time RT-PCR reactions (section 2.4.4) were prepared in duplicate for each gene of the 8 housekeeping genes (HK) to be compared (Table 2.1) in both cell types. A negative control reaction was performed for each gene in which no cDNA was added. The samples were assessed by performing real-time RT-PCR in the Bio-Rad icycler iQ™ according to the thermal protocol shown in Figure 2.4.

The resulting cycle threshold (C_t) values obtained for each HK gene for both cell types were $\log_2$ transformed and had their expression ratios calculated using the formula:

$$2^{(\text{control-sample})}$$

The control used in this case was the lowest expressed sample (the highest C_t value) to ensure all ratios had a positive expression value. Ratios were imported into GeNorm software which calculated the most stable HK gene by stepwise removal of genes after analysis of pair-wise variation of all possible combinations to leave the two most stable genes with a stability value *M* (Vandesomple *et al,* 2002). The two HK genes determined to be the most stably expressed across cell types were used as reference transcripts for quantifying relative expression values of all genes of interest.

Figure 2.5 shows the melt curves that were obtained from initial real-time RT-PCR experiments investigating reference gene stability. B2M, GAPDH, HPRT1 and SDHA (A-D respectively) were left out of GeNorm analysis as they showed poorer product specificity in the samples tested. ACTB, RPL13A, UBC and YWHAZ (E-H respectively) were included in the GeNorm analysis and the software carried out stepwise removal of genes, after analysis of pair-wise variation of the reference transcripts to generate an *M* value. An attributed *M* value of *<1.5* is considered acceptable for a transcript to be used as a reference transcript in real-time RT-PCR (Vandesompele *et al*, 2002). UBC and YWHAZ were found to be the most stably expressed transcripts with an *M* value of *0.64* which coupled with their good real-time RT-PCR expression profiles and product specificity made then suitable for use as reliable reference transcripts to quantify gene expression.

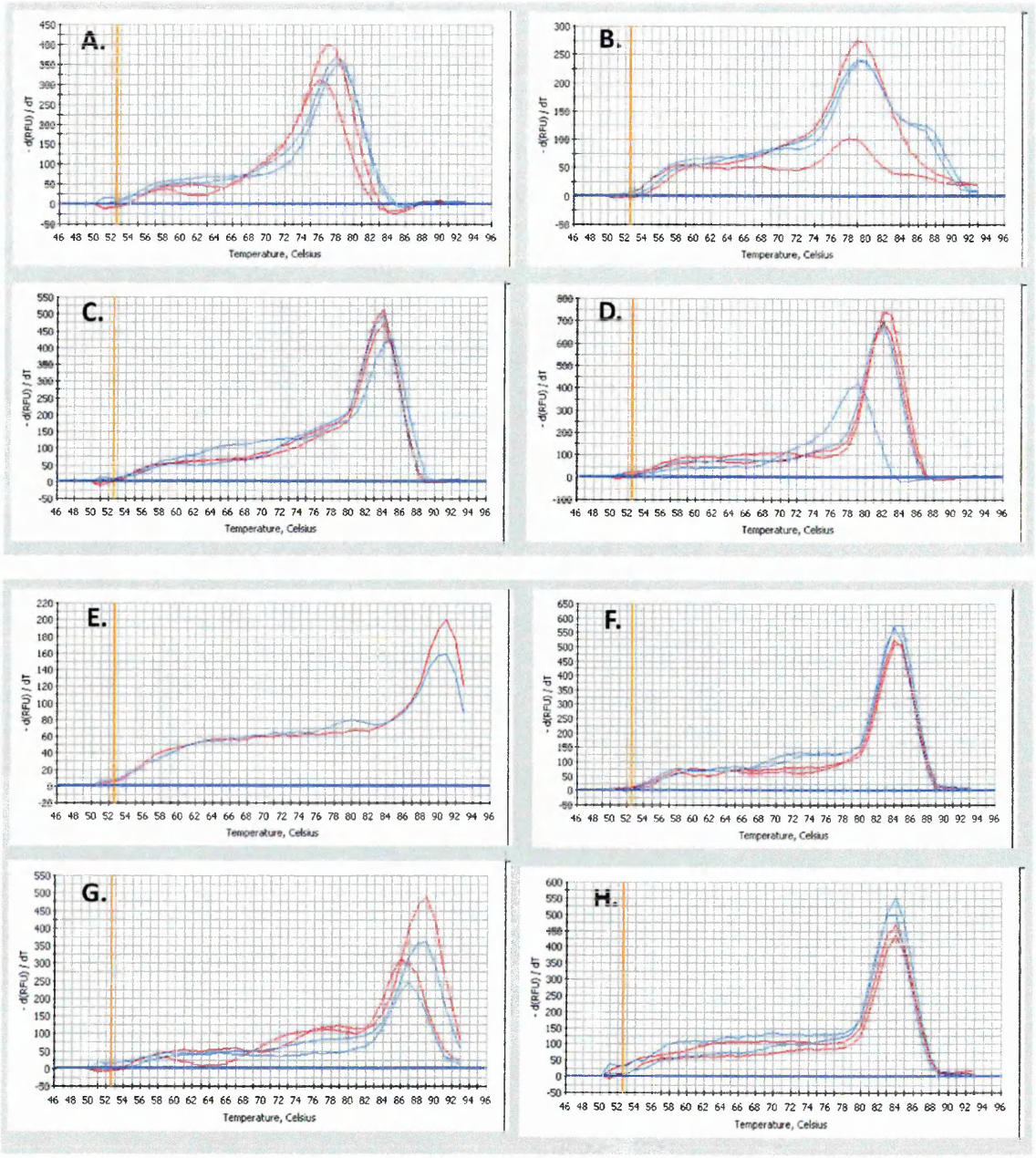


Figure 2.5: Melt curves obtained for reference transcripts assessed for stability in Ishikawa and HEC-1-B endometrial cells

The figure shows melt curves produced for **A**. B2M (beta-2-microglobulin), **B**. GAPDH (glyceraldehyde-3-phosphate dehydrogenase), **C.** HPRT (hypoxanthine phosphoribosyl-transferase 1), **D.** SDHA (succinate dehydrogenase complex, subunit A), **E**. ACTB (Beta actin), **F.** RPL13A (ribosomal protein L13a), **G.** UBC (ubiquitin C) and **H.** YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein) in Ishikawa (blue) and HEC-1-B (red) endometrial cell lines.

To further investigate the accuracy of the SYBR® Green real-time RT-PCR data that was generated, a retrospective study of the variations in the reference gene YWHAZ was carried out across all four endometrial cell types used as ideally the reference transcript should not vary significantly between them. Also, epithelial endometrial cells were used to investigate any potential effects of incubation of cells with CXCL12 on YWHAZ expression. The use of a fixed concentration of RNA in each cDNA synthesis reaction allowed for a statistical comparison of C_t values generated for YWHAZ in all SYBR® Green real-time RT-PCR experiments.

Relative expression of YWHAZ was calculated in relation to the mean triplicate C_t value obtained for YWHAZ from one of the six epithelial cell mRNA samples to which no CXCL12 had been added. Using this value as a control, variations in YWHAZ expression were calculated using the formula:

$$Variation = 2^{-(\text{sample-control})}$$

A non-parametric ANOVA (Kruskal-Wallis test) coupled with a Dunn's multiple comparison test was used to identify any significant differences in YWHAZ expression between endometrial cell types and in response to incubation with CXCL12. As YWHAZ was used in each endometrial cell type to quantify expression levels of IL6, IL8, MMP2, MMP9 and VEGFA and these experiments were carried out on up to six separate occasions, data for up to thirty, triplicate real-time RT-PCR reactions could be included. Figure 2.6, A. shows the results for epithelial endometrial cells incubated with 0, 10 and 100 ng/ml of CXCL12. No significant difference in YWHAZ expression was found between any of the CXCL12 concentrations used (P=0.24, n=30), confirming that incubation with CXCL12 was unlikely to affect the expression of the reference transcript YWHAZ.

Figure 2.6, B. shows the results obtained for epithelial, stromal, Ishikawa and HEC-1-B endometrial cells. While there were no significant differences between the epithelial, stromal and Ishikawa endometrial cells, the HEC-1-B cells produced significantly more YWHAZ mRNA in comparison to the epithelial (*P=0.0002)* and stromal endometrial cell types (*P=<0.01, n=12-30*).

Figure 2.7 shows the results obtained when the same analysis was carried out for YWHAZ and UBC in mRNA extracted directly from endometrial biopsies throughout the menstrual cycle. No significant difference could be observed between any menstrual cycle phases for either reference transcript.

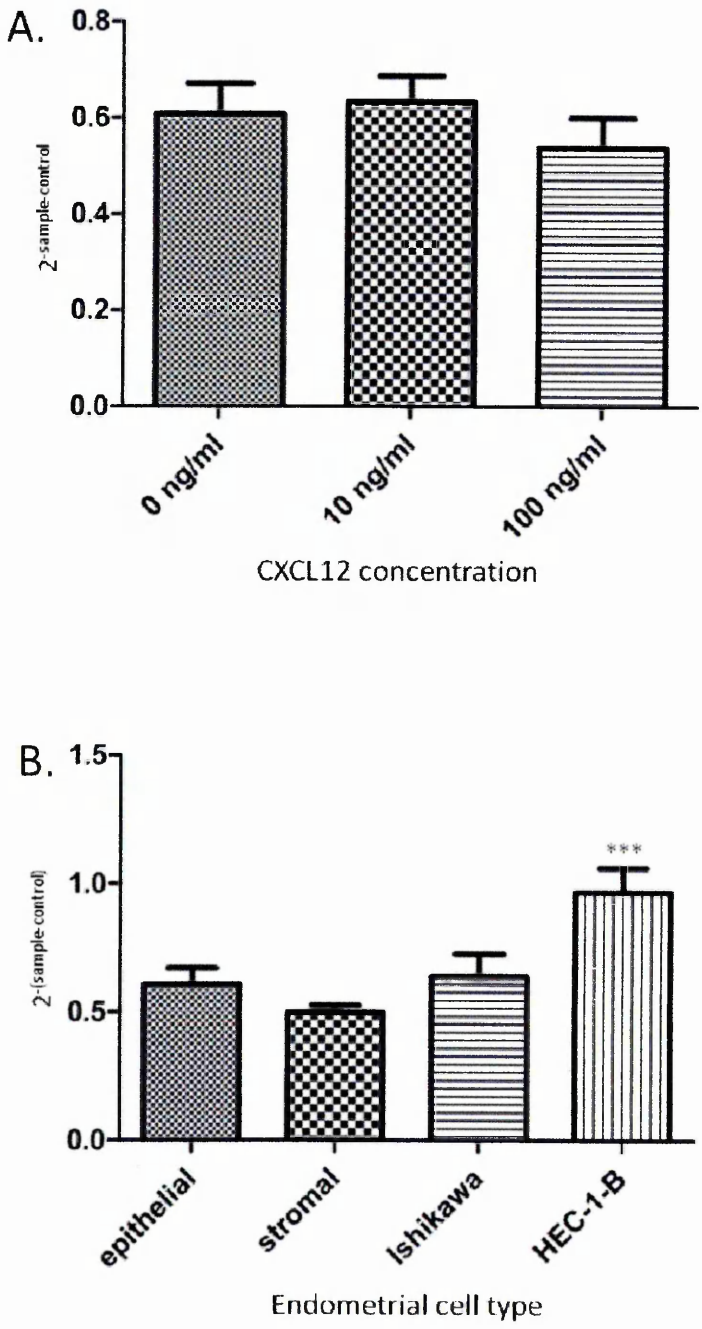


Figure 2.6: Variation in reference transcript YWHAZ mRNA in endometrial cells

A. Shows expression of YWHAZ mRNA in endometrial epithelial cells following incubation with or without CXCL12 for 24 hours. B. Shows expression of YWHAZ mRNA in epithelial, stromal, Ishikawa and HEC-1-B endometrial cells.

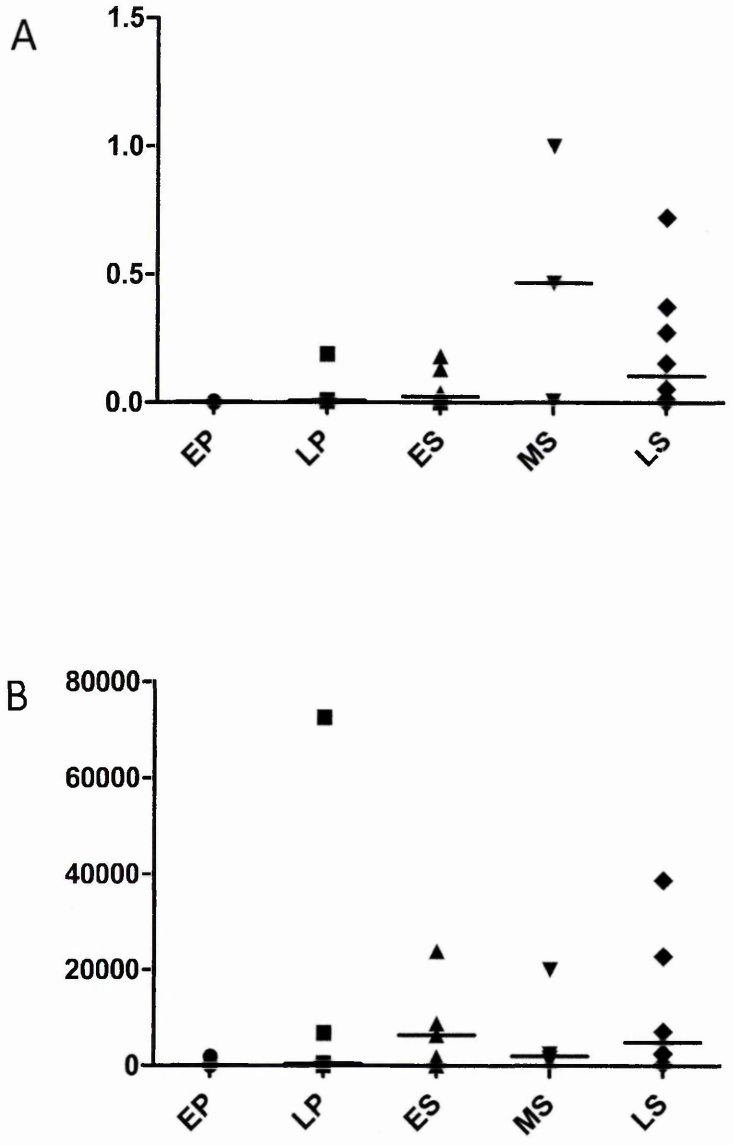


Figure 2.7: Variation in reference transcripts UBC and YWHAZ mRNA in endometrial biopsies collected throughout the menstrual cycle

A. Shows expression of UBC through the menstrual cycle. Although variation was visible, there was no statistical difference in the expression levels between menstrual cycle phases.

B. Shows expression of YWHAZ through the menstrual cycle. Although variation was visible, there was no statistical difference in the expression levels between menstrual cycle phases.

2.5.6 Measuring primer efficiency

The efficiency of the primers used in the SYBR® Green real-time RT-PCR experiments were assessed to ensure they performed comparably and effectively. Six serial dilutions of cDNA from RNA extracted from primary epithelial cell cultures, which had been reversed transcribed according to section 2.4.1 were used to assess the efficiency of each primer. Serial dilutions were made by addition of appropriate volumes of nuclease-free water to cDNA (Table 2.4). 2 µl of each serially diluted cDNA sample was plated out in triplicate into an iCycler iQ™ 96-well PCR plate for all primer sets used. SYBR® Green real-time RT-PCR was carried out by addition of 23 µl of SYBR® Green RT-PCR master mix to each cDNA sample for each primer set to be assessed. PCR plates were placed into the Bio-Rad iCycler iQ™ instrument and the standard thermal protocol performed.

Dilution	Dilution concentration	cDNA required (µl).	Nuclease-free water required (µl).
A	Neat	10	0
B	1 in 5	2 (A)	8
C	1 in 10	1 (A)	9
D	1 in 100	1 (C)	9
E	1 in 500	2 (D)	8
F	1 in 1000	1 (D)	9
G	1 in 10000	1 (F)	9

Table 2.4: Preparing serial dilutions of primary epithelial endometrial cDNA to calculate real-time RT-PCR primer efficiency

Serial dilutions of cDNA reverse transcribed from extracted primary epithelial endometrial RNA was carried out using the above method for all reference transcripts and transcripts of interest used in this study.

The C_t values for each sample assessed were recorded and the primer efficiency of each oligonucleotide primer set was calculated by the method described by Pfaffl (2001):

$$Efficiency = 10^{(-1/slope)}$$

The slope was calculated by plotting cDNA concentration along the horizontal axis of a scatter graph versus determined C_t values for each concentration on the vertical axis in Microsoft Excel software. Primer efficiencies between 90-110% were considered acceptable for use.

The efficiencies of each of the primer sets used in SYBR® Green real-time RT-PCR were calculated, the efficiencies were: CXCL12 = 106%, IL6 =95%, IL8 =91%, MMP2 =89%, MMP9 =95%, VEGFA =90%, YWHAZ =92% and UBC =91%. The ideal range for real-time RT-PCR primer efficiency is between 90 and 110%. All the primer sets used fell within this range with the exception of MMP2 at 89% which was included as this 1% deviation was not expected to significantly alter the amplification of this transcript's cDNA. Figure 2.8 shows graphs of recorded C_t values as a function of cDNA concentration for all SYBR® Green primer sets used to calculate their primer efficiencies.

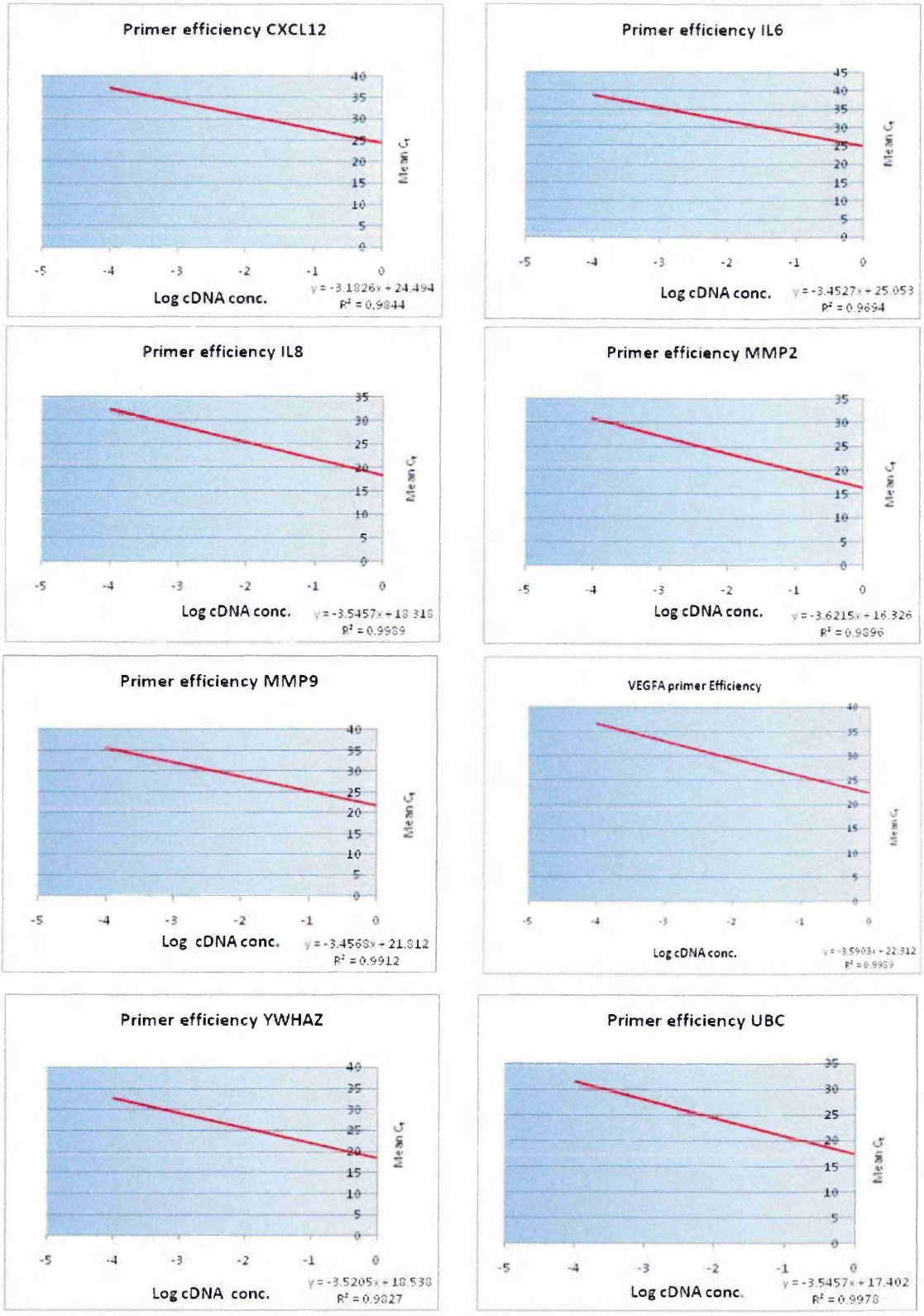


Figure 2.8: Assessing efficiencies of primers used in SYBR® Green real-time RT-PCR

Efficiencies of all primers used in SYBR® Green Real-Time RT-PCR were calculated from the *y* value obtained from each serial dilution performed. R^2 values are displayed on each chart. Primer efficiencies: CXCL12 = 106%, IL6 =95%, IL8 =91%, MMP2 =89%, MMP9 =95%, VEGFA =90%, YWHAZ =92% and UBC =91%.

2.5.7 Identification of CXCR4 RT-PCR product by polyacrylamide gel electrophoresis

Initial SYBR® Green real-time RT-PCR experiments, which identified the presence of CXCR4 mRNA in cell lines used polyacrylamide gel electrophoresis to confirm the presence of a specific PCR product at the correct size. Following confirmation of this subsequent SYBR® Green real-time RT-PCR experiments used melt-curve analysis to confirm the presence of specific PCR products.

All reagents were obtained from Sigma-Aldrich, UK unless otherwise stated. 10X TBE buffer solution was prepared by adding 108 g of TRIS base, 55 g of boric acid and 40 ml of 0.5 M EDTA (pH 8.0) (Sigma-Aldrich, UK) and made up to 1 L with distilled water. The final pH was adjusted to 8.3 and the buffer stored at room temperature. 6% polyacrylamide gels were prepared by addition of 2 ml of 30% (w/v) acrylamide:bisacrylamide solution (19:1 ratio) to 6 ml of 1X TBE buffer. To initiate polymerisation, 150 µl of 10% (w/v) ammonium persulphate (APS) solution and 10 µl of TEMED were added immediately prior to pouring between gasket sealed pre-prepared mini-gel casting plates. A plastic comb was placed into the gel to form loading wells. The polyacrylamide gels were allowed to set at room temperature.

5µl of each PCR product to be assessed was removed from the RT-PCR plate by pipette and mixed thoroughly with 1 µl of 6X stained loading buffer (Promega, UK) in micro-centrifuge tubes. A 25 base-pair (bp) DNA ladder (Promega, UK) was prepared by adding 5 µl of ladder to 1 µl of 6X loading buffer and mixed by pipette.

The polyacrylamide gels were placed into an electrophoresis tank (Biorad, UK). The tank was filled with 1X TBE buffer and each 6 µl PCR product sample and the 25 bp DNA ladder was loaded into the wells. Electrophoresis was carried out at 100V for approximately 1 hour until the dye in the loading buffer had reached close to the bottom of gel ensuring appropriate separation of the DNA ladder and PCR products.

Gels were removed from their glass plates and placed into a plastic weighing boat containing 20 ml of nuclease-free water. 10 µl of 10 mg/ml ethidium bromide solution (Sigma-Aldrich, UK) was added and the gel was placed on an orbital shaker at low speed for 10 minutes. To remove excess ethidium bromide, the gel stain was discarded and the gel was de-stained for 10 minutes by addition of 20 ml of nuclease-

free water. The gel was then removed from the weighing boat and viewed under a CCD camera fitted with a UV transilluminator and exposed until a clear image was obtained.

The analysis of CXCR4 in Ishikawa and HEC-1-B cells repeatedly showed the presence of a specific RT-PCR product for CXCR4 in both cell lines (Figure 2.9). Polyacrylamide gel electrophoresis of the PCR product showed clear bands at the expected size of 151 base pairs (Figure 2.10). Negative controls showed no PCR product. The use of a polyacrlyamide gel to separate the DNA present in the completed PCR reaction, allowed discrete bands to be observed that can sometimes be seen due to primer dimer formation and would not be visible if agarose was used. The result shows that transcripts of CXCR4 mRNA were being expressed by the endometrial cell lines tested and therefore suggests that they were an appropriate model for studying the effects of CXCL12 in endometrial function.

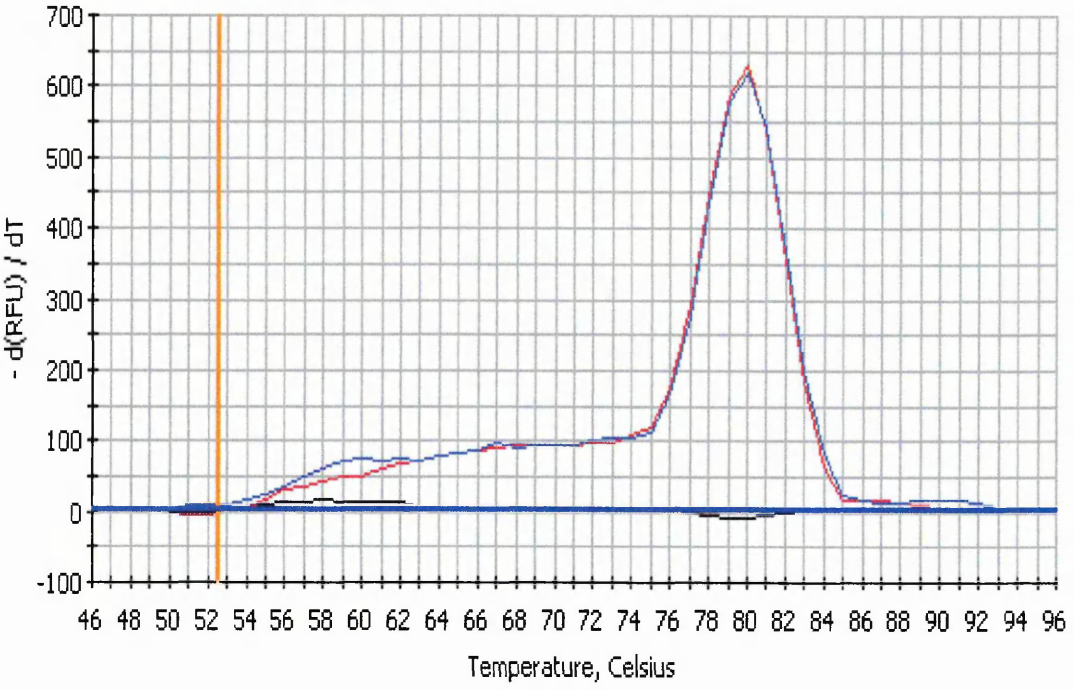


Figure 2.9: Melt curve analysis of CXCR4 real-time RT-PCR product in mRNA extracted from Ishikawa and HEC-1-B andenocarcinoma cell lines

A clear peak at 80°C for CXCR4 can be observed in the figure for Ishikawa (red) and HEC-1-B (blue) cDNA. The negative control (black) showed no recorded fluorescence at any temperature confirming no DNA was present in the reaction.

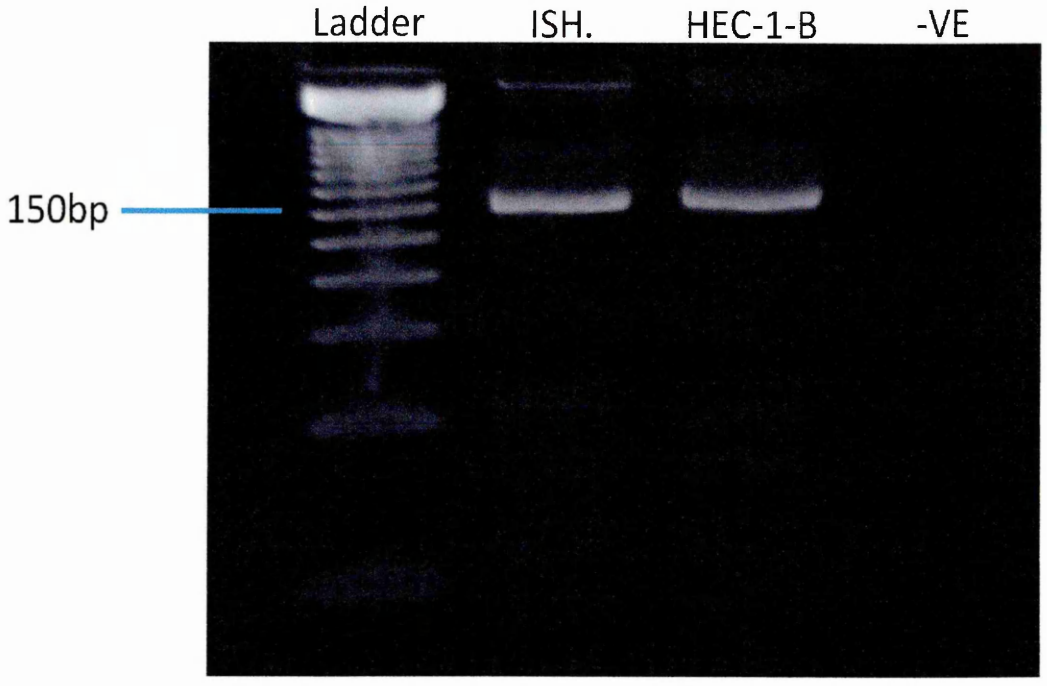


Figure 2.10: Polyacrylamide gel electrophoresis of CXCR4 RT-PCR product

A single band at 151 base pairs was seen for mRNA extracted from Ishikawa and HEC-1-B cells. The negative control showed that no DNA and no PCR amplification occurred when cDNA was not added to the Real-Time RT-PCR reaction.

2.5.8 Assessing the reproducibility and specificity of SYBR® real-time RT-PCR

Melt curve analysis was used to determine the specificity PCR products for IL6, IL8, MMP2, MMP9, VEGFA, CXCL12, YWHAZ and UBC produced using SYBR Green® real-time PCR. Melt curves produced for these transcripts all showed product specific peaks indicating the presence of a single PCR product for each transcript studied (see Figures 2.11 and 2.12). The approximate melt temperatures (°C) for the transcripts studied were: IL6=89, IL8=85, MMP2=90, MMP9=91, VEGFA=89, YWHAZ=84, UBC=84 and CXCL12=87. All transcripts of interest were clearly distinguishable from their reference gene used to calculate their expression. Figure 2.11 shows melt curves obtained from an experiment measuring the expression of IL8 (blue) mRNA in primary epithelial endometrial cells. The IL8 and YWHAZ (reference transcript) (gold) exhibit the smallest difference in peak melt temperature of any transcripts included in the same SYBR® Green real-time RT-PCR assay. Despite their similarity, specific products for the transcripts are clearly visible.

Figure 2.13 shows the typical results generated for negative controls for both transcripts in which cDNA was replaced with water. YWHAZ (green) primers did not generate any detectable product and therefore shows a flat line. IL8 (black) primers generated non-specific PCR product which can occur due to primer dimerisation and a lack of suitable template. These non-specifc products can be detected by SYBR® Green but are clearly distinguishable from the specific PCR products and indicates the presence of no contamination in the samples. Melt curve peaks in negative or –RT control samples that could not be easily distinguished from the experimental samples indicated the presence of contaminating factors such as genomic DNA, present either from RNA which was not completely pure or introduced from the surroundings. If this was observed the resulting C_t values for the experimental samples could not be considered accurate and were discarded.

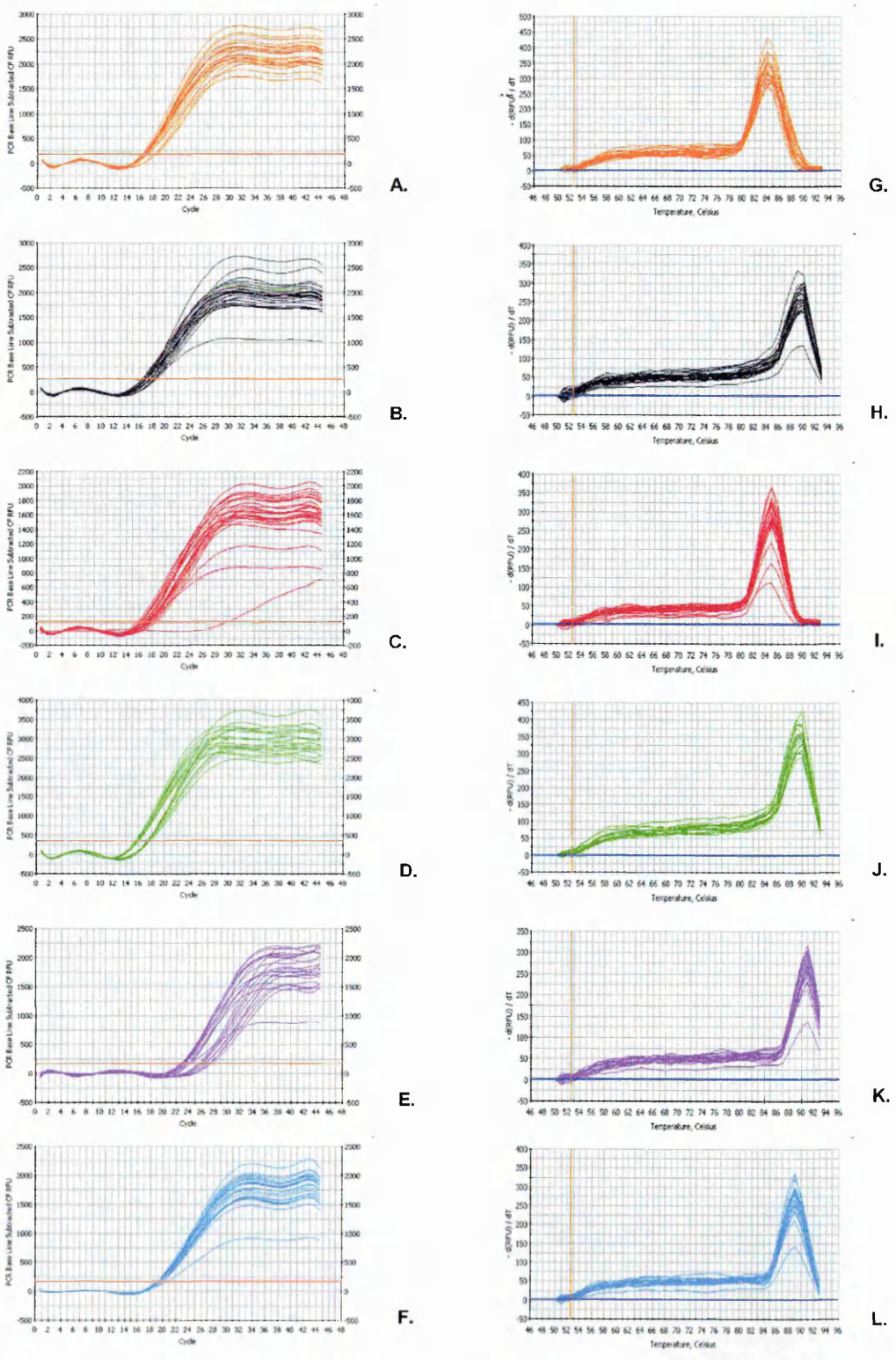
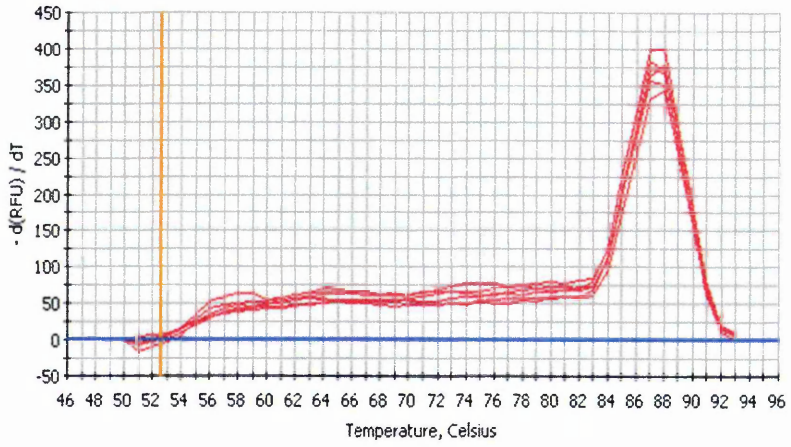


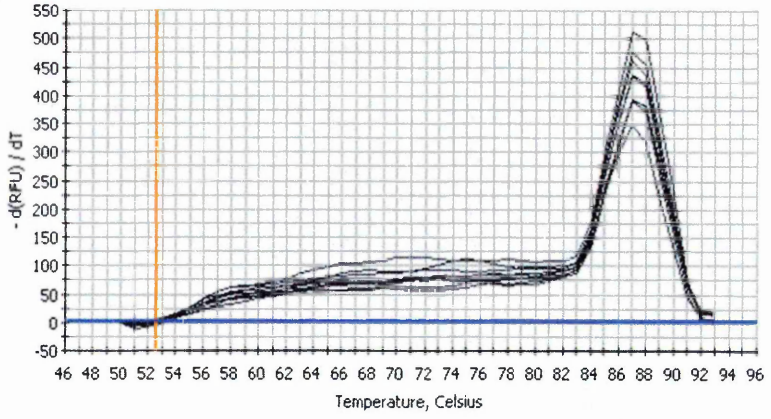
Figure 2.11: Assessing SYBR® Green real-time RT-PCR reproducibility and specificity

A-F shows the amplification graphs obtained for YWHAZ (orange), IL6 (black), IL8 (red), MMP2, (green), MMP9 (purple) and VEGFA (blue) for a number of samples of mRNA extracted from primary epithelial endometrial cells. The automatically applied cycle threshold line can be seen on these amplification plots in orange. G-L shows the associated melt curve produced for each real-time RT-PCR experiment.

A.



B.



2.12: Assessing SYBR® Green real-time RT-PCR reproducibility and specificity for CXCL12 and UBC using melt curve analysis

The figure shows examples of typical melt curves obtained for CXCL12 (A) and UBC (B) real-time RT-PCR products.

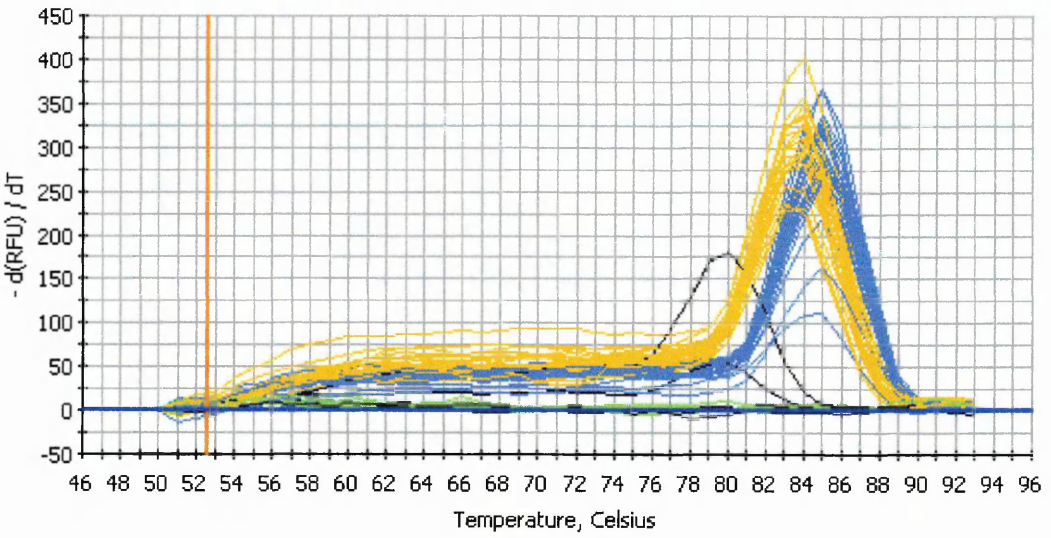


Figure 2.13: Identification of specific and non-specific real-time RT-PCR products using melt-curve analysis

Shows a melt-curve generated in a typical SYBR® Green real-time RT-PCR experiment. IL8 is shown in blue, YWHAZ in gold, IL8 negative control in black and YWHAZ negative control in green.

2.6 Measuring expression of IL6, IL8, MMP2, MMP9, VEGFA and CXCL12 using SYBR® Green real-time RT-PCR

Amounts of mRNA expression for: IL6, IL8, MMP2, MMP9 and VEGFA in Ishikawa, HEC-1-B, primary epithelial and stromal cell cultures were determined in the presence and absence of CXCL12 using SYBR® Green real-time RT-PCR. Experiments were repeated on three separate occasions for Ishikawa and HEC-1-B cells. Experiments on primary epithelial and stromal cell cultures were repeated six times using endometrial tissue obtained from different women. All cell types were incubated with 0, 10 and 100 ng/ml of CXCL12 for 24 hours prior to reaching expected confluence according to section 2.2.5. RNA was extracted using the Genelute™ Mammalian Total RNA Miniprep Kit by the method described in 2.3.1. 500 ng of each RNA sample was reverse transcribed into cDNA according to 2.5.1. Expression levels of IL6, IL8, MMP2, MMP9 and VEGFA were quantified using YWHAZ as the reference transcript.

The amount of CXCL12 expression was also determined in the presence and absence of CXCL12 in primary epithelial and stromal cells, which was repeated 3 times using endometrial tissue obtained from three different women. Cells were incubated with 0, 10 and 100 ng/ml of CXCL12 for 24 hours according to the previously described methods and UBC was used as the reference transcript.

Individual 25 µl SYBR® Green real-time RT-PCR reactions were plated out in triplicate for each of the six genes and reference transcripts for RNA extracted from each cell type for each concentration of CXCL12. Negative controls and –RT controls were also included on each real-time RT-PCR plate. A typical plate set-up is shown in Figure 2.14.

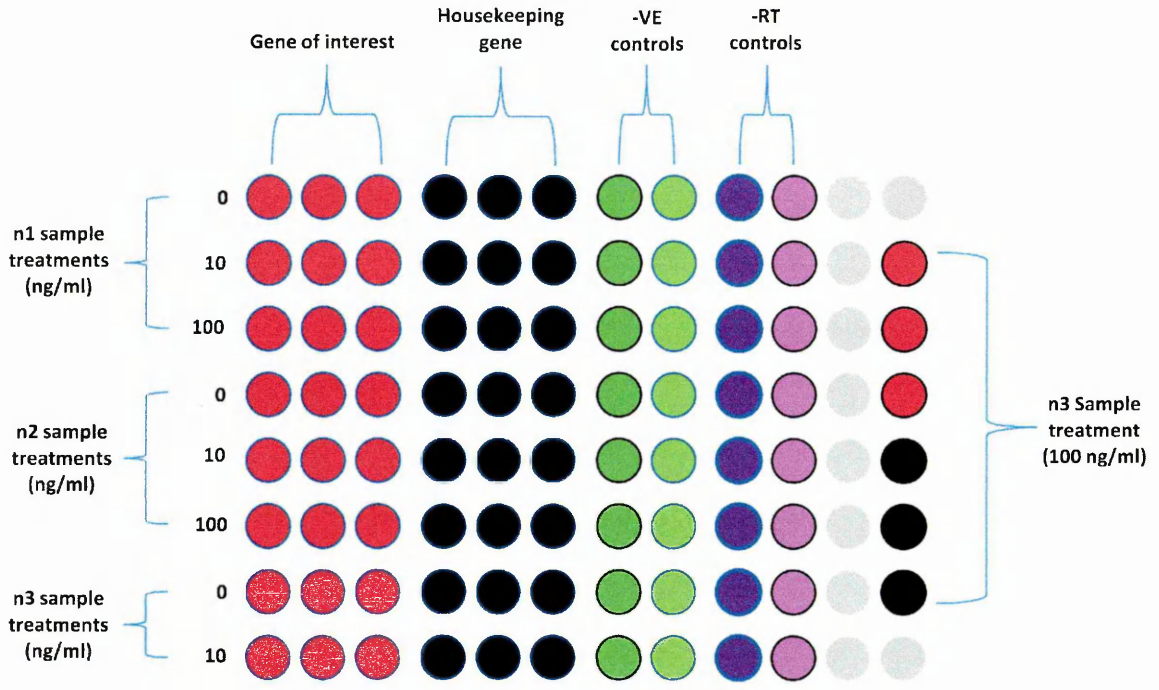


Figure 2.14: A typical plate set-up for a real-time RT-PCR experiment

Each well contains cDNA prepared from mRNA extracted from one particular cell type after incubation with CXCL12 at 0, 10 and 100 ng/ml. Primers for the gene of interest were added to red wells, while primers for reference gene were added to the black wells. Negative controls for both the gene of interest and HK gene in which no cDNA was added to the reaction are shown in light and dark green. –RT controls (dark and light purple) for both the HK gene and gene of interest in which no reverse transcriptase was added to cDNA synthesis reactions are shown in purple. Unused wells are shown in grey.

2.7 Measuring expression of CXCR4 in endometrial cell cultures using Taqman® real-time RT-PCR

Amounts of CXCR4 mRNA in epithelial and stromal primary cell cultures and Ishikawa and HEC-1-B cells incubated in the presence (10 and 100 ng/m) and absence of CXCL12 were measured using Taqman® real-time RT-PCR. Experiments in epithelial and stromal cells were repeated on six different occasions, while those in HEC-1-B and Ishikawa cells were repeated three times.

For each sample, 500 ng of total RNA extract was reverse transcribed according to 2.5.1. Taqman® assay probes for CXCR4 (assay: Hs00237052_m1) and YWHAZ (assay: Hs00237047_m1) along with Taqman® Fast Universal Master Mix were obtained from Applied Biosystems, UK. A ROX™ passive reference dye allowed normalisation of individual reactions on a MicroAmp® Fast Optical 96-well Reaction Plate (Applied Biosystems, UK). 1µl of cDNA reverse transcribed from RNA extracted from a given cell type was added to wells in triplicate on the reaction plate for both CXCR4 and YWHAZ.

Two separate master mixes for CXCR4 and YWHAZ were prepared for the number of individual reactions required, plus one to account for potential pipetting error and sample evaporation. An individual reaction contained 5 µl of Taqman® Fast Universal Master Mix, 0.5 µl of either the CXCR4 or YWHAZ Taqman® assay probes and 3.5 µl of nuclease-free water. 9 µl of the appropriate master mix was pipetted into each reaction well of the reaction plate. The total volume of an individual reaction was 10 µl. Negative controls containing nuclease-free water instead of cDNA and –RT controls were also prepared for each sample type. Plates were set up in a manner similar to that shown in Figure 2.14. Plates were sealed with MicroAmp™ Optical Adhesive Film (Applied Biosystems, UK) and placed in the Applied Biosystems StepOnePlus real-time PCR System and subjected to the thermal protocol shown in Figure 2.15. An overview of TaqMan® chemistry is shown in figure 2.16.

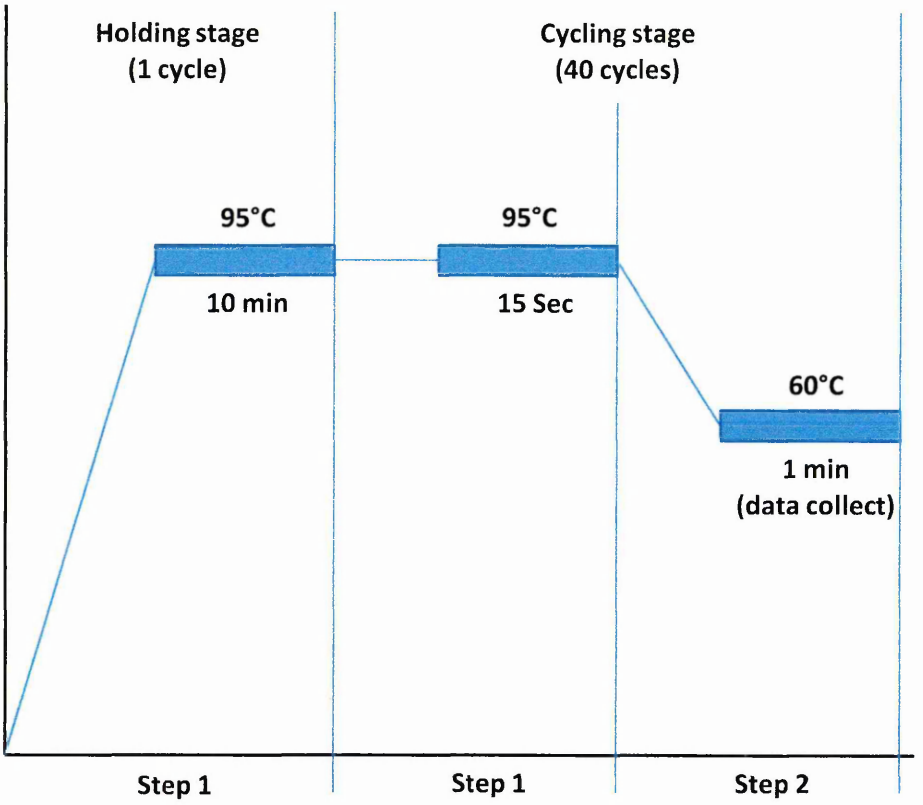


Figure 2.15: Thermal protocol used for TaqMan® real-time RT-PCR

The diagram shows the thermal protocol used for TaqMan® real-time RT-PCR gene expression analysis. After the initial holding step, target cDNA was amplified by application of 40 cycles at the stated temperatures and times. The Applied Biosystems StepOnePlus real-time PCR System collected data in the second step of the cycling stage to obtain C_t values.

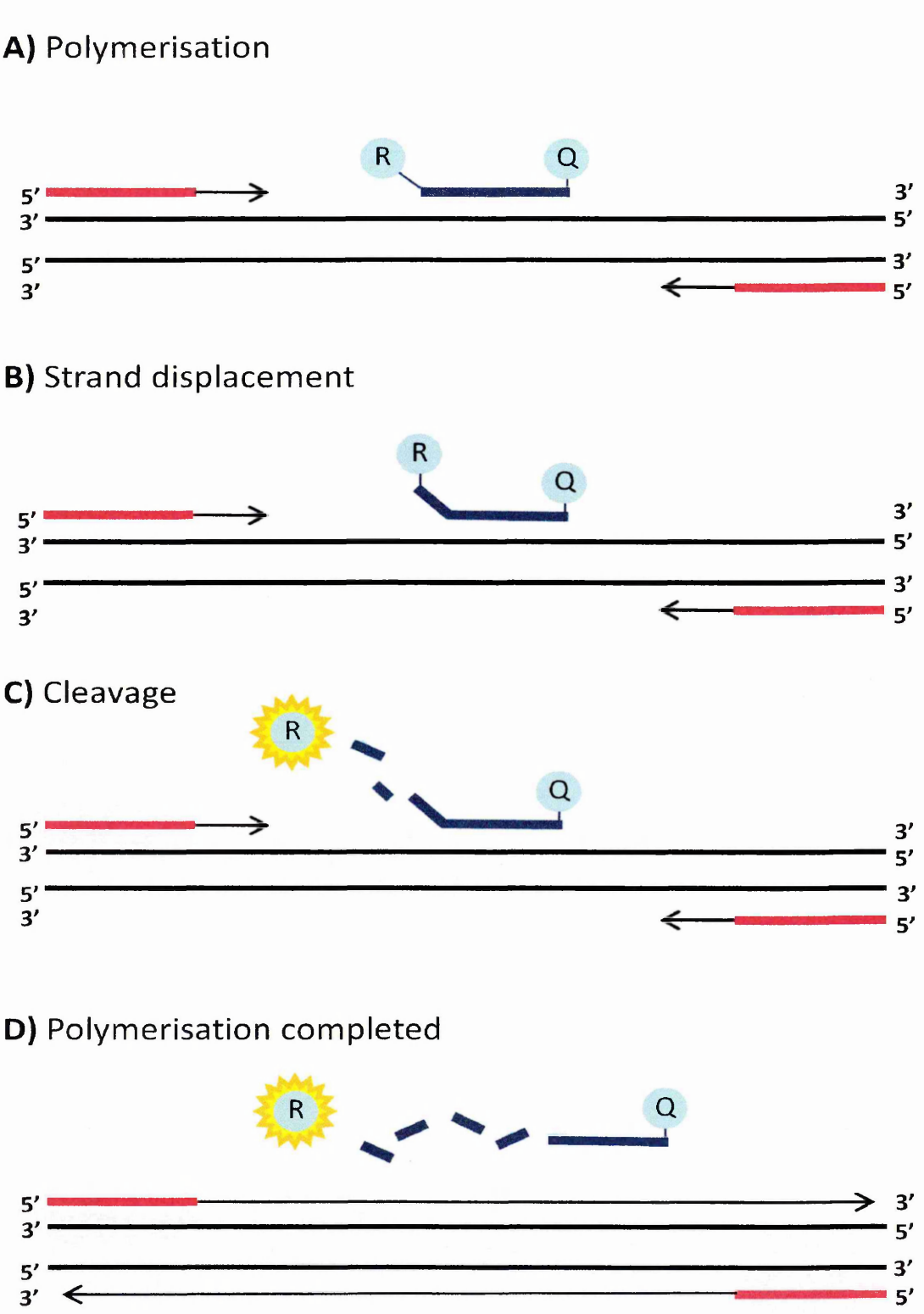


Figure 2.16: Overview of TaqMan® Gene Expression Assay chemistry

The diagram shows the unique steps involved in a TaqMan® Real-Time PCR reaction. Adapted from Applied Biosystems TaqMan® Gene Expression Assay protocol (www.appliedbiosystems.com).

2.8 Measuring levels of CXCL12 and CXCR4 mRNA in endometrial biopsies obtained throughout the menstrual cycle

2.8.1 Taqman® real-time RT-PCR to investigate expression of CXCR4

RNA extracted from thirty one endometrial tissue samples obtained throughout the menstrual cycle using TRI REAGENT™ as described in section 2.3.2 was reverse transcribed to cDNA according to the method described in section 2.5.1. CXCR4 expression was quantified in each cDNA sample using the method described in section 2.7 using YWHAZ as the reference transcript. The layout for a typical real-time RT-PCR plate is shown in Figure 2.17.

2.8.2 SYBR® Green real-time RT-PCR to investigate expression of CXCL12

RNA extracted from thirty one endometrial tissue samples obtained throughout the menstrual cycle using TRI REAGENT™ as described in section 2.3.2 was reverse transcribed to cDNA according to the method described in section 2.5.1. CXCL12 expression was quantified in each cDNA sample using the method described in section 2.5.4 using UBC as the reference transcript. A typical real-time RT-PCR plate set-up for these experiments is shown in Figure 2.17.

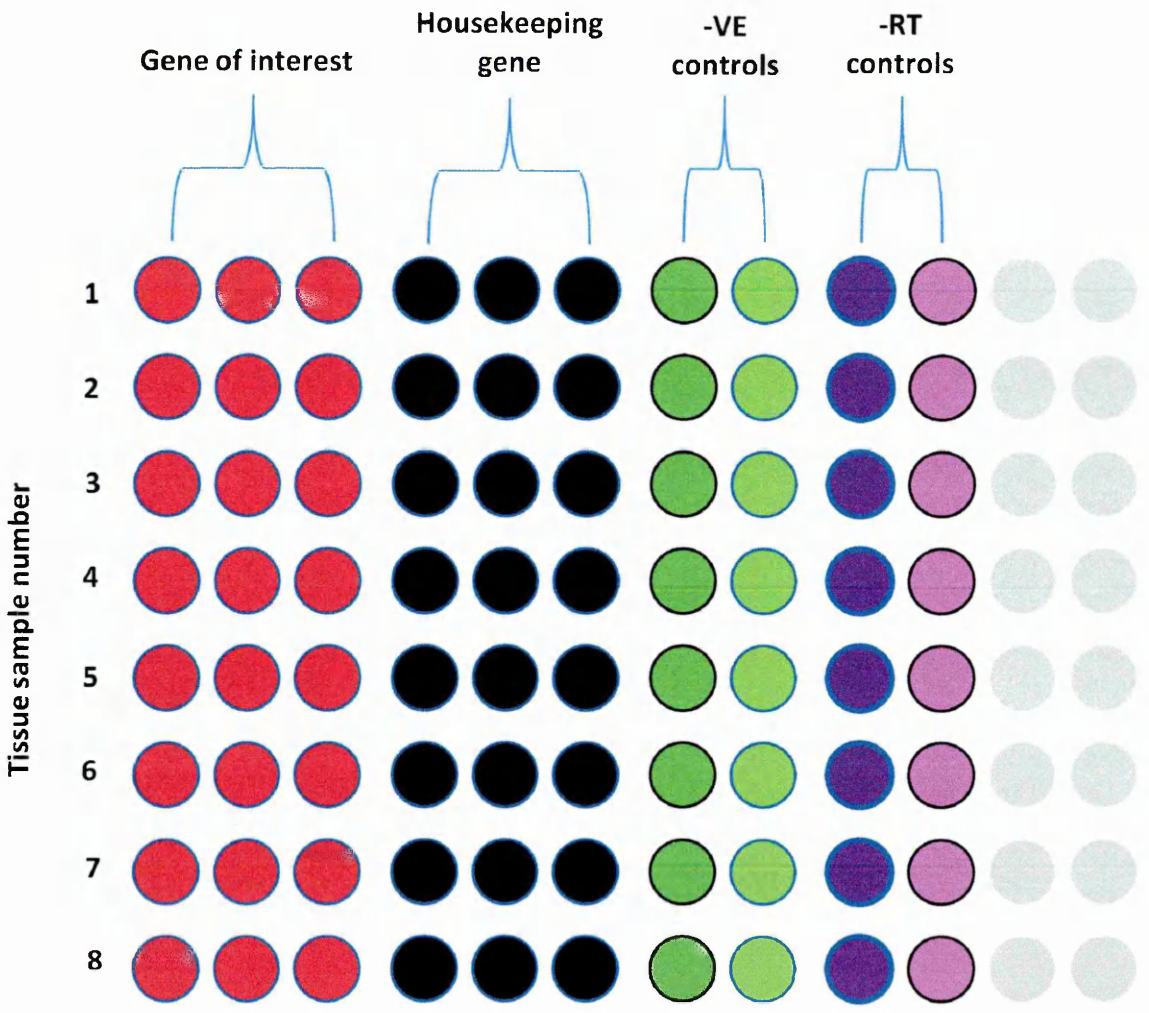


Figure 2.17: Typical plate set-up for real-time RT-PCR experiments investigating the expression of CXCR4 and CXCL12 throughout the menstrual cycle

cDNA from mRNA extracted from eight individual tissue samples can be analysed in triplicate on an individual plate. Wells occupying the gene of interest are shown in red. Wells occupying the reference gene are shown in black. Corresponding negative controls and –RT controls for both the gene of interest (dark green and dark purple) and the reference gene (light green and light purple) could also be accommodated. Unused wells are shown in grey.

2.8.3 Calculating real-time RT-PCR gene transcript expression levels

Expression of CXCL12, CXCR4, IL6, IL8, MMP2, MMP9 and VEGFA mRNA in Ishikawa, HEC-1-B, primary epithelial and stromal endometrial cells were calculated using the $2^{-(\Delta Ct)}$ method relative to the reference transcript. Levels of CXCL2 and CXCR4 mRNA expression from endometrial samples obtained throughout the menstrual cycle were calculated in the same way.

The effects of CXCL12 on the expression of each gene of interest were calculated using the $2^{-(\Delta\Delta Ct)}$ method relative to the reference transcript and further normalised relative to control samples to which no CXCL12 had been added (Livak and Schmittegen, 2001).

2.9 DNA Microarrays

DNA microarrays consist of a solid surface, usually a glass microscope slide onto which DNA molecules have been chemically bonded. The purpose of a DNA microarray is to detect the presence and quantity of labelled nucleic acids from a biological sample that bind to the DNA molecules on the array by Watson and Crick duplex formation (Stekel, 2003). Developments in the production of microarray slides have a provided a means to quantify tens of thousands of genes in a single assay including low copy number gene transcripts and highly similar sequences including single nucleotide polymorphisms (SNPs) and RNA splice variants. Gene expression profiling is one of the most widespread uses of microarray analysis and has been applied to investigation of gene functions, classification of clinical samples, pathways analysis and drug evaluation (DeRisi *et al.*, 1997; Schena *et al.*, 1995).

The volume of quantitative data produced by microarray analysis is both its major advantage and its most significant challenge and relies on the use of Bioinformatics which incorporates information technology, mathematics and statistical analysis to allow handling and subsequent interpretation of the data it produces (Bilban *et al*, 2002 and Stekel, 2003).

2.9.1 Microarray production

The first DNA microarrays were made by amplifying DNA from a clone library using highly parallel PCR to create the reporter probes for the array. The probes are spotted

onto a chemically modified glass surface using a robot after addition of an amine group to the 5' end of the reporter probe to allow attachment to chemical linkers on the glass surface (Stekel, 2003).

In 2001, Hughes *et al.* reported a method to produce DNA microarrays using oligonucleotide reporters synthesised *in-situ* directly onto the glass slide by using a modified inkjet printer in which the four colours used by the printer were replaced by the four bases of DNA (adenine, thymine, cytosine and guanine). Reporter probes were built up base by base by formation of a hydroxyl group from a protective group on the 5' end of the lengthening reporter probe. The 3' end of the next nucleotide was covalently bonded to the previous nucleotide following chemical deprotection (using acid or light). The function of the protective groups was to prevent the addition of an incorrect nucleotide to the reporter probe (Hughes *et* al, 2001 and Stekel 2003).

Perhaps the greatest advantage of this technology was that reporter probe synthesis was completely computer controlled allowing the user to define the sequences of the oligonucleotides at the time of synthesis with a computer file created from publically available sequence data published by GenBank and EMBL. This allowed the production of custom arrays to fit the needs required by any user and a simple point of reference for subsequent data interpretation. Also, inkjet arrays produced the highest quality features (a spot of reporter probes on the array for one specific gene) and allowed reproducible detection of single copy number genes and RNA splice variants. The most recent commercially available arrays have employed this method to produce numerous copies of the entire expressed human genome on a single microscope slide (Hughes *et al*, 2001 and Stekel, 2003).

2.9.1.1 Microarray probe design

Commercially or custom produced DNA microarrays must have oligonucleotide reporter probes which are considered to be sensitive, specific and isothermal to accurately measure the target genes in the mRNA extracted from a biological sample. Microarray probes are usually between 20 and 60 oligonucleotides long. A sensitive probe returns a strong signal when the complementary gene target is present in the sample. Sensitivity is achieved by avoiding probes which have internal secondary structure or that bind to adjacent identical probes within an array feature. Also, a

sensitive probe must be able to access its complementary target sequence which potentially could be unavailable as a result of secondary structure present within that sequence.

A specific probe returns a weak signal when the target gene is absent from the sample by avoiding cross-hybridisation and non-specific binding as a result of Watson-Crick base pairing. Isothermal probes behave in a similar manner during the hybridisation of the target to the reporter probes. This means all probe-target duplexes on the array must have similar melting temperatures. Computer algorithms are employed to produce suitable probes for the microarray to filter out low complexity repeating sequences, predict potential cross-hybridisation to related genes, ensure comparable melting temperature of nucleic acid duplexes and eliminate probe secondary structure that may potentially self-hybridise (Stekel, 2003 and Sugimoto *et al.*, 1995).

2.9.1.2 Microarray sample preparation and labeling for gene expression microarrays

The type of DNA microarray platform to be used determines the procedure for sample preparation and the labeling of mRNA extracted from the biological samples. The aim of performing a gene expression microarray experiment is to measure the differences in mRNA production between two or more biological samples. In all cases the first step is to perform RNA extraction from the tissues samples to be analysed. It is important that the RNA to be used is of good integrity to facilitate effective hybridisation and that it can be accurately quantified to ensure equal amounts of sample are used in the analysis. To measure the presence and quantities of the extracted mRNA present in the samples they must undergo a labeling step to allow detection by the microarray scanner once hybridisation to the array has taken place (Stekel, 2003).

For Affymetrix® microarray platforms the labeling step uses Biotin labeled cRNA. Both the control and experimental samples undergo labeling with biotin and are hybridised to separate identical microarray slides. Following slide scanning and data extraction, the ratios of labeled cRNA in each sample for each gene represented on the array are

calculated to quantify differences in expression between the experimental and control samples.

The Agilent™ microarray platform uses a single array slide to which both the control and experimental samples are combined and simultaneously hybridised to the array. To discriminate between the two samples, RNA is reverse-transcribed to cDNA using reverse transcriptase and then employs a T7 RNA polymerase to produce cRNA. It is during this step of cRNA production that labeling takes place. In addition to the nucleic acids (NTPs) added to the cRNA reaction, a proportion of the CTPs added are covalently labeled with one of two fluorescent dyes. The two most commonly used fluorescent dyes are Cyanine 3 (Cy3) which is excited by a green laser at 550 nm and has an emission wavelength of 581 nm and Cyanine 5 (Cy5) which is excited by a red laser at 649 nm and has an emission wavelength of 670 nm. One dye is used to label cRNA from the control sample and the other to label cRNA from the experimental sample. Following cRNA synthesis, a proportion of the 'C's in the cRNA products have Cyanine fluorophores attached to them. Following microarray slide scanning and data extraction, the ratio of hybridisation of the Cy3 and Cy5 labeled cRNAs that have bound to the reporter probes within an array feature can be calculated. Figure 2.18 summarises the sample preparation and labelling steps used to perform two-colour gene expression microarray analysis.

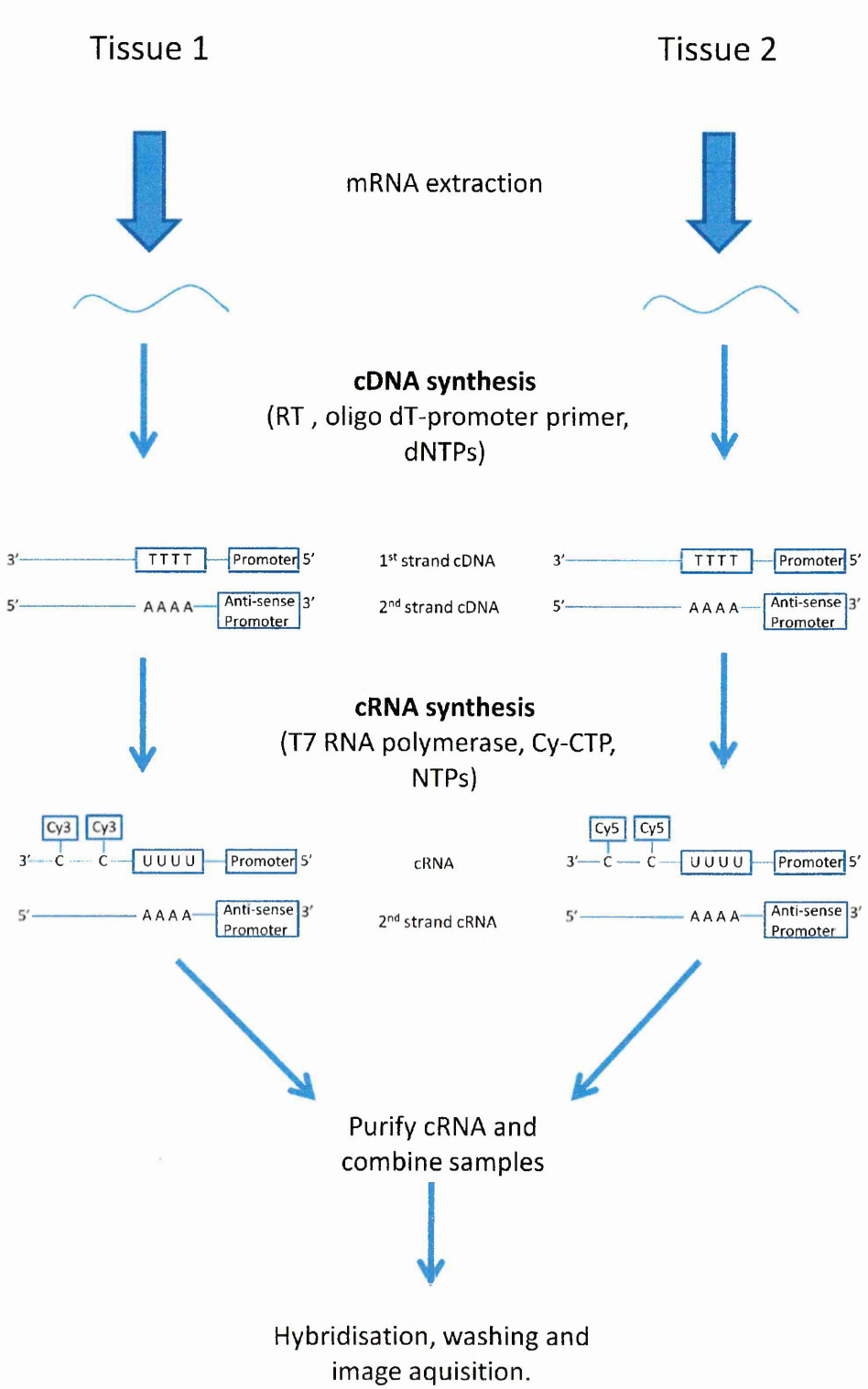


Figure 2.18: Summary of the sample preparation and labeling steps required to perform two-colour gene expression microarray analysis

This diagram shows the steps involved in performing Agilent™ two-colour gene expression microarray analysis (Adapted from the Agilent™ Two-Colour Microarray-Based Gene Expression Analysis Protocol Version 6.0, 2009. www.agilent.com).

2.9.1.3 Microarray hybridisation

Hybridisation is the process by which the DNA reporter probes on the glass microarray slide form heteroduplexes with the labeled cRNA from the biological samples by Watson-Crick base-pairing. Hybridisation is affected by a number of conditions including temperature, humidity, salt concentrations and formamide concentration. Manual hybridisation uses a hybridisation chamber. A glass cover slide with a fixed gasket has the hybridisation solution containing the combined and labeled cRNA samples injected onto its surface. The array slide is placed onto the cover slide and sealed within the chamber. The chamber is placed into a rotating hybridisation oven which maintains the correct temperature for hybridisation to take place and mixes the sample to distribute it evenly over the microarray surface. Hybridisations are typically performed between 45 and 65°C for a period between 12 to 24 hours (Agilent™ Two-Colour Microarray-Based Gene Expression Analysis Protocol, Version 6.0, 2009 and Stekel, 2003).

2.9.1.4 Microarray washing

There are two reasons to perform microarray washing after hybridisation has taken place. The first is to remove excess hybridisation solution from the array ensuring only specifically bound labeled cRNA remains on the microarray surface. The second is to increase the accuracy of the analysis by reducing cross-hybridisation. This can be achieved using a high temperature or low-salt wash to ensure only cRNA complementary to the reporter probe features remains bound to the array (Stekel, 2003).

2.9.1.5 Microarray image acquisition

The final laboratory step of performing two-colour microarray expression analysis is to produce an image of the hybridised array. The microarray slide is placed in a scanner which contains two lasers which can read the surface of the array by exciting the Cyanine labeled cRNAs causing fluorescence which is recorded by a photon multiplier tube (PMT) to produce a digital image. As the lasers shift across the array a pixilated digital image is produced. The intensity of each pixel produced represents the intensity of fluorescence at that point of the array from the labeled cRNA that has

hybridised to the reporter probes within each microarray feature. Whole genome arrays require a pixel resolution of 5 μm. Each laser scans the slide separately and the scanner produces two monochrome images representing the fluorescence produced by the Cy3 and Cy5 dyes bound to the cRNA. These images are combined to produce a red-green false colour image. The intensity of each pixel on the array is stored as a 16-bit number which is used to calculate the intensity of Cy3 and Cy5 fluorescence on each feature of the microarray (Agilent™ Two-Colour Microarray-Based Gene Expression Analysis Protocol, Version 6.0 and Stekel, 2003). Figure 2.19 summarises how a microarray scanner acquires an image.

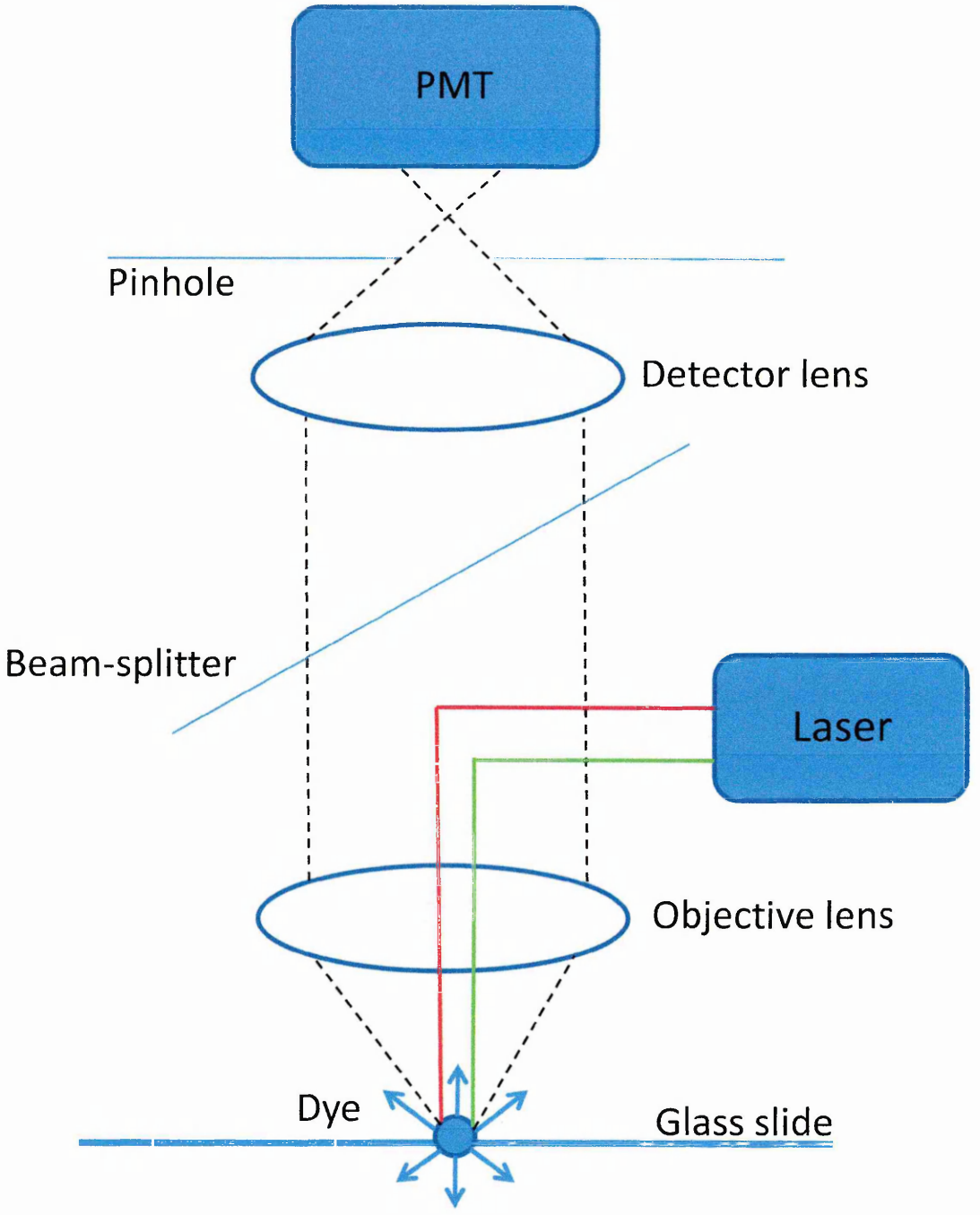


Figure 2.19: Summary of how a microarray scanner works

A microarray scanner uses two lasers that excite Cyanine dyes present in the heteroduplexes on the array at their appropriate wavelengths (Cy3 at 550 nm and Cy5 at 649 nm). This causes emission of light for Cy3 at 581 nm and Cy5 at 670 nm which is focussed through an objective lens and beam splitter onto a detector lens which focuses the fluorescence through a pinhole to be recorded by a photon multiplier tube (PMT) converting florescence into a digital signal. The whole slide is scanned either by movement of the slide or the optics within the scanner (adapted from Stekel, 2003).

2.9.2 Two-colour microarray-based gene expression analysis experiments

All Reagents were obtained from Agilent™ Technologies, UK unless otherwise stated. Agilent™ Ge45 4x44k whole genome arrays were used. The Agilent™ Two-Colour Microarray-Based Gene Expression Analysis Protocol (Version 5.5) was followed. Experiments were carried out at Oxford Gene Technologies, UK.

2.9.2.1 Microarray sample preparation

Ishikawa cell lines were incubated with 0 and 5 ng/ml of CXCL12 according to the methods stated in section 2.2.5. Each experiment was repeated four times.

2.9.2.2 Preparation of positive control RNA Spike Mixes A and B

Stock solutions of RNA spike mix A for cyanine 3 and spike B mix for cyanine 5 were mixed thoroughly on a vortex mixer and briefly centrifuged to drive contents to the bottom of the tube. A 1:20 dilution of both stock solutions was carried out in 1.5 ml tubes labeled A and B. 38µl of provided dilution buffer was added to each tube. 2 µl of each concentrated spike mix was added to their respective tubes (A and B), were mixed well and spun in a micro-centrifuge.

A further 1:40 dilution of each spike was carried out by adding 2 µl of each 1:20 diluted spike solution to 78 µl of dilution buffer in a fresh tube.

2.9.2.3 Preparing the labeling reaction

5000ng of RNA extracted from cells incubated with (experimental) and without (control) CXCL12 were added to labeled micro-centrifuge tubes. 2 µl of 1:40 diluted spike mix A was added to each of the four experimental RNA tubes and 2 µl of spike B was added to each of the four controls. 1.2 µl of T7 Promoter Primer from the Agilent™ Low RNA Input Linear Amplification Kit was added to all eight sample tubes. 0.3 µl of water was added to bring the volume of all tubes to 11.5 µl. The T7 primer and RNA template was denatured by incubating the samples at 65°C in a heating block for 10 minutes. The samples were placed on ice.

A cDNA master mix was prepared by adding the components listed in Table 2.5 in order. The 5X first strand buffer was heated at 80°C for 3 minutes to adequately re-suspend the buffer components. 8.5 µl of cDNA master mix was added to each RNA sample tube and gently mixed by repeat pipetting. The samples were then incubated at 40°C for 2 hours. This was followed by further incubation at 65°C for 15 minutes and storage on ice.

An RNA transcription master mix was prepared by adding the components listed in Table 2.6 in order. The transcription master mix requires addition of Cyanine 3-CTP or Cyanine 5-CTP fluorescent label. Experimental samples were labeled with Cyanine 3-CTP and control samples with Cyanine 5-CTP. 60 µl of transcription master mix was pipette into each sample tube and mixed gently. The samples were incubated for 2 hours at 40°C.

Component	Volume (µl) per reaction	Volume (µl) per 9 reactions
5X First Strand Buffer	4	36
0.1 M DTT	2	18
10mM dNTP mix	1	9
MMLV-RT	1	9
RNaseOut	0.5	4.5
Total Volume	8.5	76.5

Table 2.5: Components of the cDNA synthesis reaction performed using the Agilent™ Low RNA Input Linear Amplification Kit

The supplied kit reagents were prepared as shown above to perform reverse transcription of RNA to cDNA.

Component	Volume (µl) per reaction	Volume (µl) per 5 reactions
Nuclease-free water	15.3	76.5
4X Transcription Buffer	20	100
0.1 M DTT	6	30
NTP mix	8	40
50% PEG	6.4	32
RNaseOut	0.5	2.5
Inorganic pyrophosphatase	0.6	3
T7 RNA Polymerase	0.8	4
Cyanine 3-CTP or Cyanine 5-CTP	2.4	12
Total Volume	60	300

Table 2.6: Components of the cDNA synthesis reaction performed using the Agilent Low RNA Input Linear Amplification Kit

The supplied kit reagents were prepared as shown above to perform synthesis of cRNA from cDNA and to allow labeling of cRNA with the appropriate Cyanine 3 or Cyanine 5 fluorescent dyes.

2.9.2.4 Purifying the labeled/amplified RNA samples

RNeasy spin columns (Qiagen, UK) were used to purify the amplified cRNA samples. 20 µl of nuclease free water was added to each cRNA sample to make a final volume of 100 µl per sample. 350 µl of provided RLT buffer was added to each sample and mixed by pipetting. 250 µl of ethanol was added to each sample tube and mixed by pipetting. The resulting 700 µl samples were then transferred to individual RNeasy mini-columns which were placed into 2 ml collection tubes. The tubes were centrifuged in a mini-centrifuge at 16, 000 x g and 4°C for 30 seconds.

Each cRNA bound column was transferred to a new collection tube and 500 µl of ethanol-containing RPE buffer was added to each column. Samples were centrifuged again for 30 seconds at 16, 000 x g and 4°C. The resulting flow through was discarded from the collection tubes and the tubes reused to perform a repeated second wash using 500 µl of RPE buffer and centrifugation for 30 seconds. Cleaned cRNA samples were eluted from the columns by transferring columns to fresh collection tubes and adding 30µl of nuclease free water directly to the column membrane. After 60 seconds the samples were centrifuged for 30 seconds at 16,000 x g and 4°C. The resulting cRNA sample flow-through from each column was stored on ice.

2.9.2.5 Quantifying the purified cRNA samples

Samples were quantified using the NanoDrop ND-1000 UV-VIS Spectrophotometer described in section 2.3.1. After starting the NanoDrop software and selecting the microarray measurement tab, the instrument was initialised by adding 1 µl of nuclease-free water to the sample loading area and clicking OK. RNA-40 was selected as the sample type using the drop down menu. After blanking the instrument with nuclease-free water, each sample was measured for labeled cyanine 3-CTP (Cy3) or cyanine 5-CTP (Cy5) dye concentration (pmol/µl), RNA absorbance ratio and cRNA concentration.

The yield and specific activity of each reaction was determined by:

(*concentration of cRNA in ng/µl*) X 30 µl (*elution volume of cRNA sample*)

/ 1000 = µg of cRNA

(*Concentration of Cy3 or Cy5 in pmol/µl*) / (*concentration of cRNA in ng/µl*) X 1000

= pmol of Cy3 or Cy5 per µg of cRNA

After ensuring a yield of greater than 825 ng and a specific activity of greater than 8.0 pmol for each Cy 3 or Cy5 cRNA sample, the samples were prepared for hybridisation.

2.9.2.6 Hybridisation of samples to Agilent™ Ge45 4x44k whole genome arrays

10X blocking agent was prepared by adding 500 µl of nuclease-free water to lyophilised 10X blocking agent provided with the Agilent™ Gene Expression Hybridisation Kit. To ensure the pellet completely entered solution, the solution was mixed gently and heated at 37°C for 5 minutes. This was followed by brief centrifugation.

The fragmentation mix was prepared by combining 825 ng of each Cyanine labeled cRNA sample from each corresponding experimental and control sample to create 4 tubes containing combined experimental and control RNA samples *n* 1 to 4. 11 µl of 10X blocking agent was added to each of the 4 tubes and made up to a volume of 52.8 µl using nuclease-free water. Finally, 2.2 µl of 25X fragmentation buffer was added to each sample mixed gently with a vortex mixer and incubated at 60°C for exactly 30 minutes to facilitate the fragmentation of the cRNA.

The fragmentation of cRNA was stopped by adding an equal volume (55 µl) of 2X GE hybridisation buffer HI-RPM to each combined cRNA sample. Mixing was carried out gently using repeat pipetting avoiding bubble formation, which may lead to poor hybridisation to the array surface. To further aid bubble reduction, samples were centrifuged at 16, 000 x g for 1 minute in a micro-centrifuge.

A gasket slide was placed face up into an assembled SureHyb microarray hybridisation chamber and the total 500 µl of each sample (*n 1-4*) was dispensed into 4 separately sealed gasket chambers in a drag and dispense manner with care to avoid bubble formation. A Ge45 4x44k array slide was placed with its active side down onto the gasket slide containing the samples. The array was sealed to the gasket slide by closing and tightening the hybridisation chamber. After ensuring there were no stationary

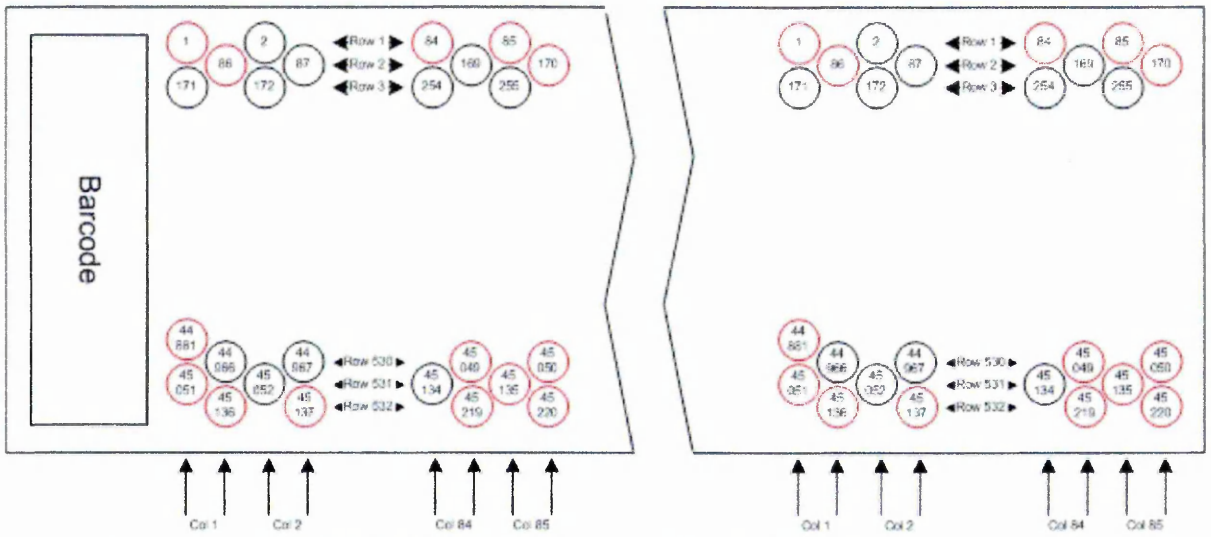
bubbles, the hybridisation chamber containing the sandwiched array was placed into a rotating hybridisation oven (Agilent™, UK) at 10 rpm for 15 hours.

2.9.2.7 Microarray wash

Array slides were washed in Gene Expression wash buffers 1 and 2 provided with the kit. Gene expression wash buffer 2 was warmed overnight at 37°C prior to the day of use. Clean (washed five times with sterile distilled water) and designated slide chambers were used for holding the washing buffers. Slide dish 1 was completely filled with room gene expression wash buffer 1 at room temperature. The hybridisation chamber containing the hybridised array was removed from the hybridisation oven. The chamber was disassembled and the gasket/array sandwich removed. The sandwiched slides were separated under submersion in slide dish 1 containing wash buffer 1. The array was carefully removed to a slide tray and placed in previously prepared wash buffer 1 contained in slide dish 2. This wash buffer in slide dish two was stirred for 1 minute using a magnetic stirrer and the slide tray containing the hybridised array was transferred to a third slide dish containing wash buffer 2 at 37°C. The wash buffer was stirred on a magnetic stirring plate for 1 minute. The slide tray was slowly removed from wash buffer 2 to minimise droplet formation on the slide surface. The slide was immediately placed in an Agilent™ Technologies Scanner G2505B to minimise impact of environmental oxidants on signal intensity.

2.9.2.8 Microarray scanning and feature extraction

Microarray slide scanning was carried out using an Agilent Technologies Scanner G2505B in conjunction with Agilent™ Feature Extraction Software (v9.5). A photon multiplier tube (PMT) gain of 10 at a resolution of 5 microns was used to scan the microarray slide and obtain an image. Figure 2.20 shows the design of the Agilent™ Ge45 4x44k slide. Once an image was obtained the appropriate feature extraction grid for the array design (Agilent™ Grid 014850_D) was imported into the feature extraction software to facilitate spot finding and data extraction. The appropriate feature extraction protocol (GE2-v5_95) for the Ge45 4x44k microarray slide design was used to obtain, normalise and statistically analyse the image. Table 2.7 summarises the feature extraction protocol used.



Feature, Row, and Column numbering in DNABack, BarcodeLeft orientation for the Agilent 4 X 45,220 feature microarray.

The center two arrays are omitted for clarity.

Approximately 150 probes in each corner, not including the BrightCorner features, are filled with DarkCorner probes. This is to ensure that no biological probes are cut off by the hybridization apparatus.

Figure 2.20: Design of the Agilent™ Ge45 4x44K microarray slide

The diagram above shows the layout of the human whole genome expression array slide. Each array slide contains probes (features) for the entire expressed human genome as well as positive control probes which bind the RNA spike-ins, negative control probes and dark corner probes. The positive and negative control probes are used to normalise the array data while multiple copies of features for some genes are distributed across the array to aid spatial distribution normalisation. Full probe information for the GE45x44K slide is published for reference at www.agient.com (image: www.agilent .com).

Protocol Step	Parameter		Default Setting/Value (v.0.5)
Place Grid	Array Format	For any format automatically determined or selected by you, the software uses the default Placement Method listed below.	Automatically Determine [Recognized formats: Single Density (11k, 22k), 25k, Double Density (44k), 95k, 185k (5 and 10 u), 244k (5 and 10 u) and Third Party]
	Placement Method	The parameters and values for placing the grid differ depending on the format, but you can't see the differences because the values are hidden.	Allow Some Distortion
Find Spots	Spot Format	Depending on the format selected by the software or by you, the default settings for this step change. See the rows below for the default values for finding spots.	Automatically Determine [Recognized formats: same as those listed above]
		Use the Nominal Diameter from the Grid Template	True (All Formats)
		Spot Deviation Limit	1.50 (All Formats)
		Calculation of Spot Statistics Method	Use Cookie (All Formats)
		Cookie Percentage	0.650 (Single Density, 25k)
			0.561 (Double Density, 95k)
			0.700 (185k, 244k)
		Exclusion Zone Percentage	1.200 (All Formats)
		Auto Estimate the Local Radius	True (All except 185, 244)
			False (185k, 244k)
		LocalBGRadius	100 (when False for 185k, 244k)
	Pixel Outlier Rejection Method		Inter Quartile Region (Automatically Determine and All Formats)
		RejectIQRFeat	1.42 (All Formats)
		RejectIQRBG	1.42 (All Formats)
	Statistical Method for Spot Values from Pixels		Use Mean/Standard Deviation (Automatically Determine and All Formats)
Flag Outliers	Compute Population Outliers		True
		Minimum Population	10
		IQRatio	1.42
		Background IQRatio	1.42
		Use Ttest for Small Populations?	True
	Compute Non Uniform Outliers		True
	Scanner	The values for the parameters change depending on the scanner used for the image. See below for differences.	Automatically Determine
	Agilent scanner		
	Automatically Compute OL Polynomial Terms		True
		Feature – (%CV)^2	0.04
		Red Poissonian Noise Term Multiplier	20
		Red Signal Constant Term Multiplier	1
		Green Poissonian Noise Term Multiplier	20

Protocol Step	Parameter		Default Setting/Value (v 9.5)
		Green Signal Constant Term Multiplier	1
		Background – (%CV)^2	0.09000
		Red Poissonian Noise Term Multiplier	3
		Red Background Constant Term Multiplier	1
		Green Poissonian Noise Term Multiplier	3
		Green Background Constant Term Multiplier	1

Table 2.7: GE2-v5_95 feature extraction protocol

The table summarises the settings used to feature extract microarray data from the Ge45 4x44K array using the appropriate 014850_D feature extraction grid with the Agilent™ Feature Extraction Software (v9.5) (source: Agilent™ Feature Extraction Software v9.5 Reference Guide).

2.9.2.9 Microarray data normalisation and statistical analysis

The Agilent™ Feature Extraction Software produced text files for each of the four array repeats. Each file contained the data acquired for each of the 45, 220 features within each experimental repeat. The data files included the raw signal data obtained for each feature on the array and data for the normalisation procedures carried out which used predefined complex algorithms employed by the Agilent™ Feature Extraction Software. These normalisation steps adjust the raw data to take into account systematic errors. This is followed by background fluorescence subtraction and then uses a LOWESS non-linear regression algorithm to correct for dye and laser bias. These procedures results in the production of processed signal data for each microarray feature, which is used to calculate the $\log_{10}$ ratio between the Cy3 and Cy5 signals showing the observed changes in gene expression between the control and experimental samples. Microarray features which fall outside the software defined parameters necessary to produce accurately normalised data are highlighted using a flagging system. The Agilent™ Feature Extraction Software then attributes statistical significance to the normalised data by applying an algorithm based on a two-sided *t* test to produce *P* values for each microarray feature.

The text files produced were imported into Microsoft Excel to organise, search and check data validity to find genes of interest which showed statistically significant differential expression of mRNA in cells incubated with and without CXCL12.

Chapter 3
Expression of CXCL12 and CXCR4 mRNA in human endometrium

3.1 Hypothesis

Human endometrium shows differential expression of CXCL12 and CXCR4 mRNA throughout the menstrual cycle and between endometrial cell types.

3.1.1 Expression of CXCL12 in human endometrium

Little is currently known about the expression of CXCL12 in human endometrial cells though its expression has been identified in monkey and bovine endometrium (Ace and Okulicz, 2004 and Mansouri-Attia *et al.,* 2009). Expression of CXCL12 throughout the menstrual cycle of non-pregnant human endometrium has not previously been demonstrated, though CXCL12 has previously been detected by immunohistochemistry during the proliferative phase of the menstrual cycle (Kitaya *et al.,* 2004). Expression of CXCL12 in human decidual cells has also been previously demonstrated and CXCL12 has also shown to be expressed by human placenta and invading trophoblast (Hanna *et al.,* 2003).

3.1.2 Expression of CXCR4 in human endometrium

Expression of CXCR4 mRNA throughout the human menstrual cycle has previously been investigated by Dominguez *et al.* (2003) who identified a trend of decreasing expression of CXCR4 mRNA during the proliferative phase of the cycle and a significant increase in expression during the mid secretory phase when implantation occurs. CXCR4 is also expressed by human trophoblast and placenta (Hanna *et al.,* 2003). CD56+ uNK cells express CXCR4 and have been shown to respond via chemotaxis to trophoblast supernatant containing CXCL12 and also recombinant CXCL12 (Wu *et al.,* 2005).

3.1.3 The function of CXCL12 and CXCR4 in human endometrium

The available evidence suggests an important role for the chemokine ligand CXCL12 and its receptor CXCR4 in human female reproductive function, which may involve the trafficking of uNK cells to decidualising endometrium and sites of trophoblast invasion during the initiation of placentation (Hanna *et al.,* 2003; Wu *et al.,* 2005 and Zhou *et al.*, 2008). Furthermore, the significant increase observed in CXCR4 mRNA expression by endometrial tissue during the mid secretory phase of the menstrual cycle implicates a role for CXCL12 and CXCR4 in the initial stages of implantation (Dominguez *et al.*, 2003). This is further supported by an *in vitro* model of apposition which showed increased levels of CXCR4 immunohistochemical staining in endometrial cells in the presence of blastocyst, which was found to express CXCL12 protein but not its receptor CXCR4 (Valles and Dominguez, 2006). Unpublished data from our laboratory has shown expression of CXCL12 at the protein level throughout the menstrual cycle and has found to be predominantly located to the glandular and luminal epithelium (Figure 3.1).

There remains a lack in knowledge regarding the expression and function of CXCL12 and CXCR4 throughout the human non-pregnant menstrual cycle and within specific endometrial cell types. No study to date has investigated fully the expression of CXCL12 throughout the human menstrual cycle. The distribution of CXCL12 and CXCR4 between different endometrial cell types also remains unknown. The differential expression of CXCL12 and CXCR4 throughout the menstrual cycle and between different endometrial cells types could provide further evidence of how the chemokine ligand and receptor carry out their functions in endometrium. The current evidence potentially indicates roles for CXCL12 and CXCR4 in uNK cell recruitment and cross-talk between the endometrial surface and the blastocyst during the implantation window. However, chemokine ligand and receptors, including CXCL12 and CXCR4 have the potential to carry out a wide range of cellular functions such as contributing to cell proliferation and differentiation (Busillo and Benovic, 2006). These additional potential functions may be of particular relevance in endometrium, which undergoes dynamic cycles of growth and differentiation during the menstrual cycle.

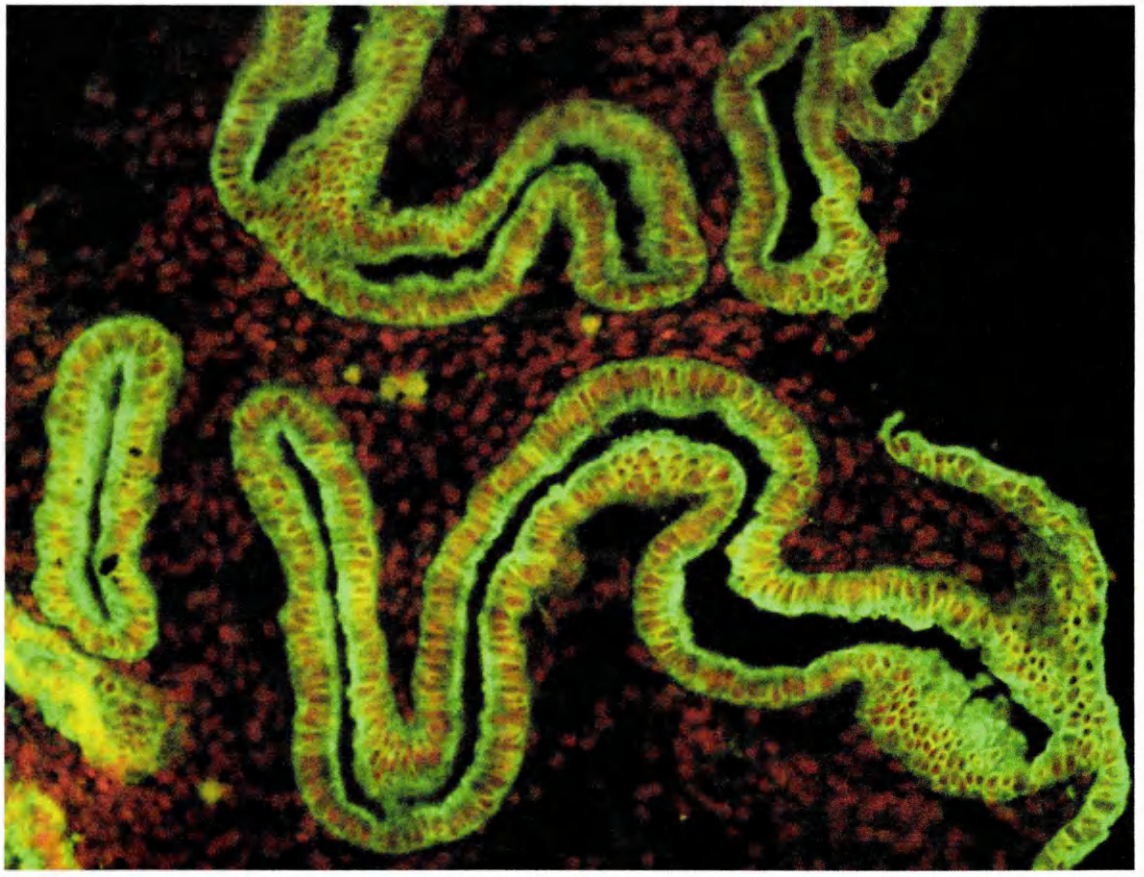


Figure 3.1: Detection of CXCL12 in human endometrium using immunohistochemistry (Unpublished laboratory data courtesy of Mariam El-Sheiki)

Detection of CXCL12 in the human endometrium, CXCL12 staining appears to be predominantly confined to the luminal and glandular epithelium.

3.1.4 Aims

To test our hypothesis, that CXCL12 and CXCR4 mRNA may be differentially expressed at key stages of the menstrual cycle in the human endometrium, we aim to quantify levels of CXCL12 and CXCR4 mRNA in endometrial biopsies obtained throughout the menstrual cycle. This would allow identification of potentially important differences at key stages of the menstrual cycle, which may be of functional importance.

To test our hypothesis that different endometrial cell types may potentially express different levels of CXCL12 and CXCR4 between specific endometrial cell types, we aim to quantify expression levels of CXCL12 and CXCR4 in primary cultures of epithelial and stromal endometrial cells.

It was also our aim to quantify expression levels of CXCL12 and CXCR4 in the Ishikawa and HEC-1-B cell lines to allow their suitability to act as a model in comparison to their epithelial counterparts to be assessed.

To achieve our aims, real-time RT-PCR will be used to accurately measure mRNA expression levels of CXCL12 and CXCR4 in tissue obtained from healthy volunteers throughout the menstrual cycle from which, either levels of CXCL12 and CXCR4 were directly quantified or the tissue was used to prepare pure epithelial and stromal cell cultures.

3.2 Methods

Endometrial biopsies were obtained from normal fertile women (*n=31*) at different times in the menstrual cycle attending the Jessop Hospital for Women in Sheffield. All samples were obtained with informed consent as described in section 2.1.

RNA was extracted from the samples and quantified as described in sections 2.3.2 and 2.4.1. Expression of CXCL12 mRNA was quantified using SYBR® Green real-time RT-PCR and using UBC as the reference transcript as described in section 2.8.2. Levels of CXCR4 mRNA expression were quantified using TaqMan® real-time RT-PCR and using YWHAZ as the reference transcript as described in section 2.8.1. Different types of real-time RT-PCR assays were used with different reference transcripts as they

provided the most accurate means available for quantifying each individual mRNA transcript.

The biopsies were dated according to the day of the last menstrual period followed by confirmation of sample date by histological staging and divided into early proliferative (days 1-9), late proliferative (days 10-14), early secretory (days 15-18), mid secretory (days 19-23) and late secretory (days 24-28) phases of the cycle. Grouping samples into these menstrual phases allowed direct comparision of results to studies such as that by Dominguez *et al.*, (2003). A Kruskall-Wallis test was used to determine statistical differences in expression of CXCL12 and CXCR4 mRNA at different times in the cycle.

Primary epithelial and stromal endometrial cells were prepared according to section 2.2.4 and grown to near confluence. Primary cultures used for investigating CXCR4 expression were prepared from six different women and CXCL12 expression was investigated from cultures prepared from three different women. Experiments in cell lines were repeated on three separate occasions. Following RNA extraction and reverse transcription (according to sections 2.3.1 and 2.5.1) levels of CXCL12 mRNA were quantified using SYBR® Green Real-Time RT-PCR using reference transcript YWHAZ. Levels of CXCR4 mRNA were quantified using Taqman® real-time RT-PCR with reference transcript YWHAZ. Mean expression levels were calculated using the $2^{-(\Delta Ct)}$ method. A Mann-Whitney U test was used to assess levels of statistical significance.

3.3 Results

3.3.1 Expression of CXCL12 mRNA in endometrium obtained throughout the menstrual cycle

CXCL12 mRNA was detected in 28 out of the 31 mRNA samples obtained (Figure 3.2). The observed values ranged from 0.0037 to 0.15 relative to the UBC reference gene (expression = 1). There appeared to be no differential expression in biopsies obtained at different times in the cycle.

The lack of a change during the cycle was confirmed by analysis of samples grouped according to the phases of the menstrual cycle (Figure 3.3). Mean expression values for CXCL12 mRNA were 0.0086 in endometrium from the early proliferative, 0.022

during the late proliferative, 0.037 during the early secretory, 0.023 during the mid secretory and 0.04 during the late secretory phases of the cycle.

3.3.2 Expression of CXCR4 mRNA in endometrium obtained throughout the menstrual cycle

CXCR4 was detected in 30 of the 31 mRNA samples extracted from endometrial tissue, (Figure 3.4). The observed values ranged from 0.16 to 12.01 relative to YWHAZ (expression=1). Median expression values for CXCR4 were 10.07 during the early proliferative, 2.58 during the late proliferative, 1.75 during the early secretory, 3.82 during the mid secretory and 1.83 during the late secretory phases. From the individual biopsy data there appeared to be an increase in CXCR4 expression in endometrium obtained during the early proliferative phase of the cycle.

Application of a Kruskall-Wallis test to all groups representing phases of the menstrual cycle failed to show any significant difference between the data. However, significant differences between CXCR4 mRNA expression was observed when the early proliferative phase was compared individually with the other phases of the menstrual cycle using a Mann-Whitney U test. The expression level of CXCR4 mRNA was found to be significantly higher in endometrium obtained during the early proliferative phase of the menstrual cycle compared to the late proliferative (p=0.035), early secretory (p=0.022) and late secretory (p=0.026) phases. Expression of CXCR4 in endometrium from the early proliferative phase was not significantly different to endometrium obtained in the mid secretory phase of the cycle (p=0.067) (Figure 3.5).

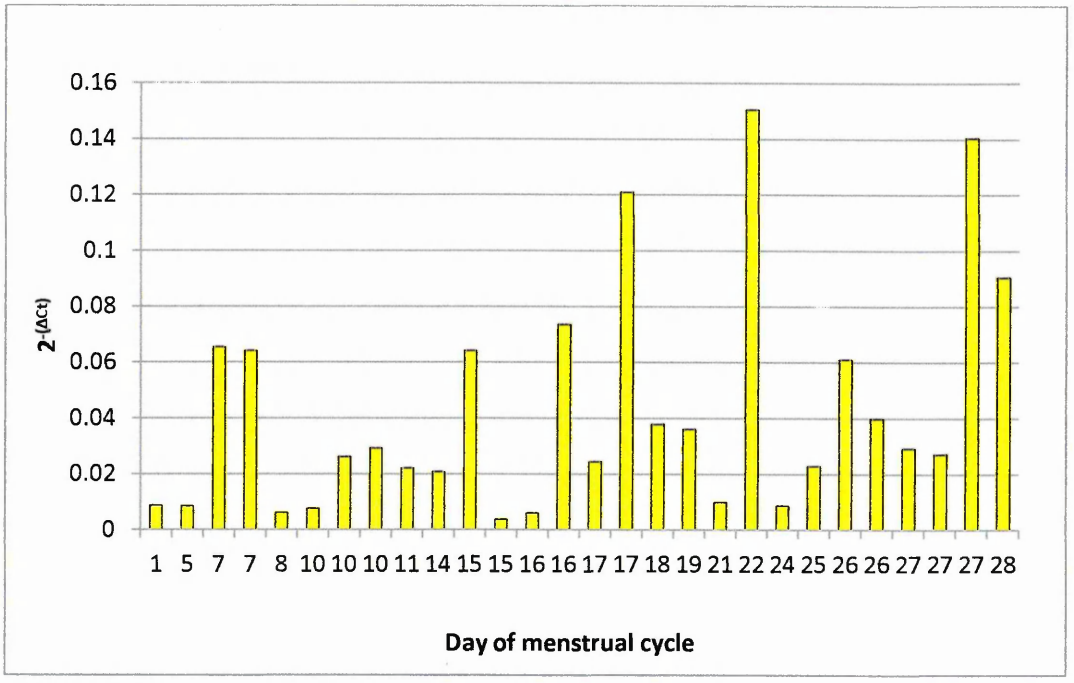


Figure 3.2 Relative expression of CXCL12 mRNA in individual biopsies obtained throughout the menstrual cycle

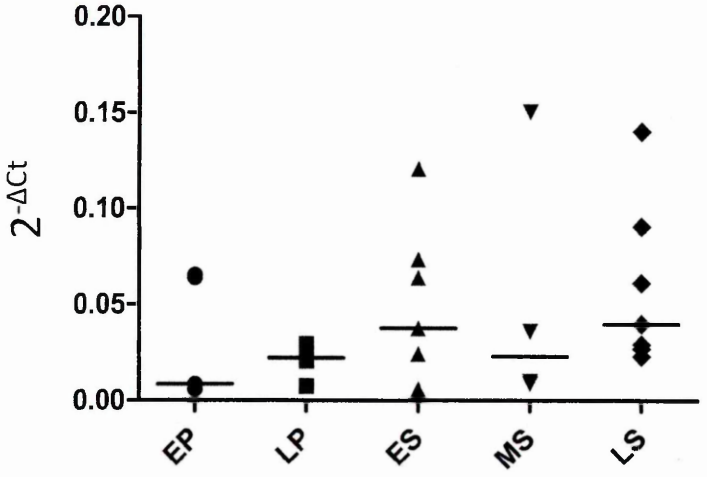


Figure 3.3 Relative expression of CXCL12 mRNA in endometrium obtained during the phases of the menstrual cycle

The points shown are from each individual volunteer samples plus the median. EP = early proliferative (days 1-9) (*n=5*), LP = late proliferative (days 10-14) (*n=5*), ES = early secretory (days 15-18) (*n=7*), MS = mid secretory (days 19-23) (*n= 4*), LS = late secretory (days 24-28) (*n=7*).

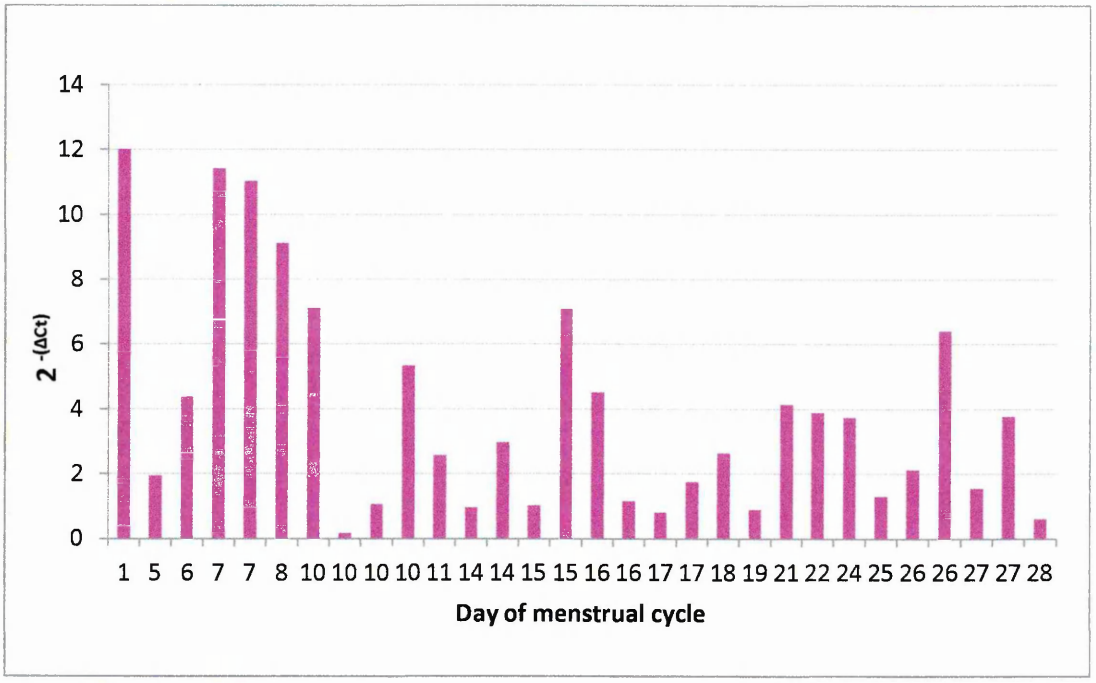


Figure 3.4 Relative expression of CXCR4 mRNA in individual biopsies obtained throughout the menstrual cycle

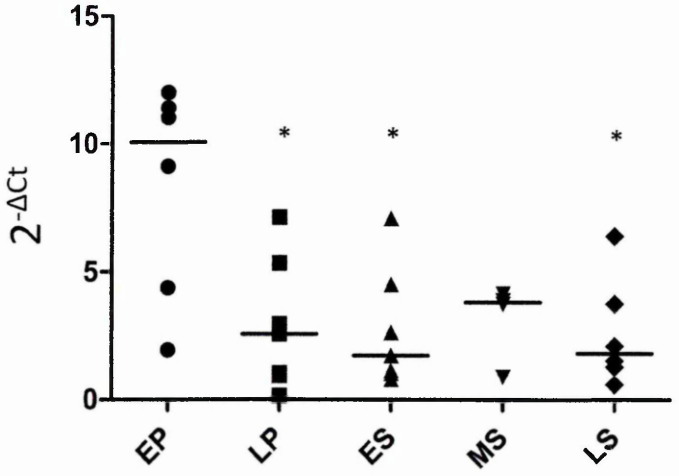


Figure 3.5 Relative expression of CXCR4 mRNA in endometrium obtained during the phases of the menstrual cycle

The points shown are from each individual volunteer samples plus the median. EP = early proliferative (days 1-9) (*n=6*), LP = late proliferative (days 10-14) (*n=7*), ES = early secretory (days 15-18) (*n=7*), MS = mid secretory (days 19-23) (*n= 4*) LS = late secretory (days 24-28) (*n=6*). * Significantly different to early proliferative phase at $P<0.05$.

3.3.3 Expression of CXCL12 mRNA in endometrial cell cultures

Both primary epithelial and stromal endometrial cells expressed CXCL12 *in vitro*, but levels were low compared to the reference transcript. Epithelial cell expression of CXCL12 was lower than stromal cell expression but these results were not significantly different from each other (Figure 3.6). Experiments attempting to measure expression of CXCL12 in Ishikawa cell lines (*n=3)* could not conclusively identify any level of expression that could be considered different to non-specific amplification observed in negative controls experiments (data not shown). Therefore, measurement of CXCL12 mRNA expression in cell lines was not continued.

3.3.4 Expression of CXCR4 mRNA in endometrial cell cultures

CXCR4 was expressed by all cell types. CXCR4 mRNA expression was lowest in HEC-1-B cells, while median CXCR4 expression in Ishikawa cells was considerably higher. Primary epithelial endometrial had the highest levels of CXCR4 mRNA expression, which was higher than the reference transcript (expression = 1). Levels of epithelial CXCR4 mRNA expression was more than twice that seen in stromal cells but expression levels were not significantly different from each other (*P=0.056*). Expression of CXCR4 in Ishikawa cells Ishikawa cells was comparable levels of CXCR4 expression to their primary epithelial counterparts, while expression in HEC-1-B cells was considerably lower than primary epithelial cells (Figure 3.7).

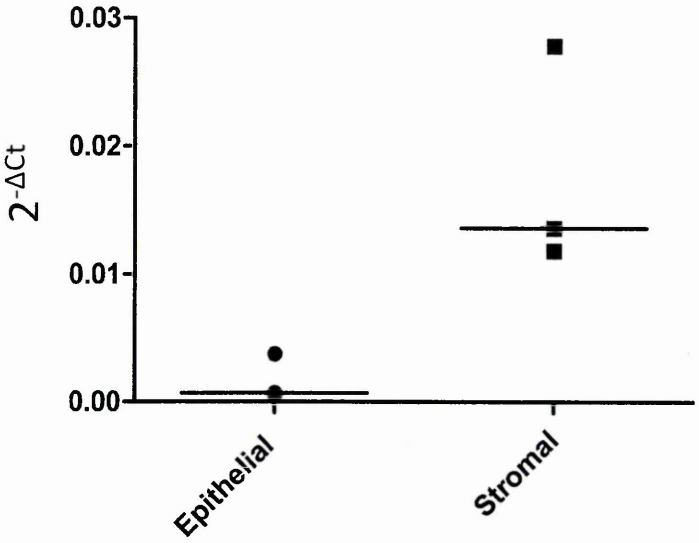


Figure 3.6: Relative expression of CXCL12 mRNA in primary endometrial cells

Median relative expression values: **Epithelial** = 6.9^{-04} and **Stromal** = 0.014. *n=3* for epithelial and stromal cells.

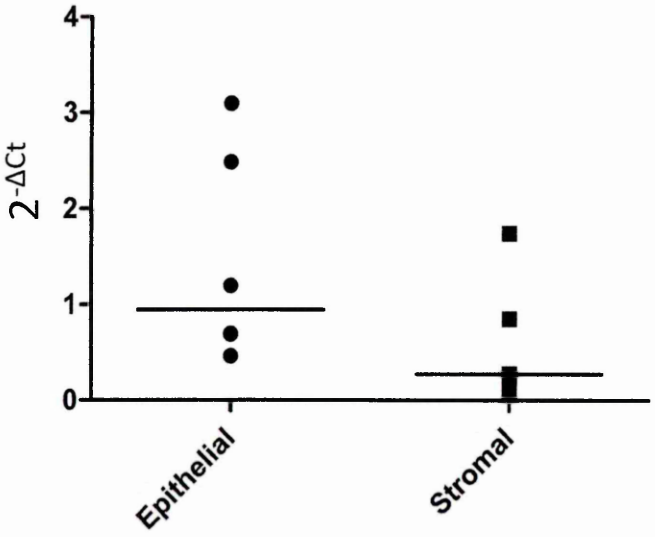


Figure 3.7: Relative expression of CXCR4 mRNA in primary endometrial cells

Median relative expression values: **Epithelial** = 0.95 and **Stromal** = 0.28. *n=6* for epithelial and stromal cells.

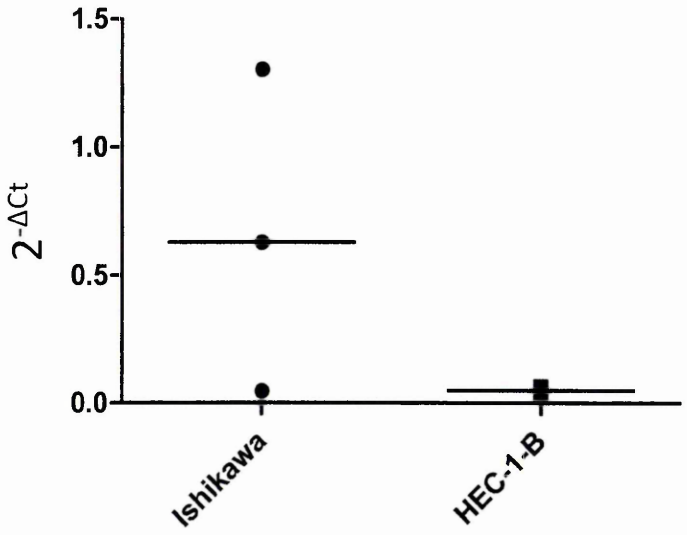


Figure 3.8: Relative expression of CXCR4 mRNA in Ishikawa and HEC-1-B cell lines

Median relative expression values: **Ishikawa** = 0.63, **HEC-1-B** = 0.05. *n=3* for Ishikawa and HEC-1-B cells.

3.4 Discussion

It was our hypothesis that CXCL12 and CXCR4 may be differentially expressed in human endometrium both throughout the menstrual cycle and between different endometrial cell types present within the tissue. Identification of differential expression of CXCL12 and CXCR4 throughout the menstrual cycle or between cell types within the endometrial tissue provides the basis of a possible mechanism by which CXCL12 and CXCR4 carries out their endometrial functions.

3.4.1 Expression of CXCL12 mRNA throughout the menstrual cycle

Few studies have investigated the expression of CXCL12 and CXCR4 in human endometrium through the human menstrual cycle. One immunohistochemical study of CXCL12 expression in the human endometrium detected the presence of CXCL12 during the proliferative phase of the menstrual cycle but did not detect CXCL12 during other phases (Kitaya *et al.*, 2004). A further study which investigated expression of CXCL12 and other chemokines in human endometrium through the menstrual cycle using gene arrays could not detect the presence CXCL12 mRNA during any phase of the menstrual cycle (Jones *et al.,* 2004). In contrast this current study did show the expression of CXCL12 mRNA in the endometrium throughout the non-pregnant menstrual cycle which was comparatively low compared to the reference gene UBC. The levels of its expression did not appear to vary during the phases of the menstrual cycle. This suggests that the steroid hormones do not affect the expression of CXCL12 as levels increase through the proliferative and secretory phases of the menstrual cycle. In contrast to this observation, a recent study has demonstrated that addition of estradiol to human endometrial stromal cell lines increased expression of CXCL12 in a time and dose-dependent manner, which could be blocked by addition of an estradiol antagonist (Tsutsumi *et al.*, 2011).

3.4.2 Expression of CXCR4 mRNA throughout the menstrual cycle

A study by Dominguez *et al.* (2003) investigated the expression of CXCR4 mRNA through the phases of the menstrual cycle using SYBR® Green real-time RT-PCR. The study showed an apparent decrease in expression levels of CXCR4 mRNA through the proliferative phase of the menstrual cycle which was in agreement with this study.

However, this study found the levels of CXCR4 mRNA to be significantly higher in the early proliferative phase of the menstrual cycle in comparison to the late proliferative, early and late secretory phases. Dominguez *et al.* (2003) showed levels of CXCR4 to peak significantly during the mid secretory phase in comparison to all other phases. The results displayed in Figure 3.5 show a slight increase in the levels of CXCR4 mRNA expression in the mid secretory endometrium in comparison to the early and late secretory phases, this difference was not found to be significantly higher than the ES and LS phases. It is possible that the presence of a blastocyst could cause an increase in MS CXCR4 levels.

Both our study and that of Dominguez *et al.* (2003) showed a decrease in CXCR4 levels through the menstrual cycle suggesting regulation by the steroid hormones. Ruiz *et al.* (2010) recently showed both estradiol and progesterone caused down-regulation of CXCR4 at the mRNA level in endometrial epithelial cell lines adding further support to this idea. Little is known about how and if steroid hormones regulate the expression of CXCR4 in the endometrium, though the recent evidence suggests it is likely to occur.

While both studies investigated CXCR4 expression in human endometrium from normal fertile patients using real-time RT-PCR, this study used a TaqMan probe to identify CXCR4 mRNA, while Dominguez *et al.* (2003) used SYBR® Green chemistry. The main difference between the current study and that of Dominguez *et al.* (2003) is that their study used tissues from 15 subjects while our group size was considerably larger.

3.4.3 Expression of CXCL12 and CXCR4 in endometrial cell cultures

The expression levels of CXLC12 transcripts were low in both primary epithelial and stromal cells in comparison to the reference transcript which was also observed in the results obtained from intact tissue from throughout the menstrual cycle. It was surprising that stromal cells exhibited higher CXCL12 mRNA levels in comparison to epithelial cells as unpublished data from our laboratory showed protein expression of CXCL12 to be highest in the glandular and luminal epithelium. However, this result is in agreement with that of Ruiz *et al.* (2010) and Tsutsumi *et al.* (2011) which found endometrial stromal cell lines to express higher levels of CXCL12 in comparison to epithelial derived cell lines. The study by Tsutsumi *et al.* (2011) also confirmed our

observation that Ishikawa cell lines do not produce CXCL12 but are CXCR4 positive. Interestingly, addition of CXCL12 to Ishikawa cells induced proliferation leading to the suggestion that a function of stromal CXCL12 in the endometrium is to control epithelial cell growth.

3.4.4 Potential functions of CXCL12 and CXCR4 in endometrium

It is possible that CXCL12 expression in the endometrium functions to recruit leukocytes. The apparently increased expression of CXCL12 in endometrial stromal cells in comparison to epithelial cells supports this idea, as secretion of the chemokine ligand would occur close to blood vessels through which leukocytes such as uNK cells could infiltrate, expression CXCL12 in the epithelial glands is more likely to be secreted into intraluminal spaces on the innermost surface of the uterus to which the blastocyst attaches during implantation. CXCL12 could potentially have an alternate role at this interface. Although recruitment of leukocytes such as uNK cells to the endometrium by CXCL12 seems possible (Hanna *et al.*, 2003; Wu *et al.*, 2005), our study could not identify any differential expression of CXCL12 during the non-pregnant menstrual cycle. uNK cells appear in greatest numbers in the endometrium during the mid-secretory phase, if CXCL12 secretion was responsible for this it would be expected that an increase in CXCL12 mRNA production would have been observed close to this time.

Other studies such as that by Tsutsumi *et al.* (2011) have suggested that stromal CXCL12 may serve as a growth factor for epithelial cell proliferation thus accounting for increased CXCL12 levels. Conversely, Diagram 3.1 shows immunohistochemical localisation of CXCL12 to the endometrial epithelium and not stoma but it is possible that CXCL12 is receptor bound to epithelial cells following secretion from the stroma. It is also possible that post-translational regulation may affect the amount of CXCL12 protein actually produced as increased levels of CXCL12 in endometrial stroma in comparison to epithelial cells has so far only been observed at the mRNA level.

CXCR4 has been shown to be maximally expressed in human endometrium during the MS phase of the menstrual cycle and presence of CXCL12 positive blastocyst has been shown to increase CXCR4 expression in endometrial cell culture models (Dominguez *et al.*, 2003). This suggests a role in mediating the cross-talk between the blastocyst and endometrium during the initial stages of implantation. Our Larger study found

maximal expression of CXCR4 during the early proliferative phase of the menstrual cycle when the endometrium begins to rapidly grow. Combined with evidence that CXCL12 can cause proliferation of epithelial endometrial cell lines (Tsutsumi *et al.*, 2011) it is possible that a major role for this chemokine and receptor is in the paracrine control of endometrial proliferation. This does not rule out a potential role in mediating the early stages of implantation as a slight increase in MS CXCR4 levels was observed by our study.

CXCL12 and CXCR4 are also known to be involved with the control of angiogenesis, stem cell migration and cell differentiation (Busillo and Benovic, 2006). All these processes are of great potential importance to the endomtrium. The maximal expression of CXCR4 during the early proliferative phase and the unchanging presence of CXCL12 throughout the menstrual cycle could also be consistent with these functions also.

3.4.5 Limitations

Direct measurement of CXCL12 and CXCR4 from biopsies obtained from human volunteers using real-time RT-PCR allows *ex vivo* quantification of chemokine and receptor levels which is useful for observing any potential changes throughout the menstrual cycle. The technique is most limited by its inability to distinguish in which cell types the factors are expressed. Endometrium consists of not only epithelial and stromal cells but also vascular cells and leukocytes which may contribute to the levels of mRNA expression observed. To overcome this to some degree, levels of CXCL12 and CXCR4 were quantified in primary epithelial and stromal cultures but this in itself does not fully account for all potential expression of CXCL12 and CXCR4 in the endomtrium.

Levels of variability of CXCL12 and CXCR4 between individual biopsy samples appeared high. As each sample was from a different human volunteer it is likely that natural variations between individuals could produce this effect. This is supported by use of reference genes that were shown not to significantly vary between the phases of the menstrual cycle into which our data was grouped (Figure ???). This highlights the importance of using as many samples a possible to obtain robust averages for each menstrual cycle phase, increasing the likelihood of significant differences between the phases being observed if present. Degradation of mRNA during collection of the

sample and its storage along with reverse transcription may also potentially account for some sample variability, although attempts to minimise this were made and samples were treated consistently.

The primary cell cultures were prepared from biopsies taken throughout the cycle. As CXCR4 was shown to be differentially expressed throughout the menstrual cycle it is possible that this may affect the levels of CXCR4 produced by the individual cultures. However as the samples were taken throughout that cycle, median values were likely to be a good representation of the amount of CXCL12 and CXCR4 present in terms of investigating the distribution between epithelial and stromal cells. Furthermore, the process of removing a sample and preparing it for cell culture is highly invasive, degrading extracellular matrix and potentially causing trauma to cells which become isolated from their normal environment and endocrine control. Therefore it is inherent in primary cell culture experiments such as these that expression levels of many factors may be different to the true *in vivo* levels and in particular relation to the endometrium, individual cultures may exhibit levels of CXCL12 and CXCR4 which do not directly relate to the stage of the menstrual cycle from which they are taken.

The cell culture conditions themselves may also affect the expression levels primary cultured cells. FCS was used to aid cell adhesion and supplement growth but contains many factors which could potentially interact with the cells in culture and alter gene expression. This culture model did not use steroid hormones which would normally be present to some degree in endometrial cell environments. Overcoming some of these limitations would potentially improve the accuracy of the experiments and reduce some of variability present in the data shown.

3.4.6 Summary

It was our hypothesis that CXCL12 and CXCR4 mRNA is differentially expressed during the non-pregnant menstrual cycle and may differ between endometrial cell types. Both CXCL12 and CXCR4 mRNA was found to be expressed throughout the menstrual cycle but only CXCR4 mRNA was found to be differentially expressed, exhibiting maximal levels during the early proliferative phase of the menstrual cycle. Stromal endometrial cells expressed higher levels of CXCL12 than epithelial cells in culture, while CXCR4 was higher in epithelial cells compared to the stromal. While CXCL12 may

potentially recruit uNK cells to endometrium and epithelial CXCR4 expression may contribute to controlling implantation on the maternal side, our findings along with recent studies by Ruiz *et al.* (2010) and Tsutsumi *et al.* (2011) suggests stromally secreted CXCL12 could act as a growth factor for endometrial epithelial cells during the early stages of the menstrual cycle and may potentially also contribute to local angiogenesis, cell differentiation and stem cell migration.

Finally, Ishikawa cells were found to express high levels of CXCR4 but CXCL12 could not be detected. This was also recently confirmed by Tsutsumi *et al.* (2011). Therefore this cell line could be a particularly good endometrial model for studying the effects of exogenously added CXCL12 on an endometrial cell line.

Chapter 4
Effects of CXCL12 on the expression of IL6, IL8, MMP2, MMP9 and VEGFA mRNA in cultured endometrial cells.

4.1 Hypothesis

CXCL12 regulates expression of IL6, IL8, MMP2, MMP9 and VEGFA in endometrial cell types.

4.2 Effects of CXCL12 on IL6, IL8, MMP2, MMP9 and VEGFA in non-endometrial cell types

Effects of CXCL12 on endometrial cell function have not been reported. However, many studies have shown the ability of this chemokine ligand to up-regulate the expression of IL6, IL8, MMP2, MMP9 and VEGF in various other human cells.

CXCL12 has been shown to up-regulate IL6 production in human primary cultured microglia (Lu *et al.*, 2009), basal cell carcinoma cell lines (Chu *et al.*, 2008) and oral cancer cells (Tang *et al.*, 2008). Both IL6 and IL8 (CXCL8) production has been shown to be increased by CXCL12 in PC3 prostate cancer cell lines (Wang *et al.*, 2005) and fibroblast-like synoviocytes from patients with rheumatoid arthritis (Nanki *et al.*, 2001). IL8 production has been shown to be increased by CXCL12 in human retinal pigment epithelial cells (Crane *et al.*, 2000), mast cells (Lin *et al.*, 2000) and T cell acute lymphoblastic leukemia cells (Scupoli *et al.*, 2008).

CXCL12 has been shown to increase production of MMP2 and MMP9 protein in PC3 prostate cancer cell lines (Singh *et al.*, 2004). CXCL12 has been shown to increase MMP9 expression in U87 malignant glioma cells (Kenig *et al.*, 2010), adult neural proginator cells (Barkho *et al.*, 2008), lung cancer cell lines (Tang *et al.*, 2008), Hela cells (Brule *et al.*, 2006), head and neck squamous carcinoma cells (Samara *et al.*, 2004), cultured chondrocytes (Kanbe *et al.*, 2004), primary human monocytes (Klier *et al.*, 2001) and megakaryocytes (Lane *et al.*, 2000). Both MMP2 and MMP9 have been shown to be increased at the mRNA level by CXCL12 in human ovarian cancer cells (Yuecheng and Xiaoyan, 2007). VEGF has been shown to be up-regulated by CXCL12 in malignant glioma cells (Yang *et al.*, 2005), human arterial endothelial cells (Neuhaus *et*

al., 2003), lymphohaematopoietic cells (Kijowski *et al.*, 2001) and microvascular endothelial cells (Mirshahi *et al.*, 2000).

Expression of IL6, IL8, MMP2, MMP9 and VEGFA mRNA was measured in four endometrial cell types: primary epithelial cells, primary stromal cells and the Ishikawa and HEC-1-B adenocarcinoma cell lines. This chapter investigates the expression of these factors in the endometrium; in particular their cellular distribution between endometrial epithelial and stromal cells. It also considers the appropriateness of using HEC-1-B and Ishikawa cell lines as a model to study these factors in relation to endometrial function. This is explored further in chapter 7, which investigates the effect of CXCL12 on the production of these factors in each cell type.

The precise functions of the chemokine ligand CXCL12 and its receptor CXCR4 in the endometrium, remains unknown. In other tissues CXCL12 has been shown to up-regulate IL6, IL8, MMP2, MMP9 and VEGF in various human cell types. This chapter reviews the evidence for the ability of CXCL12 to up-regulate these important endometrial factors in non-endometrial cell types and investigates the effects of CXCL12 on the mRNA levels of IL6, IL8, MMP2, MMP9 and VEGFA in HEC-1-B and Ishikawa cell lines and primary cell cultures of endometrial epithelial and stromal cells.

4.3 Aims

To investigate our hypothesis that CXCL12 may regulate the expression of IL6, IL8, MMP2, MMP9 and VEGFA in endometrial cells, it is our aim to measure changes in the mRNA expression levels of these factors in endometrial cell cultures in response incubation with recombinant CXCL12. To achieve this, real-time RT-PCR will be used to accurately measure the amounts of mRNA present in primary cell cultures and cell lines both in the presence and absence of CXCL12.

4.4 Methods

To assess basal mRNA expression, Ishikawa, HEC-1-B cells and primary cultures of epithelial and stromal cells were prepared as described in sections 2.2.2, 2.2.3 and 2.2.4 respectively. RNA was extracted from the cells (section 2.3.1) and levels of IL6, IL8, MMP2, MMP9 and VEGFA mRNA were quantified using SYBR® Green real-time RT-PCR (as described in sections 2.5 and 2.6). Expression levels of the gene transcripts of

interest were quantified using the $2^{-\Delta Ct}$ method relative to reference gene YWHAZ as described in section 2.8.3. RNA was extracted from endometrial epithelial and stromal cells prepared from endometrial biopsies, obtained throughout the menstrual cycle and prepared from 6 different women. Experiments using HEC-1-B and Ishikawa cells were carried out on 3 separate occasions. The Kruskal-Wallis and Mann-Whitney U test was used to determine any statistical differences in mRNA levels between cell types.

To assess the effects of CXCL12 on IL6, IL8, MMP2, MMP9 and VEGFA in endometrial cell cultures, Ishikawa, HEC-1-B cells, primary endometrial epithelial and stromal cells were prepared and grown as described in sections 2.1.3, 2.1.4 and 2.1.5 respectively. All endometrial cell types were incubated with 0, 10 and 100 ng/ml of recombinant CXCL12 for 24 hours prior to expected confluence as described in section 2.16. Following RNA extraction and reverse transcription (sections 2.2.1 and 2.4.1), SYBR® Green real-time RT-PCR was carried out to quantify the expression levels of IL6, IL8, MMP2, MMP9 and VEGFA relative to reference transcript YWHAZ (described in section 2.4). Incubation with CXCL12 was repeated 3 times in HEC-1-B and Ishikawa cells and with epithelial and stromal cells prepared from tissue obtained from 6 different women. The effects of CXCL12 on the expression levels of CXCR4 and CXCL12 mRNA were also assessed. To investigate potential effects of CXCL12 on the mRNA production of CXCR4, the effects of CXCL12 on CXCR4 mRNA expression levels was quantified in all four endometrial cell types using Taqman® real-time RT-PCR and reference transcript YWHAZ. To assess any effects of CXCL12 on CXCL12 mRNA production, the effects of CXCL12 treatment on CXCL12 mRNA production was assessed in primary epithelial and stromal cells to using SYBR® Green real-time RT-PCR and UBC as a reference transcript. This experiment was repeated 3 times. Resulting C_t values were quantified relative to the relevant reference transcript and compared to levels in mRNA extracted from cells incubated without CXCL12 to give relative expression values using the $2^{-(\Delta\Delta Ct)}$ method. Mean expression values for levels of mRNA for IL6, IL8, MMP2, MMP9, VEGFA, CXCR4 and CXCL12 were calculated and differences in expression between control cells and cells incubated with 10 or 100 ng/ml of CXCL2 was assessed using a Kruskal-Wallis test.

4.5 Results: basal expression of IL6, IL8, MMP2, MMP9 and VEGFA in endometrial cell cultures

4.5.1 IL6

IL6 mRNA expression was highest in primary epithelial cells, while expression in the stromal cells was less than half that of the epithelial cells. There was no statistical difference in levels of IL6 mRNA expressed in the two cell types. Expression of IL6 mRNA in the endometrial adenocarcinoma cell lines was found to be considerably lower than their primary epithelial counterparts with Ishikawa cells showing higher levels of IL6 expression in comparison to the HEC-1-B cells which were found to express significantly less IL6 mRNA in comparison to primary epithelial cells using a Kruskal-Wallis test (Figure 4.1).

4.5.2 IL8

IL8 mRNA expression in primary endometrial epithelial cells was found to be approximately equal to that of reference transcript YWHAZ. Expression of IL8 mRNA in the primary stromal cells was significantly lower than mRNA expression in primary epithelial cells when the two cell types were directly compared with a Mann-Whitney U test (*P=0.002*). Expression of IL8 mRNA in Ishikawa and HEC-1-B cell lines was found to be several orders of magnitude lower than their primary epithelial cell counterparts and was significantly lower than epithelial cells when all groups were compared using Kruskal-Wallis test (Figure 4.2).

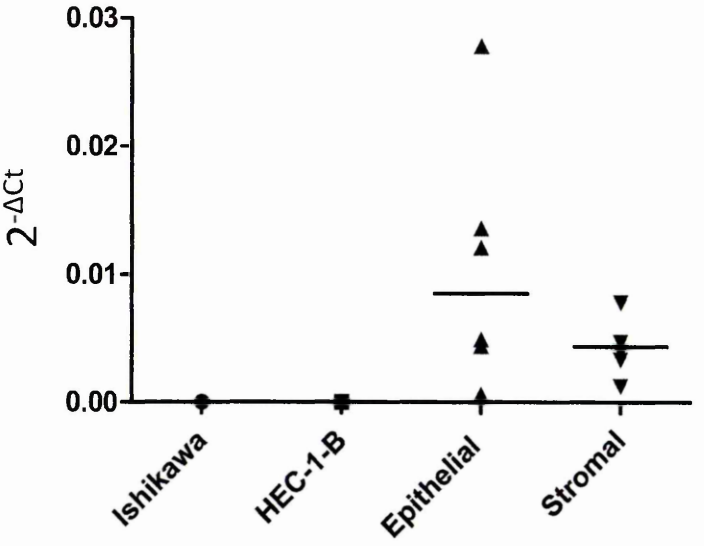


Figure 4.1: Relative expression of basal IL6 mRNA in cultured endometrial cells

Median relative expression values: **Ishikawa** = 1.39^{-05}, **HEC-1-B** = 1.18^{-05}, **Epithelial** = 8.52^{-03} and **Stromal** = 4.34-^{03}. *n=6* for epithelial and stromal cells, *n=3* for Ishikawa and HEC-1-B cells.

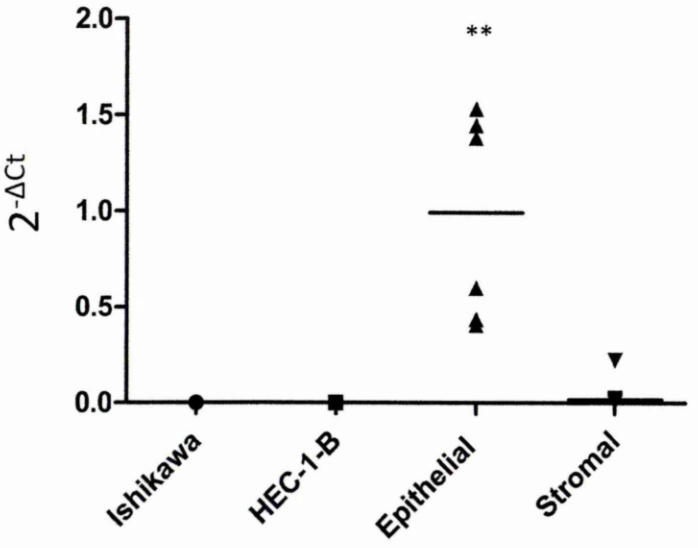


Figure 4.2: Relative expression of basal IL8 mRNA in cultured endometrial cells

Median relative expression values: **Ishikawa** = 1.32^{-06}, **HEC-1-B** = 5.73^{-04}, **Epithelial** = 0.99 and **Stromal** = 0.017. *n=6* for epithelial and stromal cells, *n=3* for Ishikawa and HEC-1-B cells. ** Significantly different from stromal cells when compared directly to epithelial cells using a Mann-Whitney U test (*P <0.01*).

4.5.3 MMP2

Endometrial stromal cells were shown to express the highest levels of MMP2 mRNA, which was found to be approximately twice that of reference transcript YWHAZ. Expression of MMP2 mRNA in primary epithelial cells was approximately equivalent to that of the reference transcript YWHAZ. Despite stromal levels of MMP2 mRNA appearing to be twice that of epithelial cells, levels of expression in each cell type were not significantly different from each other ($P > 0.05$) when directly compared to each other using a Mann-Whitney U test. Levels of MMP2 mRNA expression in the Ishikawa and HEC-1-B cell lines were several orders of magnitude lower than their primary epithelial counterparts and found to be significantly lower than stromal cells when all groups were compared using a Kruskal-Wallis test (Figure 4.3).

4.5.4 MMP9

Primary endometrial epithelial cells expressed the highest levels of MMP9 mRNA and levels were found to be significantly higher than those in stromal cells ($P=0.026$) when both groups were compared directly to each other using a Mann-Whitney U test. Both Ishikawa and HEC-1-B cell lines expressed levels of MMP9 mRNA several orders of magnitude lower than their primary epithelial counterparts. Ishikawa cells were found to express significantly lower MMP9 mRNA than epithelial cells when all groups were compared using a Kruskal-Wallis test. Levels of expression of MMP9 by HEC-1-B cultures showed considerable variation in the three different experiments (Figure 4.4).

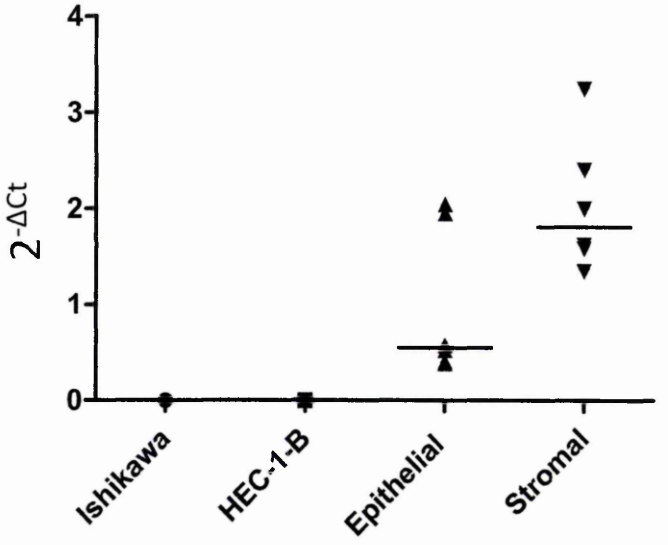


Figure 4.3: Relative expression of basal MMP2 mRNA in cultured endometrial cells

Median relative expression values: **Ishikawa** = 4.73^{-05}, **HEC-1-B** = 2.06^{-05}, **Epithelial** = 0.55 and **Stromal** = 1.81. *n=6* for epithelial and stromal cells, *n=3* for Ishikawa and HEC-1-B cells.

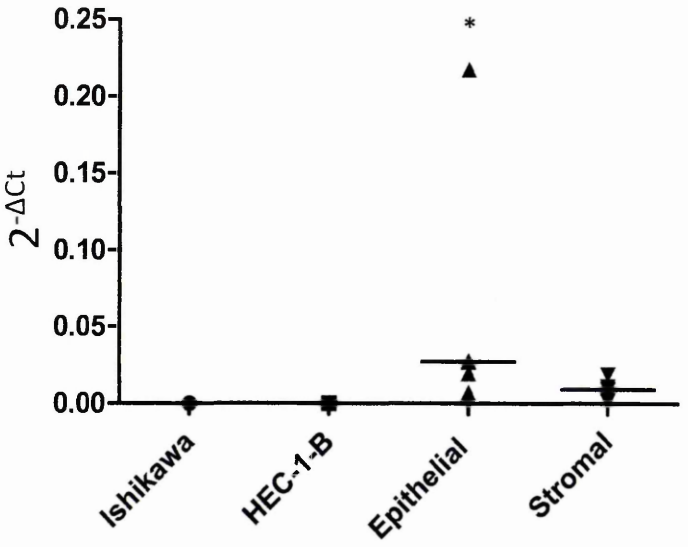


Figure 4.4: Relative expression of basal MMP9 mRNA in cultured endometrial cells

Median relative expression values: **Ishikawa** = 1.59^{-06}, **HEC-1-B** = 2.09^{-06}, **Epithelial** = 0.027 and **Stromal** = 0.009. *n=6* for epithelial and stromal cells, *n=3* for Ishikawa and HEC-1-B cells. * Significantly different from stromal cells when compared directly to epithelial cells using a Mann-Whitney U test (*P <0.05*).

4.5.5 VEGFA

Primary endometrial stromal cells were found to express significantly higher levels of VEGFA mRNA when directly compared to primary epithelial cells using a Mann-Whitney U test (*P=0.004*). In contrast to other factors VEGFA was expressed by HEC-1-B and Ishikawa cells in amounts comparable to their primary cell counterparts. Expression of VEGFA in Ishikawa cells was greater than in HEC-1-B cells (Figure 4.5).

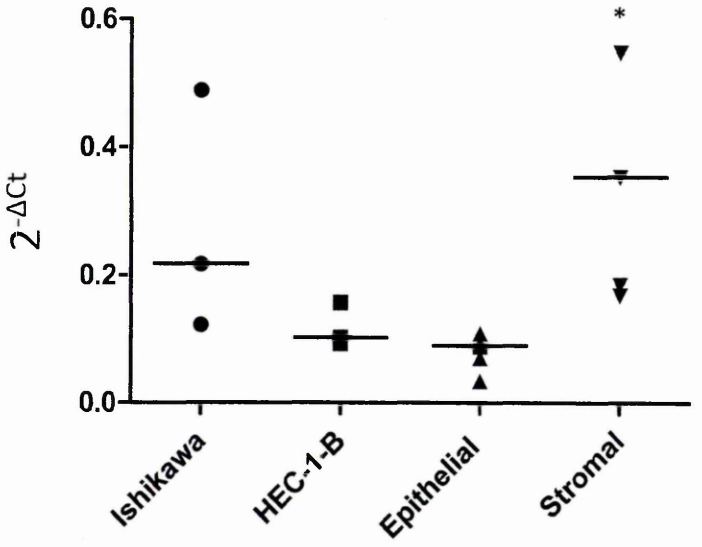


Figure 4.5: Relative expression of basal VEGFA mRNA in cultured endometrial cells

Median relative expression values: **Ishikawa** = 0.22, **HEC-1-B** = 0.10, **Epithelial** = 0.09 and **Stromal** = 0.29. *n=6* for epithelial and stromal cells, *n=3* for Ishikawa and HEC-1-B cells. * Significantly from epithelial cells when compared directly to stromal cells using a Mann-Whitney U test ($P < 0.05$).

4.6 Results: effects of CXCL12 on expression levels of CXCR4 and CXCL12 mRNA in endometrial cell cultures

4.6.1 CXCR4

CXCL12 appeared to cause a dose-dependent decrease in CXCR4 mRNA expression in Ishikawa cells. However, levels of CXCR4 mRNA in cells incubated with CXCL12 at 10 and 100 ng/ml were not significantly different from those to which no CXCL12 was added. CXCL12 at 10 and 100 ng/ml appeared to have no effect on CXCR4 expression in HEC-1-B, epithelial and stromal endometrial cells (Figure 4.6 and 4.7).

4.6.2 CXCL12

Exogenously added CXCL12 had no effect on CXCL12 mRNA expression in primary epithelial and stromal cells (Figure 4.8). The effects of exogenous CXCL12 on CXCL12 mRNA expression in Ishikawa and HEC-1-B cells was not investigated as no basal CXCL12 mRNA expression could be identified in Ishikawa cell lines.

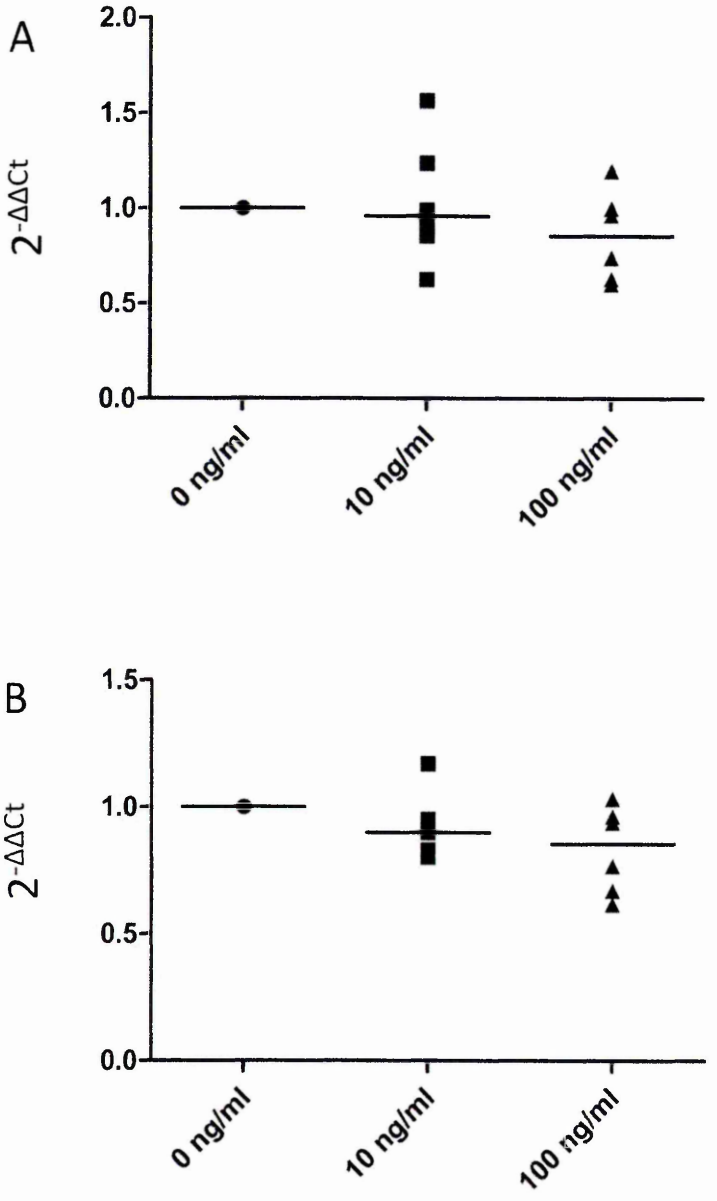


Figure 4.6: Effects of CXCL12 on CXCR4 mRNA expression in primary endometrial cells

Relative expression of CXCR4 in endometrial (A) epithelial cells (Medians: 0 ng/ml = 1.00, 10 ng = 0.96, 100 ng/ml = 0.85) and (B) stromal cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.90, 100 ng/ml = 0.85). *n=6* for epithelial and stromal cells.

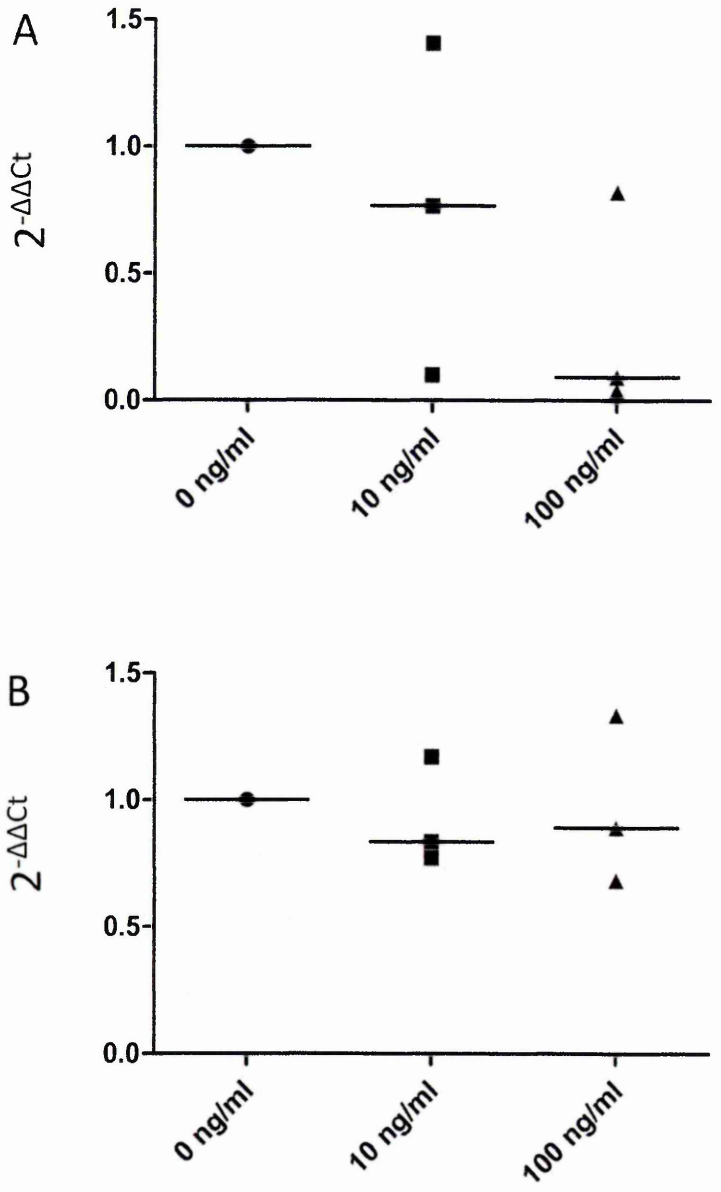


Figure 4.7: Effects of CXCL12 on CXCR4 mRNA expression in endometrial cell lines

Relative expression of CXCR4 in endometrial (A) Ishikawa cells (Medians: 0 ng/ml = 1.00, 10 ng = 0.77, 100 ng/ml = 0.09) and (B) HEC-1-B cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.84, 100 ng/ml = 0.89). *n=3* for Ishikawa and HEC-1-B cells.

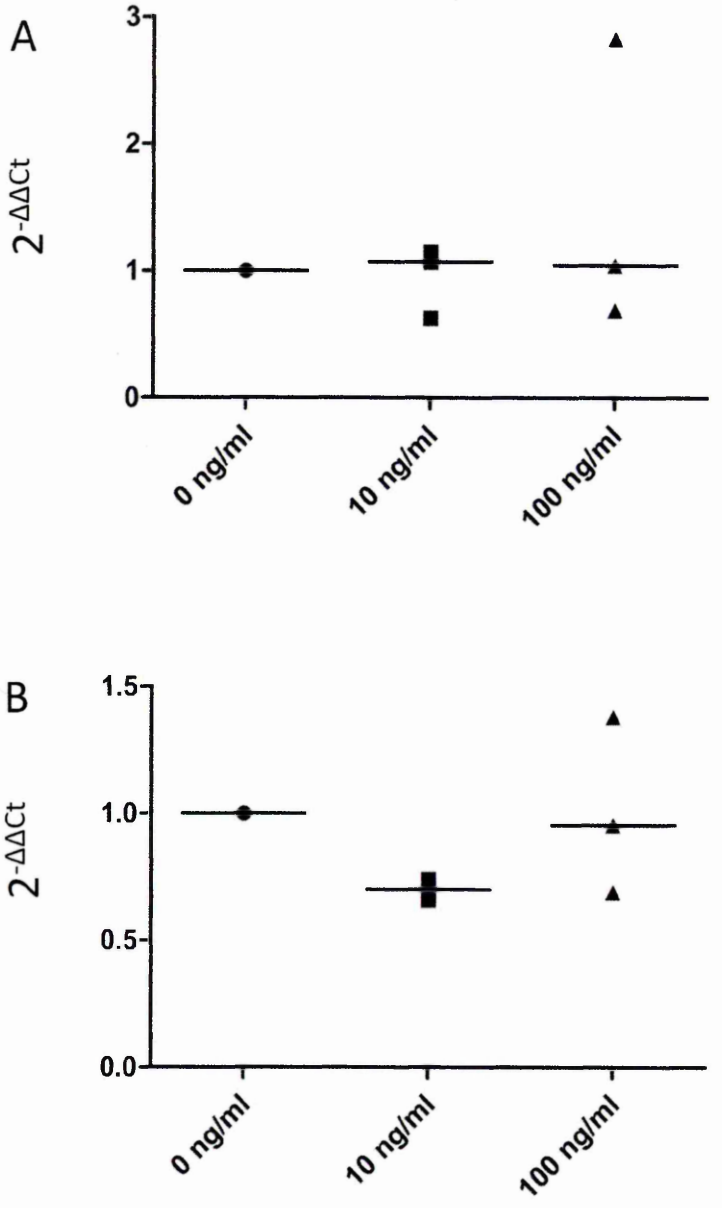


Figure 4.8: Effects of CXCL12 on CXCL12 mRNA expression in primary endometrial cells

Relative expression of CXCL12 in endometrial (A) epithelial cells (Medians: 0 ng/ml = 1.00, 10 ng = 1.07, 100 ng/ml = 1.05) and (B) stromal cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.70, 100 ng/ml = 0.95). *n=3* for epithelial and stromal cells.

4.7 Results: Effect of CXCL12 on expression levels of IL6, IL8, MMP2, MMP9 and VEGFA mRNA in endometrial cell cultures

4.7.1 IL6

CXCL12 appeared to cause a dose-dependent increase in IL6 mRNA expression in primary epithelial cells. However, levels of IL6 mRNA in cells incubated with either concentration of CXCL12 were not significantly different to cells in which no CXCL12 was added. CXCL12 at 10 and 100 ng/ml appeared to have no effect on IL6 expression in Ishikawa, HEC-1-B and stromal endometrial cell types (Figure 4.9 and 4.10).

4.7.2 IL8 (CXCL8)

CXCL12 at 10 or 100 ng/ml did not significantly alter the expression of IL8 mRNA in the Ishikawa and HEC-1-B endometrial cell lines or in primary epithelial and stromal endometrial cell types (Figure 4.11 and 4.12).

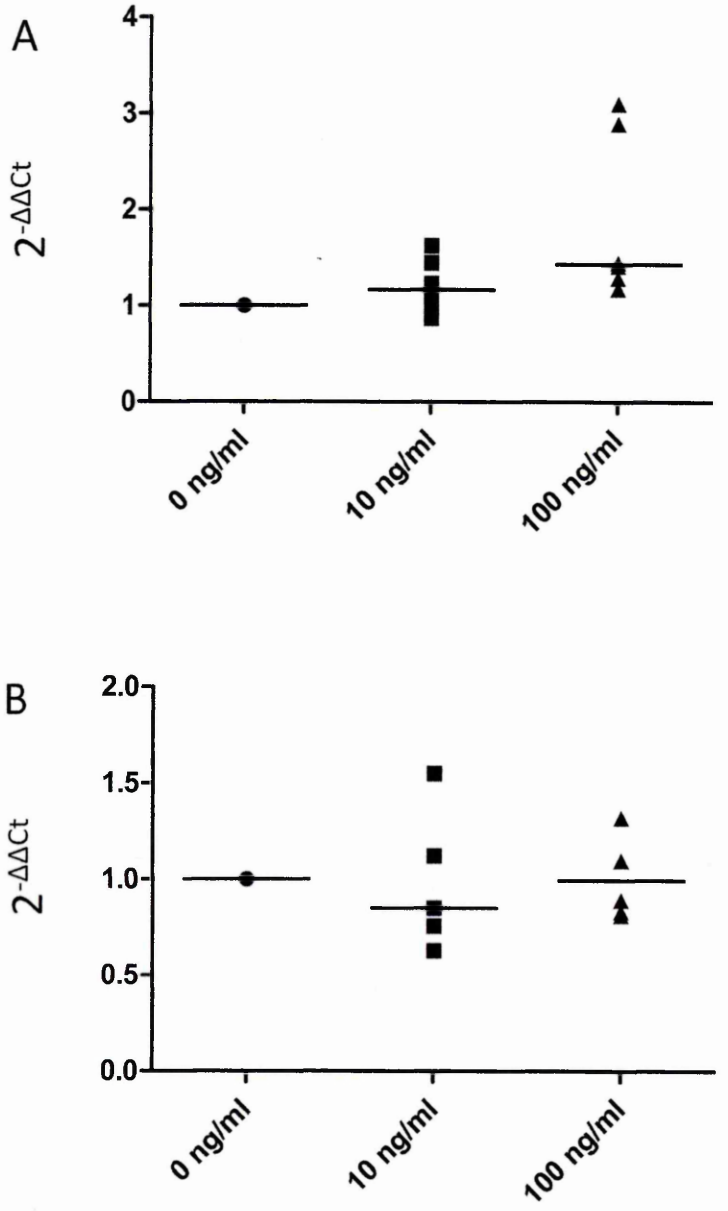


Figure 4.9: Effects of CXCL12 on IL6 mRNA expression in primary endometrial cells

Relative expression of IL6 in endometrial (A) epithelial cells (Medians: 0 ng/ml = 1.00, 10 ng = 1.16, 100 ng/ml = 1.43) and (B) stromal cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.85, 100 ng/ml = 0.99). *n=6* for epithelial and stromal cells.

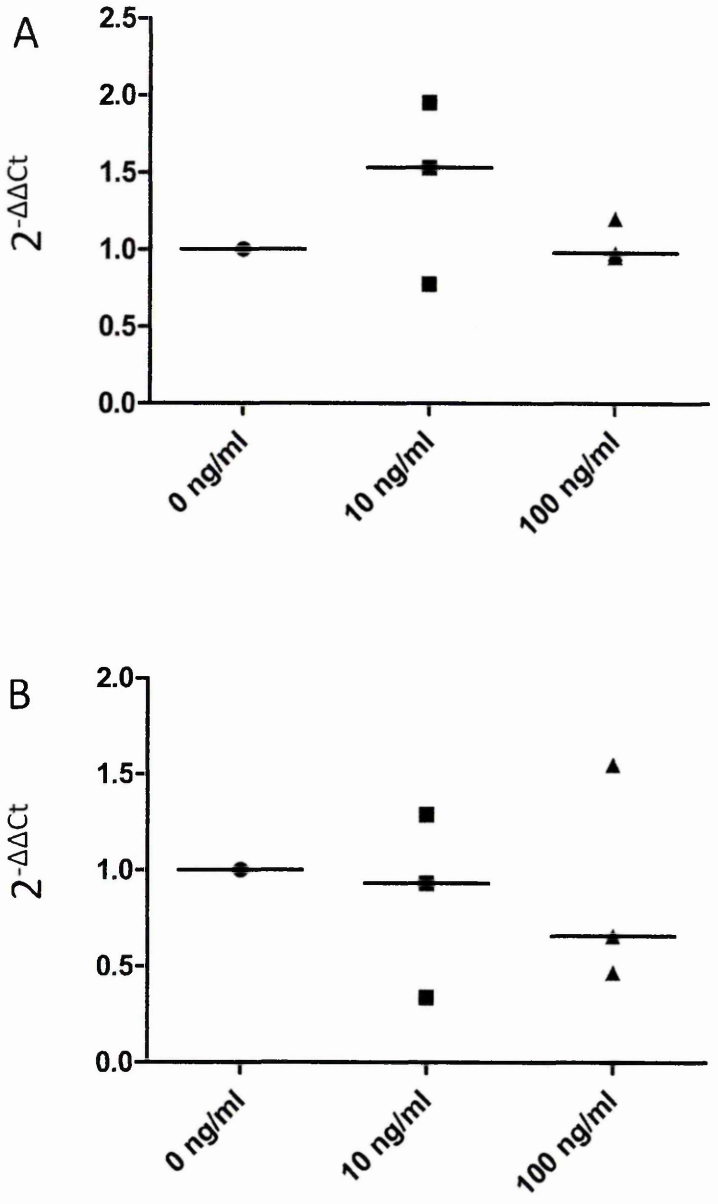


Figure 4.10: Effects of CXCL12 on IL6 mRNA expression in endometrial cell lines

Relative expression of IL6 in (A) Ishikawa cells (Medians: 0 ng/ml = 1.00, 10 ng = 1.53, 100 ng/ml = 0.97) and (B) HEC-1-B cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.93, 100 ng/ml = 0.85). *n=3* for Ishikawa and HEC-1-B cells.

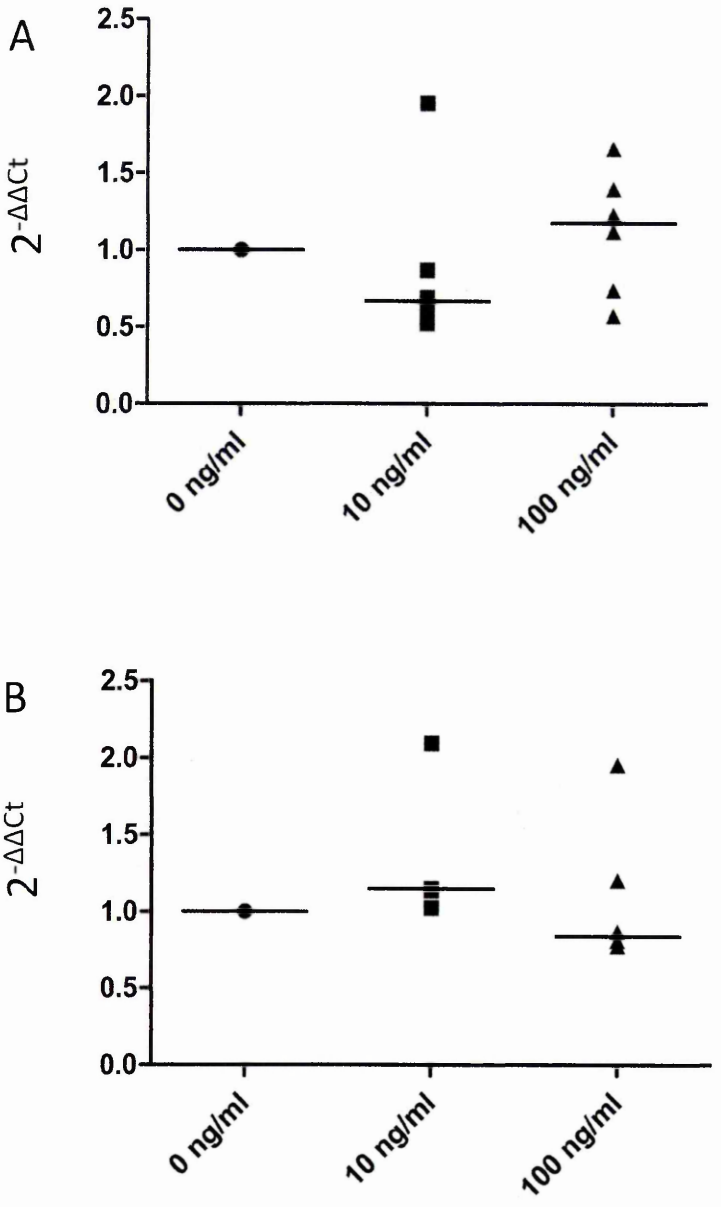


Figure 4.11: Effects of CXCL12 on IL8 mRNA expression in primary endometrial cells

Relative expression of IL8 in endometrial (A) epithelial cells (Medians: 0 ng/ml = 1.00, 10 ng = 0.67, 100 ng/ml = 1.18) and (B) stromal cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 1.15, 100 ng/ml = 0.99). *n=6* for epithelial and stromal cells.

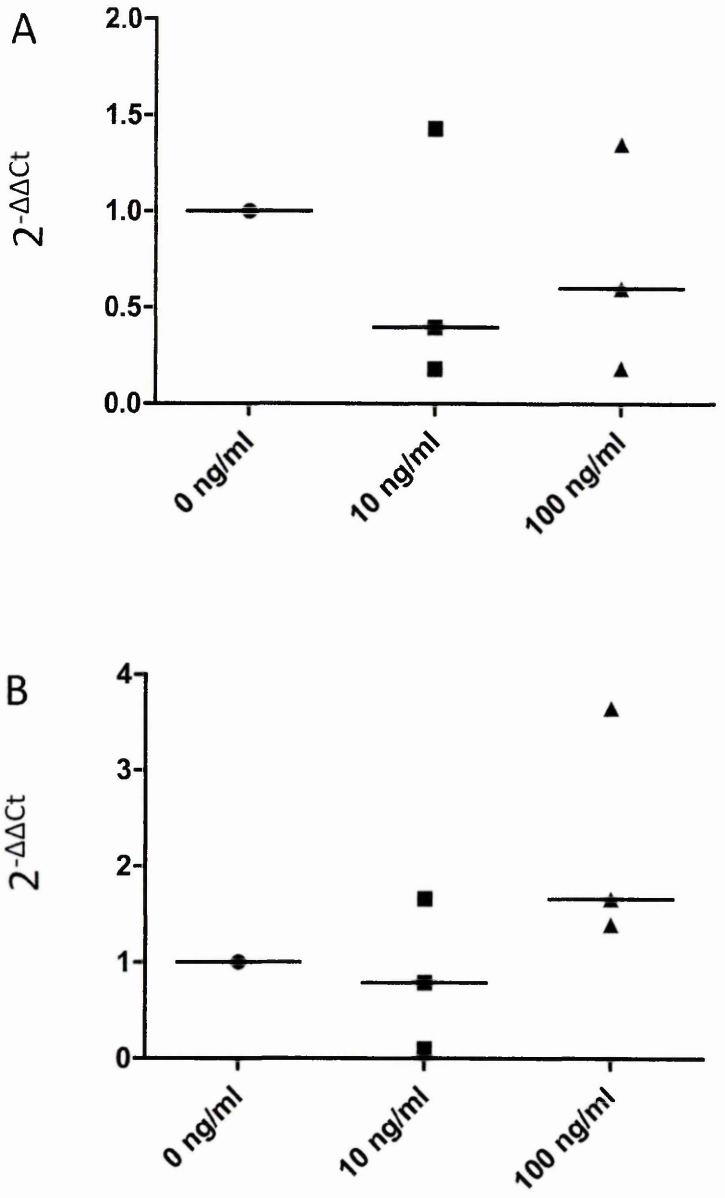


Figure 4.12: Effects of CXCL12 on IL8 mRNA expression in endometrial cell lines

Relative expression of IL8 in (A) Ishikawa cells (Medians: 0 ng/ml = 1.00, 10 ng = 0.39, 100 ng/ml = 0.60) and (B) HEC-1-B cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.71, 100 ng/ml = 1.66). *n=3* for Ishikawa and HEC-1-B cells.

4.7.3 MMP2

CXCL12 at 10 or 100 ng/ml did not significantly alter the expression of MMP2 mRNA in the Ishikawa and HEC-1-B endometrial cell lines or in primary epithelial and stromal endometrial cell types (Figure 4.13 and 4.14).

4.7.4 MMP9

CXCL12 appeared to cause a dose-dependent increase in MMP9 mRNA expression in primary epithelial cells and a dose-dependent decrease in MMP9 mRNA expression in Ishikawa cells. However, levels of MMP9 mRNA in either epithelial or stromal cells incubated with either concentration of CXCL12 were not significantly different to cells in which no CXCL12 was added. CXCL12 at 10 and 100 ng/ml appeared to have no effect on MMP9 expression in HEC-1-B or primary stromal endometrial cell types (Figure 4.15 and 4.16).

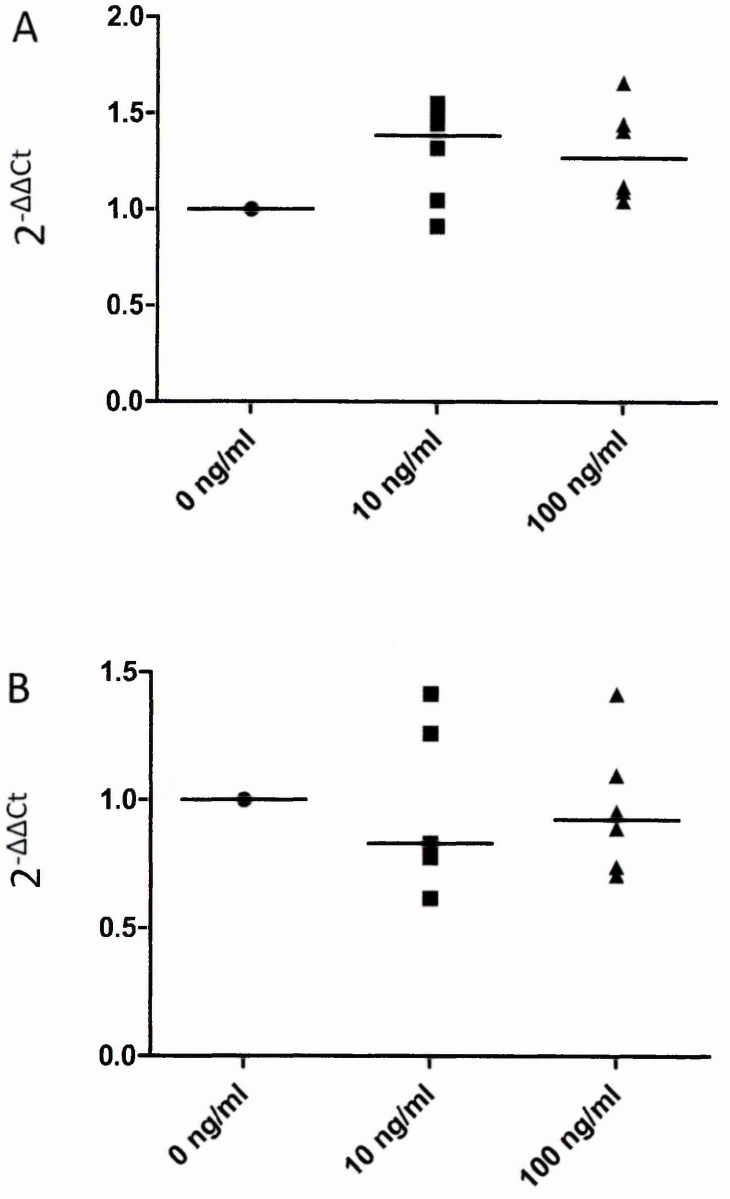


Figure 4.13: Effects of CXCL12 on MMP2 mRNA expression in primary endometrial cells

Relative expression of MMP2 in endometrial (A) epithelial cells (Medians: 0 ng/ml = 1.00, 10 ng = 1.38, 100 ng/ml = 1.27) and (B) stromal cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 1.10, 100 ng/ml = 0.92). *n=6* for epithelial and stromal cells.

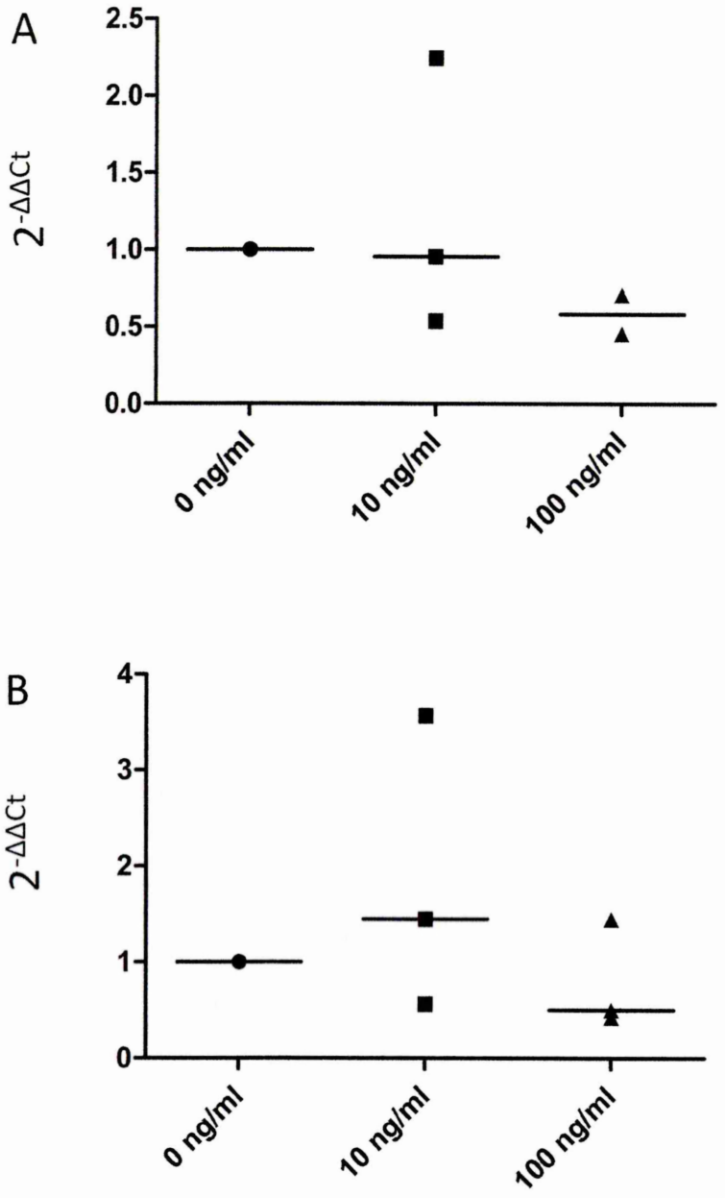


Figure 4.14: Effects of CXCL12 on MMP2 mRNA expression in endometrial cell lines

Relative expression of MMP2 in (A) Ishikawa cells (Medians: 0 ng/ml = 1.00, 10 ng = 0.95, 100 ng/ml = 0.70) and (B) HEC-1-B cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 1.45, 100 ng/ml = 0.5). *n=3* for Ishikawa and HEC-1-B cells.

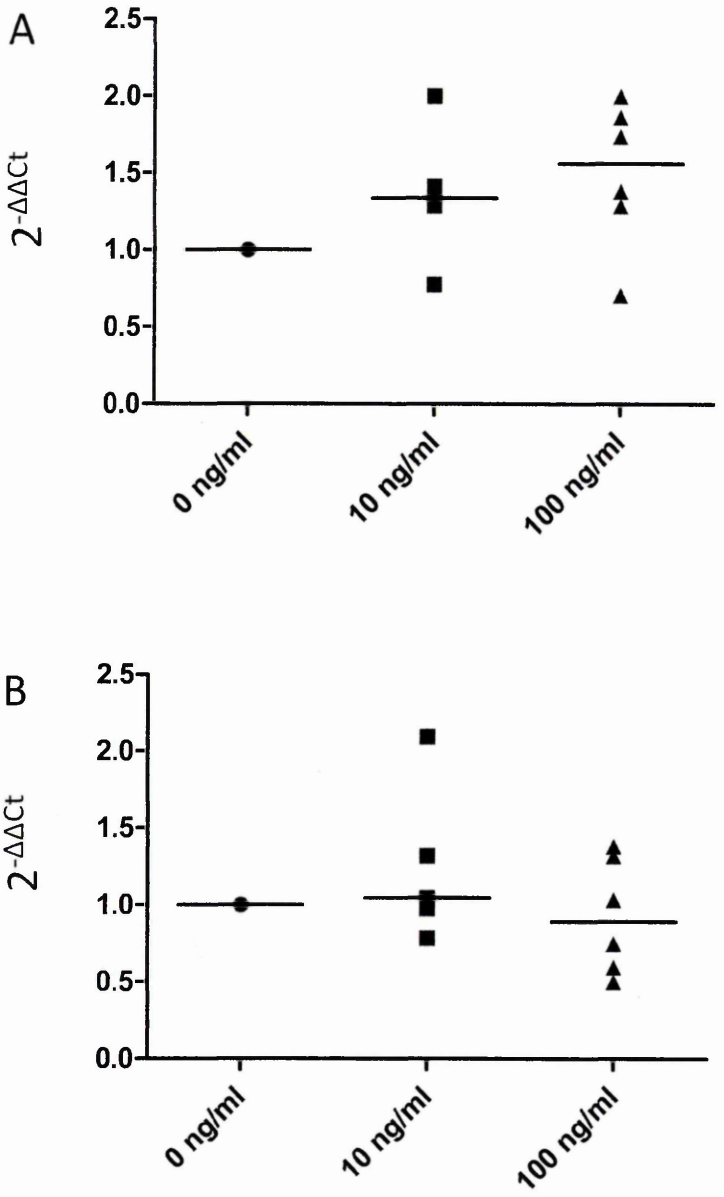


Figure 4.15: Effects of CXCL12 on MMP9 mRNA expression in primary endometrial cells

Relative expression of MMP2 in endometrial (A) epithelial cells (Medians: 0 ng/ml = 1.00, 10 ng = 1.34, 100 ng/ml = 1.56) and (B) stromal cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 1.04, 100 ng/ml = 0.89). *n=6* for epithelial and stromal cells.

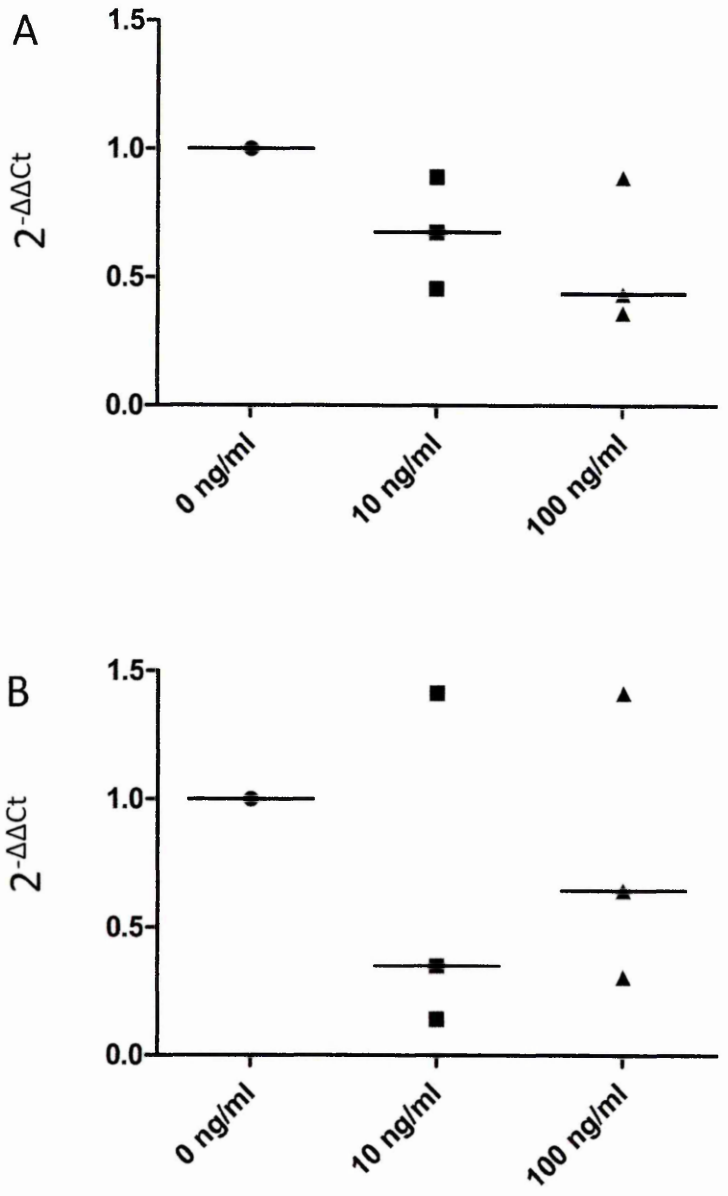


Figure 4.16: Effects of CXCL12 on MMP9 mRNA expression in endometrial cell lines

Relative expression of MMP9 in (A) Ishikawa cells (Medians: 0 ng/ml = 1.00, 10 ng = 0.68, 100 ng/ml = 0.0.44) and (B) HEC-1-B cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.39, 100 ng/ml = 0.64). *n=3* for Ishikawa and HEC-1-B cells.

4.7.5 VEGFA

CXCL12 appeared to cause small dose-dependent increases in VEGFA mRNA in Ishikawa and HEC-1-B cell types. However, levels of VEGFA mRNA in cells incubated with either concentration of CXCL12 were not significantly different to cells in which no CXCL12 was added. CXCL12 at 10 and 100 ng/ml appeared to have no effect on VEGFA mRNA expression in primary epithelial or stromal cell types (Figure 4.17 and 4.18).

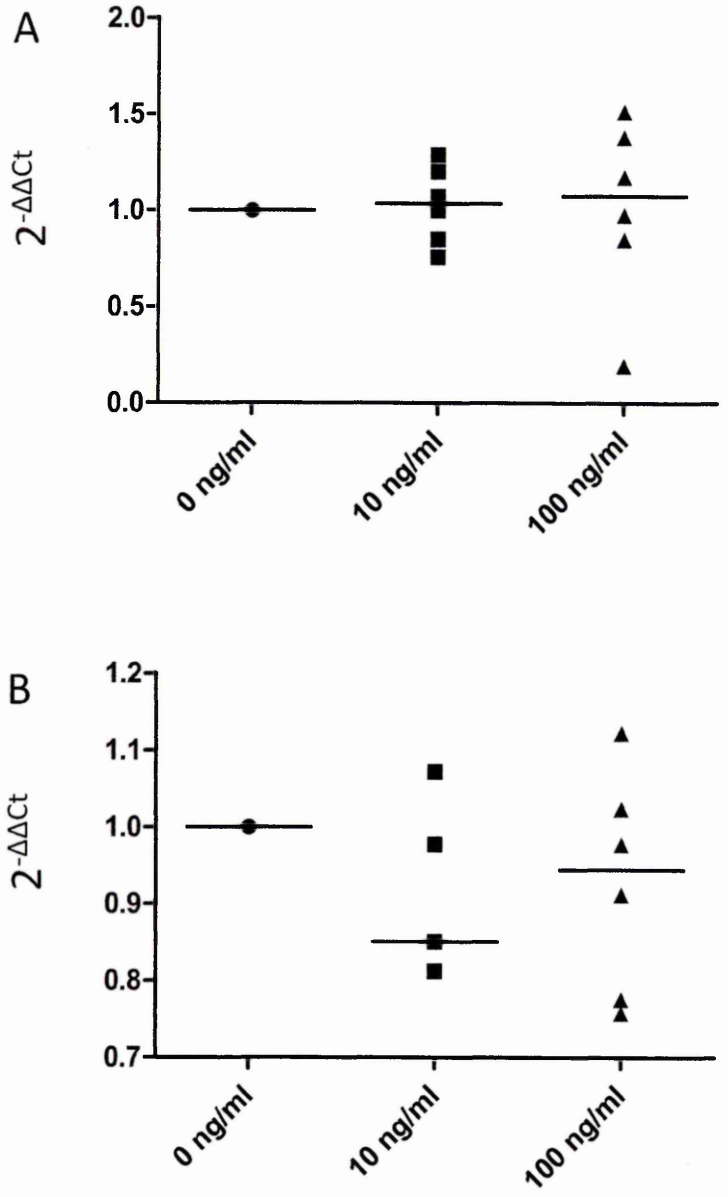


Figure 4.17: Effects of CXCL12 on VEGFA mRNA expression in primary endometrial cells

Relative expression of VEGFA in endometrial (A) epithelial cells (Medians: 0 ng/ml = 1.00, 10 ng = 1.04, 100 ng/ml = 1.07) and (B) stromal cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.85, 100 ng/ml = 0.94). *n=6* for epithelial and stromal cells.

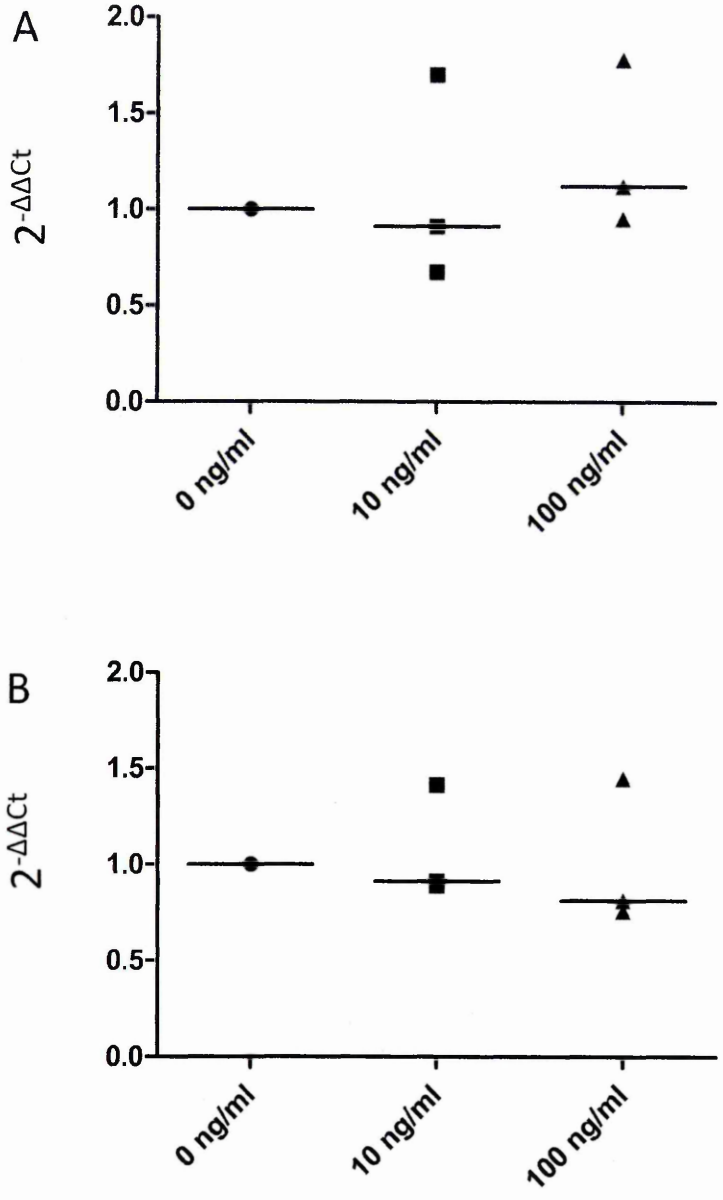


Figure 4.18: Effects of CXCL12 on VEGFA mRNA expression in endometrial cell lines

Relative expression of VEGFA in (A) Ishikawa cells (Medians: 0 ng/ml = 1.00, 10 ng = 0.91, 100 ng/ml = 1.12) and (B) HEC-1-B cells (Medians: 0 ng/ml = 1.00, 10 ng/ml = 0.91, 100 ng/ml = 0.81). *n=3* for Ishikawa and HEC-1-B cells.

4.8 Discussion

4.8.1 Basal expression of IL6, IL8, MMP2, MMP9 and VEGFA in endometrial cell cultures

Real-time RT-PCR was used to identify and quantify IL6, IL8, MMP2, MMP9 and VEGFA mRNA expression in four endometrial cell types. Specific PCR products for each of these gene transcripts, was observed in primary epithelial and stromal cells and also in the Ishikawa and HEC-1-B adenocarcinoma cell lines. Differential expression of the transcripts of interest was seen in the cell types studied.

The most striking difference between the endometrial cell types was the apparent low expression of the majority of the transcripts in the cell lines in comparison to their primary epithelial cell counterparts. The expression of IL6, IL8, MMP2 and MMP9 was many orders of magnitude lower in the cell lines. While cell lines provide a useful and practical *in vitro* tool for the study of endometrial cells, the results obtained here clearly suggest that the Ishikawa and HEC-1-B cell lines express different levels of important endometrial factors compared to primary endometrial epithelial cells. Levels of VEGFA mRNA were more comparable to the primary epithelial cells, though this high expression could potentially be due to their cancerous nature rather than representing normal endometrial function. It was surprising that MMP2 and MMP9 production was lower in the adenocarcinoma cell lines than primary cells. Cancer cells are usually more invasive than normal cells and part of this invasiveness is due to the production of MMPs.

The expression of IL6 mRNA was found to be higher in the epithelial cells than the stromal cells though this difference was not significant. Greater expression of IL6 mRNA in endometrial epithelial cells compared to stromal cells is in agreement with previous studies of IL6 expression in the endometrium (Laird *et al.*, 1993; Tabibzadeh *et al.*, 1995 and Vandermolen and Gu, 1996). The expression of IL8 was significantly higher in the endometrial epithelial cells than in the stromal cells and expression of IL8 mRNA in both cell types was considerably higher than IL6. This result is in agreement with previous studies that have investigated the distribution of IL8 in the endometrium, which have shown expression of IL8 to be highest in the endometrial epithelium (Arici *et al.*, 1998b and Garcia-Valesco and Arici, 1999). Expression of IL8 in

the endometrial epithelium confirms the importance of IL8 at the endometrial surface and may well relate to its proposed functions in leukocyte recruitment during the menstrual cycle.

MMP2 mRNA was the most abundant transcript of those studied in both epithelial and stromal cells. The apparently higher expression of MMP2 in the stromal cells compared to epithelial cells is consistent with previously reported studies and with its proposed functions in the control of menstruation and may relate to the invasion of trophoblast through the endometrial stroma during implantation (Salamonsen and Woolley, 1996).

In contrast to MMP2, MMP9 mRNA expression was significantly higher in the epithelial cells rather than the stromal cells but was lower in both cells types compared to MMP2. The increased expression of MMP9 mRNA in epithelial cells is in agreement with immunolocalisation studies which have found MMP9 expression to be located within the luminal and glandular epithelium (Jeziorska *et al.*, 1996). These results suggest potentially differing functions of MMP2 and 9 within the endometrium, though it is likely as previous studies have suggested, that these and other MMPs act in a concerted manner in the endometrium to achieve their functions (Salamonsen, 1999 and Salamonsen and Woolley 1999).

Angiogenesis of spiral arteries occurs during every menstrual cycle and is important in endometrial function and VEGF is involved in the control of angiogenesis. This study found VEGFA mRNA expression to be significantly higher in endometrial stromal cells in comparison to epithelial cells. VEGFA mRNA was expressed in substantial quantities in both endometrial epithelial and stromal cells, which is in agreement with Punyadeera *et al* (2006). This suggests that both endometrial stromal and epithelial cells may play direct roles in the production of VEGFA and thus in the control of angiogenesis in the endometrium.

Overall the results have shown considerable differences in the expression of endometrial factors in HEC-1-B and Ishikawa cells lines and primary endometrial epithelial and stromal cells. The results suggest that care should be taken in extrapolating the results seen in cell lines to the *in vivo* situation.

4.8.2 Effect of CXCL12 on expression of IL6, IL8, MMP2, MMP9 and VEGFA mRNA in endometrial cell cultures

This study investigated the effects of CXCL12 on the expression of mRNA for IL6, IL8, MMP2, MMP9 and VEGFA in endometrial Ishikawa, HEC-1-B, primary epithelial and stromal cells *in vitro*. In contrast to studies in non-endometrial cells, CXCL12 did not significantly alter any of the transcripts even at high concentrations (100 ng/ml). When a relatively low concentration (5 ng/ml) of the recombinant CXCL12 chemokine was used with the same methodology for the same incubation time (24 hr) in the Ishikawa cell line, in genome wide microarray experiments, statistically significant and reproducible alterations in gene transcript expression was observed (see chapter 5). This strongly suggests that the recombinant CXCL12 used was functional. This raises the possibility that CXCL12 does not regulate the expression of these genes in the endometrium.

CXCR4 dependent signal transduction has been shown to occur through the MAPK/ERK1/2 and PI3K/Akt pathways leading to the activation of the nuclear-factor kappa B (NF_KB) transcription factors (Chinni *et al.*, 2006; Huang *et al.*, 2009 and Tang *et al.*, 2008). The activation of NF_KB via the MAPK/ERK1/2 and PI3K/Akt signal transduction pathways has been shown to cause the up-regulation of IL6 (He *et al.*, 2008 and Tagashira *et al.*, 2009)), IL8 (Chen *et al.*, 2008), MMP2 (Chang *et al.*, 2007 and Zhang *et al.*, 2010), MMP9 (Van Themsche *et al.*, 2007 and Zhang *et al.*, 2010) and VEGF (Kazi and Koos, 2007) in endometrial cell types. While this suggests that CXCL12 and CXCR4 could potentially cause the up-regulation of the factors studied through these signal pathways in the endometrium, this effect was not observed. This suggests that CXCL12/CXCR4 signaling may occur through alternative pathways in endometrial epithelial and stromal cells.

CXCL12 and CXCR4 are known to affect a wide range of cellular activity in the human. It is implicated in leukocyte recruitment, haematopoiesis, angiogenesis, cellular differentiation and apoptosis (Busillo and Benovic, 2006). All these functions are of particular importance in the endometrium. If CXCL12 and CXCR4 are involved in any or all of these functions in the endometrium it is likely that precise control of both the ligand and the receptor is required to carry out such diverse functions. CXCL12 is

known to be able to oligomerise, interact with glycosaminoglycans in aiding gradient formation and produce differing cellular effects in a concentration dependent manner (Majika *et al.*, 2000 and Wysoczynski *et al.*, 2005). These factors may affect downstream responses upon receptor binding and effect function. The receptor CXCR4 can undergo both transcriptional and translational control which may affect its functional responses. Receptor glycosylation, homodimerisation, heterodimerisation, extracellular tyrosine sulfation and internal phosphorylation patterns of the CXCR4 receptor may all contribute to functional heterogeneity (Babcock *et al.*, 2003; Basmaciogullari *et al.*, 2006; Chabot *et al.*, 2000; Farzan *et al.*, 2002 and Issafras *et al.*, 2002). Understanding these complex interactions within the endometrium may be essential to reveal endometrial function of the chemokine and its receptor.

4.8.3 Limitations

These experiments are subject to the same inherent potential sources of error and variation as the cell culture real-time RT-PCR experiments discussed in 3.4.5. Of particular importance is the potential effects of progesterone withdrawal on the cells in culture to which no steroid hormones was added. A known effect of this is the upregulation of MMP expression in endometrial cells (Lockwood *et al.,* 1998). Our study did identify high levels of MMP2 in the primary epithelial cells in comparison to other factors measured but levels of MMP9 in primary cells was comparable to other factors and cell lines expressed low levels of MMP mRNA in comparison to primary cells even though all cells were exposed to the same culture conditions.

This study could not identify any effects of CXCL12 on the regulation of IL6, IL8, MMP2, MMP9 and VEGFA. It is possible that the inherent variation in real-time RT-PCR and cell culture models used contributed to this by masking clear statistically significant effects of CXCL12 on the factors measured. However, no cell cultures exhibited dramatic changes in mRNA levels in response to CXCL12 (e.g. ± 5 fold change) in any case. Investigating receptor activation of CXCR4 using an antagonist or downstream pathways activated by CXCR4 may have provided more information on what is precisely occurring in the cultures models. It is possible that the factors investigated are critically regulated at the protein level and thus changes in mRNA levels become

less significant to overall cell function. With more time, all these aspects could be investigated.

A further limitation of this study is the separation and isolation of the endometrial epithelial and stromal cells from one another. Studies such as that by Albrecht *et al.* (2003) have shown the potential importance of the cross-talk that may occur between these cell types in which are important in controlling their function and that of the endometrium. Co-culture models of these cell types coupled with *in vitro* steroid hormone treatment may more accurately replicate the function of each cell type *in vivo*. However, the cell culture models used in this study does allow measurement of the potential molecular effects of a single endometrial factor on individual cell types, which is useful for determining the molecular effects of the chemokine ligand CXCL12 and its receptor CXCR4.

4.8.4 Summary

The hypothesis could not be confirmed. Despite evidence that IL6, IL8, MMP2, MMP9 and VEGFA are regulated by CXCL12 in non-endometrial cell types, this may not be the case in endometrial cells. This may be due to the activation of different signaling pathways following activation of CXCR4 by CXCL12 in endometrial cell types.

Chapter 5
Gene expression microarray analysis

5.1 Hypothesis

CXCL12 may regulate the gene expression of factors that remain unknown in endometrial cells.

5.2 Aims

So far, this study has investigated the effects of CXCL12 on factors which are known outside of the endometrium to be upregulated by CXCL12 using a targeted search of the literature. As CXCL12 and CXCR4 may carry out various activities in the endometrium beyond that of leukocyte chemotaxis and other established functions, a global approach will be used to identify any gene expression potentially caused by CXCL12 within the genome of Ishikawa cell lines. The advantage of using this cell type is that the cell line expresses high levels of CXCR4 and no CXCL12 (Tsutsumi *et al.,* 2011) allowing the effects of adding known concentrations of CXCL12 to be quantified. This will be achieved using two-colour gene expression microarray analysis of the entire known expressed human genome.

5.3 Methods

Ishikawa cells were incubated with and without CXCL12 at 5 ng/ml for 24 hours. mRNA was extracted from the cells and its integrity checked as described in section 2.4. The mRNA extracted from the cells incuabted without CXCL12 (control) were labeled with Cy5 while that from the cells incubated with CXCL12 was labeled with Cy3. The hybridisation of the mRNA to the microarray and subsequent analysis of the data provided was carried out as described in section 2.9. The mRNA extracted from Ishikawa cells incubated with CXCL12 on four different occasions was analysed in this way.

5.4 Microarray results

For each individual whole human genome block on the Agilent™ Ge45 4x44K microarray (*n=4*), data was extracted for each feature according to the protocol to produce individual spreadsheets containing raw data, normalisation factors, statistical analysis and the log ratio ($base_{10}$) of the processed signal from the Cy3 and Cy5 channels. The log ratio of the processed signals gives the fold change of gene expression for each feature on the array and indicates the effects of exogenous CXCL12 (5 ng/ml for 24 hours) on Ishikawa cells. It is calculated as follows:

$$\text{Log Ratio} = \text{Log}_{10}(\text{Cy3 processed signal} / \text{Cy5 processed signal})$$

5.4.1 Microarray quality control reports

Agilent™ Quality control (QC) reports were generated for each microarray block. These reports show the feature extraction protocol used to generate array data, the normalisation factors applied and the number of differentially expressed features on the array as well as their spatial distribution. Figure 5.1 shows an example of spot finding by the feature extraction software which is included in the Agilent™ QC reports demonstrating successful spot finding by the feature extraction software. Positive control probes for the Agilent™ spike-ins allow the user to confirm the success of the experiment and that feature extraction software has successfully found the features on the array. The distinct probe pattern on each corner of each array ensures the correct orientation of the slide. Spot finding was successful in all cases and exhibited the correct pattern in the correct orientation.

Figure 5.2 shows MA plots of each array block, which plots the log ratio of a microarray feature (M) on the *Y* axis and the average log processed signal intensity (A) on the *X* axis. This allows visualisation of differentially expressed features on the arrays for each experimental repeat and the spread of the normalised data. As expected, the number of up-regulated and down-regulated features detected by this method was only a small proportion of the total on the array. Figure 5.3 shows the spatial distribution of differentially expressed features on each array block representing each experimental repeat. There was a greater number of up-regulated features compared to down regulated.

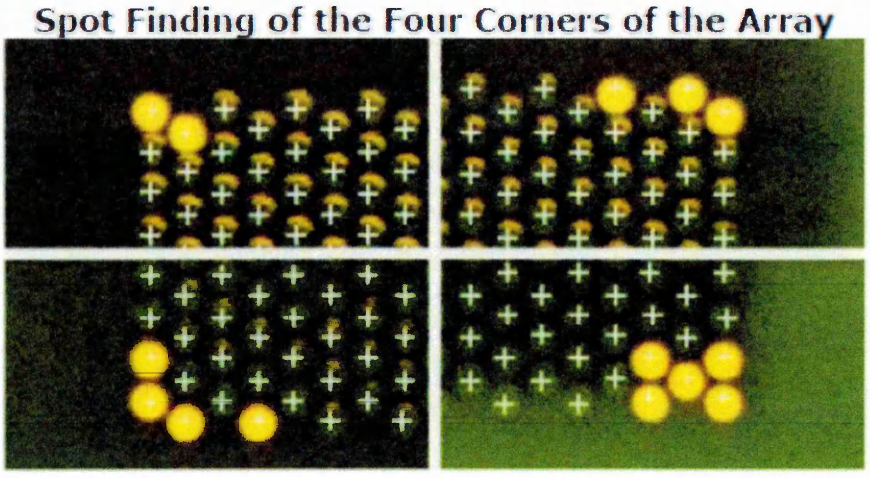


Figure 5.1: Spot finding of microarray features

The Agilent™ feature extraction process produces quality control reports for each microarray (*n=4*). The above example is from block 1 of 4 on the array slide.

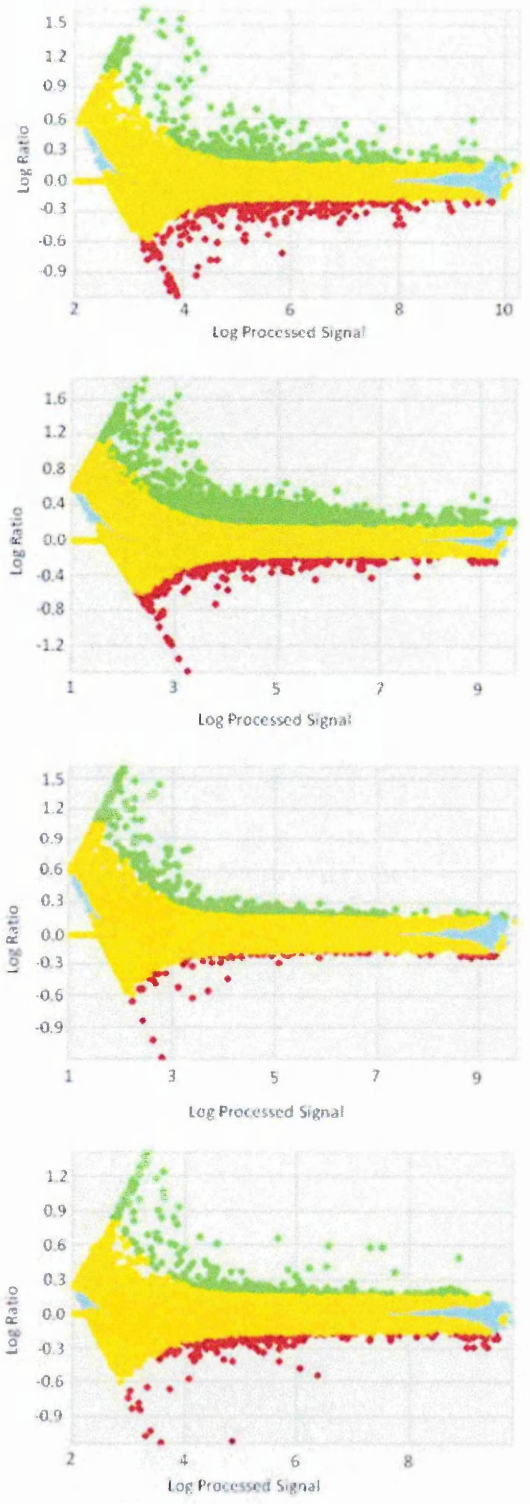
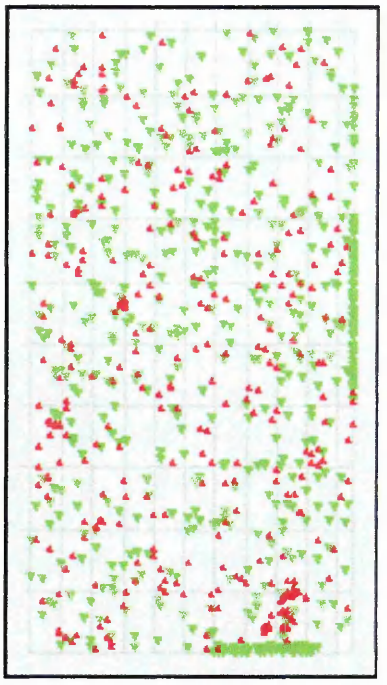


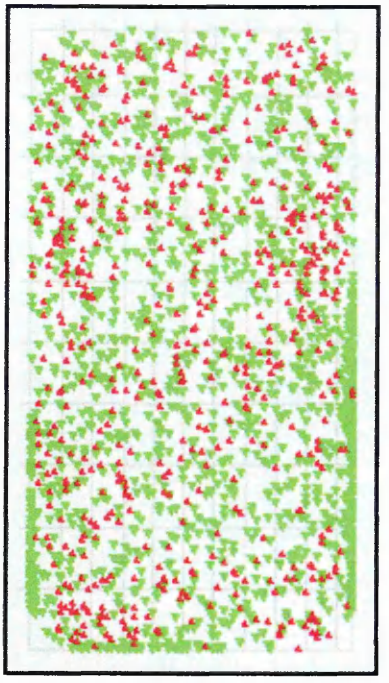
Figure 5.2: MA plots of processed feature extracted microarray data

Agilent™ QC reports produce MA plots as a convenient way to view processed data. A MA plot was generated for each array repeat (repeat 1 at the top and 4 at the bottom).

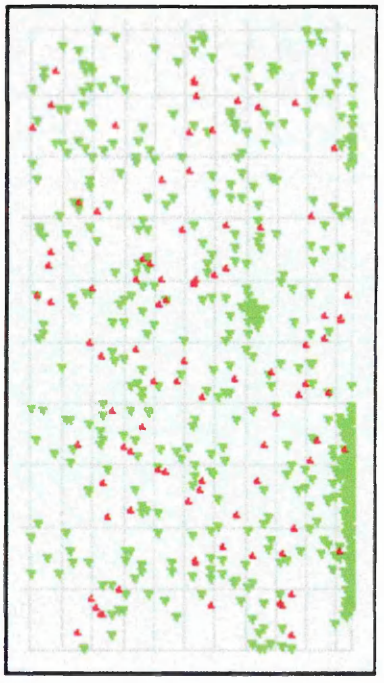
Significantly up-regulated features are highlighted in green, significantly down-regulated features are highlighted in red. Features which show no differential expression are shown in yellow, while features used to normalise the data are blue.



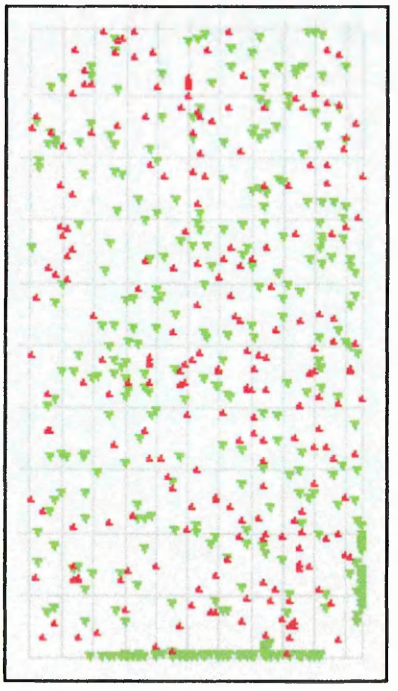
A) Block 1, # up-regulated features 577, # of down-regulated features 339.



B) Block 2, # up-regulated features 1539, # of down-regulated features 587.



C) Block 3, # up-regulated features 549, # of down -regulated features 95.



D) Block 4, # up-regulated features 357, # of down-regulated features 230.

Figure 5.3: Spatial distribution of differentially expressed microarray features

The Agilent™ QC reports produce diagrams of the spatial distribution of significantly up- and down-regulated features on each array repeat in all four array repeats.

5.4.2 Comparing microarray data from experimental repeats

Each feature on each array is subject to potential inherent error from RNA labeling bias, poor or cross-hybridisation, high background signal and compromised spot finding by feature extraction software. This results in application of high normalisation factors leading to the generation of log ratios which are biologically inaccurate. Boolean flags in the data spreadsheets highlight features where this is likely to have occurred allowing the user to ignore them from further analysis. Also, features where experimental error has had an effect will show high *P* values, which in the most extreme cases are = *1* indicating that no confidence can be applied to log ratio value.

To ensure accurate and manageable data was generated when comparing experimental repeats a number of steps were taken. For each experimental repeat, genes which showed <5-fold change in expression were ignored. If a feature on any block showed a fold change greater than this it was compared to its corresponding repeats on the other three arrays. If a >5-fold change was not seen in any of the other experimental repeats it was excluded from further analysis. If a >5-fold change for any feature was seen on 2 or more arrays, all four corresponding features were directly compared to assess the quality of the data generated in all cases and to investigate significance. To eliminate the chance of false positives occurring in the data a stringent *P* value of $1x10^{-06}$ or less was used to attribute a significant result as the number of features expected to give a false-positive result on an 44K array when using this *P* value was 0.04% giving a high level of stringency to the data included.

Tables 5.1 and 5.2 list the gene transcripts that were up-regulated >5-fold and occurred on two or more of the experimental repeats with the required stringent *P* values necessary to ensure significance. The tables also includes log ratios and *P* values for counterparts in experiments where no significant change was seen that confirm that in each case, these features were likely to have been affected by inherent experimental errors and not conflicting biological data.

Table 5.1 includes known genes and cDNA clones with full Coding Sequence (CDS) information. 37 known genes or cDNA clones with fully identified Coding Sequences (CDS) were shown to be significantly >5-fold up-regulated in 2 or more experimental repeats from separate cell cultures (*n=4*). In no cases were genes significantly up-

regulated in all experimental repeats and was up-regulated in 3 out of 4 repeats in 3 cases (U50277, SNX15 and PSCD4). However, in all 37 cases, features which did not show a log ratio of >0.5 did not exhibit a significant result and in many cases a *P* value of 1.00 was attributed indicating that there was no confidence in the value of the log ratio. This indicates that feature was subject to inherent experimental errors and was therefore attributed a Log Ratio of 0 by the feature extraction software. In most of the remaining cases, features had high error values and the *P* values generated were many orders of magnitude away from the required $1x10^{-06}$ value required to minimise possible false positive results also indicating that the feature was likely to be affected by inherent errors in probe hybridisation or high background signal. In exceptional cases where *P* values were closer to the accepted value, the log ratio was in good general agreement with the significant values in corresponding experimental repeats. The gene ETS1 on chromosome 11 is a good example of these principles as block 1 has a Log Ratio of 0.00 and a *P* value of 1.00 indicating a feature subjected to poor hybridisation or high background fluorescence. Block 2 has a significant log ratio of 0.78 indicating a 7.8 fold increase ($P= 5x10^{-11}$), block 3 has a significant log ratio of 0.80 indicating an 8.0 fold increase ($P= 8x10^{-10}$). While block 4 does not quite exhibit the highly stringent *P* required to confirm significance ($3x10^{-05}$), it is still unlikely to be a false-positive result and is in good general agreement with the log ratios of the two significant results. There are no exceptions to these principles in any of the genes identified.

Table 5.2 includes cDNA clones with partial CDS information and completely unknown genes. 14 genes with cDNA clone partial CDS sequences or that are completely unknown were shown to be significantly >5-fold up-regulated in 2 or more experimental repeats from separate cell culture treatments (*n=4*). In no cases were genes significantly up-regulated in all experimental repeats and were up-regulated in 3 out of 4 repeats in 2 cases (THC2385437 and A_32_P147500). There are no conflicting results between repeats using the principles described for Table 8.1 in any case.

When this procedure was carried out for down-regulated genes, no genes were found to be significantly down-regulated by 5-fold or below in two or more repeats so further analysis concentrated on the up-regulated genes identified.

Chromosome	Gene Name	Systematic Name	Log ratio	Log ratio error	*P* Value
1	ADORA3	NM_020683	0.00	0.82	1.00
			1.20	0.16	$3x10^{-14}$
			1.48	0.21	$1x10^{-12}$
			0.97	0.25	$1x10^{-04}$
1	CR600872	CR600872	0.73	0.10	$1x10^{-13}$
			-0.11	0.08	0.17
			0.78	0.09	0.38
			0.68	0.12	$2x10^{-11}$
2	FLJ38377	NM_152698	-0.008	0.44	0.99
			0.65	0.11	$1x10^{-09}$
			0.67	0.12	$1x10^{-08}$
			0.69	0.17	$1x10^{-05}$
2	ARHGEF4	NM_015320	0.98	0.15	$1x10^{-10}$
			-0.03	0.22	0.90
			0.16	0.22	0.49
			0.65	0.14	$5x10^{-06}$
2	ENST00000244221	ENST00000244221	0.28	0.32	0.38
			0.67	0.11	$1x10^{-09}$
			-0.2	0.35	0.56
			0.61	0.14	$6x10^{-06}$
3	DOC1	NM_182909	0.00	0.81	1.00
			1.54	0.27	$9x10^{-09}$
			1.28	0.22	$8x10^{-09}$
			0.00	0.65	1.00
3	GHRL	NM_016362	0.00	0.51	1.00
			0.73	0.11	$4x10^{-11}$
			0.65	0.11	$7x10^{-09}$
			0.66	0.15	$1x10^{-05}$
3	LRRC2	NM_024512	0.70	0.31	0.02
			0.68	0.11	$1x10^{-10}$
			-0.31	0.29	0.27
			0.60	0.13	$1x10^{-06}$
3	CACNA2D3	NM_018398	0.00	0.81	1.00
			1.28	0.18	$4x10^{-13}$
			1.56	0.24	$3x10^{-11}$
			1.01	0.25	$3x10^{-05}$
4	MGC27016	NM_144979	1.32	0.29	$7x10^{-06}$
			1.46	0.21	$1x10^{-12}$
			0.00	0.82	1.00
			0.25	0.64	0.69
4	AF130075	AF130075	0.00	0.81	1.00
			1.49	0.18	$1x10^{-16}$
			1.49	0.25	$1x10^{-09}$
			0.85	0.65	$4x10^{-03}$
6	OFCC1	AF520801	1.56	0.18	$1x10^{-18}$
			0.00	0.84	1.00
			0.82	0.62	0.19
			1.42	0.19	$1x10^{-14}$

Chromosome	Gene Name	Systematic Name	Log ratio	Log ratio error	*P* Value
7	U50277	U50277	0.00	0.81	1.00
			1.59	0.23	$4x10^{-12}$
			1.43	0.26	$4x10^{-08}$
			1.13	0.22	$1x10^{-07}$
7	DPP6	NM_130797	0.00	0.81	1.00
			1.31	0.17	$1x10^{-14}$
			1.34	0.29	$2x10^{-06}$
			1.02	0.24	$2x10^{-05}$
8	TRHR	NM_003301	0.00	0.81	1.00
			1.02	0.21	$1x10^{-06}$
			1.38	0.24	$7x10^{-09}$
			0.00	0.65	1.00
8	AF426412	AF426412	0.00	0.81	1.00
			1.23	0.22	$3x10^{-08}$
			1.49	0.25	$1x10^{-09}$
			0.00	0.65	1.00
8	LY6K	NM_017527	1.46	0.17	$2x10^{-17}$
			0.00	0.84	1.00
			0.53	0.77	0.49
			1.29	0.20	$4x10^{-11}$
8	DEFA6	NM_001926	0.00	0.81	1.00
			0.82	0.14	$1x10^{-08}$
			1.10	0.20	$5x10^{-08}$
			0.00	0.65	1.00
10	COL13A1	NM_005203	1.08	0.13	$2x10^{-16}$
			-0.44	0.27	0.10
			-0.05	0.25	0.8
			0.94	0.13	$2x10^{-13}$
10	KCNMA1	NM_002247	0.38	0.17	0.02
			0.62	0.09	$8x10^{-11}$
			0.12	0.13	0.37
			0.78	0.12	$3x10^{-10}$
10	SH2D4B	NM_207372	0.84	0.56	0.13
			1.33	0.12	$1x10^{-12}$
			0.00	0.83	1.00
			1.13	0.22	$4x10^{-07}$
11	ETS1	NM_005238	0.00	0.55	1.00
			0.78	0.12	$5x10^{-11}$
			0.80	0.13	$8x10^{-10}$
			0.90	0.22	$3x10^{-05}$
11	FAM111A	NM_022074	0.38	0.22	0.09
			0.63	0.10	$1x10^{-10}$
			-0.23	0.23	0.32
			0.86	0.13	$9x10^{-11}$
11	SNX15	NM_147777	-0.06	0.42	0.90
			0.73	0.11	$1x10^{-11}$
			0.83	0.11	$1x10^{-13}$
			0.71	0.15	$5x10^{-06}$

Chromosome	Gene Name	Systematic Name	Log Ratio	Log Ratio Error	*P* value
11	SLCO2B1	NM_007256	1.64	0.23	$6x10^{-13}$
			0.70	0.78	0.37
			0.68	0.75	0.36
			1.07	0.23	$5x10^{-06}$
13	TNAP	AF463496	0.00	0.81	1.00
			1.11	0.20	$2x10^{-08}$
			0.94	0.19	$9x10^{-07}$
			0.75	0.34	0.03
13	AF305819	AF305819	1.64	0.23	$1x10^{-12}$
			0.00	0.84	1.00
			0.72	0.72	0.32
			1.12	0.22	$3x10^{-07}$
15	MAP1A	NM_002373	0.59	0.26	0.02
			0.83	0.12	$1x10^{-12}$
			-0.19	0.37	0.62
			1.20	0.17	$7x10^{-13}$
17	KRTAP3-3	NM_033185	1.43	0.20	$3x10^{-13}$
			-0.11	0.39	0.79
			0.00	0.74	1.00
			1.09	0.23	$1x10^{-06}$
17	AF090926	AF090926	0.00	0.77	1.00
			1.14	0.15	$1x10^{-13}$
			0.75	0.13	$5x10^{-09}$
			0.98	0.25	$9x10^{-05}$
17	PITPNM3	NM_031220	0.00	0.81	1.00
			1.41	0.20	$3x10^{-13}$
			0.00	0.83	1.00
			1.08	0.23	$3x10^{-06}$
17	KIF19	NM_153209	0.00	0.81	1.00
			0.98	0.21	$1x10^{-06}$
			1.31	0.29	$6x10^{-06}$
			0.00	0.65	1.00
17	CV390614	CV390614	0.00	0.81	1.00
			1.52	0.20	$1x10^{-14}$
			1.58	0.23	$8x10^{-12}$
			0.00	0.65	1.00
19	EBI3	NM_005755	0.00	0.81	1.00
			1.14	0.16	$4x10^{-13}$
			1.61	0.23	$4x10^{-12}$
			0.00	0.65	1.00
20	BIRC7	NM_022161	0.00	0.81	1.00
			1.21	0.20	$7x10^{-10}$
			1.21	0.20	$3x10^{-09}$
			0.00	0.65	1.00
21	BX414807	BX414807	1.19	0.15	$2x10^{-15}$
			0.49	0.15	0.001
			0.26	0.27	0.34
			1.02	0.15	$2x10^{-12}$

Chromosome	Gene Name	Systematic Name	Log ratio	Log ratio error	*P* Value
22	PSCD4	NM_012285	1.54	0.20	$5x10^{-15}$
			1.13	0.15	$1x10^{-14}$
			0.00	0.83	1.00
			1.39	0.19	$1x10^{-13}$

Table 5.1: Known genes up-regulated by addition of CXCL12 (5 ng/ml for 24 hours) in the Ishikawa cell line

The genes are ordered in the table by chromosome location followed by the gene name and systematic name. The four log ratio values, error and *P* values for each gene listed are ordered by the array block number (1-4). The log ratio shows the fold change of the experimental sample versus the control and is in $base_{10}$, meaning genes with a log ratio of 0.5 have a 5 fold increase (light green) and genes with a Log Ratio of 1.0 have a 10 fold increase (dark green). Significant values are highlighted in yellow.

chromosome	Gene name	Systematic name	Log ratio	Log ratio Error	P value
1	AB015616	AB015616	0.00	0.81	1.00
			1.52	0.27	$2x10^{-08}$
			1.45	0.26	$1x10^{-08}$
			0.00	0.65	1.00
1	A_23_P136392	A_23_P136392	0.71	0.11	$2x10^{-11}$
			-0.06	0.09	0.52
			0.10	0.10	0.32
			0.56	0.11	$1x10^{-07}$
2	AK055428	AK055428	0.00	0.81	1.00
			1.42	0.21	$2x10^{-11}$
			1.54	0.24	$6x10^{-11}$
			0.00	0.65	1.00
3	THC2272949	THC2272949	1.22	0.14	$2x10^{-17}$
			0.04	0.26	0.87
			0.82	0.14	$1x10^{-09}$
			1.08	0.15	$1x10^{-12}$
4	THC2385437	THC2385437	0.00	0.82	1.00
			1.35	0.16	$1x10^{-16}$
			1.44	0.17	$1x10^{-17}$
			1.16	0.21	$4x10^{-08}$
5	ENST00000381528	ENST00000381528	0.00	0.78	1.00
			0.70	0.14	$6x10^{-07}$
			0.93	0.19	$5x10^{-07}$
			1.36	0.18	0.46
5	AK025252	AK025252	0.00	0.81	1.00
			1.75	0.22	$6x10^{-15}$
			1.51	0.24	$4x10^{-10}$
			0.00	0.65	1.00
6	A_24_P648813	A_24_P648813	0.00	0.81	1.00
			1.41	0.30	$3x10^{-06}$
			1.53	0.24	$1x10^{-10}$
			0.00	0.65	1.00
7	A_32_P147500	A_32_P147500	0.00	0.81	1.00
			1.11	0.13	$1x10^{-17}$
			1.20	0.23	$9x10^{-08}$
			1.18	0.21	$1x10^{-08}$
7	THC2350949	THC2350949	1.36	0.16	$1x10^{-17}$
			-0.18	0.36	0.61
			0.64	0.24	$6x10^{-03}$
			1.25	0.16	$2x10^{-15}$
11	THC2349421	THC2349421	1.33	0.16	$2x10^{-17}$
			0.000	0.74	1.00
			0.77	0.25	$1x10^{-03}$
			1.33	0.18	$4x10^{-13}$
12	A_24_P943657	A_24_P943657	0.86	0.55	0.12
			1.33	0.18	$2x10^{-13}$
			0.00	0.83	1.00
			1.23	0.20	$1x10^{-09}$

				Error	
14	A_32_P63630	A_32_P63630	0.00	0.81	1.00
			1.50	0.28	$6x10^{-08}$
			1.39	0.27	$3x10^{-07}$
			0.00	0.65	1.00
17	THC2315277	THC2315277	0.00	0.50	1.00
			0.82	0.11	$8x10^{-14}$
			0.58	0.14	$5x10^{-05}$
			0.86	0.15	$4x10^{-09}$

Table 5.2: Unknown genes up-regulated by addition of CXCL12 (5 ng/ml for 24 hours) in the Ishikawa cell line

The genes are ordered in the table by chromosome location followed by the gene name and systematic name. The four log ratio, error and *P* values for each gene listed are ordered by the array block number (1-4). The log ratio shows the fold change of the treated sample versus the control and is in $base_{10}$, meaning genes with a log ratio of 0.5 have a 5 fold increase (light green) and genes with a Log Ratio of 1.0 have a 10 fold increase (dark green). Significant values are highlighted in yellow.

5.5 Attributing gene function to up-regulated genes

Internet based searches using the EntrezGene and Swiss-Prot databases (via: www.genecards.org) were carried out to try and attribute functions to known gene transcripts that were shown to be up-regulated by addition of CXCL12 to Ishikawa cells and to identify the cellular location of the proteins they encode. EntrezGene uses RefSeq genomes while Swiss-Prot database is provided by the Swiss Institute of Bioinformatics. Table 5.3 summarises the information obtained. Genes for which there was no significant functional information available were ignored. The database search revealed that CXCL12 may up-regulate G-protein coupled receptors and peripheral membrane proteins; a secreted chemokine, hormones and a defensin; calcium and potassium dependent ion channels, anti-apoptotic protein or cell cycle regulators, protein sorting regulators, secondary messengers and a transcription factor. The localisation of the products of these genes in the cell is summarised in Figure 5.4. It shows that the products of identified genes were a) transmembrane proteins which span the cell membrane (receptors (blue), ion channels (red) and structural proteins brown), b) membrane associated signalling proteins (purple), c) cytoplasmic proteins (blue), d) proteins that shuttle between the cytoplasm and the nucleus (yellow) and secreted proteins (green).

Gene name, (accession number), description and ***cellular location***	**Mean fold-change** and (standard deviation)	Possible function
ADORA3 (NM_020683) Homo sapiens adenosine A3 receptor, transcript variant 1. G-protein coupled multipass ***cell membrane*** protein.	**13.40** (1.98)	Protein belongs to a family of adenosine receptors involved in a variety of intracellular signalling pathways, may mediate cell proliferation and cell death (EntrezGene). Possible role in reproduction (Swiss-Prot).
ARHGEF4 (NM_015320) Homo sapiens Rho guanine nucleotide exchange factor (GEF) 4, transcript variant 1. ***Cytoplasmic/peripheral membrane*** protein.	**8.15** (2.33)	Acts as a GEF for RhoA and RAC1 GTPases. May bind APC. The APC-ARGEF4 may be involved in cell migration as well as in E-cadherin mediated cell-cell adhesion (EntrezGene).
DOC1/CDK2AP1 (NM_182909) Homo sapiens downregulated in ovarian cancer 1. Specific CDK2-associated protein in the ***cytosol***.	**14.10** (1.84)	Thought to negatively regulate CDK2 by sequestering monomeric CDK2 and targeting for proteolysis. Also found to interact with DNA polymerase alpha and suggests a role in DNA replication during the S phase of the cell cycle (EntrezGene).
GHRL (NM_016362) Homo sapiens ghrelin/obestatin preprohormone. ***Secreted***.	**6.90** (0.57)	Generates ghrelin and obestatin. Ghrelin is the ligand for growth hormone secretagogue receptor type 1 (GHSR) which induces release of growth hormone from the pituitary. May play a role in appetite stimulation, induce adiposity and stimulate gastric acid secretion. Obestatin may be the ligand for GPR39, may reduce appetite (EntrezGene).
CACNA2D3 (NM_018398). Homo sapiens calcium channel, voltage-dependent, alpha 2/delta 3 subunit. ***Cell membrane***.	**14.20** (1.98)	A member of the alpha-2/delta subunit, a protein in the voltage-dependent calcium channel complex which mediates the influx of calcium ions into cells.
DPP6 (NM_130797) Homo sapiens dipeptidyl-peptidase 6, transcript variant 1. Single-pass ***cell membrane*** protein.	**13.25** (0.21)	A single pass type II membrane protein which is a member of serine protease family. Has no detectable protease activity due to the absence of a conserved serine residue normally found in the catalytic domain of serine proteases (EntrezGene). May modulate cell surface expression and activity of the potassium channel KCND2 (Swiss-Prot).

TRHR (NM_003301) Homo sapiens thyrotropin-releasing hormone receptor. G-protein coupled receptor. ***Cell membrane protein.***	**12.00** (2.55)	Activates the inositol phospholipid-calcium-protein kinase C transduction pathway upon the binding thyrotropin-releasing hormone (TRH) which is a neuropeptide synthesised in the hypothalamus (EntrezGene).
LY6K (NM_017527) Homo sapiens lymphocyte antigen 6 complex, locus K. ***Secreted*** from the cytoplasm and cell membrane.	**13.75** (1.20)	May play a role in cell growth (Swiss-Prot).
DEFA6 (NM_001926) Homo sapiens defensin, alpha 6. ***Secreted.***	**9.60** (1.98)	Defensins are microbicidal and cytotoxic peptides thought to be involved in host defense. They are abundant in granules of the neutrophils and the epithelia of mucosal surfaces (EntrezGene).
COL13A1 (NM_005203) Homo sapiens collagen, type XIII, alpha 1, transcript variant 1. Single-pass type II ***cell*** **membrane** protein.	**10.10** (0.99)	The alpha chain of one of the nonfibrillar collagens (EntrezGene). Involved in cell-matrix and cell-cell adhesion interactions and binds heparin (Swiss-Prot).
KCNMA1 (NM_002247) Homo sapiens potassium large conductance calcium-activated channel, subfamily M, alpha member 1, transcript variant 2. ***Cell membrane protein.***	**7.00** (1.13)	Potassium channel activated by both membrane depolarisation or increase inn cytosolic calcium that mediates export of potassium, thought to have numerous functions such as smooth muscle control and innate immunity (Swiss-Prot).
ETS1 (NM_005238) Homo sapiens v-ets erythroblastosis virus E26 oncogene homolog 1. Transcption factor. ***Nucleus.***	**7.90** (0.14)	Regulates numerous genes and are involved in stem cell development, cell senescence and death, and tumorigenesis. The conserved ETS domain within these proteins is a winged helix-turn-helix. DNA-binding domain that recognizes the core consensus DNA sequence GGAA/T of target genes (EntrezGene).
SNX15 (NM_147777) Homo sapiens sorting nexin 15 (SNX15), transcript variant B. ***Cytosol.***	**7.57** (0.64)	Structural protein involved in the filamentous cross-bridging between microtubules and other skeletal elements (Swiss-Prot).
MAP1A 9 (NM_002373) Homo sapiens microtubule-associated protein 1A. ***Structural*** protein.	**10.15** (2.62)	Catalyzes the transfer of phosphatidylinositol and phosphatidycholine between membranes (in vitro). Binds calcium ions (SwissProt).

PITPNM3 (NM_031220) Homo sapiens PITPNM family member 3. Peripheral ***cell membrane*** protein.	**12.45** (2.33)	Cytokine with pro- and anti-inflammatory processes that can regulate T-helper development, suppress T-cell proliferation, stimulate cytotoxic T cell activity, drives rapid clonal expansion of naive CD4 T-cells. it binds to the cytokine receptor WSX-1/TCCR. Has antiangiogenic properties by activating antiangiogenic chemokines (Swiss-Prot).
EBI3 (NM_005755) Homo sapiens Epstein-Barr virus induced gene 3. ***Secreted***.	**13.75** (3.32)	Binds caspases (EntrezGene) and supresses apoptosis by activation of MAPK8/JNK1 (Swiss-Prot).
BIRC7 (NM_022161) Homo sapiens baculoviral IAP repeat-containing 7. ***Nucleus*** and ***cytoplasm***.	**12.10** (0.00)	May play a role in apoptosis, expression is modulated by cytokines in natural killer cells (Swiss-Prot).
PDCD4 (NM_013385) Homo sapiens pleckstrin homology, Sec7 and coiled-coil domains 4. **Nucleus** and ***cytoplasm***.	**13.53** (2.07)	Protein is localised to the nucleus in proliferating cells, expression of the gene is modulated by cytokines in natural killer and T cells, may play a role in apoptosis but the specific role is not yet determined (EntrezGene).

Table 5.3: Known functions of up-regulated genes

Table of up-regulated genes for which proposed functions and cellular locations of the proteins they encode were found using the EntrezGene and Swiss-Prot databases (via www.genecards.org). The mean fold changes *n* number is *2* with the exception of SNX15 where *n=3*.

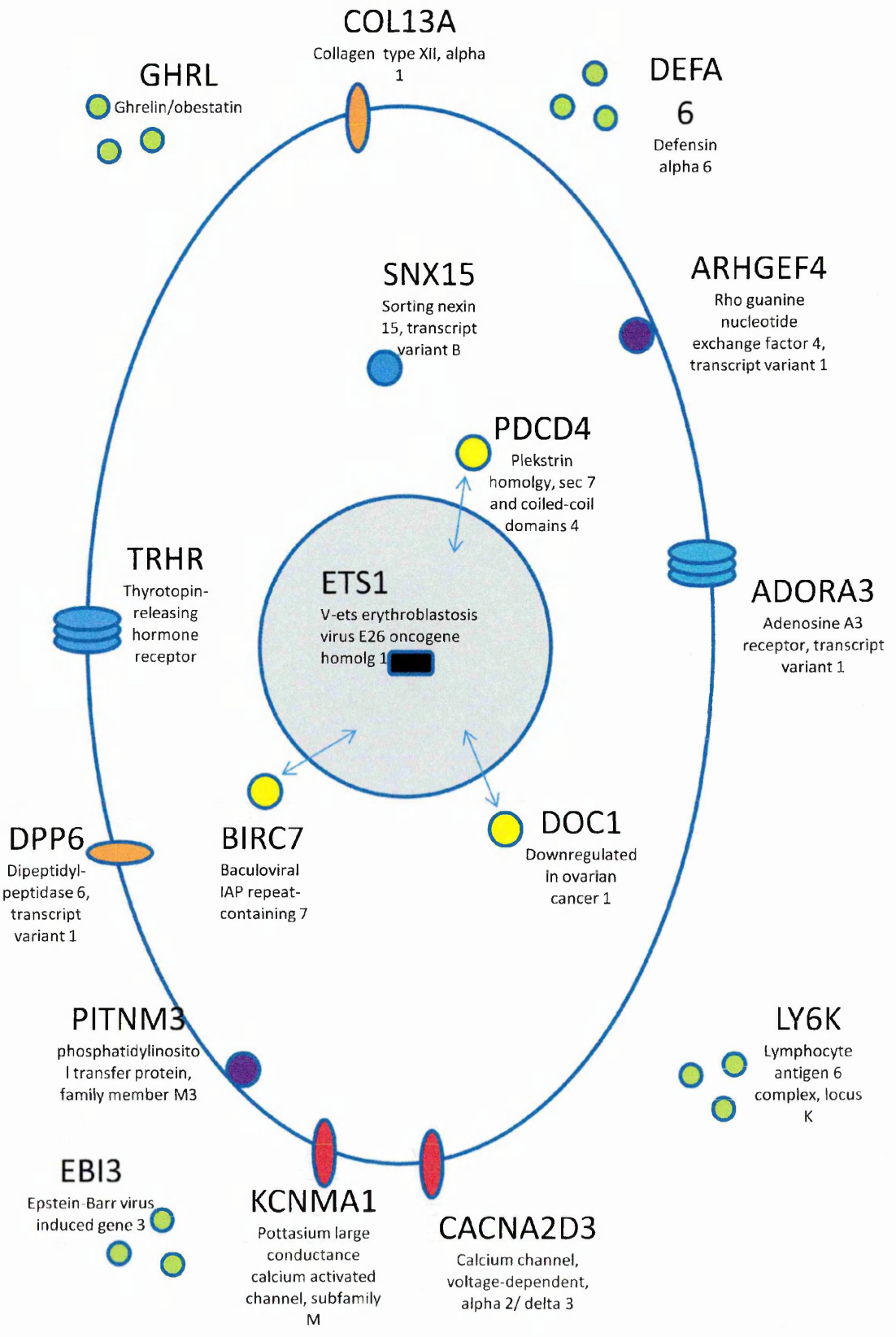


Figure 5.4: Representation of cellular location of up-regulated genes with known function

Diagram showing the probable cellular location of >5 fold up-regulated gene products in Ishikawa cells following addition of CXCL12. Proteins of functionally similar type or cellular location are attributed the same colours.

5.6 Discussion

The aim of performing genome-wide expression profiling in response to the addition of CXCL12 to Ishikawa cells was to identify previously unknown effects of CXCL12 and relate these effects to the possible role of CXCL12/CXCR4 in endometrial function. By using highly stringent *P* values to remove false-positives and comparing experimental repeats to assess potentially inaccurate results, both known and unknown gene transcripts were found to be significantly up-regulated following addition of CXCL12 for at a concentration of 5 ng/ml for 24 hours.

The study identified a total of 51 genes transcripts that were shown to be up-regulated greater than 5 fold in two or more experimental repeats, of which 14 were unknown genes transcripts. There were 37 known genes transcripts identified to be up-regulated by CXCL12 for which further functional information could be potentially identified. Using the stated criteria, CXCL12 was not found to down-regulate the expression of any transcripts in the Ishikawa cell line. Ideally the change in expression of these genes in the presence of CXCL12 should have been confirmed by real-time RT-PCR with specific primers for these genes, but time did not allow these experiments to take place.

A review of the literature has suggested that the known transcripts found to be up-regulated by addition of CXC12 and assigned a general function, several are of potential importance to female reproductive function and endometrial function.

5.6.1 Ghrelin

Ghrelin is a multifunctional hormone with both paracrine and autocrine capacities, that is known to be involved in the control of growth and metabolism. It acts via the growth hormone secretagogue receptor (GHSR) to stimulate growth hormone (GH) from the pituitary gland. Both the ligand and receptor are present in the pituitary and hypothalamus. It is also found in high levels in the stomach and many peripheral tissues including the uterus (Kojima *et al.*, 2001 and Tawadros *et al.*, 2007). Expression of ghrelin and its receptor has been demonstrated in the human endometrium, with both ghrelin and GHSR expression highest in the secretory phase of the cycle and lowest during menses. Ghrelin appears to be confined to the glandular epithelium and

stromal cells it has been suggested that it may be important in the process of decidualisation (Tawadros *et al.*, 2007). The potential importance of ghrelin in the process of decidualisation is further supported by its increase in decidualised endometrium in comparison to non-pregnant endometrium (Tanaka *et al.*, 2003). In mice, ghrelin has been shown to be present in the endometrium and in the embryo at both the morula and more advanced embryonic developmental stages. *In vitro* addition of ghrelin was found to inhibit embryo development, an effect which was abolished following addition of a GHSR antagonist. Ghrelin is thought to act as a modulator of feeding behavior and metabolism in the central nervous system and increased levels of ghrelin were identified in the uterine fluid of fasting mice in comparison to those with free access to food (Kawamura *et al.*, 2003). Although the distribution of ghrelin might suggest an important role in pregnancy, a recent study which investigated ghrelin levels in early failed and successful pregnancies following IVF treatment found it to be of no prognostic value (Vidal *et al.*, 2008). No studies to date have reported the ability of CXCL12 to up-regulate ghrelin in any cell type, but confirmatory studies are needed before conclusions can be made.

5.6.2 Adenosine A3 receptor

There is no existing available evidence that the adenosine A3 receptor (ADORA3) is expressed in the endometrium or that CXCL12 can modulate its expression, but ADORA3 expression has been identified in trophoblast and shown to be upregulated in preeclampsia. Increased expression of ADORA3 has been shown in both normal and preeclamptic villous explants in low oxygen conditions. Addition of an ADORA3 agonist in preeclamptic villous explants was found to increase MMP2 and 9 in a dose-dependent manner in low oxygen conditions (Kim *et al.*, 2008a). This microarray study has identified the presence of ADORA3 mRNA in an endometrial epithelial cell line and demonstrated increased expression in response to CXCL12. As trophoblast expresses both CXCL12 and ADORA3, it is possible that CXCL12 could influence ADORA3 in this tissue as well as the endometrium. The ability of ADORA3 to influence MMP expression is of potential interest because of the importance of MMPs in controlling trophoblast invasion into the receptive endometrium.

5.6.3 Cyclin-dependent kinase 2-associated protein 1

The tumour suppressor gene, cyclin-dependent kinase 2-associated protein 1 (CDK2AP1) is an inhibitor of cyclin-dependent kinase 2 (CDK2) and therefore prevents the G1/S transition of the cell cycle. Disruption of this gene in mice leads to embryonic lethality and suggests a role for CDK2AP1 in the early stages of embryogenesis (Kim *et al.*, 2008b). In the human, CDK2AP1 has been detected in the endometrium where it is largely found in the epithelium and known to be induced by the presence of progesterone (Cheon and Kim, 2010). Endometrial tissue goes through a series of cell proliferations during the menstrual cycle and CDK2AP1 may play a role in this process.

5.6.4 EBI3/interleukin 27

IL27 is a heterodimeric cytokine that is formed by the association of the cytokine subunit p28 with EBI3 which is a glycosylated protein related to the p40 subunit of IL12. Its expression has been shown in first trimester placenta and in syncytiotrophoblast and extravillous trophoblast. It has been shown to have the ability to act on CD4+ T cells, NK cells, macrophages and endothelial cells. It is proposed to function to regulate local immune responses and angiogenesis during human pregnancy (Coulomb-L'Herminé *et al.*, 2007 and Devergne *et al.*, 2001). IL27 is thought to be an important regulator of the T helper cell responses and in a mouse model of asthma has been shown to be involved in attenuating the Th2response (Fujita *et al.*, 2009). CXCL12 through IL27 may therefore have a role in cytokine balance which is thought to be important in pregnancy outcome (Laird *et al.*, 2003).

5.6.5 Signaling and transcription factors

This microarray study identified two factors which are known to be involved in the control of downstream CXCL12/CXCR4 signaling. The mRNA for the transcription factor E26 transformation-specific 1 (Ets1) was found to be upregulated by CXCL12 in the Ishikawa cell line. The Ets family members contain a highly conserved region of 85 amino acids which is termed the Ets domain which binds purine rich core sequences (GGAA/T) in promoter and enhancer regions of target genes. Known Ets target genes include cytokines such as granulocyte-macrophage-colony-stimulating factor (GM-CSF), interleukin 12 (IL12) and IL1; apoptosis related genes and many matrix

metalloproteinases (MMPs). Experiments in mice initially suggested the Ets genes were limited to controlling tissues undergoing morphogenesis and tissue remodeling but more widespread expression has since been identified (Kilpatrick *et al.*, 1999).

In the human endometrium, it has been suggested that Ets1 expression plays a critical role in the control of decidualisation where it has been shown that inhibition of Ets1 caused down-regulation of mRNA for specific decidualisation markers such as prolactin and insulin growth factor binding protein 1 (IGFBP-1) (Kessler *et al.*, 2006). Ets1 and 2 has been identified in endometrial glandular and luminal epithelium and expression has been shown to be low during menses and higher during the late secretory phase of the menstrual cycle at the time of decidualisation (Kilpatrick *et al.*, 1999). The mRNA for Ets1 has also been identified in the endothelial cells of villous and extravillous trophoblast where the expression has been linked to the control of angiogenesis (Luton *et al.*, 1997) which is one of the known functional effects of Ets outside of the endometrium (Leliévre *et al.*, 2001). There is evidence in the developing spinal cord that CXCL12/CXCR4 causes rapid activation of extracellular signal-related kinase 1/2 (ERK 1/2) and Ets1, which is a substrate for ERK phosphorylation. This demonstrates the possibility of CXCR4/ERK/Ets1 linked signaling though this has not been demonstrated in other tissues (Luo *et al.*, 2005). Alternative phosphorylation on one of two potential sites on Ets1 has been shown to cause activation or inhibition of the transcription factor suggesting one potential means of creating differential regulation of gene expression of MMPs and VEGFs (Dittmer *et al.*, 2003).

Programmed cell death factor 4 (PDCD4) mRNA was also shown to be up-regulated in response to addition of CXCL12 to Ishikawa lines. A study in leukaemic B cells identified PDCD4 as a previously unknown chemokine signaling target where CXCL12 was shown to cause PDCD4 phosphorylation and subsequent inhibition of the transcription factor AP-1 which is a target of the Akt signal pathway (Hayre *et al.*, 2010). This effect remains unstudied in other cell types and combined with the upregulation of PDCD4 mRNA in Ishikawa cells may represent potential CXCL12/CXCR4 signaling routes in the endometrium.

5.6.6 Other factors

There is currently limited evidence for the potential involvement of other factors identified as responding to CXCL12 in terms of potential reproductive and endometrial function. However, expression of calcium-activated potassium channel KCNMA1 has been shown in the segments of the myometrium where it is postulated to play an important role in uterine contractility during pregnancy in humans (Gao *et al.*, 2009). There is no evidence currently available of a role defensin alpha 6 (DEFA6) in the endometrium but it is an antimicrobial peptide which is linked to human host defense (Koslowski *et al.*, 2010). It is conceivable that endometrial epithelium may secrete this protein for this reason. Microtubule associated protein 1A (MAP1A) has been shown to play a role in promoting cell adhesion *in vitro* in cell lines by controlling integrin activity (Gupta *et al.*, 2006). This could therefore be an important factor in the control of integrin mediated attachment of the blastocyst to the luminal epithelium during the process of implantation.

5.6.7 Limitations

A limitation of this microarray study is the use of Ishikawa adenocarcinoma cell lines to study the effects of CXCL12, which may not accurately represent the *in vivo* function of epithelial cells in the endometrium. We initially chose this cell line rather than primary cells because of the ease of carrying out these complex experiments. The intention was to repeat the work on primary cells. However the amount of time needed to analyse the data was much greater than anticipated and time did not permit the repeat of the experiment. The Ishikawa cell line expressed CXCR4 comparable to that in cultured primary epithelial cells and does not express CXCL12, making it useful for this analysis (Tsutsumi *et al.*, 2011). However levels of IL6, IL8, MMP2 and MMP9 expression were lower (see Chapter 4). The up-regulated genes identified in this study can in several cases be related to normal endometrial function where the information is available. Validation of the effects identified at both the mRNA and protein level in primary endometrial cells and endometrial biopsies are needed to confirm the results, but if confirmed this would suggest a role for CXCL12 in endometrial function.

5.6.8 Summary

CXCL12 was shown to affect the mRNA expression of numerous genes in the endometrium. The effects have not previously been identified in the endometrium. Further work is needed to confirm the observations but the study generates several testable hypotheses (Table 5.4) relating to the potential functions of CXCL12 in the endometrium and its regulatory effects

In summary this microarray study has identified both known gene transcripts and unknown gene transcripts that respond to CXCL12 in Ishikawa cells, which in several cases can be related to current understanding of normal endometrial function. The study revealed information on the potential signal pathways through which these effects may occur and also identified possible previously unknown effects of the CXCL12 and its receptor CXCR4 to activate the transcription of a number of factors that has not previously been demonstrated in any tissue type.

CXCL12 source and CXCR4 location	Hypothesis	Potential investigative techniques
Stromal/ epithelial CXCL12 binding epithelial CXCR4	CXCL12 promotes endometrial cell proliferation potentially via lymphocyte antigen 6 complex during the early proliferative phase of the menstrual cycle.	Primary cell culture, real-time RT-PCR immunohistochemistry/ELISA, cell proliferation study.
Stromal/blastocyst CXCL12, epithelial CXCR4	CXCL12 regulates production of ghrelin/obestatin in endometrium during early proliferative and/or mid secretory endometrium.	Primary cell culture, real-time RT-PCR, immunohistochemistry/ELISA.
Stromal/blastocyst CXCL12, epithelial CXCR4	CXCL12 induces production of defensin alpha six as part of the non specific immune response during early proliferative and/or mid secretory endometrium.	Primary cell culture, real-time RT-PCR, immunohistochemistry/ELISA.
Stromal/blastocyst CXCL12, epithelial/stromal CXCR4	CXCL12 alters endometrial gene expression via the ETS1 transcription factor.	Cell culture, signal pathway analysis.
Stromal/blastosyst CXCL12, epithelial/stromal CXCR4	CXCL12 regulates calcium and potassium channel activity in endometrial cells.	Cell culture, real-time RT-PCR, immunohistochemistry.

Table 5.4: Summary of some of the hypotheses generated from microarray experiments

The table summarises potential hypotheses for the functions of CXCL12 in the endometrium generated by the microarray experiments.

Chapter 6
General Discussion

6.1 Introduction

The function of the endometrium is to accept the implanting embryo and to give rise to the maternal side of the placenta, which supports the developing embryo and fetus during pregnancy. Abnormalities in endometrial function result in reproductive failure which manifests itself as infertility and recurrent miscarriage. Therefore an increased understanding of endometrial function is important and in the long term might provide alternative treatments to women who suffer these conditions.

Although the endometrium is under the control of the steroid hormones progesterone and oestradiol, many local factors are important in bringing about the effects of these hormones in the endometrium. CXCL12 and its receptor CXC4 has been implicated in the control of trophoblast cell growth and function. However little is known about its expression and action in the endometrium. The aim of this study was therefore to investigate the potential role of CXCL12 and CXCR4 in human endometrium.

This study adopted two main approaches to investigate further the function of the chemokine ligand CXCL12 and its receptor CXCR4 in the human endometrium. The first was a targeted approach which examined the *in vivo* expression of CXCL12 and CXCR4 through the menstrual cycle coupled with an investigation into the *in vitro* effects of CXCL12 on IL6, IL8, MMP2, MMP9 and VEGFA in human endometrial cells in culture. These factors had previously been established as of importance to endometrial function and CXCL12 had been shown to affect their expression in non-endometrial tissues but had not been previously investigated in the endometrium itself. CXCR7 was not studied because at the time the work was carried out, it was not a known receptor for CXCL12 and signaling still remains controversial (Mahabaleshwa *et al.*, 2008). The precise functions of CXCL12 and CXCR4 remain unknown in the endometrium and evidence in many different tissues suggests a wider role for chemokines and their receptors beyond the chemotatic recruitment of leukocytes to sites of inflammation. To investigate this, the effects of *in vitro* addition of CXCL12 to endometrial cells and genome-wide microarray expression analysis was used to

identify differential expression of gene transcripts in response to CXCL12 to further establish its potential effects in the endometrium.

6.2 Techniques used

This study focused on the measurement of mRNA levels in endometrial tissues and cell types to gain further information on the role of CXCL12 and CXCR4 in the endometrium but complementary protein studies were also carried out in our laboratory (Laird *et al.,* 2011). Measurement of mRNA levels in a cell allows the rapid analysis of transcript expression from just one gene right through to the entire genome of an organism giving mRNA based assays an enormous range of flexibility and power to reveal valuable new information on the physiological processes that occur within cells. The expression levels of mRNA in a cell do not necessarily relate to levels of protein produced due to regulatory processes at the translational level and a complete understanding of how cells function requires investigation of the processes that control the transcription of a gene through to its activity as functional proteins structures and must take into account the regulatory processes in between. This requires both mRNA and protein based molecular biology techniques to achieve this.

This study used real-time RT-PCR and gene expression microarray analysis to investigate the expression of CXCL12 and CXCR4 in the endometrium and the potential effects of CXCL12 on endometrial function. The techniques can accurately and reproducibly measure mRNA levels, but due to their complexity several steps must be taken to maintain their validity. This included the use of high quality RNA where possible, which was accurately quantified and of great importance to the success and validity of Real-Time RT-PCR and microarray experiments. Reference genes were used to quantify transcript levels using Real-Time PCR and stringent attempts were made to ensure the stability of the reference transcript between samples. Retrospective analysis found that HEC-1-B cells consistently expressed higher levels of YWHAZ compared to the other cell types used. Therefore, a more rigorous initial study of reference genes could have been useful.

The study of the endometrium *in vivo* in humans is limited because of the difficulty in obtaining human tissue, or manipulating the tissue *in vivo* and therefore various methods of indirect study are used to help understand its function. The use of cell

culture systems is one approach that may provide important information into the control of endometrial cells. Two types of endometrial cell cultures were used: an established primary cell culture system where endometrial epithelial and stromal cells are separated from endometrial tissue and plated at a high density to establish confluency within 5 days (Cork *et al.*, 2002; Laird *et al.*, 1993 and Tuckerman *et al.*, 2000) and endometrial cell lines. The advantage of using cell lines is their ready availability; in contrast primary cell culture requires recruitment of suitable volunteers and ethical committee approval. However, this study showed that the Ishikawa and HEC-1-B cell lines did not show comparable mRNA expression profiles of important factors, such as cytokines and chemokines, MMPs and growth factors in comparison to their primary epithelial cell counterparts exhibiting very low expression of several of these factors. These results show that considerable care should be taken in relating the findings in cell lines directly to the functions of the endometrium.

6.3 Expression of CXCL12 and CXCR4 in the endometrium

Available information on the expression of CXCL12 and CXCR4 in the endometrium is limited. No previous studies have identified the presence of CXCL12 throughout the menstrual cycle in non-pregnant human endometrium, although its presence in monkey and bovine endometrium has been reported (Ace and Okuliez, 2004 and Mansouri-Attica *et al.*, 2009). This study identified the presence of CXCL12 mRNA in human endometrium throughout the menstrual cycle that was low in comparison to the reference gene used and did not significantly alter during any phases of the cycle.

CXCR4 mRNA was also shown to be expressed in human endometrium throughout the menstrual cycle. The expression levels were found to be significantly higher in the early proliferative phase of the cycle in comparison to all other phases except the mid secretory. There was an apparent increase in CXCR4 levels during the mid secretory phase of the cycle in comparison to the early and late secretory phases but this was not found to be significantly different to other phases of the cycle. The expression of CXCR4 mRNA through the normal menstrual cycle has previously been studied by Dominguez *et al.* (2003). The study identified an apparent decrease in CXCR4 mRNA expression through the proliferative phase of the cycle that was in general agreement with our findings, though the decrease was of a lower magnitude. Dominguez *et al.*

(2003) showed a significant increase in the levels of CXCR4 mRNA during the mid secretory phase of the cycle when the endometrium becomes receptive to the blastocyst. While the present study did show an apparent small increase in the levels of CXCR4 mRNA at this time, the difference was not found to be significant and expression levels were considerably lower than those of the early proliferative phase. The present study used twice the number of samples to obtain the data which could contribute to the differences observed between the studies. However, a decrease in the levels of CXCR4 mRNA through the proliferative phase of the menstrual cycle and an increase in CXCR4 during the mid secretory phase appear to be common factors between both of the studies.

As the *ex vivo* approach of quantifying the levels of CXCL12 and CXCR4 mRNA levels in the endometrium did not discriminate between the different cell types present within the samples obtained, primary cell cultures of endometrial epithelial and stromal cells were used to investigate the distribution of CXCL12 and CXCR4 between these cell types. The results showed that CXCL12 mRNA was found in both cell types studied at low levels in comparison to the reference gene used and there was no significant difference in the expression of the chemokine mRNA between the cell types. Levels of CXCR4 mRNA were found to be approximately equivalent to the reference gene used and while epithelial cell levels of CXCR4 mRNA appeared to be approximately twice that of the stromal cells, the levels of expression were shown not to be significantly different from one another. These findings suggest that endometrial epithelial and stromal cells are likely to be major sources of CXCL12 and CXCR4 in the endometrium and that this may relate to their endometrial functions. However CXCR4 is expressed by leucocytes and in particular NK cells, which are also present in the endometrium. Therefore immunohostochemical studies, which can identify the cell type from which expression originates, need to be carried out to show more conclusively precisely which cells express both CXCL12 and CXCR4.

6.4 Expression of IL6, IL8, MMP2, MMP9 and VEGFA by endometrial cells *in vitro*

The presence of mRNA transcripts for IL6, IL8, MMP2, MMP9 and VEGFA were identified in the Ishikawa, HEC-1-B, primary epithelial and stromal cell cultures used in

this study. Comparison of the levels of these transcripts between primary epithelial and stromal endometrial cells identified significant differences in their expression which may relate to their endometrial function. IL8 expression was found to be significantly higher in the endometrial epithelial cells. This result is in agreement with other studies which have shown endometrial expression of IL8 (CXCL8) to be highest in the epithelium of the endometrium and further demonstrates its potential role in the recruitment of leukocytes to the endometrium through the menstrual cycle (Arici *et al.*, 1998b and Garcia-Valesco and Arici, 1999). MMP9 mRNA expression was found to be significantly higher in the epithelial cells which is in agreement with studies such as that by Jeziorska *et al.* (1996), which showed expression of MMP9 at the protein level to be present in the luminal and glandular epithelium of the endometrium. VEGFA mRNA expression was also found to be significantly higher in the epithelial cells which is in agreement with studies such as that by Punyadeera *et al.* (2006). While the identification of differential expression of these factors between endometrial epithelial and stromal cells can be demonstrated, attributing function to them purely on the basis of their abundance is probably over-simplistic in terms of what functions they may play in the endometrium. All these factors will bring about their effects via specific cell surface receptors and therefore the distribution of their receptors is of equal importance when considering the function of these molecules in the human endometrium.

6.5 Effect of CXCL12 on the expression of IL6, IL8, MMP2, MMP9 and VEGFA

The overall aim of this study was to identify the functions of CXCL12 and its receptor CXCR4 in the endometrium and once mRNA expression of both the chemokine and its receptor throughout the menstrual cycle was established, likely targets for its effects had to be identified. While the microarray study employed a genome-wide approach to providing more information on the function of CXCL12 and CXCR4 in the endometrium, this part of the study used a targeted approach using the available current information to directly target factors that may be affected.

This study could not identify any significant alterations in the mRNA expression of IL6, IL8, MMP2, MMP9 or VEGFA in response to addition of 10 and 100 ng/ml of

recombinant CXCL12 for 24 hours in any of the cell culture types used. These transcripts were chosen for investigation because they are all factors which are established as being of importance to the control of endometrial function, have been shown in several non-endometrial cell types to respond to CXCL12 and have not previously been investigated in the endometrium in this respect.

The results suggested that CXCL12 might increaseIL6 and MMP9 mRNA in primary epithelial cell cultures, but differences between control cells and cells incubated with CXCL12 were not significant. It is possible that CXCL12 does not play a role in the regulating the expression of IL6, IL8, MMP2, MMP9 and VEGFA in endometrial cells. Our studies have shown that the receptor for CXCL12 (CXCR4) is expressed by all cell types; therefore lack of expression of the receptor is unlikely to be a reason for a lack of response to CXCL12. However it is possible that binding of CXCL12 to its receptor may activate different signaling pathways to those operating in other cell types leading to the regulation of different genes in the endometrium.

6.6 Microarray analysis

The microarray study identified 51 gene transcripts that were greater than 5-fold upregulated in response to CXCL12 treatment at 5 ng/ml for 24 hours in two or more experimental repeats. In the remaining experimental repeats, it could be demonstrated that the features representing a transcript were compromised by factors such as poor hybridization, feature saturation or high background meaning *P* values could not be accurately generated and were attributed a value of *P=1.00* by the feature extraction software. There were no cases of a transcript showing a conflicting and significant result and highly stringent *P* values were used to attribute significance to remove the possibility of a false-positive result occurring.

While the targeted Real-Time RT-PCR experiments could not identify any effects of CXCL12 on the targets chosen, the microarray experiment showed that addition CXCL12 to Ishikawa cell lines did significantly alter mRNA expression strongly suggesting the cell culture systems were functional.

The microarray analysis was carried out in Ishikawa cell lines, which this study itself demonstrates do not necessarily represent the likely *in vivo* situation due to their

cancerous nature. However, Ishikawa cells did show similar levels of CXCR4 expression to their primary epithelial cell counterparts. The use of cell lines gives the advantages of an easily accessible and reproducible source of high quality RNA which is essential for successful and accurate microarray analysis. In this case where the effects of CXCL12 alone are investigated, it provides a useful tool for revealing entirely new information about transcripts that can be potentially affected by CXCL12.

Of the 51 gene transcripts identified in this study found to respond to CXCL12, 14 were unknown transcripts and 37 were known transcripts to which potential functions could be attributed. However, before drawing definite conclusions the data needs to be verified both by using real-time RT-PCR for individual transcripts and confirming effects at the protein level by western blotting. While many of these transcripts remain unstudied in the endometrium and in most cases have not been previously shown to be affected by CXCL12 in any cell type, the accuracy of the data generated is supported by the identification of factors such as ghrelin, cyclin-dependent kinase 2-associated protein 1 (CDK2AP1), EBI3/IL27 and Ets1, which are known to be expressed in the endometrium and are implicated in playing important roles in its function. Further to this, programmed cell death factor 4 (PDCD4) was shown to be upregulated by CXCL12 and has recently been identified as playing a role in the control of CXCR4-dependent cell signaling (Hayre *et al.*, 2010).

6.7 Signaling pathways

The currently available evidence strongly suggests that CXCL12 can elicit multifunctional effects through its receptor CXCR4 (Busillo and Benovic, 2007). While CXCL12 has been shown to increase the expression of IL6, IL8, MMP2, MMP9 and VEGFA in non-endometrial cell types (see Chapter 4), this effect apparently does not occur in the endometrial cell types used in this study. Furthermore, genome wide analysis of transcripts in the Ishikawa cell line demonstrated the increase of numerous factors previously unknown to be affected by addition of CXCL12 in any cell type.

The likely explanation of how CXCL12 can exert these varying effects is by activating different intracellular signal transduction pathways in different cell types. CXCR4 has been shown to have the capacity to activate both the MAPK/ERK1/2 and PI3K/Akt signal pathways both of which can cause activation of nuclear-factor kappa B (NF_KB)

and cause transcription of NF_KB responsive genes (Chinni *et* al., 2006; Huang *et al.*, 2009 and Tang *et al.*, 2008). CXCL12 binding of CXCR4 has also been shown to activate the transcription factors Ets1 (Bu *et al.*, 2005; Grund *et al.*, 2005; Jinnin *et al.*, 2005 and Liu *et al.*, 2006), which was shown to be up-regulated by CXCL12 in our microarray study. The transcription factor AP1 can also respond to CXCR4-dependent MAPK/ERK1/2 signaling (Liu *et al.*, 2006), an effect that has recently been shown to be blocked by PDCD4 (O'Hayre *et al.*, 2010), which was another of the transcripts shown to be increased by addition of CXCL12 to the Ishikawa cell line in the microarray study (Figure 6.1).

The activation of NF_KB via the MAPK/ERK1/2 and PI3K/Akt signal transduction pathways has been shown to cause the up-regulation of IL6 (He *et al.*, 2008 and Tagashira *et al.*, 2009)), IL8 (Chen *et al.*, 2008), MMP2 (Chang *et al.*, 2007 and Zhang *et al.*, 2010), MMP9 (Van Themsche *et al.*, 2007 and Zhang *et al.,* 2010) and VEGF (Kazi and Koos, 2007) in endometrial cell types. However, addition of CXCL12 did not cause up-regulation of the mRNA for these factors in the endometrial cell types investigated in this study, despite this effect being shown in other cell types. In the case of MMP9 (Chinni *et al.*, 2006) and VEGF (Liang *et al.*, 2007) the effect of CXCL12 has been shown to occur specifically through the PI3/Akt pathway.

It is possible that CXCL12/CXCR4 may act through additional signal transduction pathways to cause its cellular effects. For example the Ets1 transcription factor was found to be up-regulated in Ishikawa cell line in response to CXCL12. This factor has been shown to be of importance to endometrial function especially with respect to process of decidualisation (Kessler *et al.*, 2006). Ets1 may represent a means of alternate signaling through ERK1/2 and may provide an explanation to why CXCL12 did not affect the expression of IL6, IL8, MMP2, MMP9 and VEGFA. Furthermore, CXCL12/CXCR4 has recently been shown to cause signaling via the transcription factor AP1. This effect has been shown to be blocked by phosphorylation of PDCD4 (O'Hayre *et al.*, 2010), which was also up-regulated in response to CXCL12 in the microarray experiment and further highlights the multiple possibilities of CXCL12/CXCR4 signal transduction. Activation of different pathways may depend on the many factors including the oligomerisation state of CXCL12 and its potential interactions with glycosaminoglycans; glycosylation of CXCR4's external structure (Majka *et al.*, 2000

and Wysoczynski *et al.*, 2005); tyrosine sulfation of CXCR4's external structure (Farzan *et al.*, 2002); homo- and hetero-dimerisation of CXCR4 (Babcock *et al.*, 2003; Basmaciogullari *et al.*, 2006 and Issafras *et al.*, 2002) and phosphorylation patterns of CXCR4's internal receptor structure (Krupnick *et al.*, 1998).

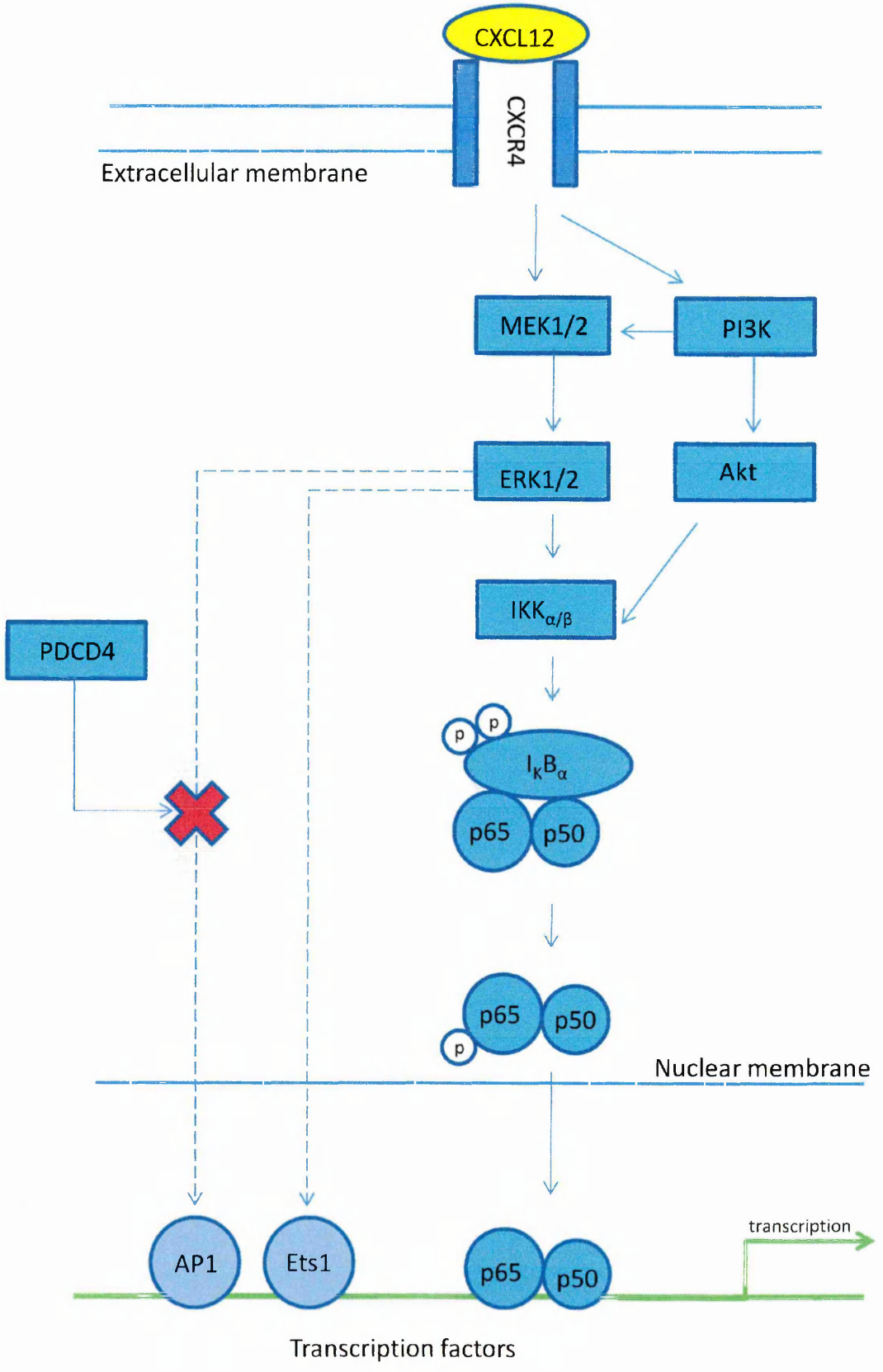


Figure 6.1: Schematic representation of the potentially important CXCL12/CXCR4 dependent signal transduction pathways in endometrial cells.
Compiled from Bu *et al.*, 2006; Chinni *et al.*, 2006; Huang *et al.*, 2009; Jinnin *et al.*, 2005; Liu *et al.*, 2006 and O'Hayre *et al.*, 2010.

6.8 Proposed functions of CXCL12 and CXCR4 in the endometrium

The precise functions of CXCL12 and CXCR4 remains unknown but it seems clear that their roles in the human are likely to be multifunctional (Bussilo and Benovic, 2007). Previous studies of CXCR4 in the endometrium focused on the implantation window when the endometrium becomes receptive to the blastocyst suggesting possible roles in the regulation of cross-talk between the endometrium and blastocyst during apposition and subsequent invasion of the trophoblast (Dominguez *et al.*, 2003). The potential of CXCL12 to recruit uNK cells to the endometrium and decidua during pregnancy has also been considered (Wu *et al.*, 2005).

This study showed that CXCR4 is most highly expressed during the early proliferative phase of the menstrual cycle and while increased levels of CXCR4 could be observed at the time of endometrial receptivity, the expression levels of CXCR4 at this time was significantly lower than during the early proliferative phase. This suggests that while CXCR4 expression could be of importance in the process of implantation, it is likely to have important endometrial functions during the early proliferative phase of the menstrual cycle. During the proliferative phase of the cycle the endometrium is lost during menses and then undergoes a phase of rapid growth and differentiation phase as endometrial thickness increases. Therefore it is reasonable to suggest that CXCR4 may play a role in the processes that occur at this time in both endometrial epithelial and stromal cells, which could include regulation of angiogenesis, cell proliferation and differentiation. A recent study shows the potential for CXCL12 and CXCR4 to fulfill this role as the chemokine ligand was found to cause proliferation in Ishikawa cell lines (Tsutsumi *et al.*, 2011).

CXCL12 was found to be expressed at the mRNA level throughout the cycle but its expression did not change during menstrual cycle phases, which has not previously been shown and is in agreement with unpublished protein studies from our laboratory. This suggests that CXCR4 expression is regulated independently of CXCL12 as they show different expression profiles. There is evidence that CXCR4 may be regulated by oestrogen either directly or indirectly and both this and Dominguez's study (2003) showed a decrease in CXCR4 mRNA expression as oestrogen increases through the proliferative phase of the cycle.

Recruitment of leukocytes and particularly uNK cells seems like an unlikely function of CXCL12 in the endometrium as there is a large increase in the numbers of leukocytes and particularly uNK cells in the endometrium during the secretory phase of the cycle. If CXCL12 was important in recruiting uNK cells to the endometrium an increase in CXCL12 levels would be expected during the secretory phase of the cycle but this was not observed.

The microarray study identified possible new effects of CXCL12 and showed that CXCL12 could increase the expression of unidentified genes, genes for which there is no available information in the endometrium and genes such as ghrelin and Ets1 which have been been linked to endometrial processes such as decidualisation and angiogenesis. Also, several factors such as EBI3, BIRC7 and PDCD4 were identified as being upregulated at the mRNA level by CXCL12. These factors could potentially play roles in controlling cell proliferation and apoptosis in the endometrium. Coupled with the knowledge that CXCR4 mRNA expression is maximal in the endometrium during the proliferative the early proliferative phase of the cycle when the endometrium is undergoing rapid growth and CXCL12 has been shown to act as a growth factor in Ishikawa cells (Tsutsumi *et al.,* 2011), it may be the case that CXCL12 acts as a paracrine growth factor in the endometrium.

Many investigations into the functions of CXCL12 and CXCR4 in the human have focused on the roles of the receptor CXCR4 in HIV infection and in the metastases of many types of cancer (Busillo and Benovic, 2007). While normal physiological roles have received less attention, studies in cancer cells have suggested that CXCL12 and CXCR4 may be important in controlling the spread of cancer cells throughout the body (Kucia *et al.*, 2005). It is thought that CXCR4 positive cancer cells are recruited to tissues by the presence of a CXCL12 gradient, causing the infiltration and establishment of cancer cells within new tissues and allowing secondary tumour development (Gargett, 2006; Gargett *et al.*, 2009 and Maruyama *et al.*, 2010). This process has been linked to an emerging physiological role for CXCL12 and CXCR4 in the recruitment of stem cells to tissues where they are required throughout the body. CXCR4 is a known stem cell marker and it has been suggested that a CXCL12 gradient could be responsible for recruiting stem cells to tissues and has been shown to occur in the pathological metastases of cancer cells (Figure 6.2).

The endometrium has a remarkable regenerative capacity, cycling through periods of rapid proliferation, differentiation and shedding. It is thought that adult stem/progenitor cells reside within the endometrium to allow this process to occur. There are several pieces of indirect evidence suggesting the presence of stem cells in the endometrium and while adult stem cells provide few markers to distinguish them from other cell populations, methods are being established to specifically identify their presence and function in the endometrium. It is conceivable that a constant level of CXCL12 expression in endometrial tissues may play a role in recruiting and maintaining the presence of adult stem/progenitor cells in the endometrium throughout the cycle.

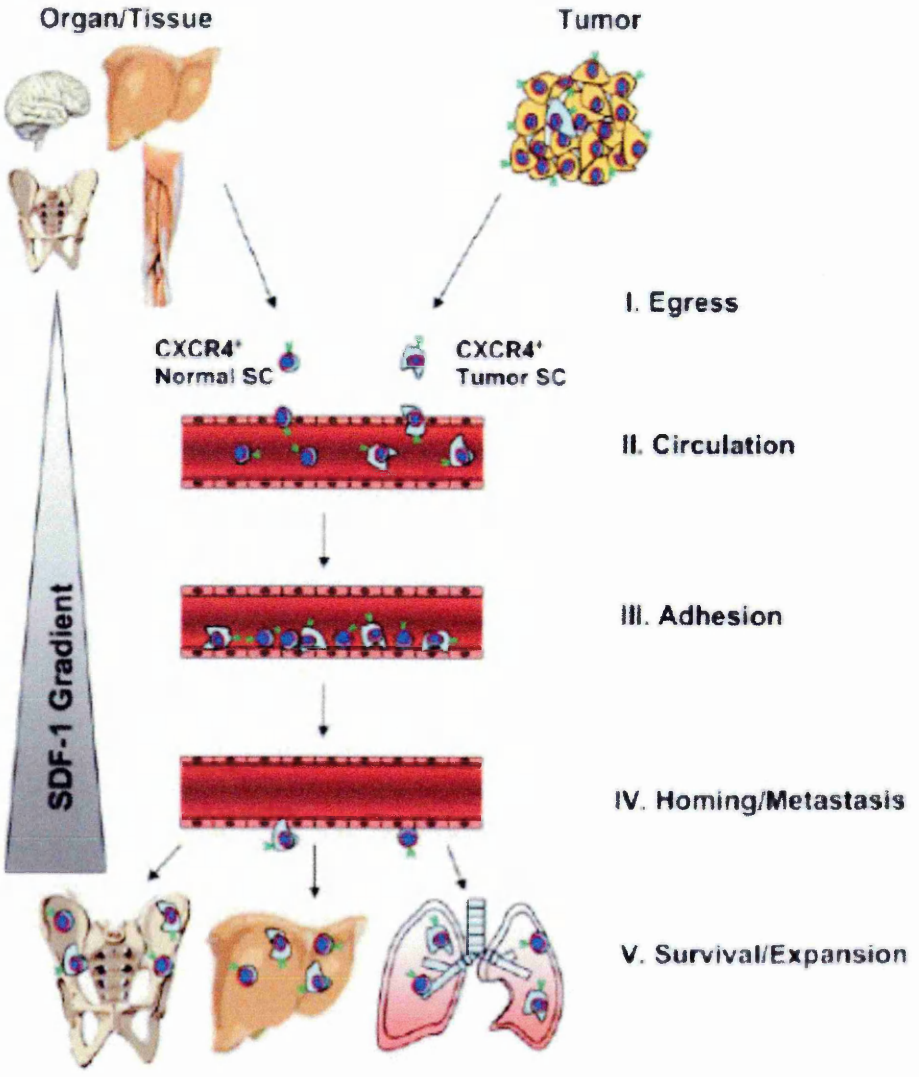


Figure 6.2: Diagram showing the potential of CXCL12 to recruit metastatic cancer cells and adult stem cells via CXCR4. (Taken from Kucia et al., 2005)

A parallel between the recruitment of stem cells via CXCL12 gradients and cancer cell metastasis can be made.

6.9 Future studies

A number of further studies need to be carried out to confirm the findings of this study and establish a role for CXCL12 and CXCR4 in the human endometrium. Validation of the effects of CXCL12 seen in the analysis of the microarray data from the Ishikawa cell lines needs to be carried by repeating this type of analysis in primary epithelial and stromal cells and the hypotheses summarised in Table 5.3 could be tested using the techniques suggested. Effects of CXCL12 on factors that are shown to be altered in the primary cell cultures needs to be confirmed by further real-time RT-PCR and protein studies using western blotting, immunostaining or analysis of secreted protein. Identification of the signaling pathways through which CXCL12/CXCR4 is functioning in the endometrium, could be studied, particularly in primary cell cultures using western blotting with antibodies specific for phosphorylated signaling proteins. Attempts could be made to represent the *in vivo* endometrium more accurately in cell culture experiments. For example, inclusion of steroid hormones may represent the *in vivo* endomtrium more accurately. A co-culture of endometrial epithelial and stromal cells may be able to determine the direction of secretion for all the paracrine factors investigated giving further information as to their mode of function. As the *in vivo* endometrium contains vascular cells and immune cell populations including uNK cells, protein studies such as immunohistochemistry may reveal the distribution of CXCL12 and CXCR4 in all endometrial cell types. As FCS can potentially alter the behaviour of cells in culture, attempts could be made to successfully grow cultures without using FCS as a cell supplement and cell culture *n* numbers could be increased to reduce the effect of experimental variation during statistical analysis of the results.

This study concentrated on the effects of one isoform of CXCL12. Several other functional isoforms of CXCL12 produced by alternative splicing have been identified (Yu *et al.*, 2006). Studying the effects of these isoforms in the endometrial cells could give more detailed information on the function of CXCL12 and CXCR4 in the endometrium. Recent studies have shown that CXCL12 may possibly function through an alternative chemokine receptor called CXCR7 (Mahabaleshwa *et al.*, 2008). Identification of this receptor in the endometrium and if present, measuring expression throughout the menstrual cycle and identifying the cell types which express CXCR7 might also help understand the roles of CXCL12 in endometrial function.

The evidence generated by this study suggests CXCR4 expression is maximal during the early proliferative phase of the endometrium and in ishikawa cells that CXCL12 may act as an endometrial cell growth factor (Tsutsumi *et al.,* 2011). As the microarray study showed CXCL12 could regulate genes involved in cell proliferation and apoptosis, primary cell cultures could be used to further investigate the potential for CXCL12 to act as a growth factor in endometrium via CXCR4. As cell differentiation and angiogenesis are also important processes occurring in the endometrium during the proliferative phase of the menstrual cycle, primary cell culture models could also be used to investigate to what extent, if at all, CXCL12 and CXCR4 contribute to this process in the endometrium. Using antagonists to CXCR4 and inhibiting its activity through intracellular signaling could be of key importance to elucidating the precise actions of this chemokine and receptor in the endometrium.

6.10 General conclusions

This study has identified the expression of CXCL12 and CXCR4 mRNA in the endometrium through the menstrual cycle and suggested little change in CXCL12 expression, but increased expression of CXCR4 in the early proliferative phase. The effects of CXCL12 on IL6, IL8, MMP2, MMP9 and VEGFA were measured in endometrial epithelial, stromal, Ishikawa and HEC-1-B cells. Despite CXCL12 affecting expression of these factors in non-endometrial cells, this effect was not observed in endometrial cells. The differential expression of CXCL12, IL6, IL8, MMP2, MMP9 and VEGFA between primary epithelial cells and HEC-1-B and Ishikawa cell lines, with low levels being expressed by the cell lines suggest that these cell lines are not good models for studying endometrial function. Microarray expression analysis showed that CXCL12 up-regulated unknown gene transcripts, known genes transcripts that have not previously shown to be importance in endometrial cell types and transcripts that have previously been shown to be of importance in the endometrium but have not been linked to CXCL12. The study therefore indicates directions of further study in order to further understand the role of CXCL12 and CXCR4 in the endometrium.

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