F-Prostanoid receptor regulation of inflammation in endometrial adenocarcinoma

Alison E Wallace

University of Edinburgh

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List of figures and tables

Declaration

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

Alison Wallace

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Publications and Presentations

A selection of the work from Chapters 3 and 4 has been published in a paper in Cancer Research, and has been included in Appendix 1 of this thesis.

Wallace, A.E, Sales, K.J, et al. (2009). "Prostaglandin $F_{2\alpha}$-F-prostanoid receptor signalling promotes neutrophil chemotaxis via chemokine (C-X-C motif) ligand 1 in endometrial adenocarcinoma." *Cancer Res* **69** (14): 5726-33.

Data obtained during this PhD has also been presented at the following conferences:
Endocrinology Annual Conference 2009, Washington DC, USA. Presidential Poster Competition (June 2009).

Society for Gynaecological Investigation Annual conference, Glasgow, UK. Poster presentation (March 2009).

Society for Reproduction and Fertility Annual Conference, Edinburgh, UK. Oral presentation prize session (July 2008).

Simpson Symposium, Edinburgh UK. Poster presentation (August 2008).

MRC Inflammation Showcase, Edinburgh, UK. Poster presentation (September 2007).

Abstract

Endometrial adenocarcinoma is the most common gynaecological malignancy in Western countries, affecting mainly post-menopausal women with a frequency of 15-20 per 100 000 women per year. Over-expression of the cyclooxygenase (COX) enzymes and prostaglandin receptors has been demonstrated in endometrial adenocarcinoma as well as other gynaecological pathologies. Increased expression of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor (FP) has been previously demonstrated in endometrial adenocarcinoma. A role for the FP receptor in the promotion of endometrial adenocarcinoma has been shown, with evidence for elevated $PGF_{2\alpha}$-FP signalling up-regulating angiogenic and tumourigenic genes, and increasing proliferation and migration of neoplastic epithelial cells. This thesis examines signalling pathways regulated by and interacting with the FP receptor that influence chemokine expression and subsequent effects in endometrial adenocarcinoma.

To investigate $PGF_{2\alpha}$-FP interactions in endometrial adenocarcinoma, an endometrial epithelial cell line of adenocarcinoma origin (Ishikawa cells) stably transfected with the FP receptor to levels seen in cancer was used (FPS cells). An antibody array identified the chemokine C-X-C motif Ligand 1 (CXCL1) as a target gene regulated by $PGF_{2\alpha}$-FP signalling in this cell line. Expression of CXCL1 and its receptor, CXCR2, were elevated in cancer tissue as compared to normal endometrium and localised to glandular epithelium, endothelium and stroma. The induction of CXCL1 expression in FPS cells and endometrial adenocarcinoma explants was determined to be by a signalling pathway involving G_q, the epidermal growth factor receptor (EGFR) and extracellular signal-regulated kinase (ERK). The infiltration of immune cells into endometrial adenocarcinoma as compared to normal endometrium was then investigated. Increased neutrophils were present in endometrial adenocarcinoma compared with normal endometrium, and the expression of CXCR2 was colocalised to neutrophils. In vitro chemotaxis assays demonstrated that conditioned media from $PGF_{2\alpha}$-treated FPS cells stimulated human neutrophil chemotaxis which could be abolished by CXCL1 protein immunoneutralisation from the conditioned media or

antagonism of CXCR2 on neutrophils. Moreover, xenograft tumours in nude mice arising from inoculation with FPS cells had higher neutrophil infiltration compared to tumours arising from wild-type cells or following treatment of mice bearing FPS tumours with CXCL1-neutralising antibody. Therefore, the up-regulation of CXCL1 by $PGF_{2\alpha}$ promoted neutrophil chemotaxis into endometrial adenocarcinoma.

The expression of a further chemokine, CC motif Ligand 20 (CCL20) was determined to be regulated by $PGF_{2\alpha}$ -FP signalling in endometrial adenocarcinoma, and expression of CCL20 and its receptor CCR6 was elevated in endometrial adenocarcinoma. The induction of CCL20 by $PGF_{2\alpha}$ -FP signalling in FPS cells was dependent on the signalling molecules G_q, EGFR, ERK, calcineurin and nuclear factor of activated T-cells (NFAT). The treatment of endometrial epithelial cells with recombinant CCL20 caused a significant increase in proliferation.

Finally interactions between the signalling pathway of another pro-inflammatory lipid, lysophosphatidic acid (LPA), and FP receptor signalling in endometrial adenocarcinoma were examined. LPA increased expression of the FP receptor and the FP target genes previously discussed in this thesis, CXCL1 and CCL20, in FPS cells. Expression of the LPA receptors (LPAR) 1, 2 and 3 was localised in endometrial tissue, and LPAR2 and 3 were found to be elevated in endometrial adenocarcinoma compared with normal endometrium, suggesting amplification of the $PGF_{2\alpha}$ -FP signalling pathway by LPA was possible.

Collectively, these data demonstrate that inflammatory cytokine signalling pathways regulated by $PGF_{2\alpha}$-FP activation can promote immune cell infiltration and proliferation of endometrial adenocarcinoma, and that interaction of LPA and $PGF_{2\alpha}$-FP signalling in endometrial adenocarcinoma may exacerbate the disease.

Commonly used abbreviations

AA	Arachidonic acid
ANOVA	Analysis of variance
AP-1	Activator-protein 1
ATX	Autotaxin
bp	Base pairs
BSA	Bovine serum albumin
cAMP	Adenosine 3',5'- cyclic monophosphate
CCR6	Chemokine (C-C) motif receptor 6
CCL20	Chemokine (C-C) motif ligand 20
COX	Cyclooxygenase
CXCL1	Chemokine (C-X-C) motif ligand 1
CXCR2	Chemokine (C-X-C) motif receptor 2
DAB	3,3'- diaminobenzidine
DMEM	Dulbeccos modified eagle medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EGTA	Ethylene glycol tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
ER	Oestrogen receptor
ERK	Extracellular signal regulated kinase
EP	E-prostanoid receptor
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FP	F-prostanoid receptor
FPS cells	Ishikawa F-prostanoid receptor sense-

	transfected cells
GPCR	G-protein coupled receptor
HB-EGF	Heparin binding EGF-like growth factor
HRP	Horse-radish peroxidase
IgG	Immunoglobulin G
IP	Inositol phosphate
IL	Interleukin
kDa	Kilodaltons
LIF	Leukaemia inhibitor factor
LPA	Lysophosphatidic acid
LPAR	Lysophosphatidic acid receptor
MCP	Monocyte chemoattractant protein
MAPK	Mitogen activated protein kinase
MEK	Mitogen associated protein kinase-extracellular regulated kinase kinase kinase
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
NFAT	Nuclear factor of activated T cells
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nM	Nano molar
NSAIDS	Non-steroidal anti-inflammatory drugs
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PG	Prostaglandin
PLC	Phospholipase C
pM	Pico molar
PR	Progesterone receptor

PTEN	Phosphatase and tensin homolog protein
PTX	Pertussis toxin
RANTES	Regulated upon activation of normal T-cell expressed and secreted
RCAN1-4	Regulator of calcineurin 1-4
RNA	Ribonucleic acid
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
S1P	Sphingosine-1-phosphate
SEM	Standard error of the mean
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TBS	Tris buffered saline
TRI	Total RNA isolation
uNK	Uterine natural killer
VEGF	Vascular endothelial growth factor
WT	Wild type

Chapter 1

Literature Review

1 Literature Review

1.1 Introduction

Endometrial adenocarcinoma is the most common gynaecological malignancy in the Western world (Doll et al. 2008). A role for the prostaglandins in this cancer has been shown, with evidence for the up-regulation of cyclooxygenase enzymes (Tong et al. 2000; Kilic et al. 2005) and the prostaglandin receptors (Sales et al. 2004; Sales et al. 2004b), and the promotion of a number of tumourigenic genes (Sales et al. 2005; Genc et al. 2007; Sales et al. 2007; Sales et al. 2008). The focus of this thesis is on the contribution of the F-prostanoid receptor to inflammatory signalling pathways in endometrial adenocarcinoma, and how these pathways may be amplified by other lipid signalling mediators. This chapter gives a brief introduction to the normal cycling uterus and a background to endometrial adenocarcinoma, including established genetic and hormonal changes which contribute to tumour progression. The concept that inflammation may play a key role in tumour progression is then introduced, and how the hormonal and genetic changes may contribute to the inflammatory microenvironment is discussed. The role that prostaglandins, chemokines and the lipid signalling mediator lysophosphatidic acid may play in inflammation in endometrial adenocarcinoma is then considered.

1.2 The normal endometrium

The uterus is a unique organ in the adult female, with a highly specialised endometrial lining that undergoes a cycle of proliferation, angiogenesis and shedding on a monthly basis in order to prepare for implantation of the embryo. The uterus is formed in the developing embryo from the Mullerian ducts, which also give rise to the epithelial lining of the cervix, ovaries and upper vagina (Cunha et al. 2002). In the adult, the uterus consists of an outer peritoneal wall over a thick myometrial layer of smooth muscle, and the internal endometrial layer. Four distinct regions make up the uterus: the fundus, corpus, isthmus and cervix, which are displayed in Figure 1.1 (Johnson et al. 2000).

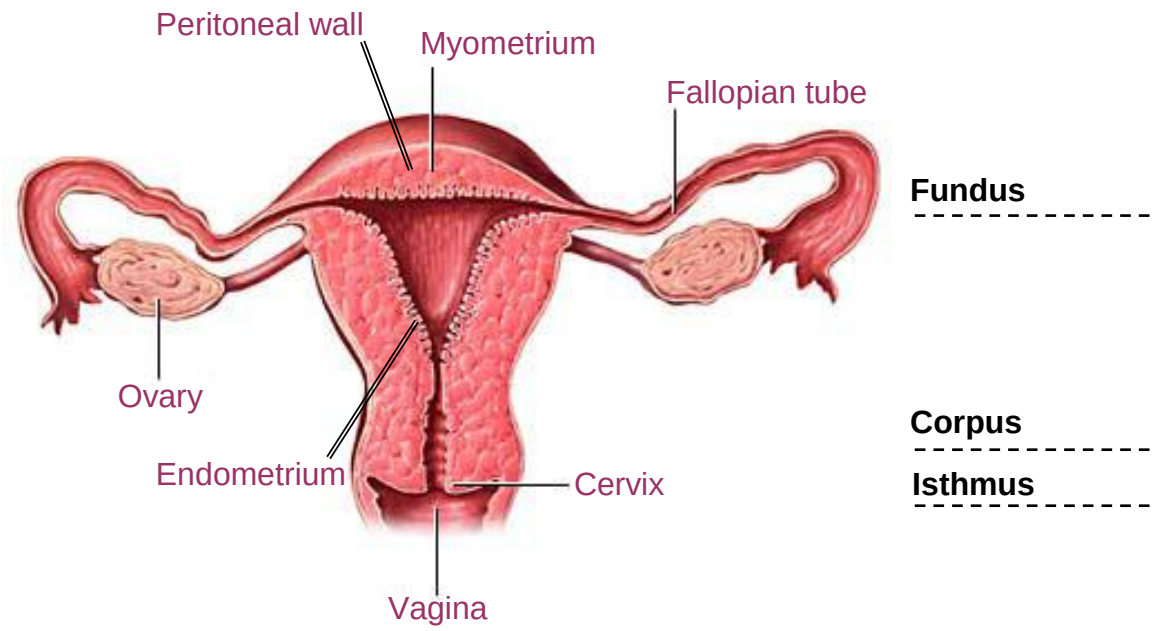

Figure 1.1 The adult uterus. The uterus is composed of four distinct regions: the upper region of the uterus named the fundus, the main body of the uterus containing the uterine cavity named the corpus, the flexible lower region named the isthmus, and the cervix. It is comprised of three layers; the outer peritoneal wall, a muscular myometrium and the internal endometrium. Adapted from http://www.nlm.nih.gov/medlineplus.

The endometrium is situated directly adjacent to the muscular myometrium with no separating tissue. It is composed of a columnar epithelium with epithelial glandular extensions throughout the stroma, and is separated into two layers known as the functionalis and basalis (Figure 1.2). In the functionalis layer, the epithelial glandular protrusions extend through the stroma from the surface epithelium. The basal regions of the glands extend through the denser stroma of the basalis layer. As well as luminal and glandular epithelial cells, the microenvironment of the endometrium contains fibroblasts, vascular endothelial cells and immune cells (Gargett 2007).

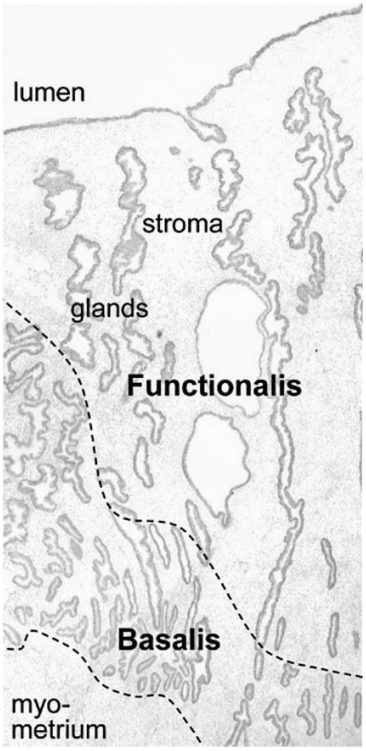

Figure 1.2 The structure of the endometrium. The endometrium lies adjacent to the myometrium and is composed of two distinct layers, the functionalis and basilis. It is comprised of an epithelium extending in glandular protrusions throughout the stroma. Adapted from (Gargett 2007).

The endometrium has the capacity to shed and completely renew itself within one menstrual cycle, a process limited to humans, old world primates including macaques and baboons, some new world monkeys (Dempsey 1939; Ortiz et al. 2005), shrews and fruit-bats (Zhang et al. 2007). In humans, this lasts an average of 28 days, being split into three phases: menses (day 1-4), the proliferative phase (day 5-14) and the secretory phase (day 14-28) (McLennan et al. 1965). The menstrual cycle takes place in response to hormonal changes which regulate the cycle of proliferation, differentiation and tissue breakdown. The human menstrual cycle was first explicitly likened to an inflammatory wound healing process in 1986 by Finn, who described the similarities of the two processes. These include increased blood flow and vessel permeability, the change to decidual tissue which resembles the granulation tissue of wound healing, and the infiltration of immune cells (Finn 1986). Subsequent

evidence has strengthened this analogy by examining in more detail the tissue remodelling, cytokine expression and leukocyte influxes that occur (Jabbour et al. 2006). In wound healing, a leukocyte influx in response to cytokine production occurs before the re-growth of tissue mediated by epidermal growth factor (EGF) and platelet-derived growth factor (PDGF). Angiogenesis is mediated by vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) (Barrientos et al. 2008). In the normal endometrial cycle, this is mirrored by the influx of leukocytes at the breakdown of tissue at menstruation, and the regrowth of the endometrium during the proliferative phase under the control of growth factors and angiogenic mediators (Critchley et al. 2001). The contribution of chemokines to the inflammatory aspects of the menstrual cycle is further discussed in Section 1.5.1.

1.2.1 The menstrual cycle

1.2.1.1 The proliferative phase

An overview of the menstrual cycle and hormonal changes involved is given in Figure 1.3. The proliferative phase of the cycle occurs after menstruation when the functionalis layer of the endometrium has been shed, and the growth factors mediating this regeneration liken this stage to the healing of a wound (Barrientos et al. 2008). At this point, only the base of the endometrial glands remains, and therefore the proliferative phase is characterised by the re-growth of epithelial glands and the stromal compartment. Under the control of oestrogen, both the epithelial cells making up the glands and the surrounding stoma begin to proliferate (Ludwig et al. 1976). At this point, the oestrogen receptor (ER) is expressed at its highest level in stromal and glandular epithelial cells (Snijders et al. 1992). Progesterone receptor (PR) is also expressed in the glandular epithelial cells (Lessey et al. 1988). The expression of both is upregulated by oestradiol from the ovary (Chauchereau et al. 1992). The glandular epithelial cells begin to undergo morphological changes and increase in size, and an increase in the mitotic index of stromal cells can be seen indicating proliferation (Johannisson et al. 1987).

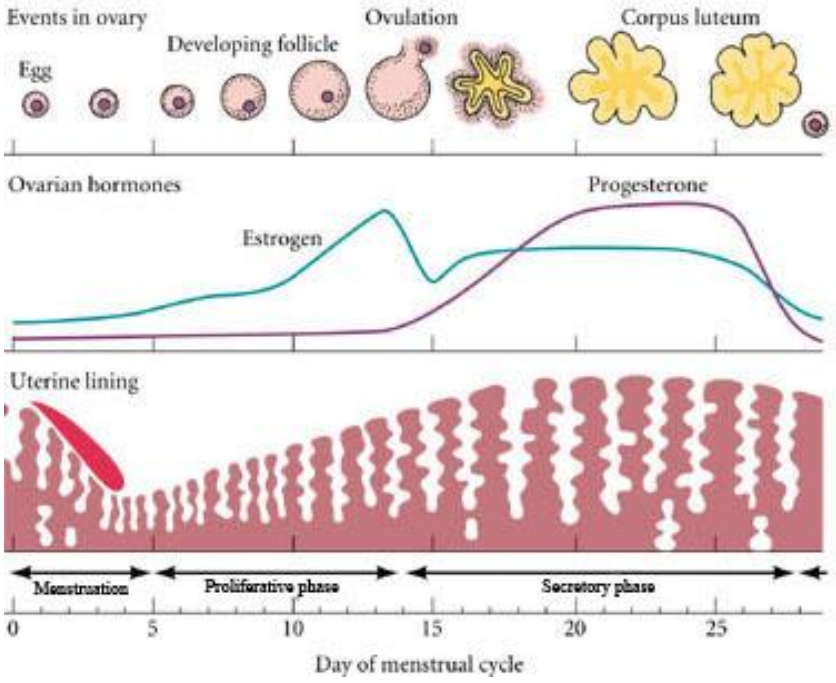

Figure 1.3 The human menstrual cycle. After menstruation and the shedding of the functionalis layer of endometrial tissue, the proliferative phase of the cycle occurs under the control of the hormone oestrogen. During this phase, cell proliferation is driven by stem cells, and increased proliferation gives rise to a thickened endometrium. At ovulation and as progesterone levels begin to increase, endometrial cells begin to differentiate and secrete proteins in preparation for implantation. This is known as the secretory phase of the cycle. Falling progesterone levels then initiate breakdown of endometrial tissue, known as menstruation. Adapted from www.*iupucanatomy.com.*

During the proliferative phase, the endometrium thickens a remarkable amount, from 1 mm to 3-4 mm (Dockery 2002). Evidence for a population of stem cells being primarily responsible for renewing the endometrium has strengthened in recent years with the development of a number of assays for stem cells. This concept was proposed decades ago due to the recognition of the huge regenerative capability of the endometrium (Prianishnikov 1978). It was subsequently supported by studies of epigenetic errors in methylation patterns which were consistent with a long lived population of stem cells dividing rather than the division of differentiated cells (Kim et al. 2005). Additionally common and persistent genetic changes in endometrial epithelial cells indicated that the cells making up glands are clonal and therefore

likely to be formed from a single cell (Mutter et al. 2001). A population of putative stem cells able to form colonies was subsequently isolated from human uterine stromal and epithelial cell explants (Gargett 2006; Gargett et al. 2009) and the murine uterus (Cervello et al. 2007). The proposed site of the endometrial stem cell niche in the endometrium is in the basalis layer and is displayed in Figure 1.4. As the proliferative phase of the menstrual cycle depends on oestrogen, it is as yet unknown if endometrial stem cells express ER, or if these cells are induced to undergo division by oestrogen-mediated signals from the stem cell niche (Gargett 2007).

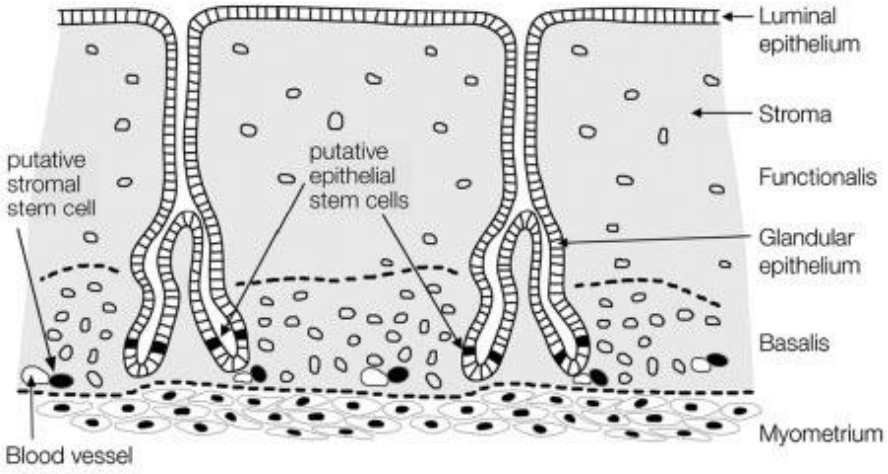

Figure 1.4 The proposed site of endometrial epithelial and stromal stem cells. The stem cell niche is proposed to exist in the basalis layer of the endometrium. Stem cells can regenerate the funtionalis layer of the endometrium after menstruation. Adapted from (Gargett 2007).

A number of growth factors are produced during the proliferative phase of the cycle, under the control of oestrogen, which are similar to those produced in an inflammatory environment (Barrientos et al. 2008). These include transforming-growth factor (TGF; Nelson et al. 1992), EGF (Nelson et al. 1991) and FGF (Fujimoto et al. 1996). The epithelial and stromal compartments of the endometrium are not the only cells undergoing regeneration, as the uterus is one of the few sites in the adult which undergoes regular angiogenesis. Angiogenesis is the generation of new vasculature from existing blood vessels, and can occur by four mechanisms:

sprouting, intussusception, elongation, or incorporation of endothelial cells from the circulation into vessels (Girling et al. 2005). The vascular structure of the endometrium is well described, with the main blood supply reaching the uterus through the myometrium from the radial arteries. The arteries then begin to split, and in the functionalis layer become the coiled spiral arteries characteristic of the endometrium. These arteries further split into the capillary plexus at the surface of the endometrium (Rogers 1996). It is the vessels in the functionalis layer that are primarily remodelled during the menstrual cycle. During the proliferative phase, the endometrial vessels proliferate and sprout (Ferenczy et al. 1979) under the control of oestrogen-induced angiogenic factors including VEGF (Li et al. 1994), EGF (Nelson et al. 1991) and platelet-derived endothelial growth factor (Zhang et al. 1997). The wave of vessel proliferation and VEGF expression has also been subsequently demonstrated in macaques, in an elegant experiment examining angiogenesis after the withdrawal of a progesterone implant (Nayak et al. 2002). Proliferation is not the only mechanism contributing to angiogenesis, and during this stage the endometrial vessels also elongate as a result of the remodelling of existing vessels (Gambino et al. 2002).

1.2.1.2 The secretory phase

The secretory phase of the menstrual cycle begins after ovulation, and is accompanied by a rise in progesterone levels produced by the corpus luteum (Figure 1.3). Progesterone receptor expression reaches a maximum at the early secretory phase in the glandular epithelial cells, and reduces progressively throughout the mid and late secretory phases. Oestrogen receptor expression is at its maximum levels in both the glandular epithelium and stroma in the proliferative phase, and declines throughout the secretory phase (Snijders et al. 1992).

During the early secretory phase, a small amount of cell proliferation occurs and very little secretory material is produced by the epithelial cells. As the early secretory phase endometrium develops into mid secretory phase endometrium, epithelial and stromal cells are under the control of progesterone rather than oestrogen and cease to

proliferate and begin to differentiate. Glandular epithelial cells increase in diameter, and begin to produce a number of secretory products and cytokines in preparation for implantation. This stage of the cycle is also known as the window of implantation (Jansen et al. 1985; Dockery et al. 1988; Dockery 2002).

The mid-secretory phase is characterised by a loss of nuclear ER and PR expression in glandular epithelial cells, while an increase in expression is observed in stromal cells (Snijders et al. 1992). The glycoproteins secreted at this stage have been well characterised and described. These include cytokines which are critical to implantation, such as interleukin-11 (Dimitriadis et al. 2000), interleukin-6 (Tabibzadeh et al. 1995) and leukaemia inhibitory factor (LIF; Cullinan et al. 1996), which may support the invasion of the trophoblast at implantation (Dimitriadis et al. 2005). Several growth factors are also upregulated at this phase of the cycle, including EGF, which is strongly expressed by the stromal cells and may play a role in trophoblast invasion (Bass et al. 1994). Heparin-binding EGF-like growth factor and insulin-like growth factor binding protein-1 are both also upregulated at this stage in the cycle (Guzeloglu-Kayisli et al. 2009).

A component of vascular remodelling occurs during this phase of the cycle, which takes the form of development of the spiral arteries and subepithelial capillary plexus (Girling et al. 2005). This is thought to occur mainly by intussusception or sprouting rather than proliferation, as an increase in branching points has been described at the secretory phase of the cycle (Gambino et al. 2002). This vascular remodelling was demonstrated to be essential to endometrial function, as the administration of an angiogenesis inhibitor to mice resulted in failures of numerous reproductive processes including decidualisation and placental formation (Klauber et al. 1997). A wave of endothelial cell proliferation was shown to occur in the human endometrium in the mid-secretory phase (Ferenczy et al. 1979). This was not confirmed in the macaque studies described earlier, which showed increased proliferation only in the mid-proliferative phase of the cycle (Nayak et al. 2002), and has also not been confirmed in subsequent human studies (Girling et al. 2005). However a

progesterone-mediated increase in an isoform of VEGF has been demonstrated in the mid-late secretory phase of the cycle, which increases vessel permeability and so may be involved in the changes in vessel structure required for implantation (Ancelin et al. 2002).

As the late secretory phase develops, the major changes in the endometrium involve the stroma, as the glandular epithelial cells begin to regress and shrink at this stage. The epithelial cells cease to secrete glycoproteins; however the glands remain full of secretory products (Dockery et al. 1990). The stromal cells begin to undergo the pre-decidual reaction, which involves a reorganisation and loosening of the cells (Dockery et al. 1990). The stromal compartment is also important in remodelling events at this stage of the cycle due to the large influx of immune cells which occurs, of which over 50% is composed of an infiltration of uterine natural killer cells (uNK). These cells are often found in a cuff around the spiral arteries (Bulmer et al. 1991). The appearance of uNK cells at this point is likely induced by the stromal decidual reaction rather than the implanting blastocyst (Ordi et al. 2006), and uNK cells are implicated in both the preparation for pregnancy by the remodelling of spiral arteries and in modulation of the maternal immune response to the foetus. The importance of uNK cells in these processes has been demonstrated in mouse models which lack NK cells. In these mice, the decidual arteries do not undergo remodelling prior to implantation (Ashkar et al. 2000). Additionally it is postulated that uNK cells may interact with HLA-G present on trophoblast cells, to modify cytotoxic responses of uNK cells and other leukocytes to the trophoblast cells (Hunt et al. 2005). Other immune cells infiltrating at this stage include T cells, macrophages (Bulmer 1994) and neutrophils mediated by IL-8 (Critchley et al. 1994).

1.2.1.3 Menstruation

In the absence of implantation, progesterone levels fall in the late secretory phase and trigger menstruation. This is supported by experiments in animal models demonstrating that prolonged progesterone concentrations inhibit menstruation (Brenner et al. 2002). The morphological changes taking place at menstruation were

first observed by Markee (Markee 1940) by explanting a piece of endometrium to the anterior eye of a rhesus monkey. Tissue regression, vasoconstriction of the spiral arteries and degradation of the extracellular matrix was observed. The process of menstruation is now known to begin with the upregulation of a number of inflammatory mediators, for example macrophage chemoattractant protein-1 (Akiyama et al. 1999) and IL-8 (Critchley et al. 1999; Milne et al. 1999). Accompanying this increase in chemokine expression is a further leukocyte influx led by uNK cells, macrophages, neutrophils and eosinophils (Critchley et al. 1999). The leukocytes, endometrial stromal cells, or both may provide the proteases and MMPs which start the breakdown of tissue at menstruation, and indeed the release of MMPs by one cell type may activate the proteases released from the other. In stromal cells, the withdrawal of progesterone can up-regulate the expression of urokinase-type plasminogen activator, MMP-1, MMP-3 and MMP-9 (Bruner et al. 1995; Singer et al. 1997; Rigot et al. 2001). MMPs can also be produced by leukocytes, as neutrophils are a source of MMP-9 (Ardi et al. 2007) and macrophages and eosinophils produce MMP-9 (Jeziorska et al. 1996). The key role of neutrophils in menstruation has been demonstrated by the impaired onset of tissue breakdown in a mouse model deficient in neutrophils (Kaitu'u-Lino et al. 2007). The role of uNK cells may be more directed toward establishment of pregnancy than menstruation, however they do produce MT-MMP-1 (Salamonsen et al. 2000).

An upregulation of the prostaglandin-synthesising enzyme COX-2 is also seen at menstruation (Critchley et al. 1999). The subsequent increase in prostaglandin expression, particularly $PGF_{2\alpha}$, is responsible for vasoconstriction of the spiral arteries and myometrial contractions (Baird et al. 1996). The vasculature is also affected by the up-regulation of VEGF by the stromal cells at this time, which is potentially mediated by hypoxia driven by vasoconstriction (Popovici et al. 1999). VEGF signalling increases vascular permeability (Popovici et al. 1999), and a loss of endothelial cell–cell junctions at this time also causes a loss of vascular integrity (Tabibzadeh et al. 1996). Menstruation ends when levels of oestrogen begin to rise to regenerate the functionalis layer of the endometrium.

1.2.1.4 The post-menopausal endometrium

The post-menopausal endometrium is atrophic, being thinned and quiescent due to the changes in the hypothalamic-pituitary-gonadal axis (Sheth et al. 1993). Oestrogen receptor is expressed in all cellular compartments, while progesterone receptor is expressed in the glandular epithelium and smooth muscle but weakly in the stroma (Snijders et al. 1992).

1.3 Endometrial adenocarcinoma

1.3.1 Epidemiology and incidence

If the normal cycling endometrium or quiescent post-menopausal endometrium is affected by an imbalance of signals promoting growth or genetic alterations, cancer of the endometrium can occur. Endometrial adenocarcinoma, the neoplastic growth of endometrial epithelial cells, is the most common gynaecological malignancy in western countries (Doll et al. 2008). Incidence is estimated at 15-20 per 100 000 women per year worldwide, with predicted data for 2009 for the United States reaching an estimated 42 160 new cases and 7780 deaths (National Cancer Institute, NIH). Endometrial adenocarcinoma most commonly affects post-menopausal women, as approximately 85% of patients are over 50 years of age (Ellenson et al. 2004). Survival rates of endometrial adenocarcinoma are approximately 85% at five years, which is relatively high compared to other gynaecological tract cancers including ovarian and cervical (Di Cristofano et al. 2007). However this survival rate is attributed to the early detection of cancer due to presentation of symptoms including abnormal uterine bleeding, and therefore a hysterectomy can be performed before spread of disease. If the cancer has metastasised beyond the uterus, chemotherapy is rarely successful and prognosis is poor (Fleming et al. 2004). Risk factors for endometrial adenocarcinoma include post-menopausal oestrogen use (Grady et al. 1995), nulliparity (Albrektsen et al. 1995), late menopause onset (Kalandidi et al. 1996) and obesity (Sturgeon et al. 1998; Reeves et al. 2007).

1.3.2 Clinical features

Endometrial tumours are divided into well, moderately or poorly differentiated cancers histologically, based on tissue architecture and the amount of solid tumour present (Ellenson et al. 2004). A study by Bokhman in 1983 proposed that endometrial adenocarcinomas could be broadly divided into two types (Bokhman 1983). Type I endometrial adenocarcinoma is associated with patients displaying increased oestrogen levels and hyperlipidemia, and is often related to obesity. These tumours are the most common, making up approximately 85% of patients, and generally display low invasion and have a good prognosis. They are usually well or moderately differentiated tumours, however can progress to invasive and poorly differentiated tumours. Type II endometrial adenocarcinoma is independent of oestrogen stimulation, and are more aggressive, poorly differentiated tumours with a morphology consisting of cells growing in papillary patterns (Hendrickson et al. 1982). This dualistic model has been strengthened by more recent studies demonstrating that the two types of endometrial adenocarcinoma can be divided by morphology and genetic mutations (Tashiro et al. 1997; Lax et al. 2000; Catasus et al. 2009). Although there are overlapping features between the two types, this thesis concentrates only on Type 1 endometrial adenocarcinoma.

1.3.3 Common genetic and cellular changes leading to development of endometrial adenocarcinoma

It was proposed in a landmark review by Hanahan and Weinberg that there are six underlying principles leading to the development of cancers, which are functional abilities acquired by cancerous cells and shared by all types of cancer (Hanahan et al. 2000). The change from normal to malignant cells is a multistep process, ending in tumours displaying all of these “hallmarks of cancer”. This theory is supported in endometrial cancer as atypical hyperplasic lesions of the endometrium have been shown to develop into endometrial cancer (Kurman et al. 1985), potentially as a result of the cells acquiring more of these characteristics. These six acquired characteristics are based on genetic changes, and are: a self sufficiency in growth signals, evading apoptosis, insensitivity to anti-growth signals, tissue invasion and

metastasis, limitless replication potential and sustained angiogenesis (Hanahan et al. 2000). Endometrial adenocarcinoma displays genetic changes which result in the above features and are discussed below. More recently, a seventh hallmark of cancer has been proposed: inflammation (Figure 1.5; Colotta et al. 2009).

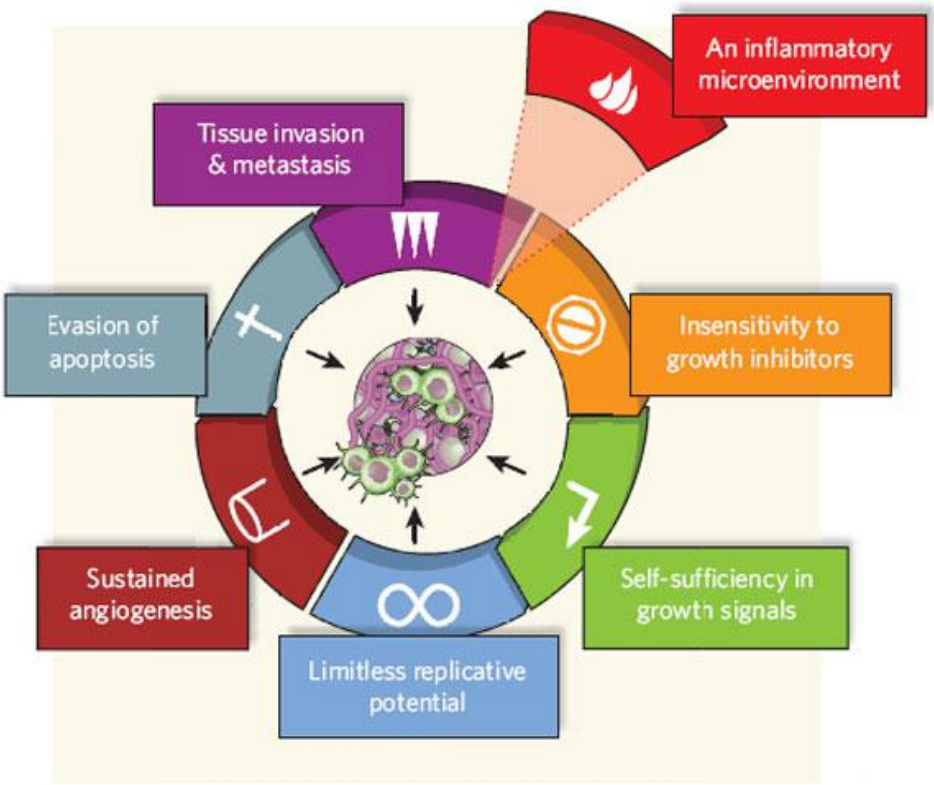

Figure 1.5 The hallmarks of cancer. A model of cancer development proposing that cells acquire six "hallmarks" during progression into cancer was proposed by Hanahan and Weinberg in 2000 (Hanahan et al. 2000). On the basis of recent evidence suggesting inflammation contributes to cancer, the seventh hallmark of an inflammatory microenvironment was recently proposed. Adapted from (Mantovani 2009).

One genetic alteration that has been linked to endometrial adenocarcinoma is a mutation in the tumour suppressor gene PTEN, leading to its inactivation (Tashiro et al. 1997). PTEN encodes the phosphatase and tensin homolog protein, which is a lipid phosphatase that downregulates phosphatidylinositol-(3,4,5)-triphosphate (PIP_3) by converting it into PIP_2. PIP_3 activates Akt signalling, therefore the common inactivation of PTEN found in endometrial adenocarcinoma can upregulate Akt signalling and subsequently impact on cellular proliferation, adhesion and migration

(Maehama et al. 1998). The mutation of this tumour suppressor gene can be seen in atypical endometrial hyperplasia, suggesting that it may occur early on or even lead to tumour progression (Levine et al. 1998). Furthermore, the conditional deletion of PTEN in the endometrium of a mouse model rapidly induced endometrial cancer formation (Daikoku et al. 2008). One of the first studies of PTEN in endometrial adenocarcinoma found that PTEN mutation was present in 50% of endometrial adenocarcinomas (Tashiro et al. 1997) and this figure is now estimated to be between 30-50% (Di Cristofano et al. 2007; Catasus et al. 2009). This is the highest reported frequency of PTEN mutation in any cancer type (Tashiro et al. 1997).

In endometrial adenocarcinoma, PTEN inactivation has been linked to an increased risk of metastasis (Salvesen et al. 2002). An additional hallmark of cancer that loss of PTEN may preclude is the evasion of apoptosis. In an *in vitro* model, the re-introduction of PTEN into endometrial adenocarcinoma cell lines including Ishikawa cells induced apoptosis, and prevented *ex vivo* tumour formation (Sakurada et al. 1999). In a mouse model, PTEN loss resulted in the upregulation of growth signals, as the increased levels of activated Akt led to an increase in ERα activation and therefore transcription of target genes which regulate proliferation (Vilgelm et al. 2006).

PTEN has been demonstrated to regulate levels of expression of the p53 protein, another tumour suppressor, by preventing its degradation. Therefore the loss of PTEN in endometrial adenocarcinoma may also disrupt p53 levels (Freeman et al. 2003). The p53 protein is a transcription factor which regulates a number of genes which can induce apoptosis in the event of cell damage. In endometrial adenocarcinoma, p53 mutation is present in approximately 15-30% of patients (Lax et al. 2000; Sakuragi et al. 2002) and is associated with a poor prognosis (Jeon et al. 2004; Catasus et al. 2009). This is potentially because p53 is more frequently mutated in poorly differentiated adenocarcinomas (Kohler et al. 1992; Lax et al. 2000). However the exact function of mutated p53 in endometrial adenocarcinoma is still unclear.

A further genetic mutation in endometrial adenocarcinoma that can lead to acquirement of many of the hallmarks of cancer is a mutation in the oncogene K-Ras (Enomoto et al. 1990). Mutations in this protein activate the extracellular signal-regulated kinase 1/2 (ERK1/2) signalling pathway, which is a mitogen-activated protein kinase (MAPK) pathway. The ERK-MAPK pathway is usually activated by signalling from G protein-coupled receptors, and is described in more detail in Section 1.4.1 and Figure 1.6. A mutation in K-Ras occurs in approximately 9-33% of endometrial adenocarcinomas (Lax et al. 2000). This mutation is also more commonly found in poorly differentiated cancers (Kohler et al. 1992). K-Ras mutations can cause constitutive activation of the ERK pathway in the absence of stimuli in endometrial adenocarcinoma, and therefore impact on downstream gene expression through the transcription of numerous genes promoting tumour progression (Mizumoto et al. 2007).The phenotypic effects of K-Ras mutations in endometrial adenocarcinoma are yet to be determined, but are likely to involve the activation of multiple pathways regulated by ERK signalling which can promote growth, migration and angiogenesis (Lax et al. 2000). Interestingly however, ERK phosphorylation has been associated with a good prognosis in endometrial adenocarcinoma (Mizumoto et al. 2007).

Other tumour suppressor genes and oncogenes commonly found to be mutated in endometrial adenocarcinoma include β-catenin (Fukuchi et al. 1998), Par-4 (Moreno-Bueno et al. 2007) and bax (Sakuragi et al. 2002).

1.3.4 Oestrogen and progesterone in endometrial adenocarcinoma

The normal endometrium undergoes regular morphological and structural changes in response to the hormones oestrogen and progesterone. These hormones have also been shown to play a role in endometrial adenocarcinoma. As described in section 1.2, oestrogen induces proliferation of endometrial epithelial cells while progesterone is thought to mainly oppose the actions of oestrogen and induce cell differentiation (Graham et al. 1997). Risk factors for endometrial adenocarcinoma mostly involve

an increase in oestrogen exposure or decrease in progesterone exposure, or both. For example, one of the major risk factors for endometrial adenocarcinoma is obesity. This is thought to increase oestrogen levels in post-menopausal women due to an increase in adipose tissue, which is rich in the enzymes promoting oestrogen formation (Akhmedkhanov et al. 2001). Other endometrial adenocarcinoma risk factors leading to prolonged oestrogen exposure include late menopause, oestrogen only contraceptives and oestrogen only hormone therapy. Factors reducing the risk of endometrial adenocarcinoma include combined contraceptive pills containing progesterone, and progestin treatments are often successfully used in the treatment of endometrial adenocarcinoma (Doll et al. 2008). Recent factors shown to decrease risk of endometrial adenocarcinoma, such as smoking, red meat consumption and coffee drinking, all promote a mechanism of oestrogen metabolism (Gunter 2009). Therefore, it may be that increased proliferation due to prolonged oestrogen exposure increases the risk of genetic mutations leading to endometrial adenocarcinoma. This has become known as the unopposed oestrogen hypothesis (Akhmedkhanov et al. 2001).

The oestrogen receptors (ER) and progesterone receptors (PR) belong to the steroid hormone nuclear receptor superfamily. There are two main isoforms of both ER and PR expressed in the endometrium: ERα, ERβ, PR-A and PR-B. Upon activation of ER α or β by ligand, the receptors translocate from the plasma membrane to the nucleus and form homo or hetero-dimers, which then bind to oestrogen response elements (ERE). This complex can than interact with other transcription factors to regulate gene transcription. The progesterone receptors act in a similar way, with hormone binding inducing dimerisation of PR-A and B and translocation to the nucleus. The progesterone receptor then binds to progesterone response elements (PRE) to initiate gene transcription. The expression of PR is also under the control of oestrogen, as oestrogen increases PR expression. Progesterone can also downregulate its own receptor (Graham et al. 1997).

In endometrial adenocarcinoma, a change in the ER α/β ratio has been reported with a decrease in ERα expression as compared to normal post-menopausal endometrium as well as a decrease from well through to poorly differentiated carcinomas (Saegusa et al. 2000; Jazaeri et al. 2001). The expression of ERα in endometrial adenocarcinoma has been associated with a less aggressive phenotype and prolonged patient survival (Ito et al. 2005), therefore the change in the ratio of receptor subtypes may be driving proliferation by altering gene transcription mediated through oestrogen. An alteration in PR isoform expression has also been demonstrated in endometrial adenocarcinoma. Most commonly, a loss of PR-A expression has been described but a decrease in PR-B expression in endometrial adenocarcinoma has also been reported (Jazaeri et al. 2001; Saito et al. 2006). However PR-B has also been recently reported to promote epithelial cell proliferation (De Vivo et al. 2002), therefore it may be that alterations in the PR-A to PR-B ratio may affect the anti-proliferative effect of progesterone.

Additionally, oestrogen signalling can activate a number of mitogenic pathways in the endometrium, and therefore potentially in endometrial adenocarcinoma. Oestrogen-mediated proliferation has been shown to be partially dependent on EGFR signalling, which can activate MAPK signalling and numerous growth pathways (Nelson et al. 1991). Oestrogen signalling can also upregulate transforming-growth factor (TGF) expression in endometrial epithelial cells, suggesting autocrine regulation of growth (Nelson et al. 1992). Oestrogen can also act through the Wnt signalling pathway in the normal endometrium, by increasing localisation of β-catenin to the nucleus and promoting growth (Hou et al. 2004). These therefore may be signalling pathways that are deregulated in endometrial adenocarcinoma.

1.3.5 Stem cells in endometrial adenocarcinoma

As discussed in section 1.2.1.1, the hypothesis that the regeneration of the endometrium after menstruation is driven by stem cells located in the basalis layer has gained strength in recent years with the isolation of putative stem cells from the endometrium (Gargett 2006; Gargett et al. 2009). The idea that cancers are initially

formed from tissue stem cells which due to genetic mutations are no longer under the control of local signals from the stem cell niche has also been strengthened with the isolation of stem cells from corneal, breast and gastrointestinal cancer (reviewed in Miller et al. 2005). Therefore, endometrial adenocarcinoma may arise from the uncontrolled growth of endometrial stem cells which have acquired the genetic mutations described above. Cells with markers of stem cells have recently been described in endometrial adenocarcinoma (Rutella et al. 2009).

1.3.6 The inflammatory microenvironment in endometrial adenocarcinoma

Recently, a seventh hallmark of cancer has been proposed: inflammation (Colotta et al. 2009). Epidemiological and molecular data has strengthened the hypothesis that chronic inflammation may initiate and worsen neoplastic transformation. Although there has been a recent revival of interest in the link between inflammation and cancer, this concept was first proposed in the 19th century by Virchow. After observing leukocyte influxes in cancers of tissues that experience chronic inflammation, he suggested that they may be the cause of tumour growth (Balkwill et al. 2001). Inflammation in cancer has now been proposed to function by two pathways – intrinsically and extrinsically. In the former, after the initiation of cancer by genetic mutations the expression of inflammatory agents is increased, leading to the promotion of tumour growth. In the extrinsic pathway, current inflammatory conditions lead to the initiation of cancer (Colotta et al. 2009).

Epidemiological data has strengthened the extrinsic inflammatory hypothesis of cancer initiation, as inflammatory diseases have been shown to predispose sufferers to certain types of cancer. For example, inflammatory bowel disease is associated with an increased risk of colon cancer (Flossmann et al. 2007), prostatitis is associated with an increased risk of prostate cancer (Sandhu 2008) and infection with certain microbes can lead to increased risk of gastric cancer (Suzuki et al. 2009). Additionally, long term intake of non-steroidal anti-inflammatory drugs (NSAIDS)

in patients at risk of developing these cancers significantly reduces cancer occurrence (Dannenberg et al. 2003; Flossmann et al. 2007).

The intrinsic pathway hypothesis states that after cancer development, several features of inflammation promote tumour progression. These include increased production of inflammatory mediators such as cytokines and prostaglandins, the presence of leukocytes, and extensive tissue remodelling including angiogenesis. These features will be discussed at a later point in this introduction. This hypothesis is strengthened by trials of antagonists of the pro-inflammatory cytokine tumour necrosis factor (TNF) in renal cancer patients. These trials have produced some promising prognostic results (Harrison et al. 2007), indicating that inflammatory pathways occurring in tumours maybe promoting their progression.

A number of studies have now linked endometrial adenocarcinoma and inflammation. Risk factors for endometrial adenocarcinoma including obesity, late menopause and reproductive disorders such as menorrhagia promote not only prolonged oestrogen exposure, but also inflammation (Modugno et al. 2005). Furthermore, the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) controls expression of a number of genes involved in the inflammatory and immune response. Increased localisation of NFκB to the nucleus has been detected in endometrial adenocarcinoma, implying an upregulation of NFκB signalling and hence inflammation (Pallares et al. 2004). Additionally, the K-Ras mutation frequently detected in endometrial adenocarcinoma has been demonstrated to promote production of inflammatory chemokines and cytokines (Sparmann et al. 2004). Finally, as previously mentioned, the menstrual cycle can be compared to a chronic inflammatory process. The production of some of the pro-inflammatory mediators normally produced in the endometrium may be increased in endometrial adenocarcinoma, and this is thought to promote inflammation (Jones et al. 1997).

1.3.7 Hormones and inflammation in the endometrium and endometrial adenocarcinoma

Oestrogen plays a key role in the development of endometrial adenocarcinoma, as demonstrated by the increase in oestrogen mediated by many of the risk factors (Akhmedkhanov et al. 2001). Oestrogen also plays a complex role in inflammation. In some cases, it is thought to act as an anti-inflammatory agent. This is based on evidence including the decreased risk of cardiovascular disease in pre-menopausal women and the decreased severity of inflammatory disease symptoms during pregnancy. However, women also show an increased incidence of autoimmune disease, indicating that pro-inflammatory functions of female sex hormones exist (Nilsson 2007). In the normal endometrium, oestrogen up-regulates a number of inflammatory cytokines. These include interleukin-6 (Jacobs et al. 1992), and production of this cytokine in an endometrial adenocarcinoma cell line after oestrogen stimulation has recently been demonstrated (He et al. 2009). Other inflammatory mediators upregulated by oestrogen include interleukin-1, TNF-α, and MMPs (Modugno et al. 2005; He et al. 2009), and it has also been shown to activate NFκB signalling, which controls a number of inflammatory pathways (Seo et al. 2004).

In the normal endometrium, progesterone can also influence production of a number of inflammatory mediators, however often with opposing anti-inflammatory actions to oestrogen. A number of *in vitro* studies demonstrate that progesterone can inhibit cytokine release from murine and human uterine cells (Ito et al. 1994; Kelly et al. 1994; Kelly et al. 1997). Many of these cytokines are under the control of NFκB. For example in a poorly differentiated endometrial adenocarcinoma cell line progesterone inhibits NFκB activation, by inducing accessory proteins which form a complex inhibiting NFκB activity (Davies et al. 2004). *In vivo* data has demonstrated that mice lacking PR have increased inflammatory responses in the uterus, with an increased infiltration of leukocytes and extensive tissue remodelling (Lydon et al. 1995). Therefore, as endometrial adenocarcinoma is characterised by increased

oestrogen to progesterone signalling, an increase in inflammatory mediators may occur which is not inhibited by progesterone, thus promoting tumour growth.

A number of these inflammatory pathways in cancer converge on the signalling pathway of the cyclooxygenase (COX) enzymes, and their production of inflammatory prostaglandins.

1.4 The prostaglandins

1.4.1 G-protein receptor coupled signalling

Before discussing prostaglandin signalling, a brief description of G-protein receptor (GPCR) coupled signalling is required. GPCRS are the largest family of cell membrane receptors, and are the target of 50-60% of all current therapeutic agents, demonstrating that irregular GPCR function is a major aspect of human disease. GPCRS have been implicated in promotion of cell proliferation, angiogenesis and inflammation in cancer (Dorsam et al. 2007). The common structure of all GPCRs consists of seven-transmembrane α-helices interacting intracellularly with a G-protein made up of 3 subunits – α, β and γ. The Gα subunit is associated with GDP, and upon ligand binding, the resulting conformational change in the receptor catalyses the transfer of GDP for GTP, leading to dissociation of Gα from βγ and the initiation of downstream signalling (Dorsam et al. 2007).

The Gα subunit is further divided into the G_q, G_s, G_i and $G_{12/13}$ families, of which more than one can couple to the same receptor. Each of these families begins signalling cascades in different manners. G_q activates the second messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG), G_s couples to stimulation of adenylyl cyclase and cAMP activation. G_i inhibits adenylyl cyclase and thus decreases cAMP production, and $G_{12/13}$ proteins couple to and activate the guanine-nucleotide exchange factors Rho (Pierce et al. 2002). Within these families further subdivision of proteins occurs, as does some degree of cross talk between pathways. For example G_q has been shown to activate the GTP-binding protein Rho by a different mechanism to that of $G_{12/13}$, leading to changes in the actin cytoskeleton mediating

cell migration (Chikumi et al. 2002). As the focus of this thesis is the FP receptor, which is G_q coupled, the key signalling pathways regulated by G_q signalling are represented in Figure 1.6 and described below.

One of the major signalling pathways deregulated in cancer and mediated by G_q-coupled receptors is the mitogen activated protein kinase (MAPK) phosphorylation cascade. This is often activated by mutated K-Ras, as described in Section 1.3.3. The signalling cascade begins with the activation of Ras, a small GTPase, by GPCR signalling. GTP-bound Ras can then begin a sequential phosphorylation cascade beginning with the serine/threonine kinase Raf, which phosphorylates MAPK ERK kinase (MEK), which phosphorylates ERK1/2. ERK translocates to the nucleus where it activates gene transcription through the regulation of a range of transcription factors including AP-1 and Ets (Roberts et al. 2007). MAPK signalling can also be activated by the epidermal growth factor receptor (EGFR). In this situation, GPCR signalling trans-activates the EGFR by initiating a tyrosine kinase (c-src) and matrix metalloprotease-mediated (Guerrero et al. 2004) cleaving of heparin bound–epidermal growth factor (HB-EGF). Src is itself activated through SH2 domain interactions with either an intracellular domain of the GPCR, the G-protein, or GPCR-associated proteins (Luttrell et al. 2004). The EGFR is a tyrosine kinase receptor which can phosphorylate the adaptor protein SHC, which forms a complex with the protein Grb2 and the nucleotide exchange factor Sos-1. Sos-1 can then activate Ras and begin the phosphorylation cascade leading to ERK activation (Pierce et al. 2001). These two main MAPK signalling pathways, and inhibitors used in this thesis which can block steps of the pathway, are outlined in Figure 1.6.

The nuclear factor of activated T cells (NFAT) family of transcription factors is linked to promotion of inflammatory gene expression due to its regulation of expression of a number of cytokines, and its functional relationship to the NFκB family of transcription factors (Hogan et al. 2003). Upon agonist stimulation of a G_q-coupled receptor, phopspholipase C mediates hydroysis of phosphatidyl inositol-bisphosphate to IP_3, which then activates IP_3 receptors on the endoplasmic reticulum

to release calcium (Ca^{2+}). Ca^{2+} binds the protein calmodulin which activates calcineurin by binding and changing its conformation, therefore releasing it from an inactive state (Klee et al. 1998). Calcineurin is a serine / threonine phosphatase which dephosphorylates and therefore activates NFAT (Klee et al. 1998). The pathway can be inhibited at this point by the actions of the protein regulator of calcineurin 1-4 (RCAN1-4), which binds to calcineurin and prevents its interaction with NFAT. RCAN1-4 can be directly phosphorylated by MEK, indicating cross talk occurs between these pathways (Harris et al. 2005). Five NFAT proteins exist, NFAT1-5, and after their dephosphorylation these rapidly translocate to the nucleus and bind to NFAT-sites on DNA to activate gene transcription. The gene activity regulated by NFAT depends in part on binding partners such as AP-1 (Macian et al. 2001).

NFκB is related evolutionarily and functionally to NFAT, as they bind to structurally highly related DNA binding domains and both activate a number of cytokines involved in the immune response (Serfling et al. 2004). The pathway leading from G_q to NFκB has been elucidated through chemical inhibitor studies, demonstrating that phosphatidylinositol (PI) 3-kinase (PI3K) and Akt signalling pathway is important in its regulation (Ye 2001). After generation of PIP_3, the pleckstin homology domain of Akt binds to this and allows phosphorylation of Akt by phosphoinositide dependent kinase-1. Akt then activates the IκB kinases (IKK) which phosphorylates the inhibitory IκB, leading to its dissociation from NFκB and the translocation of NFκB to the nucleus to initiate gene transcription (Ye 2001).

Interacting with some G_q coupled pathways is the protein kinase A (PKA). It has been demonstrated to interact directly with GPCRs, to mediate a down-regulation of their signalling by phosphorylation of the receptor to dissociate it from the G protein (Pierce et al. 2002). It has also been shown to trans-activate the EGFR by direct phophorylation (Barbier et al. 1999), and phosphorylate the IP_3 receptor to increase Ca^{2+} release (Bugrim 1999). PKA therefore has both inhibitory and stimulatory effects on GPCR signalling.

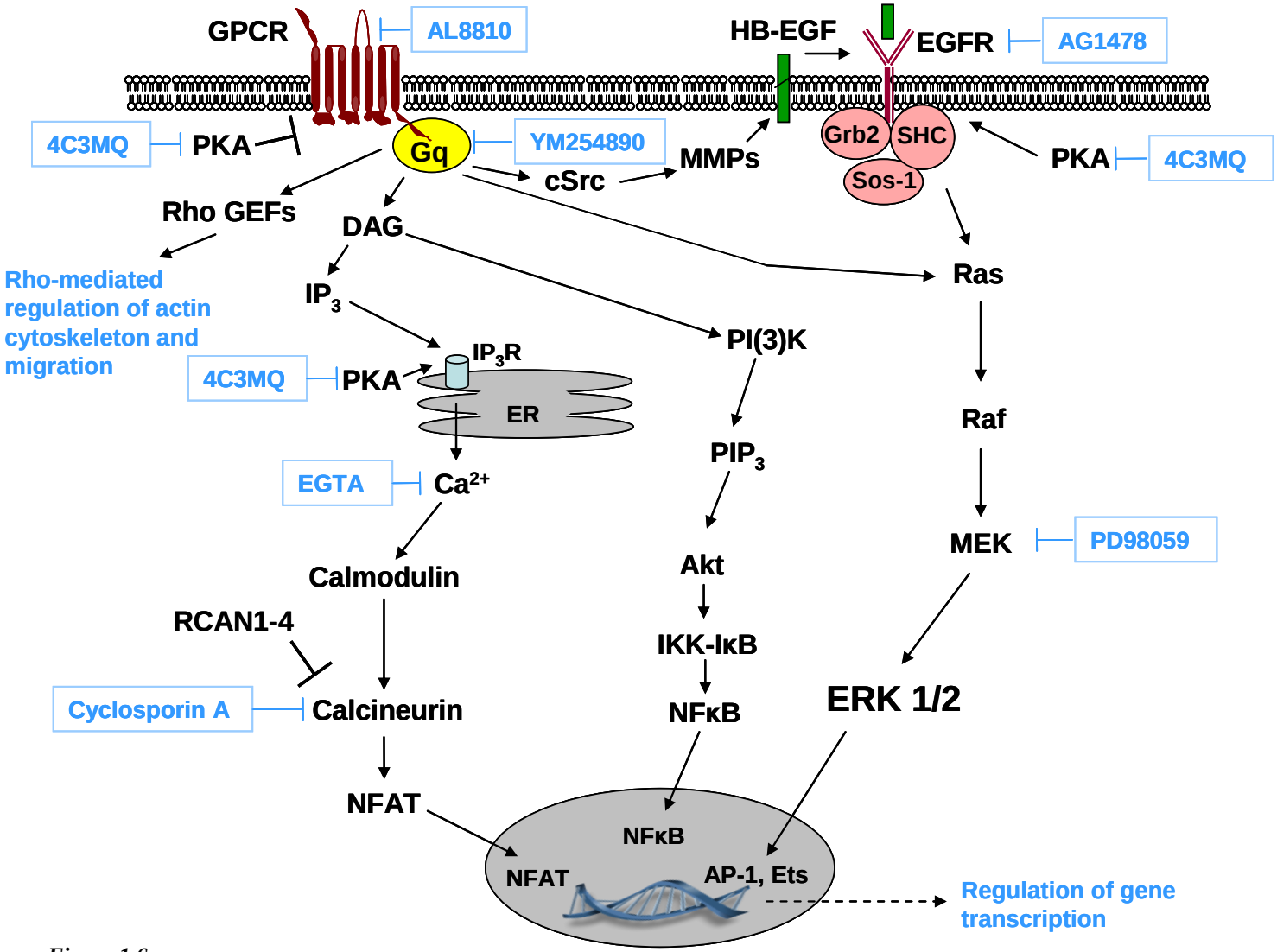
GPCR
AL8810
HB-EGF
EGFR
AG1478
4C3MQ
PKA
Gq
YM254890
MMPs
Grb2
SHC
Sos-1
PKA
4C3MQ
cSrc
Rho GEFs
DAG
Ras
Rho-mediated regulation of actin cytoskeleton and migration
IP3
IP3R
4C3MQ
PKA
PI(3)K
ER
Raf
PIP3
EGTA
Ca2+
MEK
PD98059
Calmodulin
Akt
RCAN1-4
IKK-IκB
ERK 1/2
Cyclosporin A
Calcineurin
NFκB
NFAT
NFκB
NFAT
AP-1, Ets
Regulation of gene transcription

Figure 1.6

Figure 1.6 Schematic representation of key signalling pathways activated by G_q-coupled receptor signalling. Antagonists used in this thesis are indicated in blue boxes. Agonist stimulation of G_q coupled receptors can lead to activation of the ERK 1/2, NFAT, NFκB or Rho signalling pathways. In the ERK1/2 pathway, G_q can directly activate the small GTPase Ras, which begins a phosphorylation cascade of the proteins Raf and MEK finally resulting in ERK translocation into the nucleus to initiate gene transcription. Alternatively, GPCR activation can lead to the activation of c-src and matrix metalloproteases, which cleave heparin-bound EGF to transactivate the EGFR. The phosphorylation cascade leading to ERK activation then begins. The NFAT signalling pathway begins with PLC mediated hydrolysis of phosphatidyl inositol-bisphosphate to IP_3, which then activates IP_3 receptors on the endoplasmic reticulum (ER) to release calcium (Ca^{2+}). Ca^{2+} binds the protein calmodulin which activates calcineurin through release of auto-inhibition. Calicineurin is a serine / threonine phosphatase which acts to dephosphorylate and therefore activate NFAT. The pathway can be inhibited at this point by the actions of RCAN1-4, which binds to calcineurin and prevents its interaction with NFAT. Dephosphorylation of NFAT results in translocation to the nucleus and activation of gene transcription. The gene activity regulated by NFAT depends in part on binding partners such as AP-1. NFκB is activated by the PI3K generation of PIP_3. The protein Akt binds to this which allows its phosphorylation by phosphoinositide dependent kinase-1. Akt then activates the IκB kinases (IKK) which phosphorylate IκB, leading to its dissociation from NFκB and the translocation of NFκB to the nucleus to initiate gene transcription. PKA can interact with numerous proteins in this pathway. It interacts directly with GPCRs to mediate a down-regulation of their signalling by phosphorylation. It also trans-activates the EGFR and phosphorylates the IP_3 receptor to increase Ca2+ release. G_q has recently also been shown to activate the GTP-binding protein Rho, leading to changes in the actin cytoskeleton mediating cell migration.

1.4.2 Prostaglandin formation and signalling

Prostaglandins are bioactive lipids, formed from COX enzyme mediated metabolism of the membrane fatty acid arachidonic acid. The prostaglandins are a member of the eicosanoid family, which is also made up of leukotrienes and thromboxanes.

Prostaglandins were originally discovered in 1930 in human semen, and are so named due to the belief that they originated in the prostate. It was subsequently

demonstrated that prostaglandins could be isolated from nearly all tissues, and in 1964 their formation from arachidonic acid was reported (Rouzer et al. 2005). Arachidonic acid is released from the plasma membrane or dietary fatty acids by the action of phospholipase A2 or C. Two sequential reactions are then performed by the COX enzymes which take place at two distinct sites on the enzyme. Arachidonic acid is first cyclised and oxygenated to the hydroperoxy endoperoxide prostaglandin G_2 (PGG_2). This is then reduced into the hydro endoperoxide PGH_2 (Rouzer et al. 2005). A series of prostanoid synthase enzymes then produce either PGD_2, PGE_2, $PGF_{2\alpha}$, prostacyclin or thromboxane, and are named dependent on what prostanoid they produce. The synthase enzymes are differentially expressed in different tissues, and the balance of these can determine the main prostaglandin type formed (Helliwell et al. 2004). Prostaglandins are then transported out of the cell by a prostaglandin receptor (Reid et al. 2003), and act in a paracrine or autocrine manner on their corresponding G-protein coupled receptors (DP, EP1-4, FP, IP and TP respectively). The prostaglandin receptors couple to different G-proteins, as the EP2, EP4, IP and DP receptors couple to G_s and signal using cAMP as a second messenger. EP1, FP and TP couple to G_q to activate IP_3 and calcium signalling. The G-protein coupling to EP3 appears to depend on splice variant and cell type (Breyer et al. 2001). Figure 1.7 below summarises arachidonic acid metabolism and prostaglandin signalling.

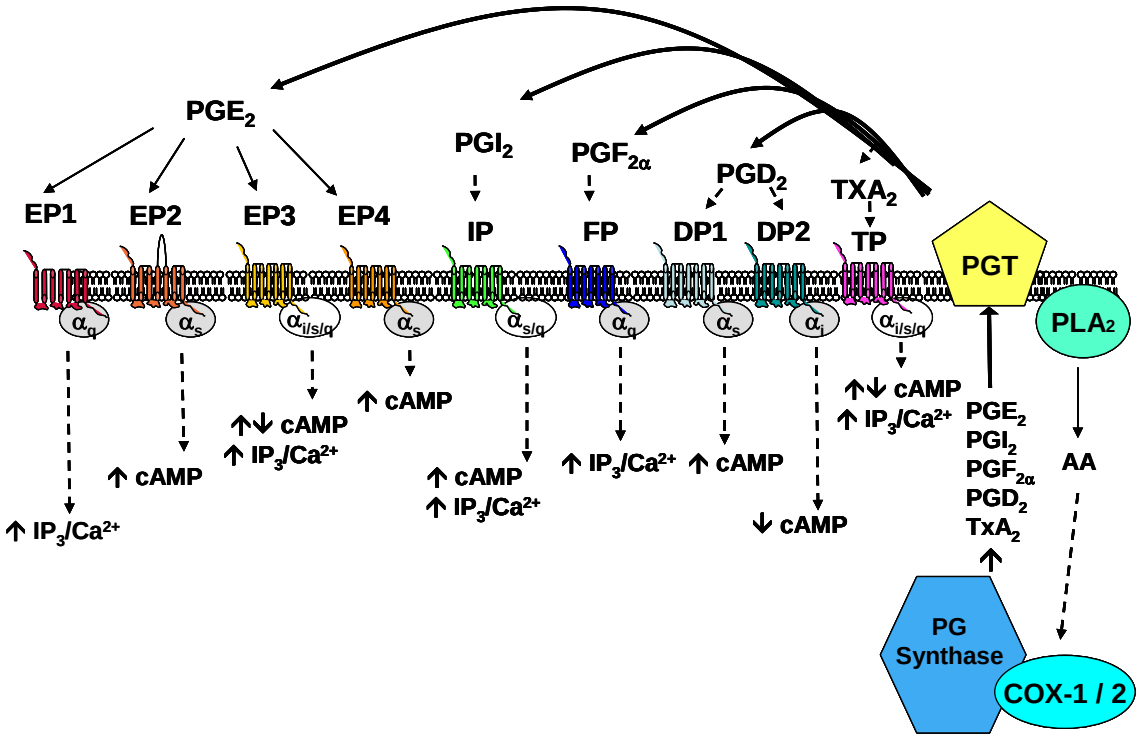

Figure 1.7 Schematic representation of arachidonic metabolism and prostanoid signalling pathways. Arachidonic acid (AA) is released from membrane lipids by the enzyme phospholipase A2 (PLA2). The enzymes cyclooxygenase (COX) 1 or 2 and specific prostaglandin synthases then catalyse reactions converting AA into prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2) or thromboxane (TXA). The prostaglandins are transported out of the cell by a prostaglandin transporter (PGT) and act in a paracrine or autocrine manner on their corresponding G-protein coupled receptors and activate the second messengers inositol trisphosphate (IP_3), calcium (Ca^{2+}) or cAMP and subsequent downstream events. Adapted from Jabbour et al. 2004.

The COX enzyme exists in two main isoforms, COX-1 and COX-2. COX-1 expression varies little throughout the body and is considered a constitutive enzyme. However, COX-2 can be regulated by growth factors, cytokines, and mechanical stress (Morita 2002). COX-2 expression is also increased in many cancer types including endometrial adenocarcinoma (Tong et al. 2000; Jabbour et al. 2001) and in

inflammation (Morita 2002). COX-1 expression is not altered in endometrial adenocarcinoma (Tong et al. 2000), however upregulation has recently been demonstrated in other cancer types including cervical cancer (Sales et al. 2002).

In the female reproductive tract, the COX enzymes and prostaglandins play an important role. The COX-2 knockout mouse (but not COX-1) displays reproductive failures at the stages of ovulation, fertilisation, implantation and decidualisation (Lim et al. 1997). Mice lacking the FP and EP receptors have demonstrated that these prostaglandins play an important role in reproduction. Knock out of EP2 results in mice which are infertile due to defects in ovulation (Kennedy et al. 1999; Tilley et al. 1999) and fertilisation (Hizaki et al. 1999). The FP receptor also plays a key role in reproduction, as demonstrated by the FP knock out mouse. Despite a normal estrous cycle and early pregnancy, the FP receptor knock out mouse fails to undergo parturition, due to a failure to initiate luteolysis which normally decreases progesterone levels (Sugimoto et al. 1997). $PGF_{2\alpha}$ is a smooth muscle contractant, and can induce contractions in the uterus and vasculature which may also play a role in parturtion (Senior et al. 1992). In the human endometrium, expression of COX-2 (Jones et al. 1997; Uotila et al. 2002) and FP receptor increases in the proliferative phase of the cycle (Milne et al. 2003). As described in section 1.2.1.1, this is coincident with a period of angiogenesis, and prostaglandin signalling through these receptors has been demonstrated to increase the expression of angiogenic factors in endometrial cells including FGF and VEGF (Sales et al. 2004; Sales et al. 2005; Sales et al. 2007).

1.4.3 The FP receptor

The human FP receptor was originally characterised in tissues such as myometrium and corpus luteum by pharmacological and functional assays studying parameters including binding affinity (Powell et al. 1976; Senior et al. 1992). It was cloned in 1994 by Abramovitz et al (Abramovitz et al. 1994), and determined to be made up of 359 amino acid residues. FP is a G_q-protein coupled receptor activating IP_3 and calcium as second messengers (Davis et al. 1987; Black et al. 1990). In sheep, a

functional splice variant of the FP receptor able to activate IP_3 was cloned in 1997 (Pierce et al. 1997). More recently, six isoforms of the FP receptor have been detected in human tissues such as heart and placenta, named altFPs (Vielhauer et al. 2004; Liang et al. 2008). These splice variants show no detectable activity and do not bind $PGF_{2\alpha}$ alone, however can form heterodimers with the FP receptor which result in IP_3 and calcium accumulation. As yet the signalling implications of this are unclear (Liang et al. 2008). Similarly to most GPCRs, continued $PGF_{2\alpha}$ signalling can result in FP internalisation (Srinivasan et al. 2002), and FP has been demonstrated to localise to plasma membrane, cytoplasm and the nuclear envelope in some cell types (Milne et al. 2003; Schlotzer-Schrehardt et al. 2002). $PGF_{2\alpha}$ can also bind with low affinity to the EP1 and EP3 receptors (Kiriyama et al. 1997), and PGD_2 can bind with low affinity to FP (Abramovitz et al. 1994).

Numerous physiological functions have been reported as a result of FP receptor signalling. In FP-transfected HEK-293 cell lines, $PGF_{2\alpha}$ signalling leads to re-organisation of the cell cytoskeleton, indicating FP signalling can lead to changes in cell morphology and movement (Pierce et al. 1999). Additionally, FP activation has been demonstrated to perturb cell cycle progression by delaying progression into mitosis, and extended activation generated aneuploidy (Chen et al. 2006). In this cell type, FP receptor signalling activates the ERK signalling cascade (Xu et al. 2008) which can then activate cross-talk with β-catenin signalling (Xu et al. 2008; Xu et al. 2009).

Deregulated $PGF_{2\alpha}$-FP signalling has been shown to contribute to endometrial pathologies including dysmenorrhoea and endometriosis. In dysmenorrhoea, elevated $PGF_{2\alpha}$ levels are present in menstrual fluid (Stromberg et al. 1984), which may play a role in affecting the vasculature and contractions of the uterus. Women with endometriosis, the growth of endometrial tissue outside of the uterine cavity, have increased COX-2 (Ota et al. 2001) and $PGF_{2\alpha}$ metabolite expression (Sharma et al. 2009), which may be resulting in the high levels of angiogenic factors seen in the peritoneal fluid (Gazvani et al. 2002). The FP receptor is expressed in prostate cancer

(Krishnan et al. 2007) and recent data from our laboratory have demonstrated that FP receptor expression is elevated in endometrial adenocarcinoma (Sales et al. 2004; Sales et al. 2005). COX-2 is also increased in endometrial adenocarcinoma, and an upregulation of COX-2 has been associated with mutated PTEN and NFκB expression in endometrial adenocarcinoma cell lines, linking genetic mutations to increased inflammation driven by the prostaglandins (St-Germain et al. 2004). One of the downstream effects of FP signalling is an increase in COX-2 expression (Fujino et al. 2003), therefore this positive feedback loop may amplify $PGF_{2\alpha}$ signalling in endometrial adenocarcinoma (Jabbour et al. 2005).

In endometrial adenocarcinoma, the increase in COX-2 and FP receptor expression leads to the activation of a number of signalling pathways which results in the upregulation of angiogenic genes. For example, the expression of VEGF (Sales et al. 2005) and FGF2 (Sales et al. 2007) is upregulated in endometrial adenocarcinoma cell lines and tissue explants after $PGF_{2\alpha}$-FP receptor signalling. This was shown to be dependent on ERK1/2 trans-activation by the EGFR (Sales et al. 2004; Sales et al. 2005; Sales et al. 2007). $PGF_{2\alpha}$ activation of its receptor also promotes proliferation, adhesion and migration of endometrial adenocarcinoma cells (Milne et al. 2003; Sales et al. 2008), demonstrating that it may promote tumourigenesis of endometrial adenocarcinoma by a number of mechanisms. A summary of FP receptor signalling in endometrial adenocarcinoma is given in Figure 1.8.

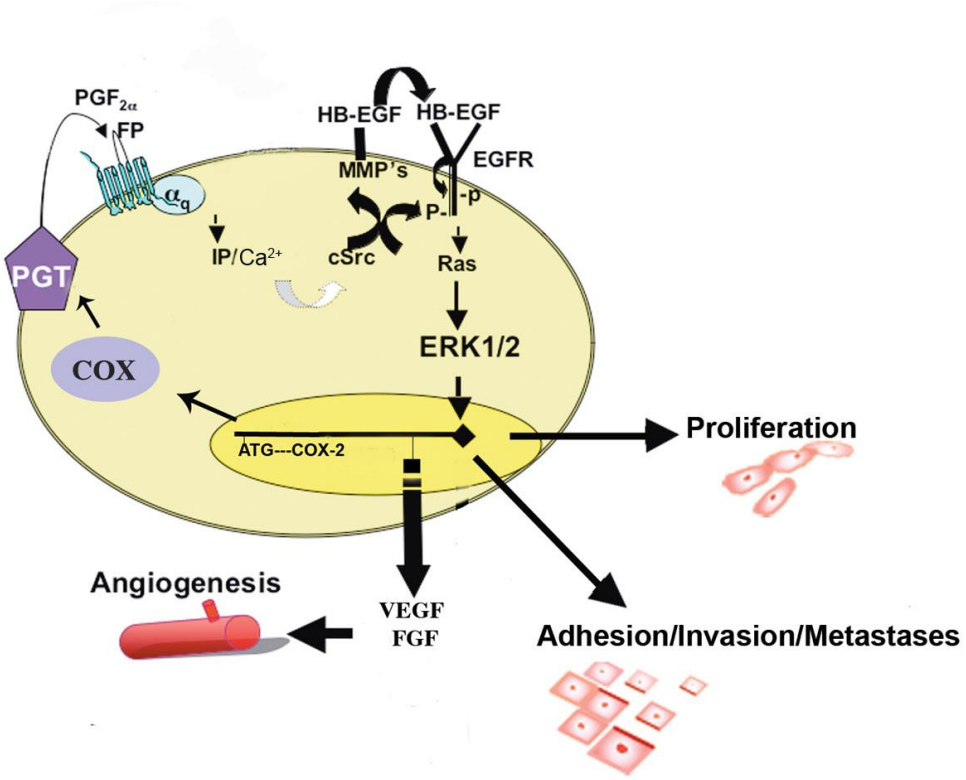

Figure 1.8 Schematic representation of FP receptor signalling in endometrial adenocarcinoma cells. $PGF_{2\alpha}$ signalling activates the FP receptor, which is coupled to G_q and activates IP_3 and calcium (Ca^{2+}) as second mesengers. A c-Src and MMP mediated mechanism then cleaves heparin-binding EGF (HB-EGF) which transactivates the EGFR and begins the ERK phosphorylation cascade leading to the transcription of genes involved in angiogenesis (VEGF and FGF), proliferation and migration (adhesion, invasion and metastases). FP receptor signalling can also upregulate expression of COX-2, leading to the formation of $PGF_{2\alpha}$ which is transported out of the cell by a prostaglandin transporter (PGT) and completes the autocrine signalling pathway. Adapted from Jabbour et al. 2006.

1.4.4 The FP receptor and inflammation

Prostaglandins are potent inflammatory mediators. The NSAIDs used to inhibit inflammation are COX-2 inhibitors, and therefore used to prevent prostaglandin formation (Cha et al. 2007). The COX-2 knock out mouse displays an altered inflammatory response, with a decreased leukocyte response to areas of necrosis (Dinchuk et al. 1995). In endometrial adenocarcinoma the activation of the transcription factor NFκB, which is associated with inflammatory pathways,

upregulates COX-2 expression and therefore prostaglandin formation in response to inflammation (St-Germain et al. 2004). The hormonal change to a low progesterone to oestrogen ratio associated with endometrial adenocarcinoma development may also promote prostaglandin formation and inflammation, as progesterone is able to inhibit inflammatory-induced $PGF_{2\alpha}$ expression (Ishihara et al. 1995).

As well as being activated by inflammation, $PGF_{2\alpha}$ – FP receptor signalling can promote an inflammatory environment. Angiogenic factors produced by $PGF_{2\alpha}$ – FP signalling can lead to tissue remodelling, which is one aspect comprising an inflammatory microenvironment (Colotta et al. 2009). Additionally, the expression of cytokines including IL-6 and IL-8 can be regulated by $PGF_{2\alpha}$ – FP signalling (Noguchi et al. 2001; Chang et al. 2009). Aspects of cytokine signalling in inflammation and cancer including leukocyte recruitment and tissue remodelling will be discussed in the next section.

1.5 Cytokines and chemokines

Cytokines are small peptides (approximately 8 kDa) released by cells that have numerous functions including acting as growth signals and chemotactic agents. Chemokines are a subfamily of cytokines, so named for their chemoattractant properties. The chemokine family is divided into four groups based on the position of two cysteine molecules (C) and any other amino acid (X) in the amino terminal of the protein. The groups are known as C, C-C, C-X-C and C-X3-C (Murphy et al. 2000). The C-X-C family is further subdivided into ELR (glutamine-leucine-arginine) positive or negative chemokines, dependent on the presence or absence of this amino acid motif. ELR+ chemokines have angiogenic properties, whilst ELR- chemokines are angiostatic (Strieter et al. 1995). Chemokines bind to GPCRs to activate signalling, and these receptors are present on immune cells as well as on cells making up other tissues of the body (Murphy et al. 2000). A link between prostaglandins and chemokines has been previously demonstrated further to the expression of chemokines mediated by FP receptor signalling, as COX-2 expression

has been colocalised with interleukin-8 and MCP-1 in the normal endometrium (Jones et al. 1997). COX-2 has also been colocalised with the expression of chemokines promoting leukocyte infiltration in bladder cancer (Chen et al. 2009).

1.5.1 Cytokines and chemokines in the normal endometrium

Cytokines and chemokines produced throughout the normal menstrual cycle are thought to be involved in tissue remodelling of the endometrium as well as the control of leukocyte influx (Kelly et al. 2001). These features mean that menstruation resembles an inflammatory process, in which chemokines play a major part (Critchley et al. 2001). The endometrial stroma is made up of a mixture of cells, including macrophages, neutrophils, natural killer (NK) cells and lymphocytes.

During the proliferative phase, a number of angiogenic chemokines are produced by the endometrium which may contribute to the large amount of vascular remodelling that occurs at this phase of the cycle. These include ELR+ chemokines such as interleukin-8, which is expressed in the epithelial cells (Arici et al. 1998) and in peri-vascular cells (Critchley et al. 1994). MCP-1 is also produced in the stroma and perivascular cells (Jones et al. 1997). CCL-5 (also known as RANTES) is chemotactic for monocytes and activated T cells, and is expressed in the proliferative endometrial stroma (Hornung et al. 1997). The upregulation of these cytokines causes the relatively low infiltration of macrophages and neutrophils seen at this stage of the cycle (Kelly et al. 1994). Macrophages secrete a range of angiogenic factors (Lin et al. 2006) which includes VEGF in the endometrium (Gargett et al. 2001), while the neutrophils at this stage of the cycle are observed adjacent to the vasculature and produce VEGF, and therefore may be involved in vascular remodelling of the endometrium (Gargett et al. 2001).

The early secretory phase is characterised by a rise in progesterone levels, and as progesterone is anti-inflammatory it suppresses the production of chemokines in the endometrium at this stage. A marked decrease in IL-8 and MCP-1 expression is observed (Jones et al. 1997). However during the mid secretory phase, also known as

the window of implantation, a number of cytokines are upregulated to aid implantation, including IL-11 (Dimitriadis et al. 2000), LIF (Cullinan et al. 1996) and IL-6 (Tabibzadeh et al. 1995). These factors are thought to aid adhesion of the blastocyst to the endometrium (Marwood et al. 2009). A gene array carried out at the mid secretory phase of the menstrual cycle demonstrated the chemokines including MCP-3 and MIP-1β that are present in the endometrium at this stage of the cycle (Jones et al. 2004). These may also play a role in chemoattraction of the trophoblast cells, however as no chemokine or chemokine receptor knock out mouse has yet displayed a reproductive phenotype, there may be a high degree of redundancy between these chemokines (Power 2003).

In the late secretory phase, the decrease in progesterone concentrations caused by the breakdown of the corpus luteum results in a further rise in chemokine expression (Jones et al. 1997). A large population of uNK cells appears in the stroma of the endometrium (King et al. 1989). This is coincident with the increase of a number of cytokines which may chemoattract uNK cells, including IL-15 (which also promotes their proliferation), macrophage inflammatory protein-1α (Kitaya et al. 2003), and MCP-1 (Jones et al. 1997). At this stage in the cycle, and during the menstrual phase, most chemokine production is driven by leukocytes (Jones et al. 2004). At menstruation degradation of the extracellular matrix occurs, mediated partly by chemokines and leukocytes. This has been demonstrated by the irregular endometrial breakdown and repair in a mouse model deficient in neutrophils (Kaitu'u-Lino et al. 2007). MIP-1α is released from the glandular epithelium which promotes infiltration of macrophages, which may contribute to the tissue destruction and promotion of apoptosis seen at menstruation (Akiyama et al. 1999). Therefore chemokine expression and leukocyte infiltration is a normal inflammatory process in the menstrual cycle, which may become deregulated in endometrial adenocarcinoma.

1.5.2 Chemokines in cancer

The upregulation and tumour-promoting effects of chemokines have been demonstrated in several types of cancer, including prostate, ovarian and breast

(Moore et al. 1999; Yang et al. 2006 ; Yao et al. 2007; Ghadjar et al. 2008). Originally, the role of chemokines in cancer was thought to be restricted to the chemo-attraction of immune cells, however recent evidence has demonstrated that chemokines also promote cell proliferation, cell migration and angiogenesis (Strieter et al. 2006). This can lead to extensive tissue remodelling and inflammation, contributing to tumour progression (Mantovani et al. 2008). A number of chemokines are upregulated in different cancers, however the key chemokines that are focused on in this thesis are chemokine (C-X-C) motif ligand 1 (CXCL1) and chemokine (C-C) motif ligand 20 (CCL20).

1.5.2.1 CXCL1

CXCL1 (also known as growth-regulated oncogene α) is an ELR+ member of the C-X-C family of chemokines with potent angiogenic, chemoattractant and inflammatory activities (Strieter et al. 2006). CXCL1 mRNA expression has previously been documented in stromal cells of the normal endometrium (Nasu et al. 2001) however its expression and localisation in endometrial adenocarcinoma has not previously been described. CXCL1 was first identified in the conditioned medium of a malignant melanoma cell line and named melanoma growth-stimulatory activity (Richmond et al. 1985), and has since been shown to be produced by several cancer types. The proteins CXCL2 and CXCL3 were subsequently isolated, and demonstrated to have 90% and 86% sequence homology respectively to CXCL1 (Haskill et al. 1990). CXCL1 binds to the CXCR2 receptor (Addison et al. 2000) to promote the recruitment of neutrophils to sites of inflammation (Moser et al. 1990). CXCR2 signalling can be inhibited by pertussis toxin, suggesting coupling to $G_{i/0}$ (Hall et al. 1999). CXCL2 and CXCL3 also bind to CXCR2, however whether they play a similar role in cancer to CXCL1 is unclear. CXCL2 may have contrasting functions as it has been demonstrated to be a negative regulator of epithelial cell growth in colon cancer (Li et al. 2004). CXCL3 promotes melanoma growth in a nude mouse model (Haghnegahdar et al. 2000) and is correlated with invasiveness in prostate cancer (Engl et al. 2006), and therefore may perform a similar function to CXCL1.

CXCL1 contributes to cancer growth in different ways, and due to the presence of the ELR motif one such mechanism is angiogenesis. In prostate cancer, CXCL1 expression is increased (Moore et al. 1999). In an *in vivo* model of prostate cancer, this increase was shown to contribute to tumour growth by increasing angiogenesis (Moore et al. 1999). CXCL1 also promotes angiogenesis in colorectal cancer (Wang et al. 2006) and melanoma (Haghnegahdar et al. 2000; Singh et al. 2009). Its expression is also upregulated as compared to normal tissue in both of these cancer types (Li et al. 2004; Haghnegahdar et al. 2000; Wen et al. 2006; Rubie et al. 2008). An increase in expression of CXCR2 can also promote tumour progression, as demonstrated by the decreased angiogenesis and tumour growth displayed in lung cancer xenografts of cell lines deficient in CXCR2 (Keane et al. 2004).

CXCL1 has also been described to perform other functions in cancer. In bladder cancer, CXCL1 expression is increased and associated with greater invasive ability (Kawanishi et al. 2008), and the increased expression in colon cancer is also associated with metastasis (Li et al. 2004). This may be attributable to its ability to downregulate the expression of fibulin-1, an extracellular matrix protein, in a colon cancer cell line, thus promoting migration (Wen et al. 2006). CXCL1 can also increase proliferation of cancer cells. It is upregulated in oesophageal cancer and acts via an autocrine signalling pathway to promote cell proliferation (Wang et al. 2006; Wang et al. 2009). In ovarian cancer xenografts, CXCL1 promotes cell proliferation via paracrine signalling to stromal fibroblasts (Yang et al. 2006). Interaction between CXCL1 and p53 has recently been demonstrated, as mutant p53 can bind to the CXCL1 promoter and increase CXCL1 expression in colon cancer cell lines (Yan et al. 2009). Several cell signalling pathways are involved in the induction of CXCL1 in different cancer types including ERK 1/2 (Wang et al. 2000; Wang et al. 2006; Yang et al. 2006) and p38 MAPK- NFκB signalling (Ward et al. 1999).

Chemokines are also potent immune cell attractants. CXCR2 is expressed highly on neutrophils and at lower levels on monocytes and macrophages (Murphy et al. 2000). The functions of immune cells in cancer will be discussed in Section 1.6.

1.5.2.2 CCL20

CCL20 (also known as macrophage inflammatory protein-3α) is a member of the CC-family of chemokines. CCL20 binds to the CC-receptor 6 (CCR6), a G_q-protein coupled receptor (Al-Aoukaty et al. 1998) that is expressed on dendritic cells, B and T lymphocytes, and tissues including spleen, lymph nodes, appendix and pancreas. CCL20 is the only chemokine known to interact with the CCR6 receptor (Schutyser et al. 2003).

Similarly to CXCL1, the expression of CCL20 and CCR6 has been described in several different types of cancer. CCL20 is over-expressed in cancers of the colon (Brand et al. 2006) liver (Rubie et al. 2006), pancreas (Campbell et al. 2005), prostate (Ghadjar et al. 2008) and Hodgkins lymphoma (Baumforth et al. 2008). The receptor CCR6 is also upregulated in colon (Brand et al. 2006), liver (Dellacasagrande et al. 2003) and prostate cancer (Ghadjar et al. 2008).

Within these cancer types, CCL20-CCR6 signalling has been proposed to influence epithelial cell migration or metastasis (Dellacasagrande et al. 2003; Campbell et al. 2005; Rubie et al. 2006) and proliferation (Brand et al. 2006; Beider et al. 2009). In pancreatic cancer tissue, CCL20 and CCR6 were co-localised to the same cells, and in a pancreatic cancer cell line this autocrine signalling caused a significant increase in cell invasion (Kimsey et al. 2004). This was discovered to be dependent on an upregulation of MMP-9 by CCL20-CCR6 signalling (Campbell et al. 2005). Increased migration was also demonstrated in colorectal cancer cells after autocrine CCL20-CCR6 signalling (Brand et al. 2006). Increased invasiveness and metastasis were also associated with CCL20 or CCR6 expression in nasopharyngeal cancer (Chang et al. 2008), prostate cancer (Ghadjar et al. 2008) and liver cancer (Rubie et al. 2006).

CCR6 expression can influence metastasis in some cancer types. Increased CCR6 expression in plasmacytoma cells in a mouse model led to a higher incidence of liver metastasis. This was found to be due to the high levels of CCL20 production by the liver causing migration of cancer cells towards this chemokine gradient, in a similar mechanism to chemoattraction of immune cells (Dellacasagrande et al. 2003). This mechanism has also been proposed as the reason for the high number of patients with colorectal cancer presenting with liver metastasis (Ghadjar et al. 2006).

CCL20 has been demonstrated to be regulated by the NFκB (Harant et al. 2001) and NFAT family of transcription factors (Pietila et al. 2007). It can also be induced via an ERK signalling pathway (Sandri et al. 2008; Kanda et al. 2009).

The expression of CCL20 has been described in an endometrial epithelial cell line and more recently primary endometrial epithelial cells (Ghosh et al. 2009), as well as primary endometrial stromal cells (Sun et al. 2002). Its expression in endometrial adenocarcinoma has previously been identified by a gene array comparing normal endometrium with adenocarcinoma samples (Wong et al. 2007).

1.6 Leukocytes in cancer

Chemokines control the influx of leukocytes into tumours. Leukocytes are a hallmark of inflammation, as well as promoters of inflammation and Virchow's original observation that cancer and inflammation may be linked was based on the observation of leukocytes in tumours (Balkwill et al. 2001). The tumour microenvironment is commonly infiltrated by several types of leukocyte including macrophages, neutrophils, T and B lymphocytes, natural killer (NK) cells and dendritic cells, and these have been shown to play contrasting roles in tumour promotion and destruction (de Visser et al. 2006).

The immune system is composed of adaptive immune cells, comprising B and T lymphocytes, and innate immune cells including mast cells, dendritic cells, NK cells,

macrophages and neutrophils. Immune cells monitor the body for disturbances such as tissue injury. The first response at a site is often the release of proteases, reactive oxygen species and cytokines by macrophages and mast cells which chemoattract a further innate immune response. Dendritic cells then migrate to the lymph nodes to present antigen to the adaptive immune system. B cells, CD4+ helper T cells and CD8+ cytotoxic T cells respond by expressing antigen-specific receptors and clonally expanding to target the disturbance. Inflammation then resolves and the tissue recovers, by pathways partly mediated by immune cells (Roitt et al. 2000).

A popular model of immune cell infiltration into tumours is similar to this system. It proposes that the first types of immune cell to infiltrate a tumour are those of the innate immune system which are chemo-attracted by pro-inflammatory cytokines secreted by the cancerous cells. After the initial infiltration of cells of the innate immune system, the activation of antigen presenting cells such as dendritic cells may result in the recruitment of cells of the adaptive immune system such as T and B lymphocytes to the tumour (de Visser et al. 2006). This model of infiltration into cancer is depicted in Figure 1.9.

The discovery of cells of the immune system within solid tumours originally led to the hypothesis that immune cells were attacking and destroying the tumour, and may eventually be used in cancer immunotherapy (Dirkx et al. 2006). This may be true in some cases, as some studies have shown that immune-compromised patients, for example AIDS and organ transplant patients, have a higher risk of hodgkins lymphoma (Clifford et al. 2009) and tobacco and alcohol related cancers of the lung, oesophagus, head and neck, and pancreas (Clifford et al. 2005; Herrero et al. 2005). Additionally, in some studies an infiltration of immune cells, particularly NK or T cells, into cancer is associated with an improved prognosis (Coca et al. 1997; Ishigami et al. 2000). There is also good evidence that these lymphocytes can contribute to tumour cell destruction by cell-mediated cytotoxicity (Nishimura et al. 1999), for example transgenic mice predisposed to cervical cancer show a decreased risk of development when an enhanced CD4+ T cell response is present (Daniel et al.

2005). Additionally, mice lacking cytotoxic T cells have an approximately four fold greater risk of skin cancer (Girardi et al. 2001). However, paradoxically tumours are often infiltrated by regulator T cells which can suppress these adaptive immune responses (Giatromanolaki et al. 2008).

However, a large body of evidence suggests that chronically activated innate immune cells can promote tumour growth, angiogenesis and cell migration (Murdoch et al. 2008). In endometrial cancer, the infiltration of macrophages (Salvesen et al. 1999; Ohno et al. 2004), natural killer cells (Hachisuga et al. 1997) and B and T lymphocytes (Yamazawa et al. 2001; Chang et al. 2005; Ohno et al. 2005; Ohno et al. 2006; Miyatake et al. 2007; Giatromanolaki et al. 2008) has previously been reported. The two cytokines discussed in this thesis activate receptors that are expressed on different immune cells. CXCL1 binds to CXCR2 which is expressed primarily on neutrophils, but also on monocytes (Weber et al. 1999; Traves et al. 2004; Smith et al. 2005). CCL20 signalling plays an important role in the chemoattraction of immature dendritic cells to sites of inflammation (Schutyser et al. 2003).

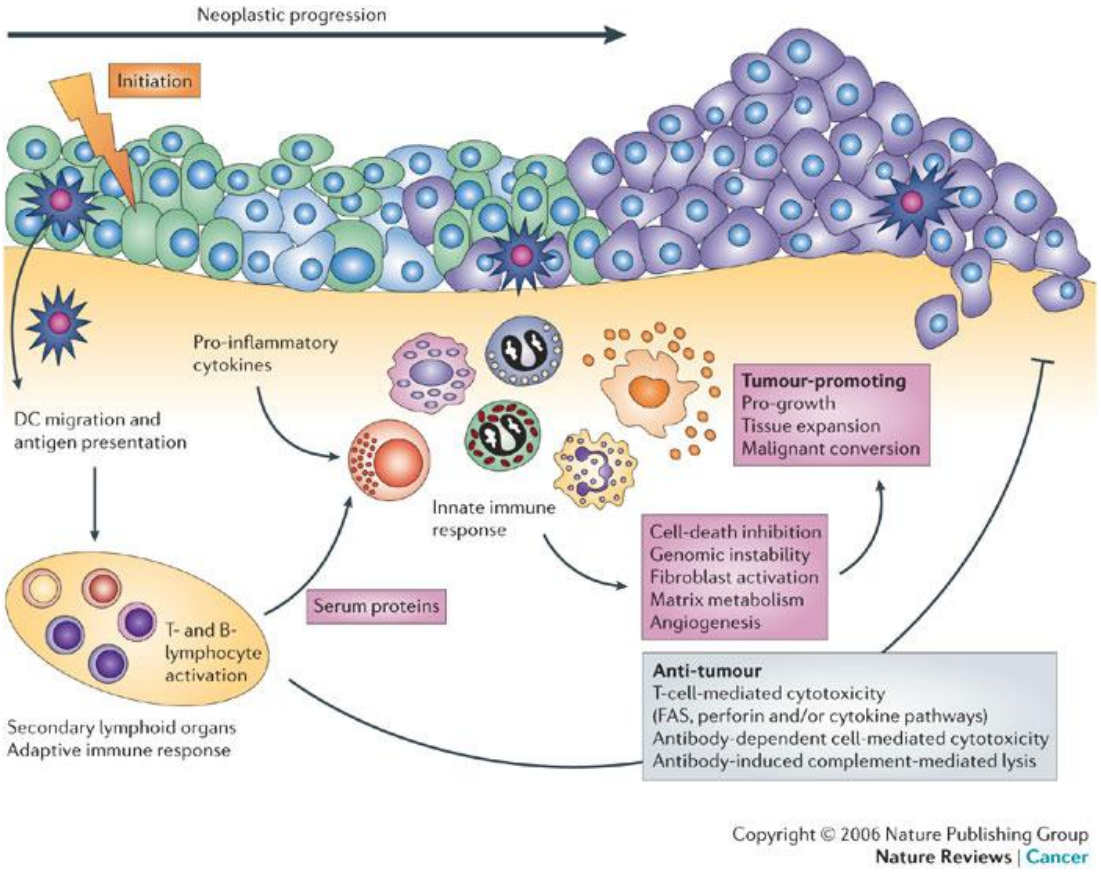

Figure 1.9 A model of immune cell infiltration into cancer. At cancer initiation, cytokines secreted by tumour cells chemoattract cells of the innate immune system. These produce a number of tumour promoting factors. Tumour antigens are also taken up by dendritic cells which activate adaptive immune responses by T and B lymphocytes, which often occur after the initial infiltration of innate immune cells. These mount cytotoxic responses against the tumour. Adapted from de Visser et al. 2006.

1.6.1 Macrophages

The most common infiltrating immune cell into cancer is often the macrophage (Dirkx et al. 2006). Tumour associated macrophages (TAM) are increased in endometrial adenocarcinoma (Hachisuga et al. 1997; Salvesen et al. 1999) as well as other cancer types including colon (Forssell et al. 2007), breast (Bingle et al. 2006), bladder (Chen et al. 2009) and melanoma (Hussein 2006). Macrophages show high plasticity, and subpopulations promoting tumour progression in different ways have been identified, for example the TIE2 subset expressing the angiopoietin receptor (Venneri et al. 2007). This plasticity may also be the reason for contrasting reports of

TAM both promoting and reducing tumour growth (Saccani et al. 2006; Forssell et al. 2007). However increased TAM are often associated with a poor prognosis (Salvesen et al. 1999; Yang et al. 2007), and primarily TAM are thought to contribute to angiogenesis within cancers. Macrophages secrete factors such as VEGF and angiopoetins which increase angiogenesis (Nesbit et al. 2001; Bingle et al. 2006; Venneri et al. 2007) and have been shown to be essential for the initiation of angiogenesis in breast cancer (Lin et al. 2007). Macrophages can also promote tumour invasion and metastasis, through the secretion of proteases including MMP12 and MMP-9 (Luo et al. 2006; Yang et al. 2007).

1.6.2 Neutrophils

Neutrophil infiltration contributes to tumour promotion in similar ways to macrophage infiltration; by the production of a number of angiogenic factors and proteases which can impact on tissue remodelling and inflammation. Neutrophils produce angiogenic factors including VEGF and FGF (Gargett et al. 2001; Scapini et al. 2004; Ai et al. 2007), and promote invasion and metastasis through the production of proteases including MMP-9 (Ardi et al. 2007). In a nude mouse model of breast cancer, an increased infiltration of neutrophils increased invasiveness of the tumour, likely due to protease production (Yao et al. 2007). In addition, the depletion of neutrophils in mouse models of fibrosarcoma and colon cancer significantly decreased metastasis in these animals (Tazawa et al. 2003; Yamamoto et al. 2008). However neutrophils may also promote tumourigenesis through means other than tissue remodelling. In an in vitro model of colon cancer, neutrophils promote cellular stress by inducing transient errors in DNA replication in epithelial cells (Campregher et al. 2008), which could ultimately lead to carcinogenesis. Neutrophils from ovarian cancer patients released higher levels of reactive oxygen species which could potentially promote cellular changes which support tumour progression (Klink et al. 2008).

Some evidence suggests that neutrophil secreted products may decrease tumour growth in some circumstances. For example, neutrophils produce the protease

neutrophil elastase. This has been demonstrated to cleave plasminogen to activate the anti-angiogenic factor angiostatin, which inhibited VEGF and FGF induced angiogenesis *in vitro.* However this only occurred at increased concentrations of elastase (Scapini et al. 2002). Neutrophil elastase has also been shown to directly degrade VEGF and FGF, and the incubation of neutrophil elastase with these factors decreased their angiogenic ability in the aortic ring assay *in vitro* (Ai et al. 2007). This action of neutrophil elastase may represent a feedback to the angiogenic factors being produced by neutrophils, and it is unclear if this mechanism is occurring in cancer.

1.6.3 B Cells

B cells are capable of producing antibody against tumour antigens (Roitt et al. 2000). However, the serum proteins secreted by B cells can chemoattract innate immune cells, and therefore may promote chronic inflammation and tumour progression (Hoebe et al. 2004). Indeed, elimination of B and T cells in a mouse model reduced innate immune cell infiltration into developing tumours and levels of tumourigenic factors including VEGF and MMP9. This caused a decrease in tumour growth. Re-introduction of B cells restored the infiltration of innate immune cells and angiogenesis to control levels (de Visser et al. 2005). As low levels of B cells are usually found in tumours (de Visser et al. 2006), their role in these tissues has not been extensively studied.

1.6.4 T cells

T cells are divided into CD4+ or CD8+ cells, on the basis of the presence or absence of these surface antigens. Broadly speaking, CD4+ T cells interact with B cells to activate proliferation and antibody production, CD8+ T cells to facilitate differentiation, or macrophages to promote inflammatory cytokine production. CD8+ T cells are cytotoxic, and can induce the death of cells infected with pathogens, or tumour cells by cell to cell interactions or secreted factors (Roitt et al. 2000). In cancer, most evidence indicates that T cells are anti-tumourigenic. For example, CD4+ and CD8+ T cells have been demonstrated to eradicate tumours from mouse

models of cancer though production of cytokines and directly adhering to tumour cells (Nishimura et al. 1999). The role played by CD4+ cells however appears to be dependent on the environment of the tumour and cancer model used (Ellyard et al. 2007). For example, subsets of CD4+ T cells can be activated in different cancer types. Regulatory FOXP3-expressing CD4+ T cells are one such subset which can suppress T cell population expansion and cytotoxicity. These are associated with increased microvascular density and angiogenic factors including VEGF in endometrial adenocarcinoma (Giatromanolaki et al. 2008). A low infiltration of this subset of lymphocytes has also been associated with increased survival in endometrial adenocarcinoma (Kondratiev et al. 2004). Different cytokines may also be produced by CD4+ T cells dependent on the tumour environment, which can play different roles in chemoattraction of innate immune cells such as macrophages (Badoual et al. 2006).

A low infiltration of CD8+ T cells has been associated with poor prognosis in endometrial adenocarcinoma (Ohno et al. 2005), and similarly a high CD8+ cell infiltration has been correlated with an increased survival rate (Kondratiev et al. 2004; de Jong et al. 2009), demonstrating that CD8+ infiltration may be destructive to the tumour. CD8+ T cells can eradicate mouse mammary tumours in the absence of CD4+ T cells (Ostrand-Rosenberg et al. 2000). The tumour-destroying properties of CD8+ T cells may be inhibited by not only the regulatory T cells described above, but also but other innate immune cells, for example the production of indoeamine dioxygenase by innate immune cells decreases T cell proliferation and promotes their apoptosis (Bronte et al. 2003). Therefore the plasticity of T cells may be modulated by innate immune cells already present within the tumour, to promote a pro-tumourigenic environment.

1.6.5 NK cells

NK cells are lymphocytes yet are components of the innate immune system. NK cells distinguish the loss of antigen, and can recognise tumour cells by the loss of MHC class I expression (Zamai et al. 2007). Upon recognition, NK cells secrete a variety

of lytic factors from specialised granules, able to lyse and promote apoptosis of targeted tumour cells. Therefore presence of NK cells in a tumour is likely to decrease tumour growth (Zamai et al. 2007). In a mouse model of fibrosarcoma, low NK cell infiltration was observed, which was due to secreted factors including granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-6 from the tumour impairing the maturation of NK cells (Richards et al. 2008). In an alternative mouse model of sarcoma, complete depletion of NK cells led to an increased rate of tumour initiation (Smyth et al. 2001). Additionally, NK cells can contribute to tumour destruction by the production of anti-angiogenic factors, including interferon-γ (Hayakawa et al. 2002).

1.6.6 Dendritic cells

Dendritic cells are the main antigen presenting cells of the immune system, which infiltrate tissues as immature cells. Upon the uptake of antigen and in response to inflammatory stimuli they differentiate into mature dendritic cells capable of activating lymphocytes (Schutyser et al. 2003). In cancer, increased numbers of immature dendritic cells in the tumour has been demonstrated to promote immune tolerance to the tumour. Immature dendritic cells taken from the tumour of a mouse model of colon cancer induced lower levels of T cell clonal expansion than mature dendritic cells (Bonnotte et al. 2004). It is possible that factors derived by the tumour promote dendritic cell immaturity or inhibit differentiation. For example VEGF (Gabrilovich et al. 1998) and the overexpression of CCL20 by colon cancer cells has been demonstrated to preferentially recruit immature dendritic cells *in vitro* (Wang et al. 2008). Similarly to other leukocytes discussed here, dendritic cells can also produce a host of angiogenic factors, including VEGF (Fainaru et al. 2008).

1.7 Other lipid mediators and their interactions with prostaglandins: Lysophophatidic acid

In a physiological setting, signalling cascades are unlikely to function alone without interaction and cross-talk with other cascades. Other lipid mediators which have been

demonstrated to play a role in cancer signalling are those derived from lysophospholipids, of which the main ligands are sphingosine-1-phosphate (S1P), lysophosphatidic acid (LPA), sphingosylphosphorylcholin, psychosine, sphingosine and lysophosphatidylcholine (Graler et al. 2002). The two most extensively studied lipid mediators are LPA and S1P. There is evidence that S1P promotes numerous functions in cancer including angiogenesis, invasion, proliferation and evasion of apoptosis (Visentin et al. 2006). However due to the proven importance of LPA in the reproductive system (Ye 2008) and in cancer (Mills et al. 2003) this thesis will concentrate on only the role of LPA in endometrial adenocarcinoma.

LPA is a water-soluble phopholipid that acts as an important extracellular signalling molecule. Its actions as a bioactive lipid were first discovered 30 years ago when it was shown to induce muscle contraction and platelet aggregation (Mills et al. 2003). LPA was postulated to signal through a GPCR, however this was not confirmed until 1996 when the first LPA receptor was cloned form neuroblast cells lines (Hecht et al. 1996), and renewed interest in this phopholipid began. LPA is formed by two main metabolic pathways, and appears to be formed extracellularly as well as intracellularly by the action of secreted enzymes. In the first pathway, the choline from lysophosphatidylcholine is removed to form lysophospholipids by autotaxin (ATX; also known as lysophospholipase D) then LPA is formed by phospholipase A1/2 (PLA1/2) (Tokumura et al. 1999; Aoki et al. 2002). In the second pathway, LPA is converted from phosphatidic acid by removal of a fatty acid chain by PLA1/2 (Aoki et al. 2002). Therefore, the availability of these enzymes also determines in some part the amount of LPA signalling within a cell. ATX was originally discovered as a melanoma-secreted factor (Stracke et al. 1992) and has now been shown to be increased in cancers including endometrial and lung (Yang et al. 1999; Tokumura et al. 2007). It promotes metastasis, invasion and angiogenesis, although whether this is via a direct effect on endothelial cells or on the tumour microenvironment is unknown (Nam et al. 2001).

LPA signalling mediates a diverse range of actions, which may depend on the GPCR which is activated. Seven GPCRs have now been identified and named LPAR1-7 (Murph et al. 2008). LPAR1-3 (which used to be known as endothelial differentiation gene 2, EDG4 and EDG7) are widely expressed in the body, while LPAR4-7 have limited expression and as yet unknown functions (Murph et al. 2006). LPAR 1-3 share high sequence homology with each other (48.8 – 53.7%; (Bandoh et al. 1999) but less than 20% homology with LPAR 4-7 (Murph et al. 2008).

LPAR-1 was cloned in 1996 from a neuroblast cell line, and identified as a GPCR for LPA by its signalling properties, and ability to be inhibited by pertussis toxin (Hecht et al. 1996). LPAR1 has been shown to couple with $G_{i/o}$, G_q and $G_{12/13}$ (Hecht et al. 1996; Fukushima et al. 1998; Ishii et al. 2000). The coupling G-protein and subsequent signalling pathways activated may differ depending on the cell type and model used. LPAR1 expression is widespread throughout the body, with the exception of the liver (An et al. 1998). LPA-LPAR1 signalling has been shown to activate common GPCR-coupled signalling pathways including NFκB (Chen et al. 2008) and ERK 1/2 (Shin et al. 2009).

LPAR2 was discovered in 1997 by a GenBank search based on the sequence of LPAR1 (An et al. 1998). LPAR2 also couples to $G_{i/o}$, G_q and $G_{12/13}$ (Ishii et al. 2000). In contrast to the widespread location of LPAR1, LPAR2 is expressed in a smaller number of tissues, including leukocytes, testis, prostate thymus and kidney (An et al. 1998). Due to the promiscuous G-protein coupling, LPAR2 also activates a range of signalling pathways including ERK1/2 (Yun et al. 2005).

LPAR3 was similarly identified by sequencing an unknown gene after amplification based on the sequences of LPAR1 and 2 (Bandoh et al. 1999). LPAR3 also couples to $G_{i/o}$ and $G_{q,}$ but not $G_{12/13}$ (Bandoh et al. 1999; Ishii et al. 2000). This difference in coupling results in some functional differences, such as the promotion of cell rounding by Rho activation which is shared by LPAR1 and 2 but not 3 (Ishii et al.

2000). However, LPAR3 activation does still activate a range of signalling molecules, including AP-1 (Hu et al. 2001) and the EGFR (Shah et al. 2005).

1.7.1 LPA signalling in reproduction

The LPAR1 knock out mouse shows defective olfactory development which manifests as a failure to suckle and therefore neonatal death (Contos et al. 2000). The LPAR2 knock out mouse shows no phenotype (Contos et al. 2002), which indicates redundancy between LPA receptors. The LPAR3 knock out mouse however is infertile, demonstrating the importance of LPA and this receptor in reproduction (Ye et al. 2005).

LPA signalling has been shown to play a key role in several reproductive processes, including fertilisation, implantation, decidualisation and parturition (Ye 2008). *In vitro*, LPA induces oocyte maturation, through an unknown receptor and an ERK and p38 signalling pathway (Komatsu et al. 2006). As the LPAR1 and 2 mice show no adverse affects on reproduction, LPAR3 is likely to play the biggest role in the female reproductive system. However LPAR1 signalling has been proposed to play a role in angiogenesis in the normal endometrium, as endometrial stromal cells stimulated with LPA produced the angiogenic chemokine IL-8 via a LPAR1 and NFκB pathway (Chen et al. 2008), and increased expression of LPAR1 has also been described in the bovine endometrium during the estrous cycle. Here it is thought to contribute to $PGF_{2\alpha}$ induced luteolysis (Woclawek-Potocka et al. 2009). Expression of LPAR 2 in the human and mouse placenta has been reported (Ye 2008).

The LPAR3 knock out mouse revealed that LPA plays a major role in implantation. In response to LPAR3 deficiency, a delay in implantation and altered positioning and numbers of blastocysts was observed. This led to increased embryo death. Interestingly, this phenotype is very close to the COX-2 knock out mouse, prompting the investigators to partially rescue the phenotype with prostaglandin exposure (Ye et al. 2005), linking the LPA and COX-2 signalling pathways.

1.7.2 LPA signalling in cancer

The increased levels of LPA and its receptors in many cancer types has indicated that it may play a role in tumour progression. LPAR1, 2 and 3 are expressed in different cancers, and the main subtype which LPA signalling occurs through may determine the outcome. Elevated LPA concentrations have been found in ascites and serum of patients with ovarian (Shen et al. 1998) and endometrial (Tokumura et al. 2007) cancer, and expression of LPAR1-3 has been documented in numerous cancers including breast (Kitayama et al. 2004), colon (Yun et al. 2005), prostate (Guo et al. 2006), gastrointestinal (Shin et al. 2009), and ovarian (Nakamoto et al. 2005; Jeong et al. 2008; Yu et al. 2008).

LPA signalling has been demonstrated to promote cell migration, cell proliferation and tissue remodelling through production of proteases and stimulation of angiogenesis (Murph et al. 2006). In gastrointestinal cancer, LPA can promote cell migration via a LPAR1-ERK 1/2 signalling pathway (Shin et al. 2009), and similarly in breast cancer cell lines, LPAR1 activation promotes cell migration (Chen et al. 2007). Migration of an ovarian cancer cell line was also promoted by LPA, an effect that could be abolished with siRNA against LPAR2 (Jeong et al. 2008). The promotion of migratory responses is commonly through LPAR1 or 2, potentially as LPAR3 does not couple to $G_{12/13}$, the main G-protein promoting cell migration (Ishii et al. 2000). LPA has also been shown to promote cell proliferation in some cancer types, including colorectal cancer (Shida et al. 2005) and prostate cancer, where overexpression resulted in increased cell proliferation *in vitro* and increased tumour growth *in vivo* (Guo et al. 2006). The expression of LPAR2 and LPAR3 on ovarian cancer cell lines also increased xenograft growth in nude mice (Yu et al. 2008).

Signalling through the LPA receptors commonly promotes expression of chemokines involved in angiogenesis and inflammation. For example, LPA promotes expression of CXCL1 in ovarian cancer via LPAR2 (Lee et al. 2006). In colon cancer, LPAR2 signalling also mediates expression of the angiogenic chemokine IL-8 (Yun et al. 2005). LPA signalling promotes VEGF expression in ovarian cancer cells, and this is

likey to be via LPAR2 or LPAR3, as the knockdown of these decreases VEGF expression (Hu et al. 2001). Over-expression of these same two receptors increased invasiveness of ovarian cancer xenografts in mice and also increased IL8, IL6 and VEGF expression (Yu et al. 2008). The expression of LPA receptors has been demonstrated in leukocytes, including macrophages (Hornuss et al. 2001), T cells (Zheng et al. 2000) and neutrophils (Tou et al. 2005). Whether LPA plays a role in the chemoattraction of these cells into cancer beyond the promotion of chemokine expression is unclear. However, LPA is known to induce ROS generation and activation of macrophages (Hornuss et al. 2001), cytokine secretion by T cells (Zheng et al. 2000; Graler et al. 2002) and degranulation by neutrophils (Tou et al. 2005).

1.7.3 LPA and COX signalling interactions

The inflammatory actions of LPA also include interaction with the COX-2 signalling pathway. As well as the LPAR3 knock out mouse, the phenotype of which could be rescued by prostaglandin administration (Ye et al. 2005), the roles of LPA and COX-2 in reproduction converge at other signalling mechanisms. In the bovine uterus, LPAR1 is the predominant LPA receptor expressed throughout the estrous cycle, and signalling through this increases prostaglandin formation (Woclawek-Potocka et al. 2009; Woclawek-Potocka et al. 2009). Similarly in the ovine uterus where LPAR1 and LPAR3 are expressed during pregnancy, LPA stimulation of cultured cells also increases prostaglandin formation (Liszewska et al. 2009). In porcine endometrium, LPAR3 expression is increased, and signalling through this directly upregulated expression of prostaglandin synthase enzymes (Seo et al. 2008). Interactions between LPA and COX-2 signalling have also been described in cancer, for example in ovarian cancer where LPA signalling induced COX-2 expression though LPAR2 (Jeong et al. 2008), and a similar increase in COX-2 is mediated by LPA in colorectal cancer, through phosphorylation of the EGFR (Shida et al. 2005).

In endometrial adenocarcinoma, LPAR2 mRNA has been previously found to be increased (Yun et al. 2005), and also expressed in a moderately differentiated

endometrial adenocarcinoma cell line (Hope et al. 2009). Here, it has been shown to promote invasion of endometrial cancer cells, via the upregulation of MMPs (Hope et al. 2009). In endometrial cells, LPA signalling can promote chemokine expression, as IL-8 expression was increased in endometrial stromal cells after LPA treatment via a G_i-MAPK-NFκB pathway (Chen et al. 2008). Therefore, LPA expression in endometrial adenocarcinoma could promote tumour progression in two different ways, firstly by directly acting upon cells expressing its receptors to promote migration, proliferation or chemokine expression, or by promoting expression of COX-2 and prostaglandins which are also upregulated in endometrial adenocarcinoma.

1.8 Aims and objectives of this thesis

This chapter has introduced the concept that chronic inflammation can initiate or promote tumour growth. In endometrial adenocarcinoma, inflammation is promoted by the oestrogen to progesterone ratio, genetic mutations activating inflammatory pathways, and by chemokines acting directly on neoplastic epithelial cells or to promote leukocyte infiltration. The increased expression of COX-2 in endometrial adenocarcinoma is mediated by some of these mechanisms, and leads to elevated prostaglandin signalling, which can result in the expression of angiogenic and inflammatory factors. Our laboratory has previously demonstrated that FP receptor expression is elevated in endometrial adenocarcinoma. We therefore hypothesised that increased $PGF_{2\alpha}$-FP receptor signalling may promote inflammation, and the crosstalk between LPA and the prostaglandins may function in endometrial adenocarcinoma to amplify this signalling.

COX-2 inhibitors were trialled in cancer treatment until 2004. However, with long term intake of these drugs the risk of cardiovascular side effects was increased, and therefore COX-2 inhibitor drugs such as Vioxx (Rofecoxib) were withdrawn from the market (Cha et al. 2007). This has led to an interest in targeting prostaglandin signalling directly at the prostaglandin receptor, however as yet only the EP receptors

have been considered (Rocca 2006). Due to the high expression of the FP receptor in endometrial adenocarcinoma, the aims of this thesis are to delineate some of the FP receptor signalling pathways which promote inflammation in endometrial adenocarcinoma. Specifically, studies described in this thesis aim to elucidate chemokine expression mediated by the FP receptor, the downstream consequences of this chemokine release, and how LPA may impact on both FP receptor and chemokine expression.

Chapter 2

General materials and methods

2 General materials and methods

2.1 Tissue Collection

2.1.1 Endometrial tissue

Normal endometrial tissue at different stages of the menstrual cycle was collected from woman undergoing minor gynaecological surgery with no underlying pathology. An endometrial suction curette (Pipelle, Laboratoire CDD, France) was used to collect tissue which was either stored in RNA Later (Ambion) at -70°C for RNA extraction, fixed in neutral buffered formalin and wax embedded for histological analysis or placed in serum free medium for tissue culture. All women had normal menstrual cycles (25-35 days) and endometrial biopsies were staged according to last stated menstrual period. Stage of cycle was confirmed histologically by a pathologist and biopsies were designated as proliferative, early secretory, mid secretory, or late secretory. Ethical approval was obtained from Lothian Research Ethics Committee under ethics number LREC/05/S1103/32 and written informed consent was obtained prior to collection.

2.1.2 Carcinoma tissue

Endometrial adenocarcinoma tissue was obtained from women undergoing hysterectomy after diagnosis of adenocarcinoma of the uterus. Diagnosis of adenocarcinoma was confirmed histologically. At collection, tissue was either stored in RNA Later (Ambion) at -70°C for RNA extraction, fixed in neutral buffered formalin and wax embedded for histological analysis or placed in complete medium for tissue culture. Endometrial adenocarcinoma biopsies were assessed by a pathologist and designated as well differentiated, moderately differentiated or poorly differentiated tumours. Ethical approval was obtained from Lothian Research Ethics Committee under ethics number LREC 1999/6/4 and written informed consent was obtained prior to collection.

2.2 Cell Culture

2.2.1 Culture conditions and passaging

The Ishikawa cell line consisting of endometrial epithelial cells originally taken from endometrial adenocarcinoma (European Collection of Cell Culture, Wiltshire, UK) was used throughout this study. Wild type (WT) Ishikawa cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) nutrient mixture F-12 with Glutamax-1 and pyridoxine supplemented with 10% FCS and 1% penicillin-streptomycin (500 IU/ml penicillin and 500 µg/ml streptomycin; complete medium). Ishikawa cells stably transfected with FP cDNA in the sense direction (FPS cells, (Sales et al. 2005)) were maintained as above with the addition of 200 µg/ml G418 antibiotic.

All cells were routinely passaged twice weekly in T162 flasks and maintained in humidified conditions at 37°C with 5% carbon dioxide. To passage, cells were washed three times with PBS to remove complete medium followed by addition of 4 ml 0.05% trypsin-EDTA. Cells were incubated at 37°C for approximately 5 minutes with regular agitation until detached from the culture flask. The trypsin was then neutralised with the addition of 8 ml complete medium and 3mls of this cell suspension was added to 30 ml complete medium and reseeded in a new flask. Cells used in experiments were counted using a haemocytometer then plated into culture dishes at the densities indicated in Table 2.1.

Culture Vessel	Cell Density / Well
96 well plate	5×10^3
24 well plate	5×10^4
12 well plate	1×10^5
6 well plate	2×10^5
60 mm dish	2.5×10^5

Table 2.1 Cell densities in different culture vessels used.

2.2.2 Primary tissue culture conditions

Normal endometrial and endometrial adenocarcinoma tissue was finely chopped in DMEM nutrient mixture F-12 with Glutamax-1 and pyridoxine medium (serum free medium) and maintained overnight in humidified conditions at 37°C with 5% carbon dioxide. For treatments, 3 to 4 pieces of chopped tissue were place in a well of a 24 well plate and 1 ml serum free medium containing the treatment was added.

2.2.3 Cell and tissue treatments

Ishikawa cells were treated with a previously optimised dose of $PGF_{2\alpha}$ of 100 nM (Milne et al. 2003). To determine gene expression in response to $PGF_{2\alpha,}$ WT and FPS cells were treated for 2, 4, 6, 8, 12, 18, 24, 48 and 72 hours with vehicle or 100 nM $PGF_{2\alpha}$.

To determine signalling pathways regulated by $PGF_{2\alpha}$, FPS cells or carcinoma tissue were treated for 8 or 24 hours with vehicle, 100 nM $PGF_{2\alpha}$ alone, or 100 nM $PGF_{2\alpha}$ and a panel of chemical inhibitors (section 2.2.4) or dominant negative constructs (section 2.3.2).

2.2.4 Chemical inhibitors

Table 2.2 below lists the chemical inhibitors used to determine signalling pathways regulated by $PGF_{2\alpha}$. The optimal concentration of all chemical inhibitors was determined empirically by titration using the manufacturer's guidelines as described in our previous studies (Sales et al. 2008). Cell viability has previously been determined for each inhibitor using the CellTitre 96 AQueous One Solution assay (Promega, Southampton, UK) as described (Sales et al. 2004; Sales et al. 2008). All treatments were carried out with a control of cells treated with inhibitor alone.

Inhibitor	Molecule inhibited	Working concentration	Supplier
YM254890	G_q	1 µM	Dr Jun Takasaki (Ibaraki, Japan)
AL8810	FP	50 µM	Sigma Aldrich (Dorset, UK)
AG1478	EGFR	200 nM	Calbiochem (Nottingham, UK)
PD98059	MEK	50 µM	Calbiochem (Nottingham, UK)
Cyclosporin A	Calcineurin	1 µM	Calbiochem (Nottingham, UK)
EGTA	Calcuim	1.5 mM	Sigma Aldrich (Dorset, UK)
4C3MQ	PKA	1 µM	Merck Biosciences (Nottingham, UK)

Table 2.2 Chemical inhibitors and working concentrations used.

The mechanisms of action of these inhibitors are described below.

YM254890 is a selective $G_{\alpha q/11}$ inhibitor which was originally purified from the broth of *Chromobacterum* species. It interferes with the exchange step of GDP for GTP rather than a direct interaction with the receptor. The specificity of YM254890 has been demonstrated by its ability to block G_q-mediated calcium mobilisation in several cell lines including an overexpressing G_q cell line. It did not affect second messenger activation mediated by $G_{\alpha i}$ or $G_{\alpha 15}$-coupled receptors (Takasaki et al. 2004).

AL8810 is a specific FP antagonist described by Griffin *et al* (Griffin et al. 1999). It is able to dose-dependently reduce IP_3 production in response to fluprostenol, a

specific FP agonist. It does not inhibit activation of EP_2, EP_4, IP or DP by specific agonists, demonstrating its specificity. It appears to act as a competitive antagonist and also displays weak agonist activity for the FP receptor at 261 ± 44 nM.

AG1478 is an EGFR inhibitor which functions by acting as an analogue of ATP. The EGFR must bind to both ATP and substrate to cause activation, and the addition of AG1478 competitively displaces ATP binding and therefore EGFR activation (Ward et al. 1994).

The extracellular-regulated kinases (ERK) 1/2 are activated by the mitogen-activated protein kinase kinase MEK. To inhibit this pathway, PD98059 was used. PD98059 was identified by screening a compound library for inhibition of ERK1/2 phosphorylation (Dudley et al. 1995). It reversibly inhibits MEK by preventing its phosphorylation at a site distinct to the active site (Alessi et al. 1995).

The nuclear factor of activated T-cells (NFAT) transcription factors are activated by a signal transduction cascade involving the protein calcineurin. Cyclosporin A forms a complex with the intracellular receptor cyclophilin which then binds to calcineurin and prevents its phosphorylation of NFAT, and therefore the translocation of NFAT to the nucleus to initiate gene transcription (Clipstone et al. 1994).

Ethylene glycol tetraacetic acid (EGTA) is a calcium chelating compound (Sigma Alrdich). 4-cyano-3-methylisoquinoline (4C3MQ) is an isoquinoline-derived potent PKA inhibitor. It binds PKA at the catalytic subunit competitively to ATP, therefore preventing the ability of PKA to phosphorylate proteins (Lu et al. 1996).

2.2.5 Adenoviral infection

A short hairpin RNA adenovirus was used to knock down expression of FP and an adenovirus was used to over-express RCAN-1-4. Both were kindly prepared by Dr Pamela Brown and Mr Stuart Reid (Biomolecular Core Facility, MRC Human Reproductive Sciences Unit, Edinburgh, UK). cDNA of RCAN-1-4 was prepared as

previously described (Maldonado-Perez et al. 2009) and fused to the pDC316 shuttle vector (Microbix Biopharmaceuticals, Toronto, Canada) to create pDC316-RCAN-1-4. FP cDNA (ORIGENE, Rockville, MD) was excised with EcoRI and Sma1 and fused to the pDC316 vector to pDC316-FP.

HEK-293 cells (Invitrogen, Paisley, UK) were seeded in 6-well plates in nutrient mixture F-12 with Glutamax-1 and pyridoxine supplemented with 10% FCS and 1% penicillin-streptomycin (500 IU/ml penicillin and 500 µg/ml streptomycin). Once at 50% confluency, cells were co-transfected with 0.75 µg pDC316RCAN-1 or pDC316-FP and 2.25 µg pBGH10 ΔE1,3 Cre adenoviral backbone in Optimem Reduced Serum Medium (Invitrogen) using Trans-IT-293 transfection reagent (Mirus Bio Corp, Madison, WI) as per the manufacturer's instructions. Cells were incubated for approximately 2 weeks with regular changes of medium until evidence of cytopathic effect (CPE) was seen, which was defined as a rounded appearance to cells and when approximately 70% of cells had lifted from the plate surface. Adenovirus was then harvested and virus released from the cells by 3 freeze-thaw cycles. Serial dilutions of the virus were performed and used to re-infect HEK-293 cells at 75-85% confluence. Wells were overlaid with 0.5% SeaPlaque Agarose (FMC Corp, Rockland, ME) to ensure homogenous plaques were obtained. Plaques were visible within 4-5 days and were picked using a P1000 tip. Plaques were freeze-thawed 3 further times and used to re-infect a further T75 flask which was incubated until CPE was observed. The virus from this flask was harvested and termed the first seed. The adenovirus was then expanded by inoculation of further flasks with the first seed. Adenovirus was purified and concentrated using the Vivapure AdenoPACK 100 purification kit (Sartorius AG, Goettingen, Germany) as per manufacturer's instructions. Virus titres were determined using the AdenoX Rapid titre kit (Clontech, France) as per manufacturer's instructions.

FPS cells were seeded in 6-well plates and incubated for 24 hours. Cells were then infected with scrambled sequence adenovirus, shFP or RCAN1-4 adenovirus at 5 viruses / cell in complete medium. After 24 hour incubation, cells were starved in

medium containing 3 µg/ml indomethacin for 18 hours before treatment with 100 nM $PGF_{2\alpha}$.

2.2.6 Lentiviral infection

A short hairpin RNA lentivirus (the kind gift of Professor Aubrey Thompson, Mayo Clinic, Florida, USA) was used to knock down expression of RCAN-1-4 and was prepared as previously described (Bush et al. 2007). FPS cells were seeded in 6-well plates and incubated for 24 hours. Cells were then infected with control scrambled lentivirus or shRCAN1-4 lentivirus at 5 viruses / cell in complete medium. After 24 hour incubation, cells were starved in medium containing 3 µg/ml indomethacin for 18 hours before treatment with 100 nM $PGF_{2\alpha}$.

2.3 Luciferase reporter assays

2.3.1 Transient transfection of CXCL1 luciferase promoter construct

Luciferase reporter assay was used to determine the promoter activity of CXCL1 after cell treatments. The gene encoding luciferase is inserted under control of the CXCL1 promoter, and CXCL1 transcriptional activity can then be measured by luminescence after addition of the luciferase substrate. The CXCL1 reporter plasmid consisting of the CXCL1 minimal promoter region inserted into the pGL2 Basic vector (Promega) fused to the firefly luciferase reporter (as described in Nirodi et al, 2001) was kindly supplied by Professor Ann Richmond (Department of Cell Biology, Vanderbilt University School of Medicine, Nashville, Tennessee). An empty vector (pcDNA3.0; Invitrogen) was used as a control. FPS cells were seeded in 12 well plates and 1.5 µg of CXCL1 construct transfected using Superfect transfection reagent (Qiagen, Crawley, UK) as per the manufacturer's instructions. This was co-transfected with 0.15 µg pRL-TK (containing the renilla luciferase coding sequence; Promega) as an internal control. After 4 hours the transfection media was removed and replaced with complete DMEM for at least 24 hours. Cells were then serum starved in medium containing 3 µg/ml indomethacin for 18 hours before pre-treatment with vehicle, AL8810 or PD98059 at the concentrations indicated in Table 2.2 for 1 hour. Cells were then treated with vehicle or 100 nM

$PGF_{2\alpha}$ alone, $PGF_{2\alpha}$ and AL8810 or $PGF_{2\alpha}$ and PD98059 for 2, 4, 6, 8 and 24 hours. Promoter activity was measured by luciferase assay as described in section 2.3.3.

2.3.2 Transient transfection of dominant negatives and CXCL1 luciferase reporter construct

In order to determine signalling pathways leading to CXCL1 promoter activation, the CXCL1 luciferase promoter construct was co-transfected into FPS cells with dominant negative constructs. Dominant negative isoforms of Ras, EGFR, NFAT and mitogen-activated protein kinase kinase (MEK) were kindly supplied by Professor Zvi Naor (Department of Biochemistry, Tel Aviv University, Tel Aviv, Israel) and modes of action are described below. FPS cells were transfected with the CXCL1 luciferase promoter construct or empty vector as described in section 2.3.1, and 1.5 µg of vector encoding either a dominant negative isoform or empty vector pcDNA3.0 was co-transfected. Transfection was performed as described in section 2.3.1 before cells were serum starved for at least 18 hours then treated with vehicle or 100 nM $PGF_{2\alpha}$ for 8 hours. Cells were then washed in ice cold PBS and lysed using NPS lysis buffer. Promoter activity was measured by luciferase assay as described in section 2.3.3.

The mutant ras protein (DN-Ras) was produced by Feig et al (Feig et al. 1988) by altering the specificity of *ras* binding to GTP and therefore its activation. A GTP-binding assay was used to identify mutants produced by random mutagenesis of the bacterial expression vector ras^H. Sequencing determined that a substitution of asparagine (Asn) for serine at position 17 in the highly conserved p21 region decreased GDP binding affinity by approximately 40 fold as compared to normal cellular ras^H. The actions of this dominant protein are demonstrated by its growth inhibitory phenotype (Feig et al. 1988; Stacey et al. 1991).

Dominant negative EGFR (DN-EGFR) was produced by the deletion of 63 amino acids from the C-terminal of the EGFR, removing 2 autophosphorylation sites (Livneh et al. 1986). EGFR cDNA was placed into a substitution SV40-based

expression vector and cloned. The resultant plasmid pLΔ NA8 construct was used to transform *E. coli* HB101 and the presence of the required deletion was tested by digestion with appropriate restriction enzymes. Dominant negative activity of pLΔ NA8 has been previously demonstrated (Livneh et al. 1986).

Dominant negative MEK (DN-MEK) was produced by the mutation of a lysine residue to an alanine residue at position 97, resulting in an inactive protein (Seger et al. 1994). MEK cDNA was inserted into *Eco*RI and *Bam*H1 sites of the pcDNA3 vector and transfected into XL-1 cells. Point mutations were made by polymerase chain reaction, and mutations were then sequenced to demonstrate the correct composition. Dominant negative ability has been demonstrated by decreased MAPK activation and cell proliferation (Seger et al. 1994; Harris et al. 2002).

NFAT binds to specific DNA sequences to activate transcription. Dominant negative NFAT was produced by Crotti *et al* by deletion of the DNA binding domain (Crotti et al. 2006). After cloning NFATc1 cDNA into the pTAT–HA vector, the domain was deleted by restriction digest with PvuII. Dominant negative ability has been previously demonstrated by inhibition of target gene expression (Maldonado-Perez et al. 2009).

2.3.3 Dual luciferase assay

CXCL1 promoter activity was determined by using the Dual-luciferase reporter assay system (Promega). Total luciferase activity was determined relative to the internal renilla control and fold increase in luciferase activity was determined as compared to vehicle treated cells.

2.4 Polymerase Chain Reaction (PCR)

2.4.1 RNA extraction

RNA was extracted from cells using TRI-reagent (Sigma Aldrich, Dorset, UK) following manufacturer's guidelines. A volume of 1 ml of TRI-reagent was added to experimental wells to lyse cells and samples were placed in Phase Lock tubes

(Flowgen Bioscience, Nottingham, UK). A volume of 200 µl bromochloropropane (Sigma Aldrich) was added and samples were shaken vigorously for 15 seconds. Samples were left to stand at room temperature (RT) for approximately 5 minutes before centrifugation at 14000 rpm for 15 minutes at 4 °C. After centrifugation the RNA remains in the upper aqueous phase above the gel layer of the Phase Lock tube, and DNA and proteins remain in the organic phase below. A volume of 500 µl isopropanol was added to the aqueous phase and again spun at 14000 rpm for 15 minutes at 4 °C to precipitate the RNA. The RNA pellet was then washed in 1 ml 70% ethanol by centrifugation at 14000 rpm for 5 minutes at 4 °C. The remaining RNA pellet was then air dried and resuspended in DEPC treated water.

RNA was extracted from tissues using the Qiagen RNeasy Mini kit (Qiagen, Crawley, West Sussex, UK). Pieces of tissue, in 2 ml microcentrifuge tubes containing a stainless steel ball, were homogenised in 600 µl RLT lysis buffer using a tissue lyser (Qiagen) for 2 x 2 minutes at 25 Hz. The lysate was then centrifuged for 3 minutes at 14000 rpm and the supernatant removed into a new microcentrifuge tube. A volume of 600 µl of 70 % ethanol was added to the supernatant which was then transferred to an RNeasy spin column and centrifuged for 15 seconds at 14000 rpm. A volume of 700 µl of wash buffer RW1 was added to the spin column which was centrifuged at 14 000 rpm for 15 seconds. On-column digestion of DNA was then performed by addition of DNAse1 (Qiagen) which was incubated at RT for 15 minutes. DNAse 1 was then removed by a further wash with wash buffer RW1. Subsequently 700 µl of buffer RPE containing ethanol, to wash the RNA, was added to the column and centrifuged at 14 000 rpm for 2 minutes. This longer centrifuge ensured all residual ethanol was removed from the columns. To elute the RNA which had remained stuck to the column throughout, 30 µl DEPC treated water was added to the columns which were centrifuged for 1 minute at 14 000 and the eluate was collected in new microcentrifuge tubes.

RNA was quantified using a Nanodrop-1000 spectrophotometer (Labtech International, East Sussex, UK) measuring at a wavelength of 260 nm and 280 nm and RNA concentration was adjusted to 100 ng / µl with DEPC-treated water.

2.4.2 Reverse transcriptase PCR

RNA samples were reverse transcribed for TaqMan quantitative PCR using the following reaction (reagents all supplied by PE Applied Biosystems, Warrington, UK):

Water:	1.75 µl
Reaction buffer	1 µl
Magnesium Chloride	2.2 µl (5.5 mM)
Deoxy (d)-NTPs	2 µl (0.5 mM)
RNAse inhibitor	0.2 µl (0.4 U)
Random Hexamers	0.25 µl (2.5 µM)
Oligo dT	0.25 µl (2.5 µM)
MultiScribe reverse transcriptase	0.25 µl

The mix was aliquoted into individual tubes (8 µl / tube) and template RNA was added (2 µl/ tube of 100 ng RNA) or 2 µl water to control for contamination. Samples were incubated for 90 min at 25 ºC, 45 min at 48 ºC and 5 min at 95 ºC. Thereafter samples were stored at -20 ºC. A tube with no reverse transcriptase was also included to control for any genomic DNA contamination.

2.4.3 TaqMan quantitative PCR

2.4.3.1 Principle of reaction

TaqMan quantitative PCR was used to accurately quantify mRNA of genes of interest in a sample. TaqMan probes have the fluorophore FAM attached to the 5' end and a quencher dye (TAMRA) attached to the 3' end. The 18S probe used in this thesis used the VIC fluorophore to distinguish from the gene of interest. After annealing to the gene of interest between the primers, Taq polymerase extends from the primer 5' to 3' and displaces FAM. This leads to a separation between the

fluorophore and the quencher, leading to an increase in fluorescence which can be measured. As the PCR reaction is exponential, the original amount of cDNA in the sample, and therefore RNA, can be quantified by measuring amount of fluorescence (Figure 2.1).

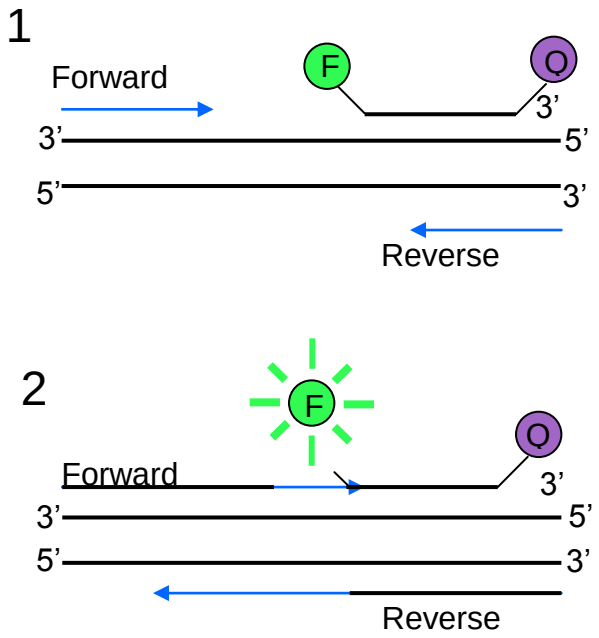

Figure 2.1 Principle of Taqman reaction. 1: Specific primers and probe anneal to the gene of interest. Taq polymerase begins to extend the primers from 5' to 3'. 2: Taq polymerase extends the primer to displace the fluorophore FAM (F) from the TAMRA quencher (Q). This results in an increase in fluorescence which can be measured, which increases as the PCR reaction continues exponentially.

2.4.3.2 TaqMan reaction

cDNA expression was measured using an ABI Prism 7900 HT Fast Real Time PCR machine (PE Applied Biosystems, Warrington UK). Primers and probes were designed using PRIMER express program Version 3.0 (PE Applied Biosystems), and were supplied by Eurogentec (Seraing, Belgium). Sequences are given in Table 2.3. Primers and probes were designed with the following features:

- Multiple repeats of a nucleotide, particularly guanosine, were avoided

- A guanosine at the 5' end of the probe was avoided to prevent quenching when cleaved
- Primer melting temperature was 58-60 °C, and was equal between the forward and reverse primers
- Primers were designed as close together as possible without overlapping the probe
- Primers and probes were designed where possible to run over an exon-exon junctions, to avoid genomic DNA contamination
- Amplicon length was designed to be 50-150 bp

The following master mix was made up (reagents supplied by PE Applied Biosystems, Warrington, UK):

TaqMan buffer (5.5 mM $MgCl_2$, 200 µm dATP, 200 µM dCTP, 200µM dGTPm 200 µM dUTP) - 25 µl
Forward primer (300 nM) – 0.6 µl
Reverse primer (300 nM) – 0.6 µl
Probe (100 nM) – 2 µl
18S mix (forward and reverse primers and probe, 50 nM) – 0.75 µl
Water – 19.05 µl
cDNA (40 ng) – 2 µl

This mix was added in duplicate to a 96 well MicroAmp fast optical reaction PCR plate (PE Applied Biosystems, Warrington UK). Reactions were incubated for 5 min at 45°C followed by 5 min at 95C, followed by PCR cycling for 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C.

Gene	Sequence of primers and probe
CXCL1: Forward	5'-GTTTTCAAATGTTCTCCAGTCATTATG-3'
Reverse	5'-CCG CCA GCC TCT ATC ACA GT-3'
Probe	5'-TTC TGA GGA GCC TGC AAC ATG CCA-3'
CXCR2: Forward	5'-TGC TCT TCT GGA GGT GTC CTA CA-3'
Reverse	5'-AGA TCT TCA CCT TTC CAG AAA TCT T-3'
Probe	5'-CCC AGC GAC CCA GTC AGG ATT TAA-3'
CCL20: Forward	5'-TCC TGG CTG CTT TGA TGT CA-3'
Reverse	5'-CCA AGA CAG TCA AAG TTG CT-3'
Probe	5'-TGC TGC TAC TCC ACC TCT GCG GC-3'
CCR6: Forward	5'-TTG GCT ATA CGA AAA CTG TCA CAG A-3'
Reverse	5'-AAG CGT AGA GCA CAG GGT TCA-3'
Probe	5'-TCC TGG CTT TCC TGC ACT GCT GC-3'
LPAR1: Forward	5'-CGT CAG GGC CTC ATT GAC A-3'
Reverse	5'-GTG CCT CTC GAT TGC AAT AGC-3'
Probe	5'-CCT GAC GGC ATC TGT GGC CAA CTTA-3'
LPAR2: Forward	5'-GTG TAC ACC CCG CAT TTT CTT C-3'
Reverse	5'-ACG CCC CCA GGA TGA TG-3'
Probe	5'-AGA GCA TGT CAG CTG CCA CCC CC-3'
LPAR3: Forward	5'-ACG TCT TGT CT CCG CAT ACA A-3'
Reverse	5'ACG CCC CTA AGA CAG TCA TCA-3'
Probe	5'TAC ATC AGC CGC CGG AGG A-3'
COX-2: Forward	5'-CCT TCC TCC TGT GCC TGA TG-3'
Reverse	5'-ACA ATC TCA TTT GAA TCA GGA AGC T-3'
Probe	5'-TGC CCG ACT CCC TTG GGT GTC A-3'
18S: Forward	5' -CGG CTA CCA CAT CCA AGG AA- 3'
Reverse	5' -GCT GGA ATT ACC GCG GCT- 3'
Probe	5' -TGC TGG CAC CAG ACT TGC CCT C- 3'

Table 2.3 Sequences of TaqMan primers and probes.

Results were analysed by the use of the comparative Ct method, where gene expression is measured relative to a reference calibrator sample, such as a sample of normal endometrium. Variation due to differing amounts of RNA and pipetting errors are taken into account by normalising results relative to an endogenous

housekeeping gene (18S). First the expression of gene of interest is normalised to that of the housekeeping gene by subtraction of the threshold cycle (Ct; the cycle number at which the gene is first detected), which is termed ΔCt. This assumes that primers and probes of 18S and the gene of interest are working at similar efficiencies (see section 2.4.3.3). The difference between each sample and the calibrator sample, the ΔΔCt, is then determined. Finally, the differences in transcript are expressed using the equation:

$$2^{\wedge} (- \Delta\Delta Ct)$$

This is derived from the equation describing the exponential amplification of PCR:

$$X_n = X_0 (1 + E_x)^{Ct,x}$$

Where X_n is the number of templates at cycle X, X_0 is the number of template copies at initiation, E_X is the efficiency of target amplification, and n is the number of cycles.

As the ΔΔCt of the control sample is subtracted against itself, its final value after applying 2^(- ΔΔCt) is 1. Therefore all other samples are expressed as fold difference relative to this.

2.4.3.3 Primer and probe validation

As genes of interest were normalised to the endogenous gene 18S, primers and probes needed to be validated to ensure they worked at similar amplification efficiencies. If amplification of the gene of interest and reference gene are similar, when plotted against dilutions of RNA (log ng RNA) the ΔCt values should plot a straight line. Primer validations were carried out for each TaqMan primer and probe set designed. An example of the validation of CXCL1 is given below (Figure 2.2).

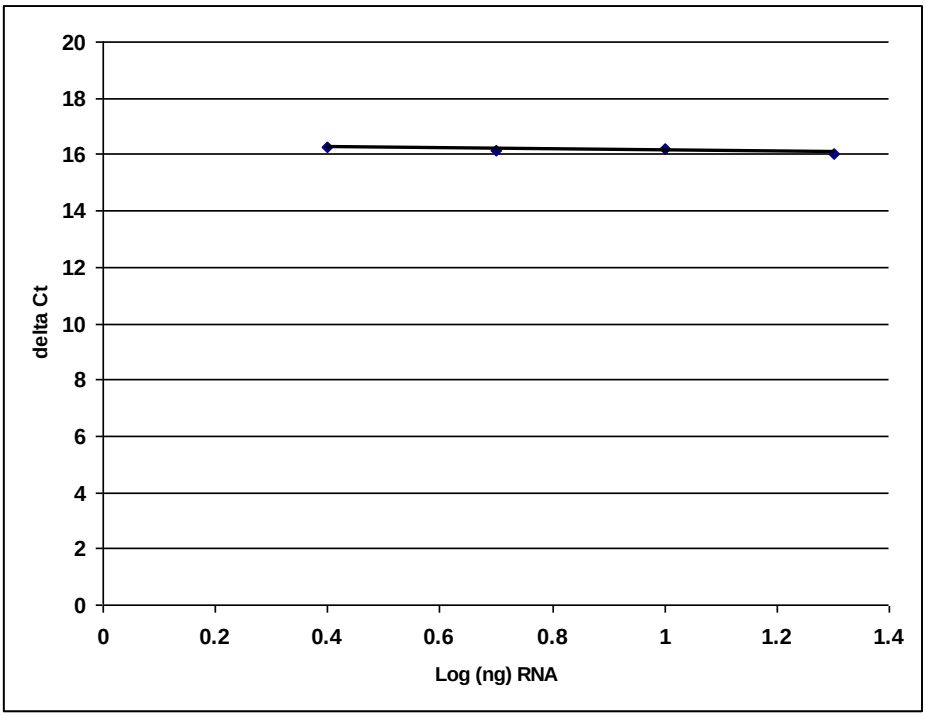

Figure 2.2 CXCL1 primer and probe validation, with log ng RNA plotted against ΔCt.

2.5 Immunohistochemistry

2.5.1 Tissue fixation and processing

Tissues were fixed immediately in 4% neutral buffered formaldehyde (NBF) for 24 hours and transferred to 70% ethanol for a further 24 hours. Tissues were subsequently wax embedded using standard protocols.

Tissues were cut into 5 µm sections and adhered to glass slides by flotation in warm water. Slides were then incubated at 37 °C for at least 24 hours to ensure complete adherence to the slide.

2.5.2 3'3- Diaminobenzidine tetrahydrochlorine immunohistochemistry

The localisation of specific proteins in tissue can be detected by the use of immunohistochemistry. A primary antibody binds specifically to an epitope of the protein of interest. The secondary antibody raised against the species of the primary antibody then binds to the primary antibody. The secondary antibody is conjugated to

biotin and so is bound by streptavidin horseradish peroxidise (HRP). This can be visualised by with the addition of 3,3- diaminobenzidine tetrahydrochlorine (DAB), which produces an insoluble brown precipitate in the presence of HRP.

Slides were dewaxed in xylene for 10 minutes followed by rehydration through graded ethanol (100%, 95%, 80%, and 70%) for 20 seconds each and a final wash in water. Antigen retrieval was performed for antibodies which required epitope unmasking. Where this was required, slides were boiled in a pressure cooker in either citrate buffer (0.01M, pH 6) or EDTA buffer (1mM, pH 8) for 5 minutes and left to stand for a further 20 minutes.

Endogenous peroxidase activity was then blocked by washing sections in 3% hydrogen peroxidase in methanol. After a brief wash in tap water slides were washed in Tris buffered saline (TBS) twice for 5 minutes each. Sections were then incubated in normal serum from the species in which the secondary antibody was raised for at least 30 minutes at room temperature. The primary antibody was then applied diluted in 2 parts serum: 4 parts TBS: 0.5% BSA at the dilutions indicated in Table 2.4 and incubated at 4 °C overnight. Control sections were included with no primary antibody, non-immune IgG or primary antibody pre-absorbed with blocking peptide (CXCL1: sc-1374-P, CCL20: sc-9775-P, Santa Cruz Biotechnology, Autogen Bioclear, UK). Following further washes in TBS, the indicated biotinylated secondary antibody was applied diluted in normal serum at 1:200 for 30 minutes at room temperature. Sections were again washed twice in TBS and subsequently streptavidin-HRP (DAKO, UK) diluted 1:1000 in TBS was added for 30 minutes at room temperature. After more TBS washes, slides were incubated with DAB (DAKO, UK) for 1-5 minutes until positive staining was identified by a brown colour under x10 magnification.

Slides were then counterstained for 1-5 minutes in haemotoxylin followed by 5-10 seconds in 1% acid alcohol to remove any non-specific staining. A blue colour was developed by a 30 second wash in Scotts Tap Water then slides were dehydrated

through graded alcohols for 20 seconds each and xylene for 2 x 5 minutes. Slides were coverslipped using Pertex (Cell Path, Hemel Hempstead, UK).

Primary Antibody and supplier	Species and dilution used	Antigen retrieval	Block	Secondary Antibody and supplier
CXCL1 (Santa Cruz, sc-1374)	Goat, 1:100	Citrate	Rabbit	Rabbit anti-goat (DAKO EO466)
Neutrophil elastase (DAKO, MO752)	Mouse, 1:100	Not required	Goat	Goat anti-mouse (Sigma, B6649)
CXCR2 (R&D Systems, MAB331)	Mouse, 1:75	Citrate	Goat	Goat anti-mouse (Sigma, B6649)
CCL20 (Santa Cruz, sc-9775)	Goat, 1:100	Citrate	Rabbit	Rabbit anti-goat (DAKO EO466)
CCR6 (Santa Cruz, sc-9697)	Goat, 1:75	Citrate	Rabbit	Rabbit anti-goat (DAKO EO466)
FP (Cayman, 101802)	Rabbit, 1:75	Citrate	Goat	Goat anti-rabbit (DAKO, E0432)
CD4 (Invitorgen, 08-1282)	Mouse, Prediltued	EDTA	Goat	Goat anti-mouse (Sigma, B6649)
CD8 (Santa Cruz, sc-70791)	Mouse, 1:50	Citrate	Goat	Goat anti-mouse (Sigma, B6649)
CD56 (Zymed, CA123C3)	Mouse, 1:250	Citrate	Goat	Goat anti-mouse (Sigma, B6649)
CD68 (DAKO, M0866)	Mouse, 1:250	Citrate	Goat	Goat anti-mouse (Sigma, B6649)
CD20cy (DAKO, M0755)	Mouse, 1:150	Citrate	Goat	Goat anti-mouse (Sigma, B6649)
CD11c (Abcam, ab62056)	Rabbit, 1:200	Citrate	Goat	Goat anti-rabbit (DAKO, E0432)
Gr-1 (R&D Systems, MAB1037)	Rat, 1:100	Not required	Rabbit	Rabbit anti-rat (Vector BA4000)
CD31 (Abcam, ab28364)	Rabbit, 1:100	Citrate	Goat	Goat anti-rabbit (DAKO, E0432)
LPAR1 (EDG-2; Abcam, ab13026)	Rabbit, 1:500	Citrate	Goat	Goat anti-rabbit (DAKO, E0432)
LPAR2 (EDG-4; Abcam, ab13349)	Rabbit, 1:250	Citrate	Goat	Goat anti-rabbit (DAKO, E0432)

Primary Antibody and supplier	Species and dilution used	Antigen retrieval	Block	Secondary Antibody and supplier
LPAR3 (Abcam, ab13350)	Rabbit, 1:300	Citrate	Goat	Goat anti-rabbit (DAKO, E0432)

Table 2.4 List of primary and secondary antibodies used for DAB immunohistochemistry.

2.5.3 Dual immunofluorescence microscopy

Dual immunofluorescence microscopy is used to determine if two or more antigens are expressed in the same cell. Specific protocols for antibodies are described in individual chapters. Protocols using two antibodies from the same species required a microwave blocking step and the use of secondary antibodies with fragment antigen binding fragments (Fab fragments). These ensure that there is no open binding site on the secondary antibody which can cross react with the next primary antibody which is added (Negoescu et al. 1994; Lan et al. 1995), and are described in detail in Section 4.2.6. Generally, slides were treated as for DAB immunohistochemistry until the addition of the secondary antibody, with the exception of carrying out washes in phosphate-buffered saline (PBS). A biotinylated or peroxidase conjugated antibody was then added, diluted in normal serum at 1:200 or 1:500 respectively for 30 minutes. Streptavidin AlexaFluor 488 or 546 (Molecular Probes) diluted 1:200 in PBS was then added to biotyinlated secondary antibodies for 30 minutes at room temperature. To peroxidase-incubated sections, tyramide-cy3 (PerkinElmer, Massachusetts, USA) diluted 1:50 in tyramide buffer was added for 10 minutes. Alternatively, a fluorophore-conjugated antibody was added diluted 1:200 in PBS (AlexaFluor 488 or 546, Molecular Probes, UK) for 1 hour. Sections were then re-blocked in normal serum and the process repeated with the second primary antibody. Nuclei were stained using ToPro (Molecular Probes, UK). Slides were coverslipped using Permafluor (Immunotech, Marseille, France).

2.5.4 Immunohistochemistry imaging

DAB immunohistochemistry was visualised on a PROVIS microscope (Olympus Optical, London, UK) and images obtained using Canon EOS image capture software (Canon, Woodhatch, Surrey, UK). Dual immunofluorescence immunohistochemistry was visulaised on a LSM 510 Meta-Confocal (Carl Zeiss, Hertfordshire, UK) and images obtained using LSM5 Image Browser.

2.6 Enzyme linked immunosorbent assays (ELISAs)

2.6.1 CXCL1

CXCL1 protein secretion by Ishikawa FPS cells in the culture medium was measured by ELISA. FPS cells were serum starved in medium containing 3 µg/ml indomethacin for at least 18 hours prior to one hour pre-incubation with selected chemical inhibitors. Cells were then stimulated with fresh serum-free media containing indomethacin with vehicle, 100nM $PGF_{2\alpha}$ (n=4) or 100nM $PGF_{2\alpha}$ and chemical inhibitors (n=5) for time points indicated by the figure legend. ELISA plates were coated with monoclonal mouse anti-human CXCL1 antibody (720 µg/ml, clone 20326, R&D systems, Abingdon, UK) and left at 4 ºC overnight. Wells were then blocked with Dry Coat solution (2% polyvinyl pyridine, 1% BSA, 0.05% sodium azide, 5mM EDTA in Tris-buffered saline) for 1 hour followed by addition of CXCL1 standard (recombinant human CXCL1, R&D Systems, Abingdon, UK) and culture medium samples in duplicate diluted in ELISA buffer (2mg / ml BSA, 2mM EDTA and 300µl Phenol Red Solution and Tween-20% in Tris-buffered saline). Plates were incubated for 3 hours at room temperature then washed in ELISA wash buffer (0.05% Tween in PBS, pH 7.2-7.4) and 50ng/ml biotinylated goat anti-human CXCL1 antibody (R&D Systems, Abingdon, UK) was added for 1 hour at room temperature. After further washing, streptavidin peroxidase (dilution of 1:2000, Roche UK) was added for 20 minutes. Colour change was detected by addition of substrate solution (1:1 mix in 100mM sodium acetate buffer, pH 6, of: 5mg/ml hydrogen peroxide in sodium acetate buffer, pH 6: tetramethylbenzidine 3mg/ml in

dimethyl formamide) and the reaction was stopped by addition of 2N sulphuric acid. Optical density of wells was read at 450nM.

2.6.2 CCL20

CCL20 protein secretion by Ishikawa FPS cells in the culture medium was measured by ELISA. FPS cells were serum starved in medium containing 3 µg/ml indomethacin for at least 18 hours prior to one hour pre-incubation with selected chemical inhibitors. Cells were then stimulated with fresh serum-free media containing indomethacin with vehicle, 100nM $PGF_{2\alpha}$ (n=4) or 100nM $PGF_{2\alpha}$ and chemical inhibitors (n=5) for time points indicated by the figure legend. CCL20 ELISA was performed using the Human CCL20/MIP-3 alpha DuoSet (R&D Systems, Abingdon, UK). Plates were coated with capture antibody at 2 µg/ml and left at room temperature overnight. Wells were then blocked with Dry Coat solution for 1 hour followed by addition of CCL20 standard and culture media samples in duplicate diluted in ELISA buffer. Plates were incubated for 2 hours at room temperature then washed in ELISA wash buffer. The detection antibody was then added to wells diluted to 50ng/ml in ELISA buffer and incubated for 2 hours at room temperature. Wells were then washed and streptavidin-HRP diluted 1:200 in ELISA buffer was added for 20 minutes at room temperature. Colour change was detected by addition of substrate solution and the reaction was stopped by addition of 2N sulphuric acid. Optical density of wells was read at 450nM.

2.7 Statistical Analysis

Where appropriate, statistical analysis was carried out using GraphPad Prism (GraphPad Software. San Diego, California, USA).

2.8 Commonly used solutions

2.8.1 Tissue Culture

Complete Medium

Dulbecco's Modified Eagles Medium (DMEM) Glutamax F-12, supplemented with:
50 mls (10%) (v/v) heat inactivated foetal bovine serum

5 mls (100 U/ml) Penicillin and (100 µg/ml) Streptomycin

200µg/ml G418 (FPS Ishikawa cells only)

Starving Medium

Dulbecco's Modified Eagles Medium (DMEM) Glutamax F-12, supplemented with:

5 mls (100 U/ml) Penicillin and (100 µg/ml) Streptomycin

200µg/ml G418 (FPS Ishikawa cells only)

Transfection Medium

Dulbecco's Modified Eagles Medium (DMEM) Glutamax

2.8.2 Luciferase reporter assays

NPS Lysis Buffer

4.4g NaCl

1.86g EDTA

25 ml 1M Tris pH 7.4

3 ml NP40

50 ml Glycerol

To 500 ml with dH_2O

2.8.3 Immunohistochemistry

NBF fixative

100 ml 40% formaldehyde

900 ml Distilled water

4 g Sodium dihydrogen phosphate monohydrate

6.5 g Disodium hydrogen phosphate anhydrous

Citrate buffer (0.1M)

42.02 g Citric acid monohydrate

900 ml Distilled water

pH to 5.5 with concentrated NaOH

Make up to 2L and pH to 6

0.5M Tris-HCl stock x10

121.14 g Tris(Hydroxymethyl)methylamine

2000 ml distilled water

pH to 7.4 with HCl

0.05M TBS

100 ml 0.5M Tris-HCl

900 ml distilled water

8.5 g sodium chloride

Peroxidase blocking solution

30 ml of 30% (3% v/v) Hydrogen Peroxide

270 ml Methanol

Blocking solution

2ml non-immune serum

8 ml PBS

0.5 g BSA

Chapter 3

$PGF_{2\alpha}$-F prostanoid receptor regulation of chemokine (C-X-C) motif ligand 1 in endometrial adenocarcinoma

3 $PGF_{2\alpha}$-F-prostanoid receptor regulation of chemokine (C-X-C motif) ligand 1 expression in endometrial adenocarcinoma

3.1 Introduction

Overexpression of the cyclooxygenase (COX) enzymes and prostaglandins has been demonstrated in endometrial adenocarcinoma (Jabbour et al. 2006) as well as a number of other cancer types such as breast, bladder and lung (Cha et al. 2007). Expression of the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor, FP, is elevated in endometrial adenocarcinoma (Sales et al. 2004). FP is a G_q coupled receptor which upon activation leads to release of inositol-1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Abramovitz et al. 1994). A role for FP in endometrial adenocarcinoma has been demonstrated with evidence for elevated FP activation leading to the upregulation of a number of angiogenic and tumourigenic genes including COX-2 (Jabbour et al. 2005), FGF2 (Sales et al. 2007) and vascular endothelial growth factor (VEGF; Sales et al. 2005). FP activation can also increase proliferation and migration of neoplastic epithelial cells (Milne et al. 2003; Sales et al. 2004; Sales et al. 2008).

The chemokine family is divided into four groups based on the position of two cysteine molecules (C) and any other amino acid (X) in the amino terminal of the protein. The groups are known as C, C-C, C-X-C and C-X3-C (Murphy et al. 2000). Chemokine (C-X-C motif) ligand 1 (CXCL1, also known as growth-regulated oncogene α) is a member of the CXC family, which is further subdivided into glutamine-leucine-arginine positive or negative (ELR+ or ELR-) chemokines, dependent on the presence or absence of this amino acid motif. ELR+ chemokines have angiogenic properties, whilst ELR- chemokines are angiostatic (Strieter et al. 1995).

CXCL1 is an ELR+ chemokine with angiogenic, chemoattractant and inflammatory activities (Strieter et al. 2006). It binds to the CXCR2 receptor, a GPCR, (Addison et al. 2000) to promote the recruitment of neutrophils to sites of inflammation (Moser et al. 1990). CXCL1 has also been implicated in tumourigenesis as it is upregulated in a number of cancer types including melanoma (Haghnegahdar et al. 2000; Wang et al. 2000), colorectal (Li et al. 2004; Wen et al. 2006; Rubie et al. 2008), and prostate (Moore et al. 1999). CXCL1 has been demonstrated to promote a variety of processes important to tumour progression. For example, increased CXCL1 expression is associated with greater invasive ability in bladder cancer (Kawanishi et al. 2008), promotion of proliferation in oesophageal cancer (Wang et al. 2006) and angiogenesis in prostate cancer (Moore et al. 1999).

Recent evidence suggests a link between prostaglandin receptor signalling and CXCL1 expression as PGE_2 signalling via the EP4 receptor induces CXCL1 expression in a colorectal cancer cell line (Wang et al. 2006). Although localisation of CXCL1 and CXCR2 has previously been described in the normal endometrium (Nasu et al. 2001; Mulayim et al. 2003), expression of CXCL1 and CXCR2 in endometrial adenocarcinoma and the potential role of FP in the regulation of CXCL1 have not been examined. This chapter therefore sought to investigate the regulation of CXCL1 by $PGF_{2\alpha}$ in an endometrial adenocarcinoma cell model expressing the FP receptor, and the expression and localisation of CXCL1 and CXCR2 in endometrial adenocarcinoma tissue.

3.2 Materials and Methods

3.2.1 Tissue collection

Normal endometrium and endometrial adenocarcinoma tissue was collected as described in sections 2.1.1 and 2.1.2. Normal endometrial tissue was collected from women undergoing surgery for minor gynaecological procedures with no underlying endometrial pathology, and endometrial adenocarcinoma tissue was obtained from women undergoing hysterectomy after diagnosis of adenocarcinoma of the uterus.

Stage of cycle and grade of cancer was assessed by a pathologist. Written informed consent was collected from all patients before surgery and ethical approval was obtained from Lothian Research Ethics Committee.

3.2.2 Cell culture

Ishikawa FPS cells were maintained as described in section 2.2.1. Primary tissue explants were maintained as described in section 2.2.2.

3.2.2.1 Cell and tissue treatments

To determine the pattern of CXCL1 expression in response to $PGF_{2\alpha}$, FPS cells were serum starved in media containing 3 µg/ml indomethacin for at least 18 hours prior to stimulation for 2, 4, 6, 8, 12, 18, 24, 48 and 72 hours with fresh serum-free media containing indomethacin and vehicle or 100 nM $PGF_{2\alpha}$ (n=5). Carcinoma tissue was serum starved in media containing 3 µg/ml indomethacin for at least 18 hours prior to treatment for 2, 4, 6, 8, and 24 hours with fresh serum-free media containing indomethacin and vehicle or 100 nM $PGF_{2\alpha}$ (n=4).

To determine signalling pathways leading to CXCL1 expression regulated by $PGF_{2\alpha}$, FPS cells or carcinoma tissue were serum starved in media containing 3 µg/ml indomethacin for at least 18 hours and treated for 8 or 24 hours respectively with vehicle, inhibitor alone, 100 nM $PGF_{2\alpha}$ alone or 100 nM $PGF_{2\alpha}$ and a panel of chemical inhibitors (n=7) or dominant negative constructs (n=5) as indicated in the figure legends.

3.2.3 Chemokine antibody array

Cytokine expression by Ishikawa FPS cells was examined using the Raybio Human Cytokine Antibody Array 3 (Raybiotech Inc, Middlesex, UK), performed according to manufacturer's instructions. FPS cells were plated in 60mm petri dishes and stimulated for 24 hours with vehicle or 100 nM $PGF_{2\alpha}$ (n=3 each treatment). Condtioned media was collected from each treatment and pooled. Antibody array membranes were blocked for 30 minutes with 2 ml blocking buffer before addition of 2 ml of conditioned medium for 2 hours at room temperature (RT) to allow binding

of the proteins in the conditioned media to the impregnated antibodies on the membrane. Membranes were then washed with wash buffer 1 for 3 x 5 minutes and wash buffer 2 for 2 x 5 minutes. The biotin-conjugated primary antibodies were diluted in 2 ml blocking buffer and added to the membranes and incubated for 2 hours at RT. Membranes were then washed as above and incubated with the HRP-conjugated streptavidin (diluted 1:1000 in blocking buffer) at 4 °C overnight. Membranes were again washed as above and incubated with the detection buffers (1:1 mix of detection buffer C and D) for 2 minutes at RT. Cytokine production was visualised by detection of the streptavidin-HRP reaction on x-ray film after a 20 second exposure.

3.2.4 Polymerase chain reaction

3.2.4.1 RNA extraction and reverse transcriptase PCR

RNA from cells was extracted as described in section 2.4.1. Briefly, 1 ml TRI-reagent was added to experimental wells and RNA extracted using the phenol method.

RNA from tissue was extracted as described in section 2.4.1 using the Qiagen minikit protocol. Pieces of tissue, in 2 ml microcentrifuge tubes containing a stainless steel ball, were homogenised in 600 µl RLT lysis buffer using a tissue lyser (Qiagen Crawley, West Sussex, UK) for 2 x 3 minutes at 25 Hz. RNA was then extracted following manufacturers guidelines (Qiagen). cDNA was prepared as described in section 2.4.2.

3.2.4.2 Taqman quantitative RT- PCR

CXCL1 and CXCR2 mRNA expression in cells and tissues was examined using Taqman quantitative PCR as described in section 2.4.3.2. Briefly, primer and probe sequences were designed to amplify CXCL1 and CXCR2 (sequences given in Table 2.2). A master mix was made up containing 18S primers and probes and 200ng cDNA added to each sample. Samples were added in duplicate to a 96 well MicroAmp fast optical reaction PCR plate and analysed using an ABI Prism 7900

HT Fast Real Time PCR machine. Expression of analyzed genes was normalised to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed as fold increase above vehicle treated cells.

3.2.5 FP adenovirus

A short hairpin adenovirus to knock down expression of FP was kindly prepared by Dr Pamela Brown (Biomolecular Core Facility, MRC Human Reproductive Sciences Unit, Edinburgh, UK) as described in section 2.2.5. FP cDNA (ORIGENE, Rockville, MD) was excised with EcoRI and Sma1 and fused to the pDC316 vector to create pDC316-FP. HEK-293 cells were seeded in 6-well plates in nutrient mixture F-12 with Glutamax-1 and pyridoxine supplemented with 10% FCS and 1% penicillin-streptomycin (500 IU/ml penicillin and 500 µg/ml streptomycin). Once at 50% confluency, cells were co-transfected with 0.75 µg pDC316-FP and 2.25 µg pBGH10 ΔE1,3 Cre adenoviral backbone in Optimem Reduced Serum Media (Invitrogen) using Trans-IT-293 transfection reagent (Mirus Bio Corp, Madison, WI) as per the manufacturer's instructions. Cells were incubated for approximately 2 weeks with regular changes of media until evidence of cytopathic effect (CPE) was seen, which was defined as a rounded appearance to cells and when approximately 70% of cells had lifted from the plate surface. Adenovirus was then harvested and virus released from the cells by 3 freeze-thaw cycles. Serial dilutions of the virus were performed and used to re-infect HEK-293 cells at 75-85% confluence. Wells were overlaid with 0.5% SeaPlaque Agarose (FMC Corp, Rockland, ME) to ensure homogenous plaques were obtained. Plaques were visible within 4-5 days and were picked using a P1000 tip. Plaques were freeze-thawed 3 further times and used to re-infect a further T75 flask which was incubated until CPE was observed. The virus from this flask was harvested and termed the first seed. The adenovirus was then expanded by inoculation of further flasks with the first seed. Adenovirus was purified and concentrated using the Vivapure AdenoPACK 100 purification kit (Sartorius AG, Goettingen, Germany) as per manufacturer's instructions. Virus titres were determined using the AdenoX Rapid titre kit (Clontech, France) as per manufacturer's instructions.

FPS cells were seeded in 6-well plates and incubated for 24 hours. Cells were then infected with 5 viruses / cell in complete medium. After 24 hour incubation, cells were starved in media containing 3 µg/ml indomethacin for 18 hours before treatment with 100 nM $PGF_{2\alpha}$ or vehicle for 8 hours (n=4).

3.2.6 Immunohistochemistry

3,3- diaminobenzidine tetrahydrochlorine (DAB) immunohistochemistry to localise CXCL1 and CXCR2 in endometrial tissue was performed as described in section 2.5.2 (for immunolocalisation of CXCL1: proliferative stage endometrium n=7, early secretory stage endometrium n=3, mid secretory stage endometrium n=4, late secretory stage endometrium n=5, well differentiated adenocarcinoma n=7, moderately differentiated adenocarcinoma n=5, poorly differentiated adenocarcinoma n=3. For immunolocalisation of CXCR2: proliferative stage endometrium n=4, early secretory stage endometrium n=2, mid secretory stage endometrium n=3, late secretory stage endometrium n=4, well differentiated adenocarcinoma n=5, moderately differentiated adenocarcinoma n=5, poorly differentiated adenocarcinoma n=5). Tissue sections (5 µm) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed, followed by blocking for endogenous peroxidase activity by washing sections in 3% hydrogen peroxidase in methanol. Sections were then blocked in normal serum of rabbit (for immunolocalisation of CXCL1) or goat (for immunolocalisation of CXCR2) followed by overnight incubation with primary antibody at the concentrations indicated in Table 2.4. Control sections were included with non-immune IgG for both antibodies and primary antibody pre-absorbed with blocking peptide (performed for immunolocalisation of CXCL1). Sections were then incubated in rabbit anti-goat (for immunolocalisation of CXCL1) or goat anti-mouse (for immunolocalisation of CXCR2) biotinylated secondary antibody and subsequently streptavidin-HRP. Sections were then incubated with DAB for 1-5 minutes until positive staining was identified by a brown colour under x10 magnification.

3.2.7 CXCL1 enzyme linked immunosorbent assay (ELISA)

CXCL1 protein released into the culture medium from treatments described in section 3.2.2.1 was measured by ELISA (n=5; R&D Systems, Abingdon, UK) as described in section 2.6.1.

3.2.8 Transient transfection of CXCL1 luciferase promoter construct

CXCL1 promoter activity was measured in FPS cells by transient transfection of the CXCL1 reporter plasmid consisting of the CXCL1 minimal promoter region inserted into the pGL2 Basic vector (Promega, Southhampton, UK) fused to the firefly luciferase reporter, as described in section 2.4.1. FPS cells were seeded in 12 well plates and 1.5 µg of CXCL1 construct co-transfected with 0.15 µg pRL-TK (containing the renilla luciferase coding sequence; Promega) as an internal control using Superfect transfection reagent (Qiagen) as per the manufacturer's instructions. After 4 hours the transfection media was removed and replaced with complete DMEM for at least 24 hours before cell treatments were carried out as described in section 3.2.2.1 (n=7). Cells were then washed in ice cold PBS and lysed in NPS lysis buffer. Promoter activity was measured by luciferase assay as described in section 3.2.10.

3.2.9 Transient transfection of dominant negatives and CXCL1 luciferase reporter construct

CXCL1 promoter activity in response to $PGF_{2\alpha}$ was examined in the presence of dominant negative isoforms of signalling molecules, as described in section 2.3.2. FPS cell transfection was performed as in section 3.2.8 with the addition of 1.5 µg of vector encoding a dominant negative isoform of EGFR, Ras, MEK, NFAT or empty vector (pcDNA3.0). Cells were then treated with vehicle control or 100 nM $PGF_{2\alpha}$, and washed in ice cold PBS and lysed in NPS lysis buffer (n=5). Promoter activity was measured by luciferase assay as described in section 3.2.10.

3.2.10 Luciferase reporter assay

CXCL1 promoter activity was determined by using the Dual-luciferase reporter assay system (Promega). Total luciferase activity was determined relative to the

internal renilla control and fold increase in luciferase activity was determined as compared to vehicle treated cells.

3.2.11 Statistical analysis

Where appropriate, data were analysed by Students t-test and one way ANOVA using GraphPad Prism (GraphPad Software. San Diego, California, USA). Data are presented as mean ± SEM.

3.3 Results

3.3.1 $PGF_{2\alpha}$ – FP receptor interaction induces CXCL1 protein secretion

In order to determine cytokines regulated by $PGF_{2\alpha}$ – FP signalling in Ishikawa FPS cells, a cytokine antibody array was used to examine protein changes in conditioned medium taken from FPS cells stimulated with vehicle or 100 nM $PGF_{2\alpha}$ (Figure 3.1). An increase in expression of the cytokines IL-6, IL-7, IL-8 and leptin was observed following $PGF_{2\alpha}$ treatment of FPS cells. Additionally, increased expression of members of the CXCL family of proteins (CXCL1, 2 and 3) was identified, as well as increased expression of CXCL1 alone.

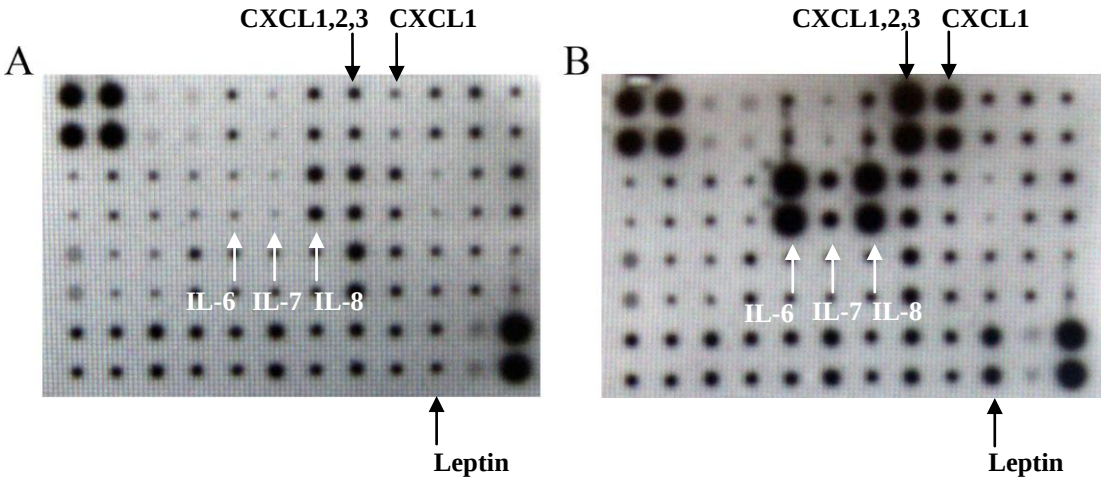

Figure 3.1 Cytokine expression in FPS cell conditioned media. A Human Cytokine Antibody Array was used to determine differences in cytokine production in the conditioned medium of **(A)** vehicle treated and **(B)** 100 nM $PGF_{2\alpha}$ treated FPS cells. An increase in the cytokines of the CXCL family, IL-6, IL-7, IL-8 and leptin was observed, and are indicated by arrowheads.

3.3.2 Expression and localisation of CXCL1 and CXCR2 in normal endometrium across the menstrual cycle and in endometrial adenocarcinoma

3.3.2.1 Expression of CXCL1 mRNA in normal endometrium and endometrial adenocarcinoma

Normal endometrium (n=45) and endometrial adenocarcinoma tissue (n=58) samples were analysed for CXCL1 mRNA expression by quantitative RT-PCR (Figure 3.2). CXCL1 expression was increased in the mid-secretory phase of the cycle however this increase was not statistically significant (Figure 3.2A). There was no significant difference in CXCL1 mRNA expression between grades of cancer examined (Figure 3.2B), however CXCL1 mRNA was found to be significantly increased by 5.9 ± 2.9 fold in pooled carcinoma samples as compared to pooled normal endometrium samples ($p<0.001$, Figure 3.2C).

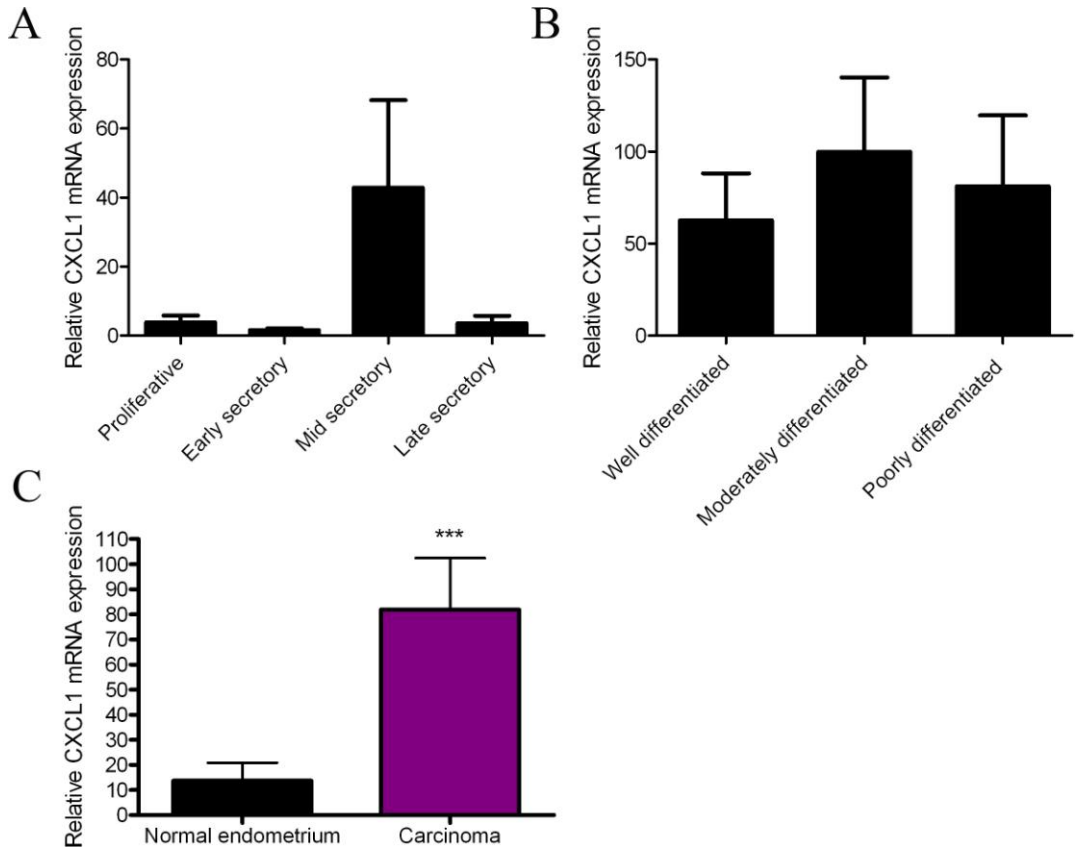

Figure 3.2 Expression of CXCL1 mRNA in normal endometrium and endometrial adenocarcinoma. A: CXCL1 mRNA was examined by quantitative RT-PCR in endometrium from the proliferative phase (n=17), early secretory phase (n=11), mid secretory phase (n=12) and late secretory phase (n=5) of the menstrual cycle. Differences in expression between different stages of the cycle were not statistically significant. **B:** CXCL1 mRNA was examined by quantitative RT-PCR in well (n=18), moderately (n=20) and poorly (n=20) differentiated endometrial adenocarcinoma. No significant difference was demonstrated between different grades of cancer. **C:** CXCL1 mRNA expression was examined by quantitative RT-PCR in pooled normal endometrial samples (n=45) and pooled adenocarcinoma samples (n=58), and demonstrated to be significantly increased in carcinoma tissue. *** denotes $p<0.001$, data are expressed relative to a normal endometrium control sample and are presented as mean ± SEM.

3.3.2.2 Expression of CXCR2 mRNA in normal endometrium and endometrial adenocarcinoma

Expression of CXCR2, the receptor for CXCL1, was also examined by quantitative RT-PCR in normal endometrium (n=45) and endometrial adenocarcinoma tissue (n= 57; Figure 3.3). CXCR2 mRNA expression was significantly increased in the late secretory phase of the cycle as compared to all other menstrual stages ($p<0.01$, Figure 3.3A). CXCR2 mRNA expression was not significantly different between different grades of cancer (Figure 3.3B) however expression was found to be significantly increased by 4.2 ± 1.04 fold in pooled cancer samples as compared to pooled normal endometrium samples ($p<0.001$, Figure 3.3C).

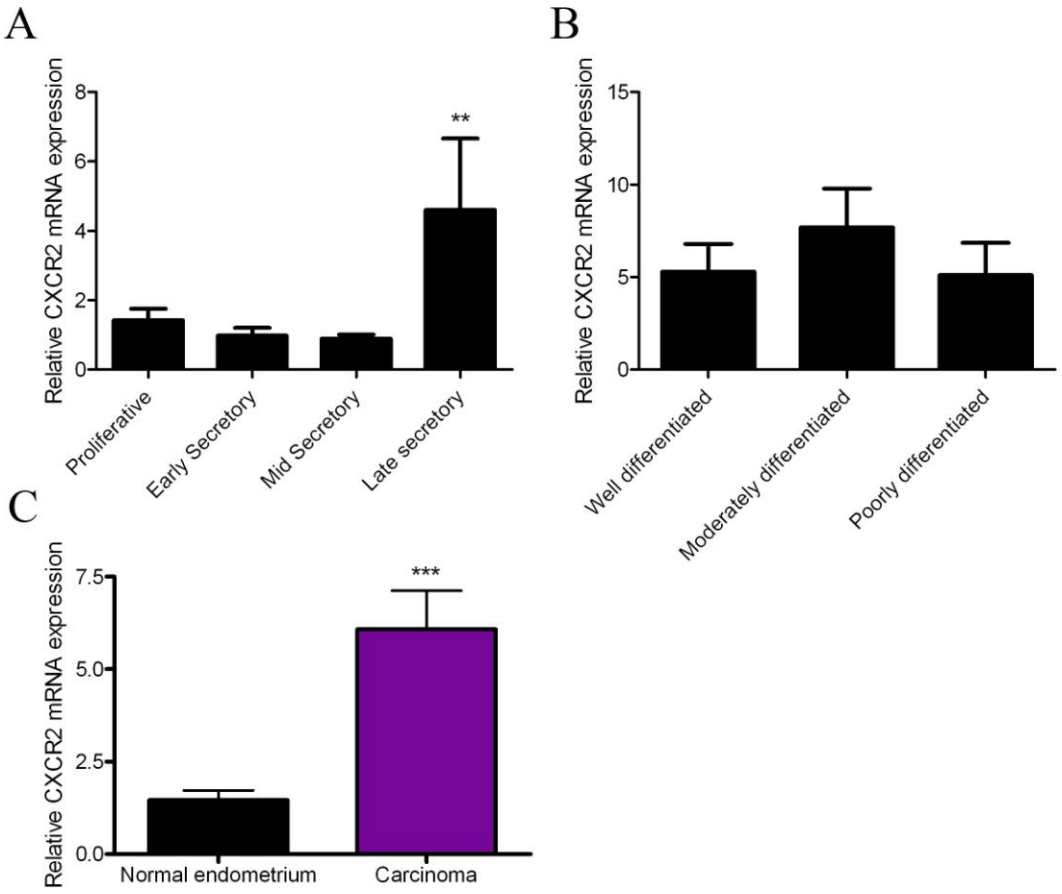

Figure 3.3 Expression of CXCR2 in normal endometrium and endometrial adenocarcinoma. A: CXCR2 expression was examined by quantitative RT-PCR in endometrium from the proliferative phase (n=18), early secretory phase (n=13), mid secretory phase (n=10) and late secretory phase (n=4) of the menstrual cycle. CXCR2

mRNA was significantly increased in the late secretory stage of the cycle. **B:** CXCR2 mRNA was examined by quantitative RT-PCR in well (n=18), moderately (n=20) and poorly (n=19) differentiated endometrial adenocarcinoma tissue, and did not significantly differ between grades of cancer. **C:** CXCR2 mRNA expression was examined by quantitative RT-PCR in pooled normal endometrium samples (n=45) and pooled adenocarcinoma samples (n=57), and was significantly increased in carcinoma tissue. **denotes p<0.01, *** denotes p<0.001, data are expressed relative to a normal endometrium control sample and are presented as mean ± SEM.

3.3.2.3 Localisation of CXCL1 in normal endometrium and endometrial adenocarcinoma

CXCL1 protein in the normal endometrium has been previously examined and localised to stromal cells in secretory endometrium (Nasu et al. 2001). In another study, CXCL1 was localised to glandular epithelium in secretory and proliferative phase endometrium (Hess et al. 2007). In this chapter, endometrial tissue from all stages of the cycle (proliferative n=7, early secretory n=3, mid secretory n=4, late secretory n=5) was examined by immunohistochemistry for CXCL1 expression. In the proliferative phase, CXCL1 staining can be seen in the cytoplasm of the glandular epithelium and immunoreactivity is rarely seen in stromal cells (Figure 3.4A). CXCL1 was also expressed in vascular endothelial cells throughout the cycle, and this is shown in the early secretory phase of the cycle (Figure 3.4B). CXCL1 was also localised to glandular epithelium and stroma at this stage of the cycle. In the mid and late secretory phases, CXCL1 expression can be seen in the stroma and is also localised to glandular epithelium (Figure 3.4C, D). Negative controls were incubated with non-immune IgG (inset; Figure 3.4A) and with primary antibody which had been pre-absorbed with CXCL1 blocking peptide (inset; Figure 3.4C). Both demonstrated no immunoreactivity.

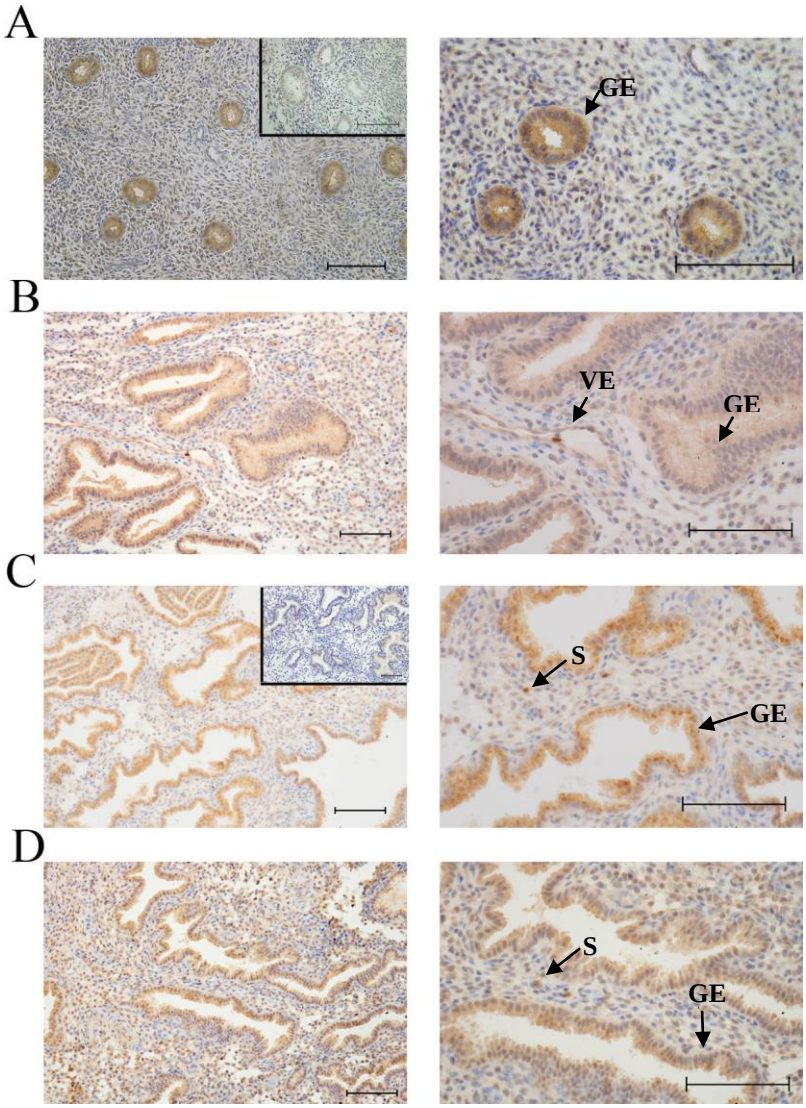

Figure 3.4 Localisation of CXCL1 protein in normal endometrium by immunohistochemistry. Representative images demonstrating that CXCL1 localises to the glandular epithelium, stroma and vascular endothelium in normal endometrium. **A:** CXCL1 localises to the glandular epithelium (GE) in proliferative phase endometrium. Negative control (inset) is incubated with non-immune IgG (left image is x200 magnification, right image is x400). **B:** In early secretory phase endometrium, CXCL1 immunoreactivity can be seen in the glandular epithelium and vascular endothelial cells (VE; left image is x200 magnification, right image is x400). **C:** In mid secretory phase endometrium, CXCL1 can be localised to glandular epithelium and stroma (S; left image is x200 magnification, right image is x400). Negative control (inset) is incubated with primary antibody pre-absorbed with CXCL1 blocking peptide. **D:** CXCL1 also localises to glandular epithelium and stroma in late secretory phase endometrium. All scale bars represent 100 µm.

CXCL1 expression was then examined in all grades of endometrial adenocarcinoma by immunohistochemistry (well differentiated n=7, moderately differentiated n=5, poorly differentiated n=3). Similarly to normal endometrium, CXCL1 could be localised to epithelium (E), vascular endothelium (VE) and stromal cells (S) in all grades of cancer studied (Figure 3.5). Negative controls were incubated with primary antibody pre-absorbed with blocking peptide (inset; Figure 3.5A) and with non-immune IgG in place of primary antibody (inset; Figure 3.5B).

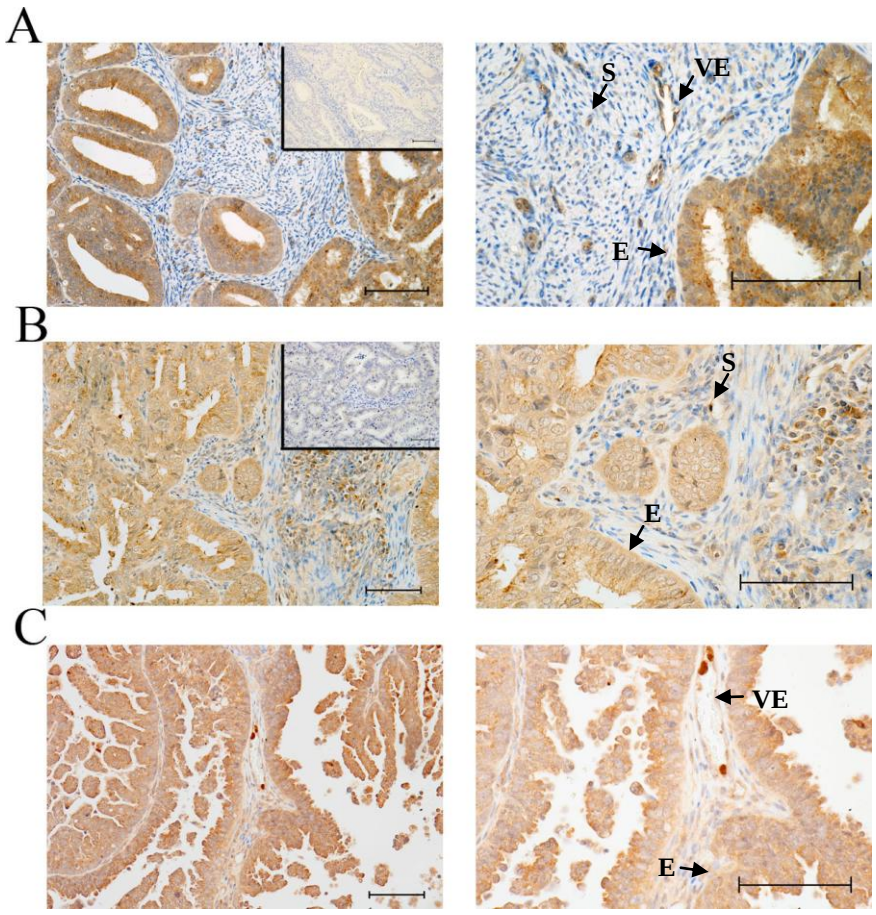

Figure 3.5 Localisation of CXCL1 protein in endometrial adenocarcinoma by immunohistochemistry. Representative images demonstrating that CXCL1 localises to the neoplastic epithelium (E), vascular endothelium (VE) and stroma (S) in **A:** Well differentiated carcinoma (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with primary antibody pre-absorbed with blocking peptide. **B:** Moderately differentiated carcinoma (left image is x200 magnification, right image is x400). Negative

control (inset) is incubated with non-immune IgG in place of primary antibody. **C:** Poorly differentiated carcinoma (left image is x200 magnification, right image is x400). All scale bars represent 100 µm.

To further support a role for the FP receptor in the regulation of CXCL1, immunohistochemistry for both proteins was carried out on serial sections of endometrial adenocarcinoma. For all grades of cancer, FP protein could be localised to a number of the same cells as CXCL1. Shown in Figure 3.6 are representative images from moderately differentiated (Figure 3.6A) and well differentiated cancers (Figure 3.6B), where localisation of FP receptor and CXCL1 can be demonstrated in the same neoplastic epithelial cells, vascular endothelial cells and many of the same stromal cells. Co-localisation is indicated by arrowheads. FP negative controls were incubated with non-immune IgG and are inset in Figure 3.6A; CXCL1 negative controls incubated with non-immune IgG and antibody pre-absorbed with blocking peptide are shown in Figure 3.5.

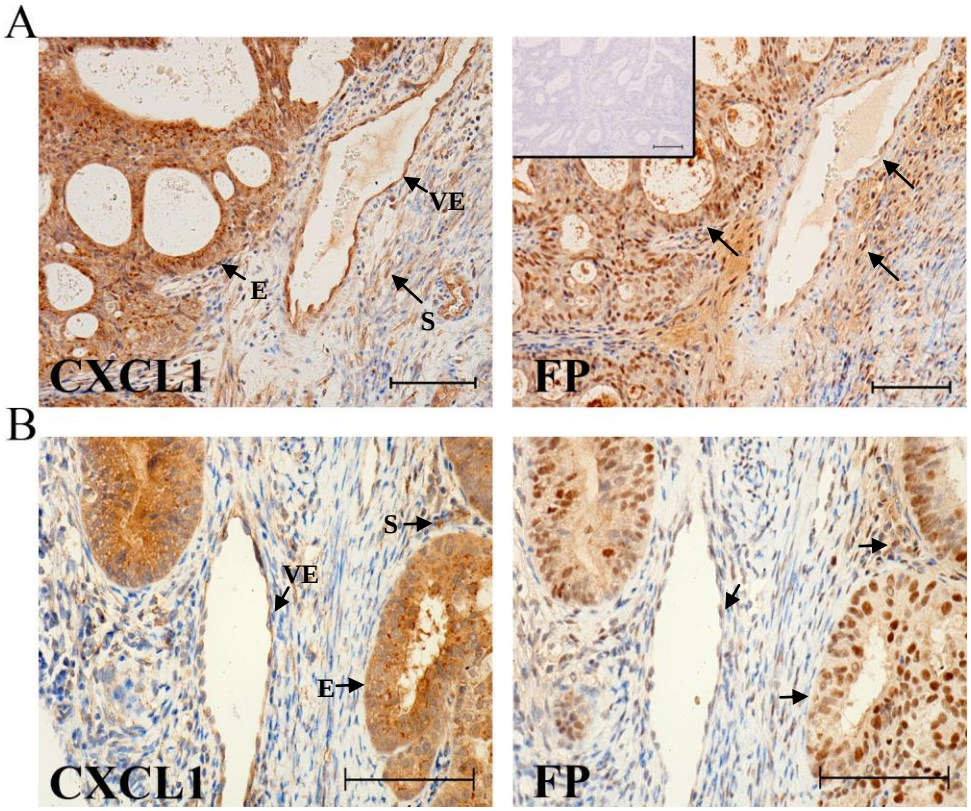

Figure 3.6 CXCL1 can be localised to the same cells as the FP receptor by immunohistochemistry of serial sections. Representative images demonstrating that

CXCL1 and FP can be colocalised to neoplastic epithelium (E), vascular endothelium (VE) and stroma (S; indicated by arrowheads) in **A:** moderately differentiated endometrial adenocarcinoma (x 200 magnification) and **B:** well differentiated endometrial adenocarcinoma (x 400 magnification). All scale bars represent 100 µm.

3.3.2.4 Localisation of CXCR2 in normal endometrium and endometrial adenocarcinoma

The localisation of the receptor for CXCL1, CXCR2, was then investigated in normal endometrium and endometrial adenocarcinoma. CXCR2 expression has been previously examined in normal endometrial tissue (Mulayim et al. 2003; Ulukus et al. 2005). However discrepancies exist in the literature about the site of expression of CXCR2, with no stromal expression observed in one study (Ulukus et al. 2005). Therefore we examined CXCR2 expression in all phases of the menstrual cycle (proliferative n=4, early secretory n=2, mid secretory n=3, late secretory n=4).

CXCR2 immunoreactivity can be seen in the cytoplasm of the glandular epithelium of proliferative phase endometrium; however stromal CXCR2 expression was rarely observed (Figure 3.7A). In endometrium from the early secretory phase, CXCR2 was also localised to glandular epithelium (Figure 3.7B). In endometrium from the mid and late secretory stages, CXCR2 remained localised to the glandular epithelium and increasingly was expressed focally in the stroma (S; representative image is shown from late secretory phase endometrium in Figure 3.7D). In some tissues from the secretory phase CXCR2 could be weakly localised to the vasculature, demonstrated in Figure 3.7C. Negative controls were incubated with non immune IgG and displayed no immunoreactivity (Figure 3.7C, inset).

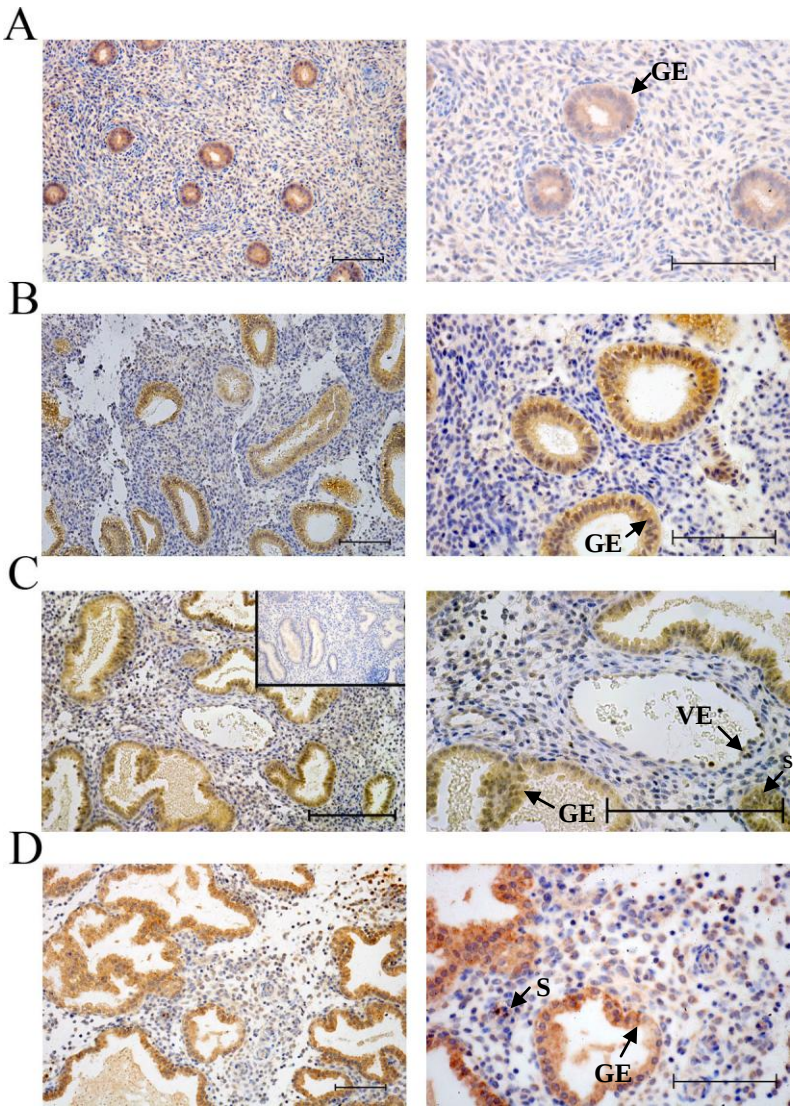

Figure 3.7 Localisation of CXCR2 protein in normal endometrium by immunohistochemistry. Representative images demonstrating that **A:** CXCR2 localises to the glandular epithelium (GE) in proliferative phase endometrium (left image is x200 magnification, right image is x400). **B:** In early-secretory endometrium, CXCR2 localised to the glandular epithelium (left image is x200 magnification, right image is x400). **C:** In mid-secretory phase endometrium, CXCR2 immunoreactivity can be seen in the glandular epithelium and vascular endothelium (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune IgG in place of primary antibody. **D:** In late secretory phase endometrium, CXCR2 can be localised to glandular epithelium and is seen more frequently in the stroma (S; left image is x200 magnification, right image is x400). All scale bars represent 100 µm.

CXCR2 localisation was then examined in all grades of cancer (well differentiated n=5, moderately differentiated n=5, poorly differentiated n=5). As in normal endometrium, CXCR2 could be localised to neoplastic epithelial cells, and also to the stroma. CXCR2 immunoreactivity could also be seen in vascular endothelial cells in all grades of cancer, and representative images of moderately and poorly differentiated cancers demonstrate this (Figure 3.8). Negative controls were incubated with non immune IgG and displayed no immunoreactivity (Figure 3.8A, inset).

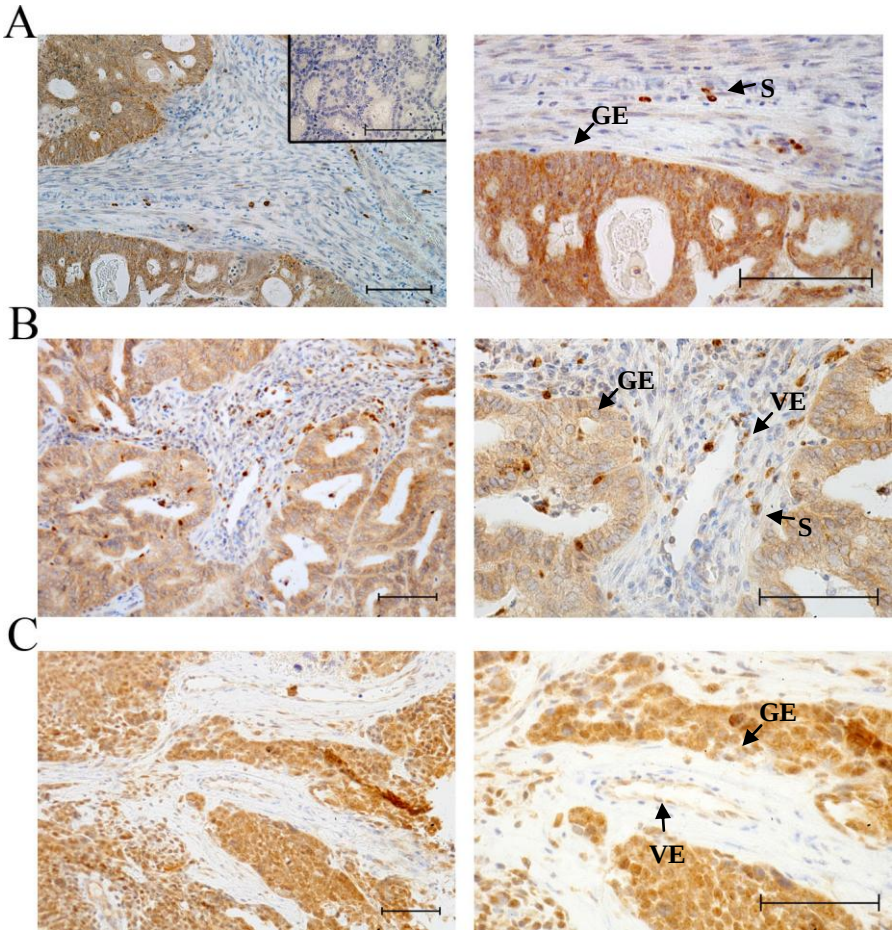

Figure 3.8 Localisation of CXCR2 protein in endometrial adenocarcinoma by immunohistochemistry. Representative images demonstrating that CXCR2 localises to the glandular epithelium (GE), vascular endothelium (VE) and stroma (S) in **A:** Well differentiated carcinoma (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune IgG in place of primary antibody. **B:** Moderately differentiated carcinoma (left image is x200 magnification, right image is x400). **C:** Poorly

differentiated carcinoma (left image is x200 magnification, right image is x400). All scale bars represent 100 μm.

3.3.3 CXCL1 mRNA expression is regulated by $PGF_{2\alpha}$ in FPS cells

CXCL1 protein expression by FPS cells after 24 hours of $PGF_{2\alpha}$ stimulation was demonstrated by cytokine antibody array (Figure 3.1). The regulation of CXCL1 by $PGF_{2\alpha}$ in FPS cells was therefore next examined by luciferase assay, quantitative RT-PCR and ELISA.

To examine CXCL1 promoter activity, FPS cells were transfected with a luciferase-conjugated CXCL1 promoter. Cells were treated for 2-24 hours with vehicle or 100 nM $PGF_{2\alpha}$ (n=4) and promoter activity was determined by luciferase assay. After 8 hours of $PGF_{2\alpha}$ treatment CXCL1 promoter activity was significantly elevated as compared to the earliest time point and remained elevated to 33.3 ± 2.3 fold above vehicle treated control after 24 hours treatment ($p<0.05$, Figure 3.9A).

CXCL1 mRNA expression was then determined by quantitative RT-PCR. FPS cells were treated for 2-72 hours with vehicle or 100 nM $PGF_{2\alpha}$ (n=5). After 4 hours $PGF_{2\alpha}$ treatment, CXCL1 mRNA expression was elevated above vehicle treated control and this induction was sustained to a maximum of 159.3 ± 62.6 at 12 hours, followed by a decrease in mRNA expression ($p<0.05$, Figure 3.9B).

CXCL1 protein concentration was then measured in medium of FPS cells treated for 2-72 hours with either vehicle or 100nM $PGF_{2\alpha}$ (n=5). As compared to vehicle treated cells, $PGF_{2\alpha}$ treatment resulted in CXCL1 protein production which was significantly elevated at 24 hours ($p<0.05$, Figure 3.9C) and remained significantly elevated after up to 72 hours of stimulation.

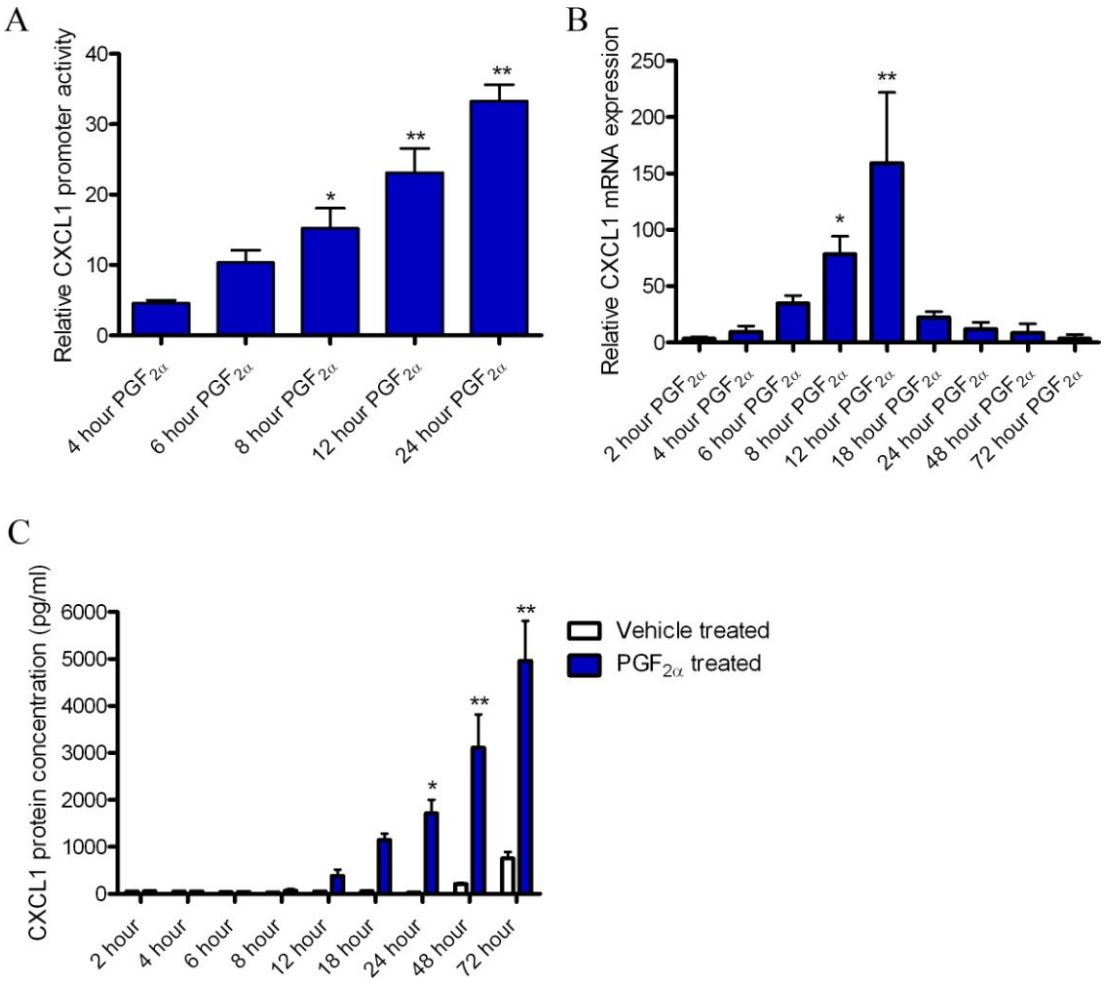

Figure 3.9 Regulation of CXCL1 expression by $PGF_{2\alpha}$ treatment in FPS cells. A: CXCL1 promoter activity was measured in FPS cells stimulated with vehicle or 100 nM $PGF_{2\alpha}$ over a period of 4 to 24 hours. $PGF_{2\alpha}$ stimulation time-dependently increased CXCL1 promoter activity. Data are expressed as fold increase in luciferase activity as compared to vehicle treated cells. **B:** CXCL1 mRNA expression in FPS cells was measured by quantitative RT-PCR after stimulation with vehicle or 100 nM $PGF_{2\alpha}$ over a period of 2 to 72 hours. Data are expressed as fold increase over vehicle treated control. **C:** CXCL1 protein secreted into media by FPS cells after stimulation with vehicle treated control or 100 nM $PGF_{2\alpha}$ over a period of 2 to 72 hours was measured by ELISA. CXCL1 protein was elevated between 24-72 hours after treatment with $PGF_{2\alpha}$. Data are presented in pg / ml. All data are presented as mean ± SEM. * denotes $p<0.05$, ** denotes $p< 0.01$.

3.3.4 Intracellular signalling pathways mediating $PGF_{2\alpha}$ regulation of CXCL1 expression in FPS cells

3.3.5 Signalling pathways mediating CXCL1 expression in FPS cells

$PGF_{2\alpha}$ binding to FP activates a range of signalling molecules via G_q-coupled pathways (Sales et al. 2005; Sales et al. 2008). To elaborate the pathway leading to CXCL1 production, FPS cells were treated with vehicle, 100 nM $PGF_{2\alpha}$ alone or 100 nM $PGF_{2\alpha}$ and a panel of chemical inhibitors (n=7; Figure 3.10). Treatment of FPS cells with $PGF_{2\alpha}$ for 8 and 24 hours induced a 91.5±8.4 and 22.3±4.7 fold increase in CXCL1 mRNA (Figure 3.10A) and protein (Figure 3.10B) expression respectively compared to vehicle treated cells. This increase was abolished by treatment of cells with a selective inhibitor of G_q (YM254890, $p<0.01$) and significantly inhibited with the FP receptor antagonist AL8810 ($p<0.05$) and inhibitors of epidermal growth factor receptor (EGFR; AG1478, $p<0.05$) and mitogen activated protein kinase kinase (MEK; PD98059, $p<0.01$). However, inhibitors of calcium (ethylene glycol tetraacetic acid, EGTA), calcineurin (cyclosporine A, CsA) and protein kinase A (PKA; 43CMQ) did not significantly alter CXCL1 mRNA or protein production. This indicates that in FPS cells $PGF_{2\alpha}$ regulates CXCL1 expression via the FP receptor and a G_q coupled signalling pathway involving components of the mitogen activated protein kinase (MAPK) pathway and the EGFR, which has been shown in our lab to mediate ERK phosphorylation following FP receptor activation by $PGF_{2\alpha}$ (Sales et al. 2005).

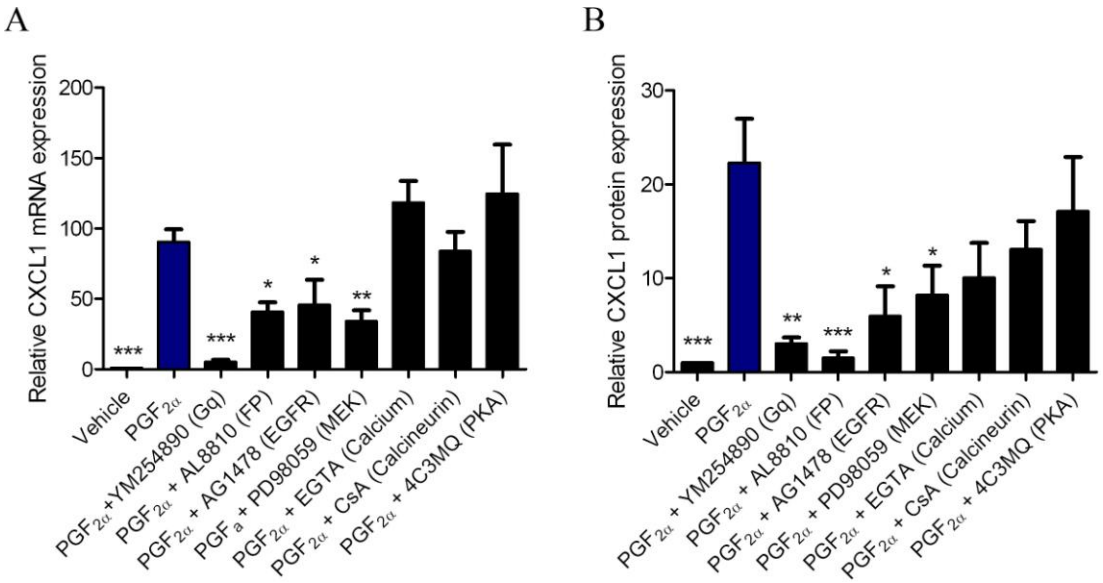

Figure 3.10 $PGF_{2\alpha}$ regulated CXCL1 expression is decreased by selected chemical inhibitors. A: FPS cells were treated for 8 hours in the presence or absence of vehicle or $PGF_{2\alpha}$ and chemical inhibitors of G_q, FP, EGFR, MEK, calcium, calcineurin and PKA. $PGF_{2\alpha}$ regulated CXCL1 mRNA expression was examined by quantitative RT-PCR and found to be significantly inhibited by inhibitors of G_q, FP, EGFR and MEK. Data are expressed as fold over vehicle treated control **B:** FPS cells were treated for 24 hours in the presence or absence of vehicle or $PGF_{2\alpha}$ and chemical inhibitors of G_q, FP, EGFR, MEK, calcium, calcineurin and PKA. CXCL1 protein was measured by ELISA and found to be significantly decreased after treatment with chemical inhibitors of G_q, FP, EGFR and MEK. Data are expressed as fold over vehicle treated control and are presented as mean ± SEM. * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$.

To further confirm a role for the FP receptor in $PGF_{2\alpha}$ mediated CXCL1 production, short hairpin RNA specifically targeted against the FP receptor (sh FP) or with a scrambled sequence as a control (sh scrambled) was transfected into FPS cells using adenoviral delivery. After transfection, FPS cells were treated for 8 hours with 100 nM $PGF_{2\alpha}$ or vehicle (n=4). In FPS cells transfected with the scrambled sequence control, CXCL1 mRNA expression after $PGF_{2\alpha}$ treatment was increased 117.5 ± 62.3 fold over vehicle treated cells at 8 hours (Figure 3.11). This was comparable to non-adenoviral infected cells (Figure 3.9B). This $PGF_{2\alpha}$-induced increase in CXCL1

mRNA expression was reduced with the addition of shRNA directed against the FP receptor to 29.7± 13.2 fold (Figure 3.11).

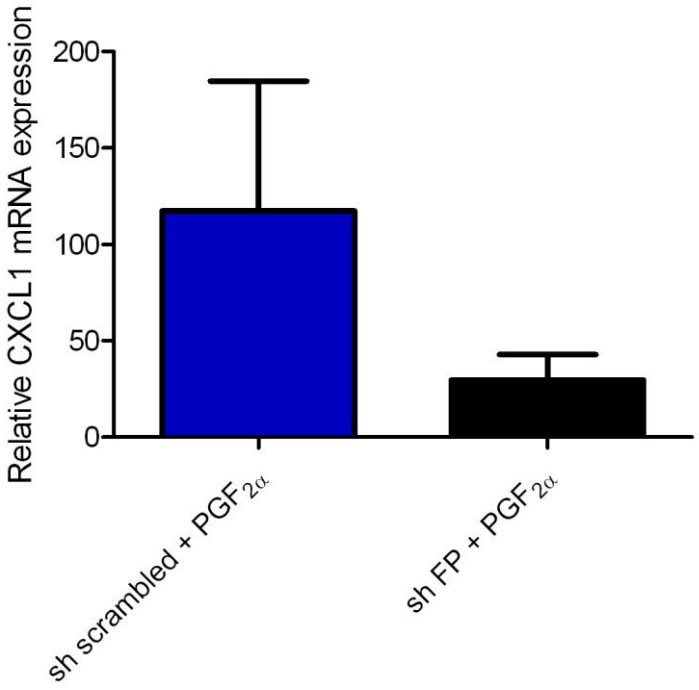

Figure 3.11 $PGF_{2\alpha}$ regulated CXCL1 mRNA expression is inhibited with shRNA directed against the FP receptor. FPS cells were transfected with adenovirus containing scrambled sequence shRNA (sh scrambled) or shRNA against the FP receptor (sh FP). Cells were treated for 8 hours with vehicle or 100 nM $PGF_{2\alpha}$. CXCL1 expression was decreased in cells infected with FP shRNA as compared to scrambled sequence shRNA. Data are expressed as fold over vehicle treated control and are presented as mean ± SEM.

In order to confirm the involvement of components of the signalling pathway identified by chemical inhibitor experiments, and particularly the involvement of the classical mitogen activated protein kinase (MAPK) pathway, cDNA encoding dominant negative (DN) mutants of signalling molecules or an empty vector were co-transfected into FPS cells with the luciferase-conjugated CXCL1 promoter. DN-EGFR, Ras and MEK were used to confirm the activation of extracellular signal-related kinases 1/2 (ERK1/2). Ras is a small heterotrimeric G protein upstream of MEK in the MAPK phosphorylation cascade. After transfection, FPS cells were

treated for 8 hours with vehicle or 100 nM $PGF_{2\alpha}$ and CXCL1 promoter activity measured by luciferase assay (n=5; Figure 3.12). In cells transfected with the empty vector, 8 hours $PGF_{2\alpha}$ treatment induced an 18.95 ± 3.6 fold increase in CXCL1 promoter activity as compared to vehicle treated control cells. This increase was significantly decreased when cells were transfected with DN-EGFR ($p<0.05$), DN-Ras ($p<0.01$) or DN-MEK ($p<0.01$). Transfection of DN-NFAT did not significantly affect CXCL1 promoter activity.

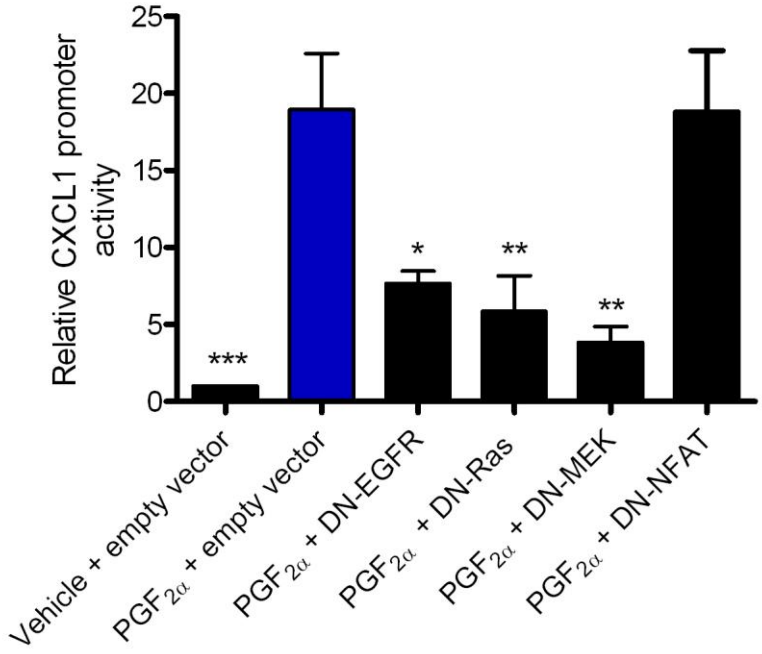

Figure 3.12 Inhibition of CXCL1 promoter activity by co-transfection of cDNA encoding dominant negative mutant forms of EGFR, Ras and MEK. FPS cells were co-transfected with cDNA encoding luciferase-conjugated CXCL1 promoter and either empty vector, DN-EGFR, DN-Ras, DN-MEK or DN-NFAT. Cells were treated for 8 hours with vehicle or $PGF_{2\alpha}$ and CXCL1 promoter activity measured by luciferase assay. CXCL1 promoter activity was increased by $PGF_{2\alpha}$ treatment. This effect could be significantly decreased by transfection of DN-EGFR, DN-Ras and DN-MEK, but not DN-NFAT. Data are expressed as fold over vehicle treated control and are presented as mean ± SEM. * denotes $p<0.05$, ** denotes $p<0.01$.

3.3.6 Signalling pathways mediating CXCL1 expression in carcinoma tissue

To confirm results obtained on the FPS cell line in human tissue, human endometrial adenocarcinoma explants were treated for 2, 4, 6, 8, and 24 hours with vehicle or $PGF_{2\alpha}$ and CXCL1 mRNA expression measured by quantitative RT-PCR. After treatment with $PGF_{2\alpha}$ for 24 hours, CXCL1 mRNA expression was significantly increased in carcinoma explants by 2.8 ± 1.2 fold as compared to vehicle treated control cells ($p<0.01$; Figure 3.13A). In another set of experiments endometrial adenocarcinoma explants were treated for 24 hours with $PGF_{2\alpha}$ alone, $PGF_{2\alpha}$ and the FP receptor antagonist AL8810 or $PGF_{2\alpha}$ and the MEK inhibitor PD98059. $PGF_{2\alpha}$ treatment increased CXCL1 mRNA expression by 5.3 ± 0.7 fold as compared to vehicle treated explants ($p<0.01$; Figure 3.13B). Co-treatment with $PGF_{2\alpha}$ and AL8810 or PD98059 significantly reduced CXCL1 mRNA expression to 2.6 ± 0.2 and 1.8 ± 0.2 fold respectively ($p<0.001$; Figure 3.13B), confirming the role of FP and MEK in $PGF_{2\alpha}$–regulated CXCL1 expression in carcinoma tissue.

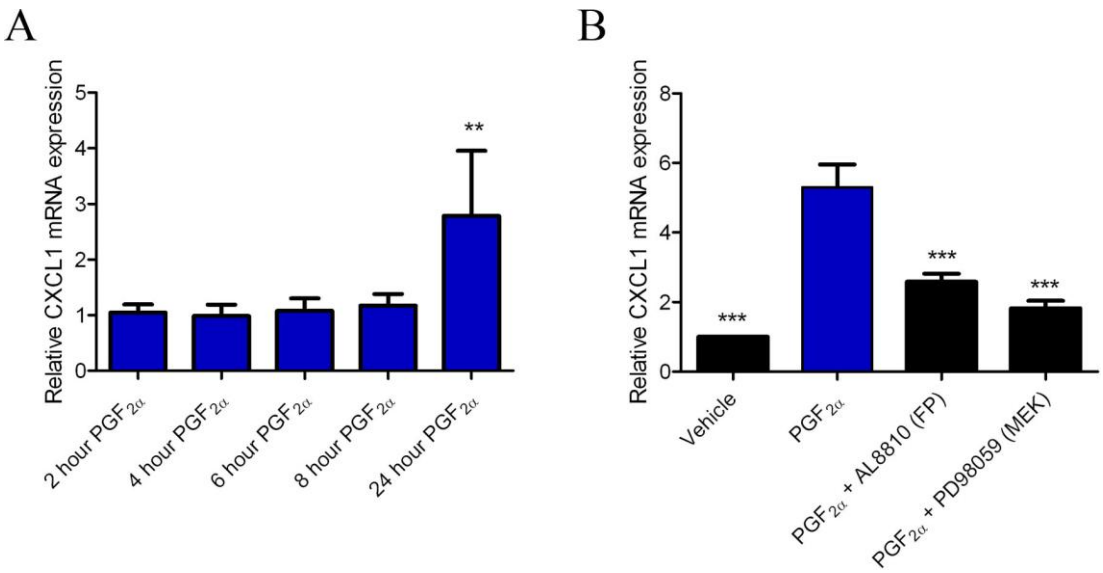

Figure 3.13 $PGF_{2\alpha}$ regulated CXCL1 expression is decreased in carcinoma explants by selected chemical inhibitors. A: Endometrial adenocarcinoma explants were treated for 2 - 24 hours with vehicle or $PGF_{2\alpha}$ and CXCL1 mRNA was measured by quantitative RT-PCR. CXCL1 mRNA was significantly increased after 24 hours of $PGF_{2\alpha}$ treatment. **B:** Endometrial adenocarcinoma explants were treated for 24 hours with vehicle, $PGF_{2\alpha}$ alone,

or $PGF_{2\alpha}$ and chemical inhibitors. Co- treatment with $PGF_{2\alpha}$ and AL8810 or PD98059 significantly reduced CXCL1 mRNA expression. Data are expressed as fold over vehicle treated tissue and are presented as mean ± SEM. ** denotes $p<0.01$, *** denotes $p<0.001$.

3.4 Discussion

The over-expression of chemokines in cancer has been implicated in the remodelling of the tumour microenvironment and promotion of tumour progression (Mantovani et al. 2008). This study identified the increased expression of CXCL1 in endometrial adenocarcinoma, and characterised the localisation of CXCL1 and its receptor CXCR2 in endometrial adenocarcinoma tissue. A role for $PGF_{2\alpha}$ in the regulation of CXCL1 was demonstrated in an endometrial adenocarcinoma cell line *in vitro* and *ex vivo* in endometrial adenocarcinoma tissue explants.

Prostaglandins have previously been demonstrated to regulate chemokine expression *in vitro.* Prostaglandin E_2 (PGE_2) secretion is elevated in many cancer types and recently has been shown to induce CXCL1 production in colon cancer cells. This can then promote network formation and migration of endothelial cells; crucial steps in the initiation of angiogenesis (Wang et al. 2006). Our laboratory has previously ascertained a role for the FP receptor and $PGF_{2\alpha}$ signalling in endometrial adenocarcinoma, as $PGF_{2\alpha}$-FP signalling was shown to regulate a number of pro-tumourigenic genes (Sales et al. 2004; Sales et al. 2005; Sales et al. 2007; Sales et al. 2008). In the present study, a chemokine antibody array identified CXCL1 as a chemokine regulated by $PGF_{2\alpha}$-FP signalling in an *in vitro* model system of the Ishikawa cell line stably expressing the human FP receptor (FPS cells).

To confirm the physiological significance of CXCL1 expression demonstrated in our cell line model, the expression of CXCL1 and its receptor CXCR2 was examined in endometrial tissue. CXCL1 mRNA expression has previously been documented in the normal endometrium (Nasu et al. 2001) and in agreement with this study our data demonstrate a possible increase in CXCL1 expression in the mid-secretory phase of

the menstrual cycle as compared all other phases. The mid-secretory phase of the menstrual cycle corresponds to the window of implantation, during which the endometrium is receptive to embryo implantation. A previous study examining proteins expressed in the mid-secretory phase by use of experimentally induced progesterone withdrawal also concluded from the resulting decrease in CXCL1 expression that this chemokine is expressed at this stage of the menstrual cycle (Catalano et al. 2007). This indicates that CXCL1 may play a role in preparation for implantation in the normal endometrium, potentially by signalling to the CXCR2 receptor expressed on trophoblast cells (Red-Horse et al. 2004), or may play a role in angiogenesis if implantation occurs (Hess et al. 2007).

Our data demonstrate that CXCR2 mRNA is present across the menstrual cycle and is significantly higher in endometrium from the late secretory phase. The elevated CXCR2 mRNA expression seen in the late secretory phase of the menstrual cycle may be due to the increased number of infiltrating leukocytes present at this stage of the cycle, as a number of leukocytes found in endometrial stroma express the CXCR2 receptor (Murphy et al. 2000; Salamonsen et al. 2000). Immunoreactivity for CXCR2 was demonstrated in glandular epithelium and stroma, and weak CXCR2 expression was also seen in some vascular endothelial cells. Expression of CXCR2 in both epithelial and stromal cells of the normal endometrium has been previously demonstrated by immunohistochemistry (Mulayim et al. 2003).

Over-expression of CXCL1 has been shown in a number of cancer types including melanoma, colorectal and prostate cancer (Haghnegahdar et al. 2000; Wang et al. 2006; Wen et al. 2006; Kawanishi et al. 2008; Rubie et al. 2008). Data presented in this chapter show increased CXCL1 and CXCR2 mRNA expression in endometrial cancer tissue as compared to normal endometrium. CXCL1 immunoreactivity was localised to neoplastic epithelial cells, vascular endothelium and stromal cells. CXCR2 could also be localised to neoplastic epithelial cells, and this indicates the potential for autocrine signalling in endometrial adenocarcinoma. In melanoma and colon cancer cell lines, inhibition of CXCR2 signalling on cancer cells inhibits cell

proliferation thus indicating that an autocrine signalling pathway is occurring (Li et al. 2004; Singh et al. 2009). Furthermore, cell migration may be promoted by CXCR2 signalling on neoplastic cells, as shown by the increased metastatic potential of colon cancer cell lines expressing high levels of CXCR2 and CXCL1 (Li et al. 2004). A similar CXCL1-CXCR2 autocrine signalling mechanism may promote proliferation and cell migration in endometrial adenocarcinoma.

To investigate a role for $PGF_{2\alpha}$-FP signalling in CXCL1 regulation as indicated by the chemokine antibody array, CXCL1 could be co-localised to the same epithelial cells and vascular endothelial cells as the FP receptor, further implicating $PGF_{2\alpha}$ signalling in its regulation. CXCL1 promoter activity, mRNA expression and protein secretion was also examined in FPS cells. All were found to be increased after $PGF_{2\alpha}$ treatment. The signalling pathways mediating the effect of $PGF_{2\alpha}$-FP receptor signalling on CXCL1 expression were then elaborated by the use of small molecule inhibitors of cell signalling pathways. A key effector pathway which has been previously shown to regulate signalling in response to GPCR activation is the mitogen-activated protein kinase (MAPK) pathway. The signalling components of this pathway in FPS cells have been identified in our laboratory, where the phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2) in response to $PGF_{2\alpha}$ was demonstrated to be mediated by cross talk with the epidermal growth factor receptor (EGFR) after its trans-activation by c-Src phosphorylation (Pierce et al. 2001; Sales et al. 2005). This study found that chemical inhibitors of G_q, FP, EGFR and the upstream kinase of ERK, MEK, could inhibit CXCL1 mRNA and protein production. Nuclear factor of activated T-cells (NFAT), a common regulator of cytokine expression (Boyd et al. 2006), has been previously shown in our laboratory to be regulated by $PGF_{2\alpha}$-FP signalling. However, a chemical inhibitor of NFAT signalling did not significantly inhibit CXCL1 mRNA or protein production. Additionally, inhibition of protein kinase A (PKA) did not significantly alter CXCL1 production in response to $PGF_{2\alpha}$ – FP receptor signalling, despite evidence that PKA can trans-activate the EGFR (Barbier et al. 1999). These data highlight that CXCL1

expression in response to $PGF_{2\alpha}$ – FP interaction is mediated via a G_q-EGFR-MEK-ERK1/2 pathway.

In agreement with the data generated using chemical inhibitors, short hairpin RNA against the FP receptor and dominant negative mutants of EGFR, Ras and MEK significantly decreased CXCL1 promoter activity and mRNA expression in response to $PGF_{2\alpha}$. These signalling data are supported by previously published evidence in colorectal adenocarcinoma cell lines where the ERK pathway was also crucial in the regulation of CXCL1 expression after stimulation with PGE_2 (Wang et al. 2006). In addition, treatment of endometrial adenocarcinoma explants with $PGF_{2\alpha}$ caused an increase in CXCL1 expression which was mediated via the FP receptor and ERK1/2 signalling pathways. This confirms the importance of this signalling cascade in regulating CXCL1 expression in endometrial adenocarcinoma tissue *ex vivo*. The signalling pathway leading from FP receptor activation to CXCL1 expression is summarised in Figure 3.14, with components of the signalling pathway examined in this chapter highlighted in red.

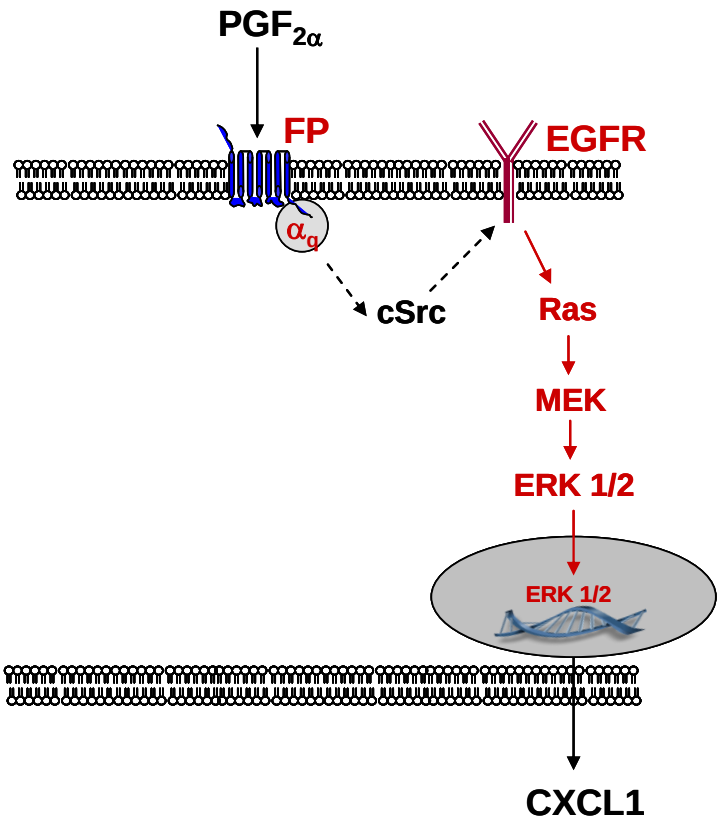

Figure 3.14 $PGF_{2\alpha}$ stimulates CXCL1 production in FPS cells and endometrial adenocarinoma via a FP-G_q-EGFR-Ras-MEK dependent pathway. Use of dominant negative mutants and chemical inhibitors of cell signalling (highlighted in red) demonstrated that CXCL1 production by $PGF_{2\alpha}$-FP signalling is dependent on a FP-G_q-EGFR-Ras-MEK signalling pathway.

Deregulation of the MAPK signalling pathway is common in many cancer types, and this has previously been shown to lead to upregulation of CXCL1 and other chemokines in cancer. CXCL1 is expressed following Ras activation in ovarian cancer epithelial cells, and stimulated growth of ovarian cancer xenografts by signalling to tumour-associated fibroblasts and altering the tumour microenvironment (Yang et al. 2006). IL-8 was expressed via this pathway in a nude mouse cervical cancer xenograft model (Sparmann et al. 2004) and IL-6 and IL-10 were elevated via the MAPK pathway in an *in vitro* model of breast cancer (Sumimoto et al. 2006).

The aspects of tumour progression associated with CXCL1 and CXCR2 expression in cancer are cell proliferation, invasion, angiogenesis and infiltration of immune cells (Keane et al. 2004; Li et al. 2004; Singh et al. 2009; Singh et al. 2009). For example, in esophogeal and prostate cancer cell lines, knockdown of constitutive production of CXCL1 decreased cell proliferation (Li et al. 2004; Wang et al. 2006). A decrease in tumour growth was also demonstrated by inhibition of CXCR2 activity in a mouse model of melanoma (Singh et al. 2009) and neutralisation of CXCL1 in a mouse model of prostate cancer (Moore et al. 1999). A role for CXCL1 in tumour cell invasion has also been demonstrated in other published literature. Increased secretion of CXCL1 is associated with increased invasive potential in bladder cancer (Kawanishi et al. 2008) and prostate cancer (Li et al. 2004). CXCL1 over-expression also decreased expression of fibulin-1, an extracellular matrix protein, in a colon cancer cell line. The breakdown of the extracellular matrix is one of the first steps in initiation of metastasis (Wen et al. 2006).

CXCR2 was also localised to vascular endothelial cells in endometrial adenocarcinoma and the presence of the ELR motif in CXCL1 indicates that it is an angiogenic chemokine (Strieter et al. 1995). The angiogenic capabilities of CXCL1 have been demonstrated in a number of cancer models. In mouse models of melanoma and prostate cancer (Moore et al. 1999; Singh et al. 2009), CXCR2 signalling increased microvascular density in xenografts and a corneal angiogenesis assay. Vascular density and tumour growth was also decreased in CXCR2-knockout mice xenografted with Lewis-lung carcinoma cells (Keane et al. 2004). In another mouse model, CXCL1 neutralisation of melanoma cells taken from tumours decreased angiogenic capability in the corneal pocket model (Haghnegahdar et al. 2000). These studies indicate that *in vivo,* CXCL1 signalling can induce angiogenesis to increase tumour growth. The expression of both CXCL1 and CXCR2 in endometrial adenocarcinoma indicates a potential angiogenic role in this cancer type.

An additional role of CXCL1-CXCR2 signalling is in the chemoattraction of immune cells. Strong CXCR2 expression was observed in a number of cells throughout the

stroma of endometrial adenocarcinomas which morphologically appeared to be immune cells. CXCR2 is highly expressed on neutrophils and on some macrophages (Murphy et al. 2000). Neutrophils in cancer have been demonstrated to contribute to tissue remodelling impacting on tumour growth (Ardi et al. 2007; Yamamoto et al. 2008) and a similar role has been shown for macrophages, particularly in the promotion of angiogenesis (Bingle et al. 2006; Lin et al. 2007). CXCL1 induced by $PGF_{2\alpha}$ in endometrial adenocarcinoma may therefore be signalling to CXCR2 positive cells in the stroma of endometrial adenocarcinoma.

Wang et al demonstrated an increase in CXCL1 expression after PGE_2 treatment in colorectal cancer cells, which was shown to promote angiogenesis *in vitro* (Wang et al. 2006). Similarly linking prostaglandins and CXCL1, data presented in this chapter show that $PGF_{2\alpha}$ can induce CXCL1 expression in our endometrial adenocarcinoma cell line and endometrial adenocarcinoma tissue. Expression of CXCL1 and its receptor CXCR2 was increased in cancer as compared to normal endometrial tissue and localised to neoplastic epithelium, vascular endothelium and stroma. Evidence presented in this chapter therefore indicates that the CXCL1 produced by $PGF_{2\alpha}$ –FP interactions in our endometrial adenocarcinoma cell model may promote angiogenesis and immune cell infiltration into tumours *in vivo*. Therefore the next chapter in this thesis concentrates on the potential role of CXCL1 in immune cell infiltration in endometrial adenocarcinoma, as well as the role of CXCL1 in angiogenesis.

Chapter 4

Chemokine (C-X-C) motif ligand 1 expression in endometrial adenocarcinoma: Effects on immune cell infiltration and angiogenesis

4 Chemokine (C-X-C motif) ligand 1 expression in endometrial adenocarcinoma: Effects on immune cell infiltration and angiogenesis

4.1 Introduction

In the previous chapter, the regulation of the chemokine CXCL1 by $PGF_{2\alpha}$ in an endometrial adenocarcinoma cell line and in endometrial adenocarcinoma explants was demonstrated. CXCR2 was localised by immunohistochemistry to a number of stromal and vascular endothelial cells within endometrial adenocarcinoma, and therefore the potential role of CXCL1 signalling to these cells in endometrial adenocarcinoma is further examined in this chapter.

The hypothesis that inflammation may drive tumour progression has received much attention, due to studies showing that long term intake of specific COX-2 inhibitors (NSAIDS) can significantly reduce cancer occurrence in pre-disposed patients (Dannenberg et al. 2003). Of the multiple cell types which make up the tumour microenvironment, several types of leukocyte commonly infiltrate and the role that these cells play in tumourigenesis is still unclear. In addition to epithelial and endothelial cells, expression of CXCR2 has been previously documented on various immune cells including neutrophils, eosinophils, monocytes, macrophages, T cells and dendritic cells (Murphy et al. 2000). Only the role of CXCL1 as a neutrophil chemoattractant is well defined, however there is some evidence that it may promote monocyte/macrophage migration into tissues (Weber et al. 1999; Traves et al. 2004; Smith et al. 2005). The function of all of these immune cells in cancer has been intensely studied, and there is contrasting evidence for a role in both tumour promotion and eradication (de Visser et al. 2006).

A popular model of immune cell infiltration into tumours suggests that the first types of immune cell to infiltrate are those of the innate immune system including macrophages, neutrophils and dendritic cells. These are chemo-attracted by pro-

inflammatory cytokines secreted by the tumour. The discovery of these cells within solid tumours originally led to the hypothesis that immune cells were attacking and destroying tumour cells, and may eventually be used in cancer immunotherapy (Dirkx et al. 2006). However, new evidence suggests cells of the innate immune system promote tumour growth, angiogenesis and cell migration (Murdoch et al. 2008). After the initial infiltration of cells of the innate immune system, the activation of antigen presenting cells such as dendritic cells may result in the recruitment of cells of the adaptive immune system such as T and B lymphocytes to the tumour. There is some evidence that these lymphocytes can contribute to tumour cell destruction by cell-mediated cytotoxicity (de Visser et al. 2006). However, paradoxically tumours are often infiltrated by regulator T cells which can suppress adaptive immune responses (Giatromanolaki et al. 2008).

In endometrial cancer, the number of infiltrating immune cells has previously been examined and compared to normal endometrium. The numbers of macrophages (Salvesen et al. 1999; Ohno et al. 2004) and B and T lymphocytes (Yamazawa et al. 2001; Chang et al. 2005; Ohno et al. 2005; Ohno et al. 2006; Miyatake et al. 2007; Giatromanolaki et al. 2008) were found to be increased in cancer, while the number of natural killer cells was similar to that of the normal endometrium (Hachisuga et al. 1997). However the infiltration of neutrophils, the main cell type known to be recruited by CXCL1 (Moser et al. 1990), has not previously been quantified in endometrial adenocarcinoma.

As discussed in Chapter 3, CXCL1 binding to CXCR2 on endothelial cells can promote angiogenesis in a variety of *in vitro* and *in vivo* models of cancer. The angiogenic properties of CXCL1 have been displayed *in vitro* by its ability to promote several functions of endothelial cells crucial to angiogenesis, including network formation (Wang et al. 2006) and endothelial cell migration (Strieter et al. 1995; Wang et al. 2006). *In vivo*, mouse models of melanoma (Haghnegahdar et al. 2000; Singh et al. 2009), lung (Keane et al. 2004) and prostate cancer (Moore et al. 1999) have decreased tumour angiogenesis after prevention of CXCL1-CXCR2

signalling. Therefore in this chapter the dual role of $PGF_{2\alpha}$-regulated CXCL1 as a chemoattractant for immune cells and a stimulator of angiogenesis is investigated.

4.2 Materials and Methods

4.2.1 Tissue collection

Normal endometrial and endometrial adenocarcinoma tissue was collected for RT-PCR analysis and immunohistochemistry as described in sections 2.1.1 and 2.1.2. Normal endometrial tissue was collected from women undergoing surgery for minor gynaecological procedures with no underlying endometrial pathology, and endometrial adenocarcinoma tissue was obtained from women undergoing hysterectomy after diagnosis of adenocarcinoma of the uterus. Stage of cycle and grade of cancer was assessed by a pathologist. Tissue collected for RT-PCR analysis was stored in RNA Later (Ambion) at -70°C for RNA extraction and tissue for immunohistochemical analysis was fixed in neutral buffered formalin and wax embedded. Written informed consent was collected from all patients before surgery and ethical approval was obtained from Lothian Research Ethics Committee.

4.2.2 Cell culture

Ishikawa FPS cells were maintained as described in section 2.2.1.

4.2.3 Cell and tissue treatments

To obtain conditioned media for the neutrophil chemotaxis experiment, FPS cells were serum starved in media containing 3 µg/ml indomethacin for at least 18 hours prior to stimulation for 48 hours with fresh serum-free media containing indomethacin and vehicle or 100 nM $PGF_{2\alpha}$. Medium from 3 experiments was pooled to use in each chemotaxis assay.

4.2.4 Immunohistochemistry

3,3- diaminobenzidine tetrahydrochlorine (DAB) immunohistochemistry was used to localise neutrophil elastase, CD68, CD4, CD8, CD20, CD56, and CD11c in human endometrial tissue (normal proliferative phase endometrium n=7, well differentiated endometrial adenocarcinoma n=10, moderately differentiated endometrial adenocarcinoma n=10, poorly differentiated endometrial adenocarcinoma n=10), and Gr-1 and CD31 in mouse xenograft tissue (wildtype xenografts n=10, FPS xenografts n=10, CXCL1 neutralised FPS xenografts n=10). Immunohistochemistry was performed as described in section 2.5.2. Tissue sections (5 µm) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed for CD68, CD8, CD20, CD56, CD31 and CD11c. Antigen retrieval in EDTA buffer (1mM, pH 8) was performed for CD4. No retrieval step was required for neutrophil elastase or Gr-1. Sections were then blocked for endogenous peroxidase activity by washing in 3% hydrogen peroxidase in methanol. Blocking in normal serum of rabbit (for immunolocalisation of Gr-1) or goat (for immunolocalisation of neutrophil elastase, CD68, CD4, CD8, CD20, CD56, CD11c, CD31) was then performed followed by overnight incubation with primary antibody at the concentrations indicated in Table 2.4. Control sections were included with non-immune rabbit or goat IgG. Sections were then incubated in rabbit anti-rat (for immunolocalisation of Gr-1), goat anti-mouse (for immunolocalisation of neutrophil elastase, CD68, CD4, CD8, CD20, CD56,) or goat anti-rabbit (for immunolocalisation of CD11c, CD31) biotinylated secondary antibody and subsequently streptavidin-HRP. Sections were then incubated with DAB for 1-5 minutes until positive staining was identified by a brown colour under x10 magnification. Slides were coverslipped and visualised as described in Section 2.5.2 and 2.5.4.

4.2.5 Stereological analyses

4.2.5.1 Immune cells

The number of immune cells in endometrial tissue was quantified using immunohistochemical staining and standard stereological techniques. Each section was examined using x40 plan apo objective from a BH2 microscope (Olympus, Tokyo, Japan) fitted with an automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK) using a video camera (HV-C20; Hitachi, Tokyo, Japan) and were analyzed with Image-Pro Plus 4.5.1 software with a Stereology 5.0 plug-in (Media Cybernetics, Wokingham, Berkshire, UK). A total of 40 randomised fields of view were examined and counted (proliferative phase endometrium n=7, well differentiated endometrial adenocarcinoma n=10, moderately differentiated endometrial adenocarcinoma n=10, poorly differentiated endometrial adenocarcinoma n=10) and data are expressed as mean ± SEM number of cells per mm^2 of tumour examined.

4.2.5.2 Microvascular density

Microvascular density was quantified in mouse xenograft tissue, using immunohistochemistry for the vascular marker CD31. A modified intra-tumour microvessel density protocol of Weidner (Weidner et al. 1993; Weidner 1995) was used. Briefly, each section (n=10 each group, as described in section 4.2.4) was examined under low power magnification using x50 plan apo objective from a BH2 microscope (Olympus, Tokyo, Japan) fitted with an automatic stage (Prior Scientific Instruments Ltd., Cambridge, UK) using a video camera (HV-C20; Hitachi, Tokyo, Japan). Sections were analyzed with Image-Pro Plus 4.5.1 software with a Stereology 5.0 plug-in (Media Cybernetics, Wokingham, Berkshire, UK). Section identities were blinded, and areas containing neovascular ‘hotspots’ were identified by CD31 staining. Three hotspots were then examined under higher power magnification (x200 plan apo objective, 0.9 mm^2 field). As defined by Weidner, any distinct CD31-positive endothelial cell or cells separate from other endothelial cells or connective tissue was counted as a microvessel. Results were then calculated as the average

number of the highest number of microvessels in any single x200 field from the three separate hotspots, and are expressed as average highest microvessel count per field.

4.2.6 Dual immunofluorescence microscopy

For colocalisation studies, the expression of CXCR2 and neutrophil elastase, CD45, CD68, CD3, CD20, CD56, CD11c, CXCL1 or CD31 was examined by dual immunofluorescence microscopy in all grades of endometrial adenocarcinoma (well differentiated n=3, moderately differentiated n=3, poorly differentiated n=3). Protocols differed depending on the species the primary antibodies were raised in, and are detailed below.

4.2.6.1 Dual immunofluorescence microscopy using two primary antibodies raised in the same species

CXCR2 and CD45, CD68, CD3, CD20 and CD56 were all raised in mouse and therefore dual immunofluorescence required a microwave blocking step and the use of secondary antibodies with fragment antigen binding fragments (Fab fragments). These ensure that there are no open binding sites on the secondary antibody which can subsequently cross react with the next primary antibody which is added. Sections (5 μm) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed for CD45, CD68, CD3, CD20, and CD56. No retrieval step was required for neutrophil elastase. Sections were blocked in 5% normal goat serum diluted in PBS with 5% BSA before incubation with mouse anti-CD45 (1:1000), neutrophil elastase (1:500), CD68 (1:1000), CD3 (1:400), CD20 (1:500), and CD56 (1:1500). Following an overnight incubation at 4ºC sections were incubated with goat-anti mouse biotinylated Fab, then tyramide signal amplification kit (TSA Fluorescein System, 1:50 dilution, Perkin Elmer, MA). Sections were then microwaved in 0.01M citrate buffer for 30 minutes and endogenous peroxidase blocked using hydrogen peroxide. Non-specific binding was blocked with 5% normal goat serum and sections were incubated with mouse anti-CXCR2 (1:500) at 4ºC overnight. The sections were again incubated with goat-anti mouse biotinylated Fab and tyramide signal amplification kit. Nuclei were

counterstained in To Pro (Molecular Probes, UK) and sections were then coverslipped and visualised as described in Section 2.5.3 and 2.5.4.

4.2.6.2 Dual immunofluorescence microscopy using two primary antibodies raised in different species

To colocalise CXCR2 with CXCL1, CD11c and CD31, sections (5 µm) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed and sections were blocked in 5% normal goat serum (CD11c and CD31) or rabbit serum (CXCL1) diluted in PBS with 5% BSA. Sections were then incubated with rabbit anti-CD11c (1:100) or CD31 (1:100), or goat anti-CXCL1 (1:100) overnight at 4ºC. Goat anti-rabbit (CD11c and CD31) or rabbit anti-goat (CXCL1) biotinylated antibody was then added, diluted in PBS at 1:200 for 30 minutes. Streptavidin AlexaFluor 546 (Molecular Probes) diluted 1:200 in PBS was then added to biotyinlated secondary antibodies for 30 minutes at room temperature. Sections were then re-blocked in normal serum and the process repeated with mouse anti-CXCR2 (1:25). Tissues were then incubated with a fluorophore-conjugated antibody diluted 1:200 in PBS (AlexaFluor 488, Molecular Probes, UK) for 1 hour. Nuclei were counterstained in To Pro (Molecular Probes, UK) and then coverslipped and visualised as described in Section 2.5.3 and 2.5.4.

To colocalise CXCL1 with CD31, sections (5 µm) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed and sections were blocked in 5% normal donkey serum diluted in PBS with 5% BSA. Sections were then incubated with goat anti-CXCL1 (1:20) overnight at 4ºC. Donkey anti-goat AlexaFluor 488 antibody was then added, diluted in PBS at 1:200 for 1 hour. Sections were then re-blocked in normal goat serum and rabbit anti-CD31 added (1:100). Tissues were then incubated with goat anti-rabbit biotinylated antibody diluted in PBS at 1:200 for 30 minutes. Streptavidin AlexaFluor 546 (Molecular Probes) diluted 1:200 in PBS was then added to biotyinlated secondary antibodies for 30 minutes at room temperature for 1

hour. Nuclei were counterstained in To Pro (Molecular Probes, UK) and sections were then coverslipped and visualised as described in Section 2.5.3 and 2.5.4.

4.2.7 Neutrophil migration assay

Neutrophil chemotaxis was analyzed using transwell inserts (5-µm pore size; Corning Life Sciences, Leicestershire, UK). Neutrophils were kindly purified as previously described (Ward et al. 1999) by Dr A Leitch (Centre for Inflammation Research, University of Edinburgh) and resuspended in serum free media. Cells (7.5 x 10^5) were added to the top chamber of the transwell insert and 600 µl of conditioned media from FPS cells treated with vehicle or 100 nM $PGF_{2\alpha}$ (Section 5.2.3; V-CM or $PGF_{2\alpha}$-CM) was added to the bottom chamber. Serum-free media alone or with 50 ng/ml recombinant CXCL1 was added as negative and positive controls, respectively. Cells were incubated at 37 °C in a 5% CO2 atmosphere for 1 hour and the plate was gently tapped to dislodge cells adhered to the underside of the membrane. Cells in the bottom chamber were collected and counted at least six times using a haemocytometer (n=4).

4.2.8 Animal xenograft model

The animal model experiments were kindly performed by Dr R Catalano and Dr M Wilson. A suspension of 5 x 10^5 Ishikawa wild-type (WT) or FPS cells in a total volume of 0.2 ml DMEM was injected subcutaneously into each dorsal flank of CD1-*Foxn1*^{nu} mice (Charles River) giving rise to two tumours per mouse. The mice bearing FPS tumours (n = 20) were divided into two groups of equal tumour size one week after engraftment. The mice were then treated twice weekly with 100 µg IgG (n=10) or CXCL1 neutralizing antibody (n=10) via intraperitoneal injection for 4 weeks. Animals bearing WT tumours (n=10) were also treated twice weekly with 100 µg IgG via intraperitoneal injection for 4 weeks. The animals were maintained under sterile conditions in individually vented cages. At the end of the experiment, animals were sacrificed and the tumours were excised. One tumour from 5 mice from each treatment group was placed in PBS for flow cytometry analysis, and RNA was extracted from one tumour of the remaining 5 mice. The second tumour from each

mouse was fixed in neutral buffered formalin and wax embedded for histological analysis.

4.2.9 RNA extraction and polymerase chain reaction

RNA from xenograft tissue was extracted as described in section 2.4.1 using the Qiagen minikit protocol. Briefly, pieces of tissue, in 2 ml microcentrifuge tubes containing a stainless steel ball, were homogenised in 600 µl RLT lysis buffer using a tissue lyser (Qiagen Crawley, West Sussex, UK) for 2 x 3 minutes at 25 Hz. RNA was then extracted following manufacturers guidelines (Qiagen). cDNA was prepared as described in section 2.4.2.

4.2.10 Taqman quantitative PCR

CXCL1 mRNA expression in xenograft tissue was examined using Taqman quantitative PCR as described in section 2.4.3.2. Briefly, primer and probe sequences were designed to amplify CXCL1 (sequence given in Table 2.2). A master mix was made up containing 18s primers and probes and 200 ng cDNA added to each sample. Samples were added in duplicate to a 96 well MicroAmp fast optical reaction PCR plate and analysed using an ABI Prism 7900 HT Fast Real Time PCR machine. Expression of CXCL1 was normalised to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed compared to normal endometrium calibration sample.

4.2.11 Flow cytometry of xenograft tissue

Xenografts from nude mice (n=5 each group) were assessed for neutrophil infiltrate using flow cytometry. Briefly, tumours were digested by collagenase treatment at 37 °C for 45 minutes. Tissue was then mechanically disrupted into a single cell solution using a syringe and 40 µm mesh and resuspended in FACS wash (PBS + 1%BSA + 2% formalin). Cells were incubated at 4 °C for 30 minutes in FACS wash containing the following monoclonal antibodies and appropriate isotype controls: CD11b-FITC, Gr-1-PE and CD11c-APC. All antibodies were obtained from eBioscience (Hatfield, UK) and used at a dilution of 1:200. Red blood cells were lysed using BD FACS lysing solution according to manufacturer's instructions (BD Biosciences, Oxford,

UK). Samples were analysed using a FACScalibur cytometer (BD biosystems) using BD CellQuest software. In xenograft tissue, neutrophils were defined by expression of Gr-1 and CD11b epitope, absence of CD11c and scatter profile.

4.2.12 Statistical analysis

Where appropriate, data were analysed by Students t-test and one way ANOVA using GraphPad Prism (GraphPad Software. San Diego, California, USA). Data are presented as mean ± SEM.

4.3 Results

4.3.1 Identification of CXCR2 expressing cells in endometrial adenocarcinoma

As described in Chapter 3, a number of stromal cells were seen in all grades of endometrial adenocarcinoma expressing CXCR2. The identity of these cells was then investigated in all grades of endometrial adenocarcinoma (well differentiated n=3, moderately differentiated n=3, poorly differentiated n=3) using dual immunofluorescence microscopy to examine the expression of CXCR2 and a number of markers of immune cells. Immune cell markers were chosen based on the morphological appearance of these cells and previous evidence of CXCR2 expression on immune cells (Murphy et al. 2000).

4.3.1.1 CXCR2 and CD45 (Leukocyte common antigen)

CD45 (also known as protein tyrosine phosphatase receptor type C) is a membrane protein tyrosine phosphatase specifically expressed in leukocytes and therefore used as a pan-leukocyte marker (NCBI 2009). Dual immunofluorescence microscopy revealed high numbers of CD45 positive cells in all grades of endometrial adenocarcinoma sections examined. A moderately differentiated cancer is shown in Figure 4.1. The expression of CD45 (Figure 4.1A) colocalised to a large number of CXCR2 (Figure 4.1B) expressing cells (Figure 4.1C), identifying the majority of

CXCR2-positive cells as leukocytes. An enlarged image of the inset box in Figure 4.1C is shown in Figure 4.1D, and colocalisation is indicated by arrowheads.

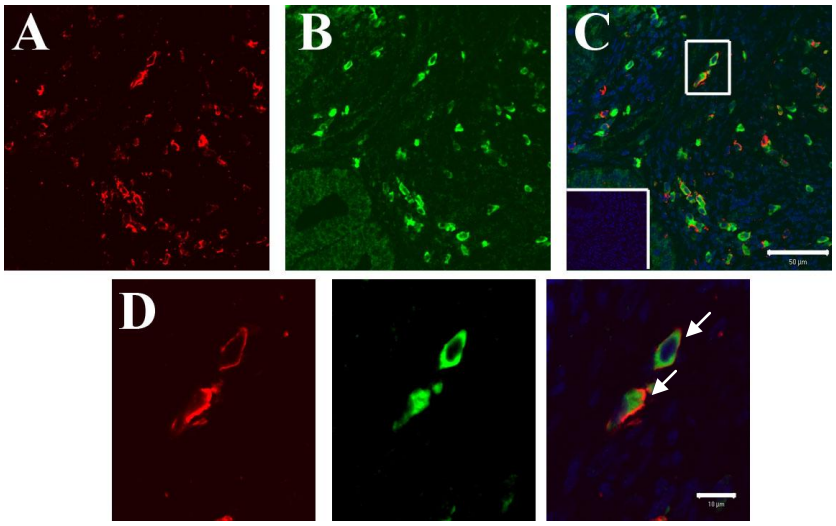

Figure 4.1 Colocalisation of CXCR2 and CD45 protein in a moderately differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. CD45 **(A)** and CXCR2 **(B)** were colocalised **(C)** to a large number of cells within the stroma of endometrial adenocarcinoma, identifying them as leukocytes. Negative control (inset) was incubated with non-immune IgG in place of primary antibody. Scale bar represents 50 µm. **D:** Magnification of the area within the white box inset in 4.1C, colocalisation is indicated by arrowheads. Scale bar represents 10 µm.

4.3.1.2 CXCR2 and neutrophil elastase

The expression of CXCR2 on neutrophils is well characterised (Murphy et al. 2000). To determine if CXCR2-expressing cells in endometrial adenocarcinoma were neutrophils, expression of neutrophil elastase, an enzyme specific to neutrophils used to hydrolyse proteins within neutrophil granules (NCBI 2009), was examined by dual immunofluorescence microscopy. Neutrophil elastase (Figure 4.2A) and CXCR2 (Figure 4.2B) colocalised within a large number of cells in all grades of endometrial adenocarcinoma, however a number of larger CXCR2-positive cells were not neutrophil elastase positive (Figure 4.2C and magnification, Figure 4.2D). Colocalisation is indicated by arrowheads. A representative image of a well differentiated adenocarcinoma is shown in Figure 4.2.

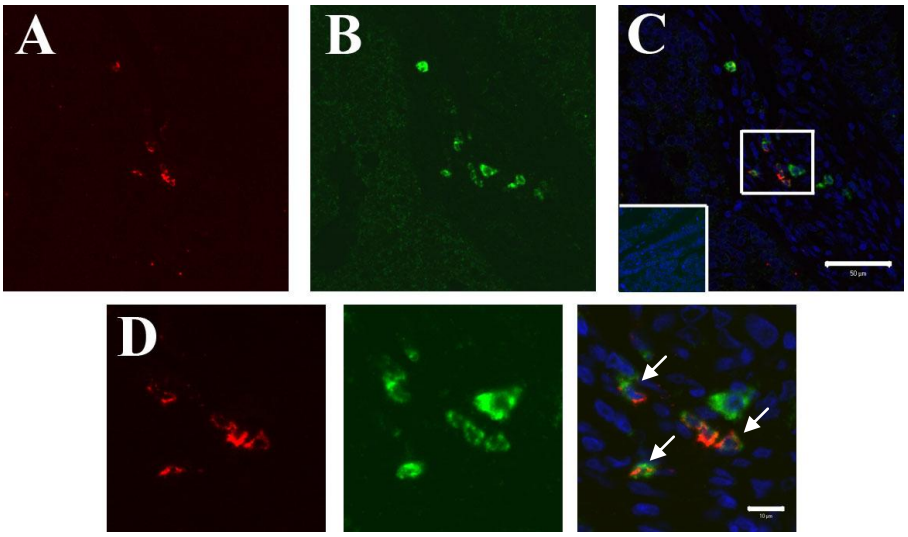

Figure 4.2 Colocalisation of CXCR2 and neutrophil elastase protein in a well differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. Neutrophil elastase **(A)** and CXCR2 **(B)** were colocalised **(C)** to neutrophils within the stroma of a well differentiated endometrial adenocarcinoma. Negative control (inset) was incubated with non-immune IgG in place of primary antibody. Scale bar represents 50 µm. **D:** Magnification of the area within the white box, inset in C. Scale bar represents 10 µm.

4.3.1.3 CXCR2 and CD68

CD68 is a membrane glycoprotein specifically expressed by macrophages and monocytes (NCBI 2009). A high number of CD68-positive cells were seen throughout the stroma of all grades of endometrial adenocarcinoma (Figure 4.3A), however colocalised to only a small number of CXCR2-positive cells (Figure 4.3B and merge, 4.3C) within endometrial adenocarcinoma using dual immunofluorescence microscopy. Colocalisation is indicated by arrowheads. A representative image of a poorly differentiated adenocarcinoma is shown in Figure 4.3.

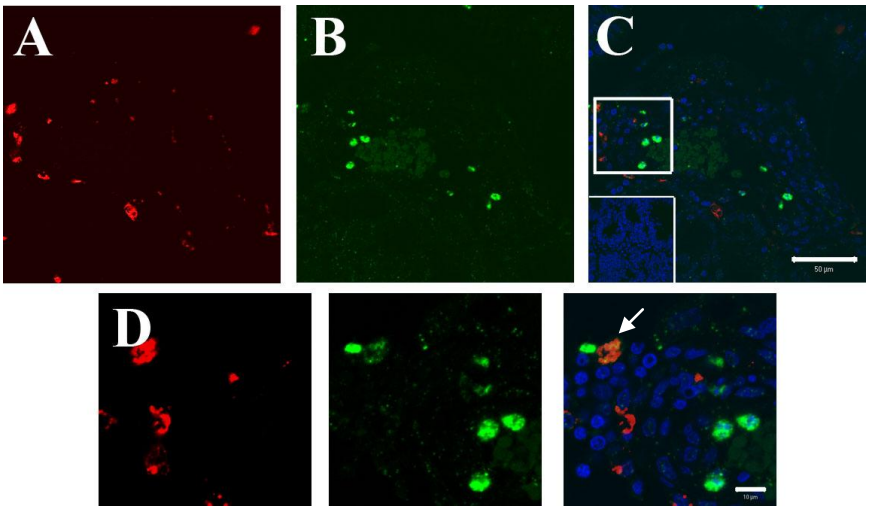

Figure 4.3 Colocalisation of CXCR2 and CD68 protein in a poorly differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. CD68 **(A)** and CXCR2 **(B)** were colocalised **(C)** to a small number of macrophages within the stroma of a poorly differentiated endometrial adenocarcinoma. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 50 µm. **D:** Magnification of the area within the white box, inset in C. Colocalisation is indicated by arrowhead. Scale bar represents 10 µm

4.3.1.4 CXCR2 and CD3

The specific T-cell marker CD3 is a protein which forms a complex with the T cell receptor to enable antigen recognition (NCBI 2009). CD3 expression (Figure 4.4A) could be seen throughout all grades of endometrial adenocarcinoma but did not colocalise with CXCR2 expression (Figure 4.4B, C). A magnification is presented in Figure 4.4D. A representative image of a well differentiated adenocarcinoma is shown in Figure 4.4.

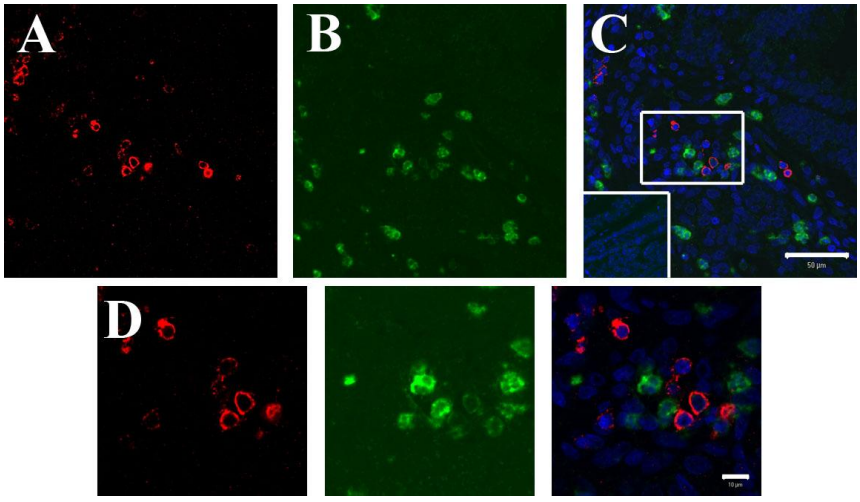

Figure 4.4 Expression of CXCR2 and CD3 protein in a well differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. CD3 **(A)**, a specific T-cell marker, and CXCR2 **(B)** were expressed in a well differentiated endometrial adenocarcinoma but did not colocalise **(C)**. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 50 µm. **D:** Magnification of the area within the white box, inset in C. Scale bar represents 10 µm.

4.3.1.5 CXCR2 and CD20

CD20 is a membrane protein specifically expressed on B cells (NCBI 2009). Similarly to T-cells, B cells were found throughout all grades of endometrial adenocarcinoma tissue in clusters (Figure 4.5A) but CD20-positive cells did not colocalise with CXCR2 expression (Figure 4.5B, C). This can be clearly seen in the magnification in Figure 4.5D. A representative image of a well differentiated adenocarcinoma is shown in Figure 4.5.

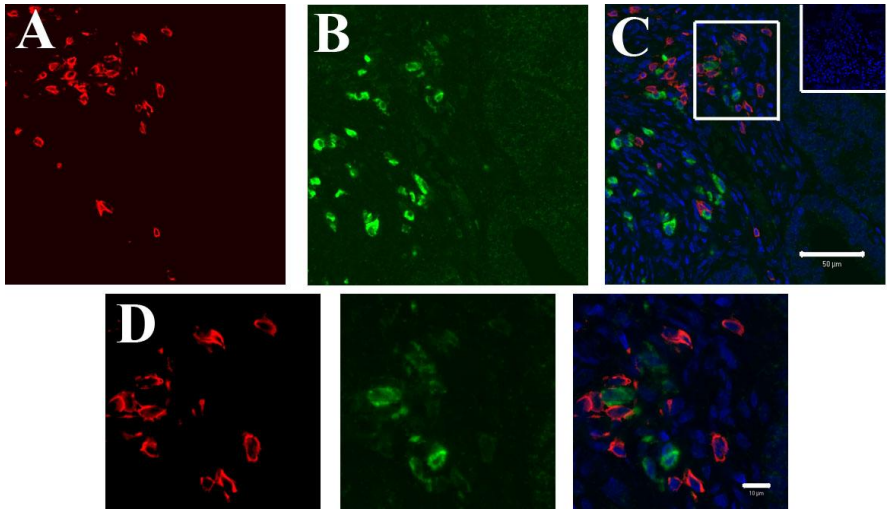

Figure 4.5 Expression of CXCR2 and CD20 protein in a well differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. CD20 **(A)**, a specific B-cell marker, and CXCR2 **(B)** were expressed in a well differentiated endometrial adenocarcinoma but did not colocalise **(C)**. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 50 µm. **D:** Magnification of the area within the white box, inset in C. Scale bar represents 10 µm.

4.3.1.6 CXCR2 and CD56

Uterine natural killer (NK) cells can be identified by expression of the adhesion molecule CD56 (also known as NCAM1) (NCBI 2009). These cells were sparsely distributed throughout all grades of endometrial adenocarcinoma sections examined (Figure 4.6A) and did not colocalise with CXCR2 expression (Figure 4.6C, D) in all grades of endometrial adenocarcinoma sections examined. A representative image of a well differentiated adenocarcinoma is shown in Figure 4.6.

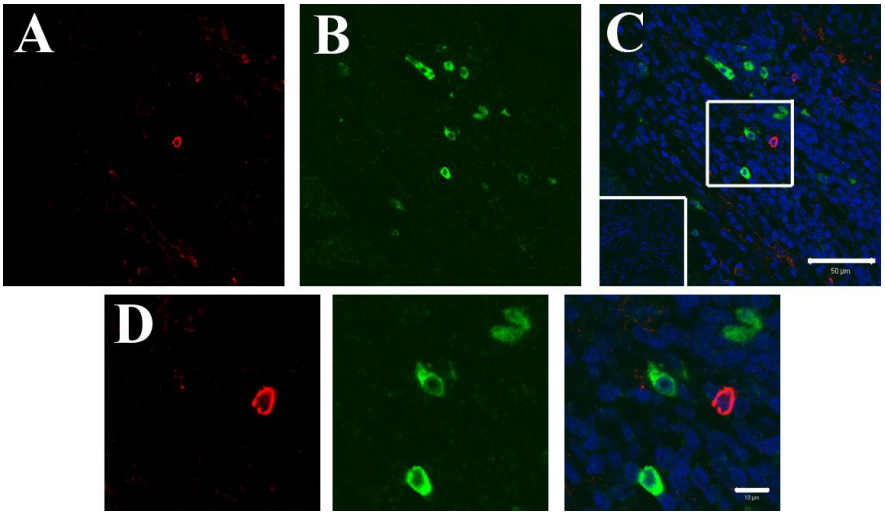

Figure 4.6 Expression of CXCR2 and CD56 protein in a well differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. CD56 **(A)**, a specific NK cell marker, and CXCR2 **(B)** were expressed in a well differentiated endometrial adenocarcinoma but did not colocalise **(C)**. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 50 µm. **D:** Magnification of the area within the white box, inset in C. Scale bar represents 10 µm.

4.3.1.7 CXCR2 and CD11c

CD11c (also known as integrin alpha-x) is an integrin expressed on immature dendritic cells and during differentiation into mature dendritic cells, so despite its expression on a small number of other leukocytes is an adequate dendritic cell marker (Coukos et al. 2005). In all grades of endometrial adenocarcinoma, CD11c-positive cells (Figure 4.7A) were seen in clusters within the stroma. CD11c expression did not colocalise with CXCR2 expression (Figure 4.7B) in endometrial adenocarcinoma sections examined (Figure 4.7C, D). A representative image of a moderately differentiated adenocarcinoma is shown in Figure 4.7.

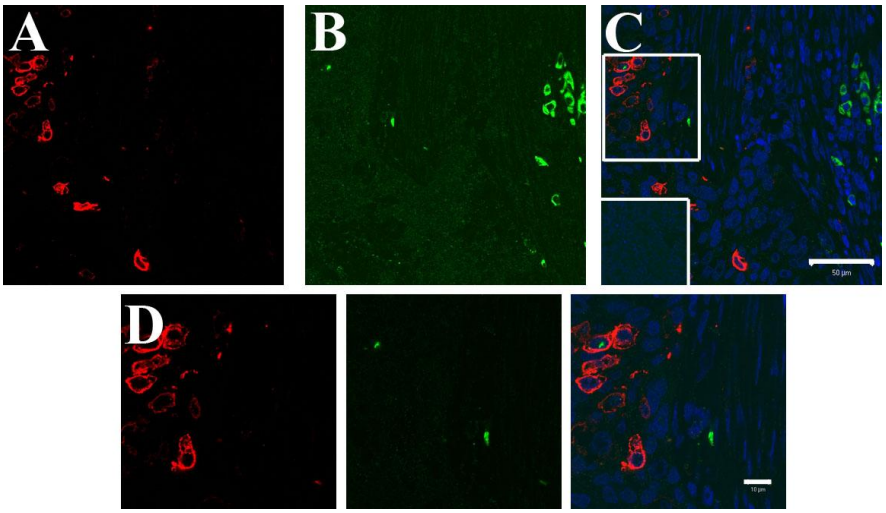

Figure 4.7 Expression of CXCR2 and CD11c protein in a moderately differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. CD11c **(A)**, a dendritic cell marker, and CXCR2 **(B)** were expressed in a moderately differentiated endometrial adenocarcinoma but did not colocalise **(C)**. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 50 µm. **D:** Magnification of the area within the white box, inset. Scale bar represents 10 µm.

4.3.2 Colocalisation of CXCR2 and CXCL1 expression in endometrial adenocarcinoma

The expression of CXCL1 by a number of stromal cells in endometrial adenocarcinoma was also noted in Section 4.2.3.3. To identify if these cells also expressed CXCR2, dual immunofluorescence microscopy was performed and revealed that CXCL1 (Figure 4.8A) colocalised with CXCR2 (Figure 4.8B) to a high number of cells within all grades of endometrial adenocarcinoma, as indicated by arrowheads (Figure 4.8 C, D). A representative image of a well differentiated adenocarcinoma is shown in Figure 4.8.

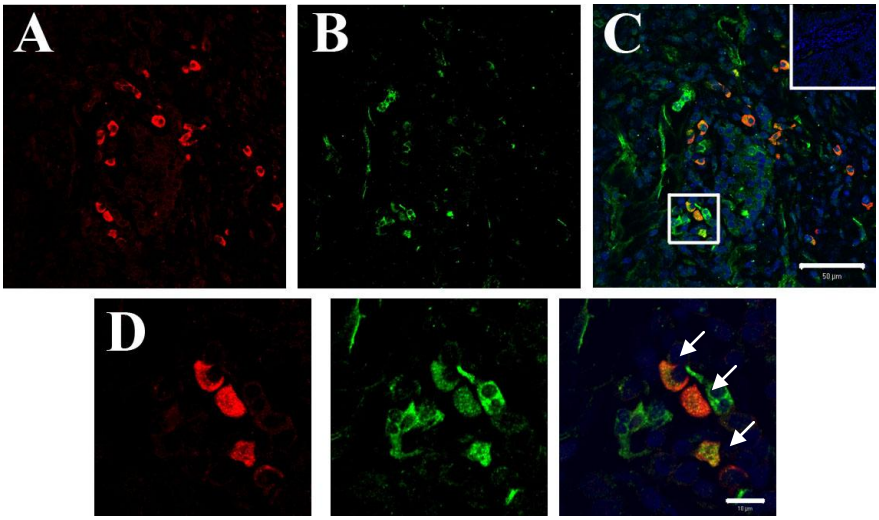

Figure 4.8 Expression of CXCR2 and CXCL1 protein in a well differentiated endometrial adenocarcinoma by dual immunofluorescence microscopy. CXCL1 **(A)** and CXCR2 **(B)** colocalised in a number of cells in a well differentiated endometrial adenocarcinoma **(C)**. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 50 µm. **D:** Magnification of the area within the white box, inset in C. Colocalisation is indicated by arrowheads. Scale bar represents 10 µm.

4.3.3 Localisation and quantification of immune cells in endometrial adenocarcinoma by immunohistochemistry

As the majority of CXCR2 positive cells in the stroma of endometrial adenocarcinoma were immune cells, notably neutrophils, the infiltration of immune

cells into endometrial adenocarcinoma as compared to normal endometrium from the proliferative stage of the menstrual cycle was localised and quantified. Immune cells were identified and localised by immunohistochemistry of specific markers and then quantified using standard stereological techniques (normal proliferative endometrium n=7, well differentiated endometrial adenocarcinoma n=10, moderately differentiated endometrial adenocarcinoma n=10, poorly differentiated endometrial adenocarcinoma n=10). Immune cells were identified by expression of the following markers: neutrophil elastase (neutrophils), CD68 (macrophages), CD4 (CD4+ T cells), CD8 (CD8+ T cells), CD20 (B cells), CD56 (uterine natural killer (uNK) cells) and CD11c (dendritic cells).

4.3.3.1 Neutrophil infiltration into endometrial adenocarcinoma as compared to normal endometrium

Neutrophils in normal endometrium from the proliferative phase of the menstrual cycle and in all grades of endometrial adenocarcinoma were measured by expression of neutrophil elastase (representative images shown in Figure 4.9). Neutrophils were seen scattered throughout the stroma, and were rarely present in normal endometrium from the proliferative phase of the menstrual cycle (Figure 4.9A). Neutrophils were more frequently distributed in all grades of endometrial adenocarcinoma (Figure 4.9B). No significant difference in neutrophil infiltration was seen between different grades of endometrial adenocarcinoma. Therefore the data was pooled, and the number of neutrophils in endometrial cancer was significantly increased as compared to normal proliferative endometrium ($p<0.01$; Figure 4.9C), by an average of 13.9 ± 2.3 fold.

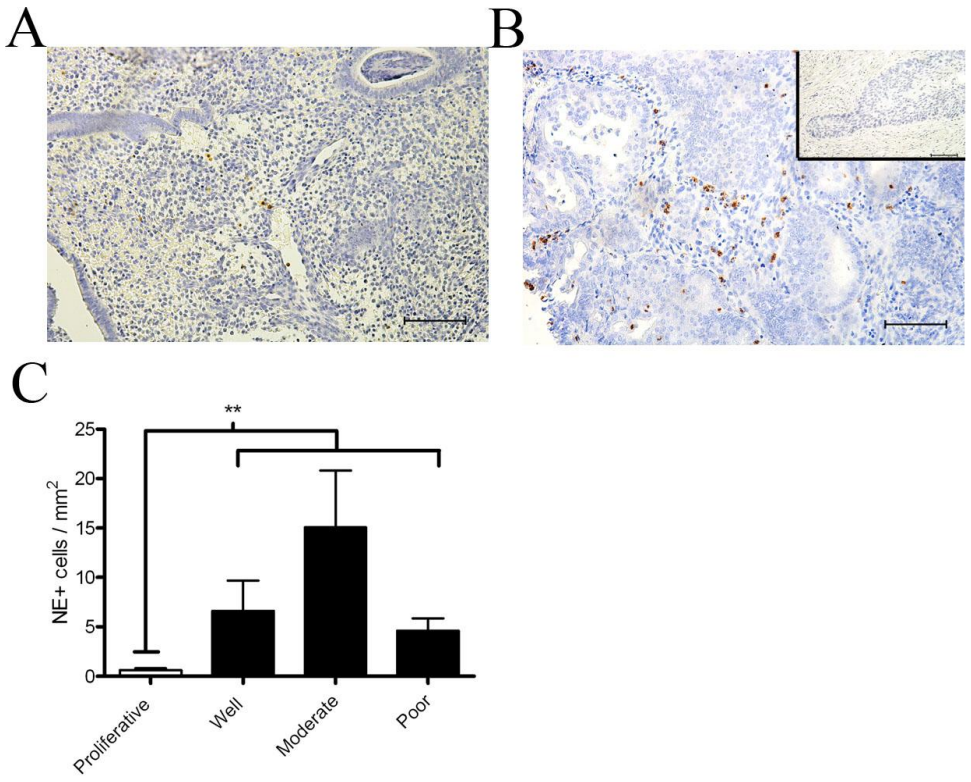

Figure 4.9 Localisation and quantification of neutrophils in normal endometrium and endometrial adenocarcinoma. A: Representative image demonstrating the localisation of neutrophil elastase expression, a neutrophil marker, in normal proliferative phase endometrium (n=7). Neutrophils are found rarely throughout the stroma. Scale bar represents 100 µM. **B:** Representative image demonstrating the localisation of neutrophils in well differentiated endometrial adenocarcinoma. Neutrophils are present throughout the stroma (well differentiated adenocarcinoma n=10, moderately differentiated adenocarcinoma n=10, poorly differentiated adenocarcinoma n=10). Negative control (inset) was incubated with non-immune IgG in place of primary antibody, Scale bar represents 100 µM. **C:** Neutrophils were significantly increased in endometrial adenocarcinoma as compared to normal endometrium from the proliferative stage of the menstrual cycle (n= as above). No significant difference was found between different grades of cancer. Data are expressed as number of neutrophil elastase positive cells/mm^2 and are presented as mean ± SEM. ** denotes $p<0.01$.

4.3.3.2 Macrophage infiltration into endometrial adenocarcinoma as compared to normal endometrium

Macrophages in normal endometrium from the proliferative phase of the menstrual cycle and in all grades of endometrial adenocarcinoma were measured by expression

of CD68 (representative images shown in Figure 4.10). Macrophages were present throughout the stroma of endometrial tissue from the proliferative phase of the menstrual cycle (Figure 4.10A) and in all grades of endometrial adenocarcinoma (Figure 4.10B). Macrophage numbers did not significantly vary between grades of endometrial adenocarcinoma examined, and hence data were pooled. Macrophages were significantly increased an average of 2.48 ± 0.73 fold in all grades of endometrial adenocarcinoma pooled as compared to normal endometrium from the proliferative phase of the menstrual cycle ($p<0.05$; Figure 4.10C).

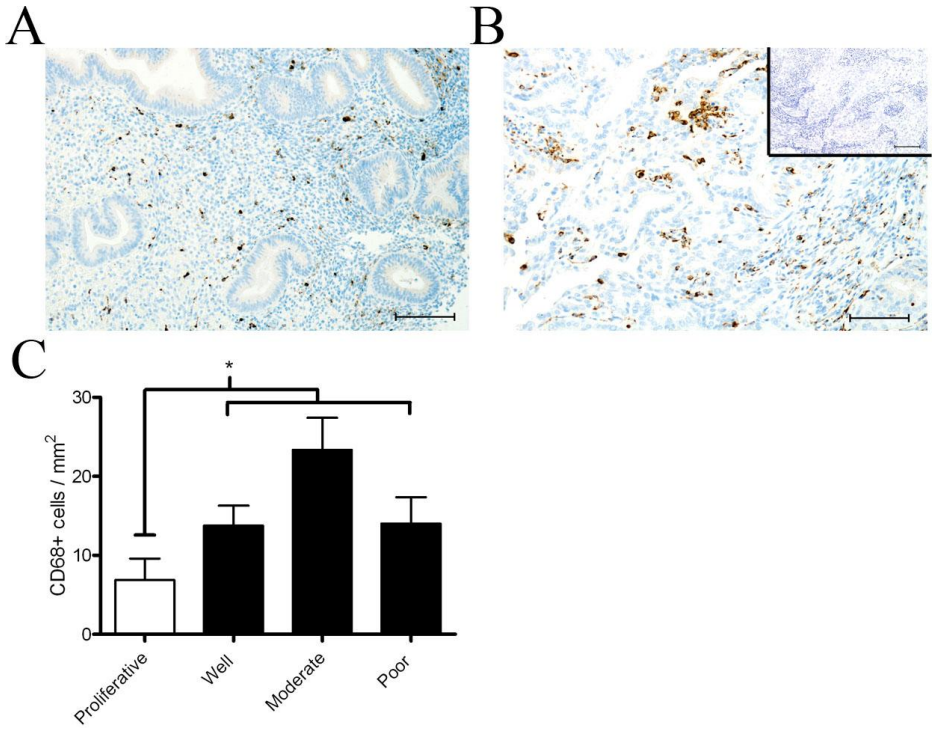

Figure 4.10 Localisation and quantification of macrophages in normal endometrium and endometrial adenocarcinoma. A: Representative image demonstrating the localisation of CD68 expression, a macrophage marker, in normal proliferative phase endometrium (n=7). Macrophages are found throughout the stroma. Scale bar represents 100 µM. **B:** Representative image demonstrating the localisation of macrophages in a moderately differentiated endometrial adenocarcinoma. Macrophages are present throughout the stroma (well differentiated adenocarcinoma n=10, moderately differentiated adenocarcinoma n=10, poorly differentiated adenocarcinoma n=10). Negative control (inset) was incubated with non-immune IgG in place of primary antibody, Scale bar represents 100 µM. **C:** Macrophages were significantly increased in endometrial adenocarcinoma as compared to normal proliferative phase endometrium (n= as above). No significant

difference was found between different grades of cancer. Data are expressed as number of CD68 positive cells/mm^2 and are presented as mean ± SEM. *denotes p<0.05.

4.3.3.3 CD4+ T cell infiltration into endometrial adenocarcinoma as compared to normal endometrium

CD4+ T cells in normal endometrium from the proliferative phase of the menstrual cycle and in all grades of endometrial adenocarcinoma were measured by expression of CD4 (representative images shown in Figure 4.11). CD4+ T cells were present throughout the stroma in normal proliferative phase endometrium (Figure 4.11A) and in all grades of endometrial adenocarcinoma, often in small clusters (Figure 4.11B). There was no significant difference in CD4+ T cell numbers between different grades of endometrial adenocarcinoma, and therefore data were pooled. No significant difference was found between infiltrations of CD4+ T cells in pooled endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the cycle, however a trend towards increased numbers in cancer was found.

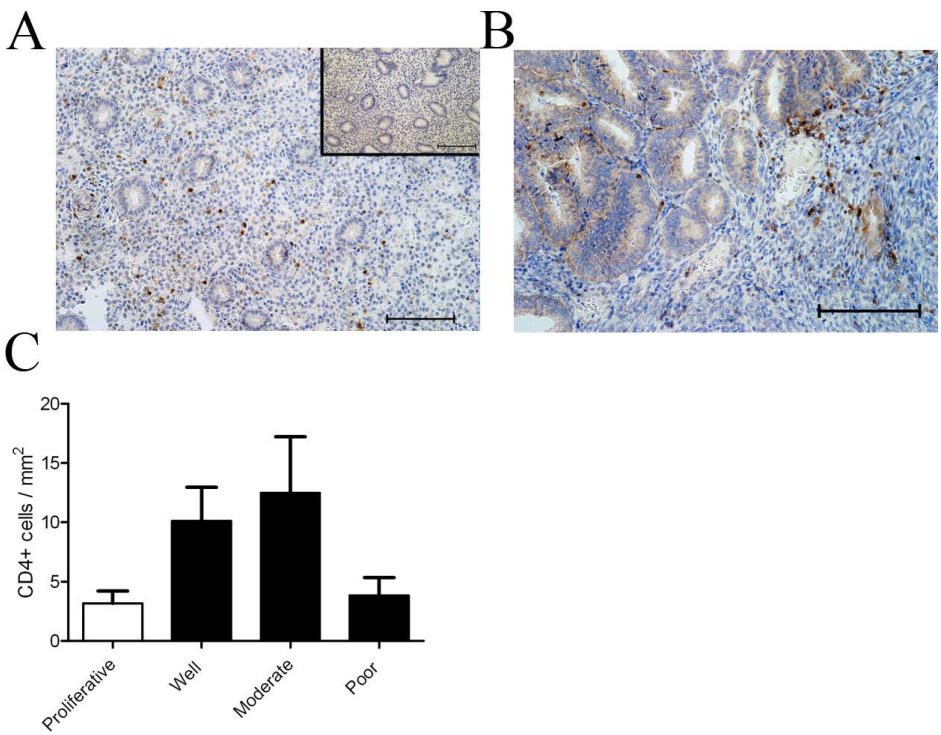

Figure 4.11 Localisation and quantification of CD4+ T cells in normal endometrium and endometrial adenocarcinoma. A: Representative image demonstrating the localisation of CD4+ T cells in normal endometrium from the proliferative phase of the menstrual cycle (n=7). CD4+ T cells are found throughout the stroma. Scale bar represents 100 μM. **B:** Representative image demonstrating the localisation of CD4+ T cells in a moderately differentiated endometrial adenocarcinoma. T cells are present throughout the stroma, often found in clusters (well differentiated adenocarcinoma n=10, moderately differentiated adenocarcinoma n=10, poorly differentiated adenocarcinoma n=10). Negative control (inset) was incubated with non-immune IgG in place of primary antibody, Scale bar represents 100 μM. **C:** CD4+ T cell numbers did not significantly differ in endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the menstrual cycle (n= as above). No significant difference was found between different grades of cancer. Data are expressed as number of CD4+ T cells cells/mm^2 and are presented as mean ± SEM.

4.3.3.4 CD8+ T cell infiltration into endometrial adenocarcinoma as compared to normal endometrium

CD8+ T cells in normal endometrium from the proliferative phase of the menstrual cycle and in all grades of endometrial adenocarcinoma were measured by expression of CD8 (representative images shown in Figure 4.12). In normal proliferative phase endometrium, CD8+ T cells were found sparsely throughout the stroma (Figure 5.12A). CD8+ T cells were also found throughout the stroma of all grades of endometrial adenocarcinoma, and a representative image of a well differentiated adenocarcinoma is presented below (Figure 5.12B). There was no significant difference in numbers of CD8+ T cells between the different grades of cancer. Moreover, CD8+ T cells did not differ between normal endometrium and endometrial adenocarcinoma (Figure 5.12C).

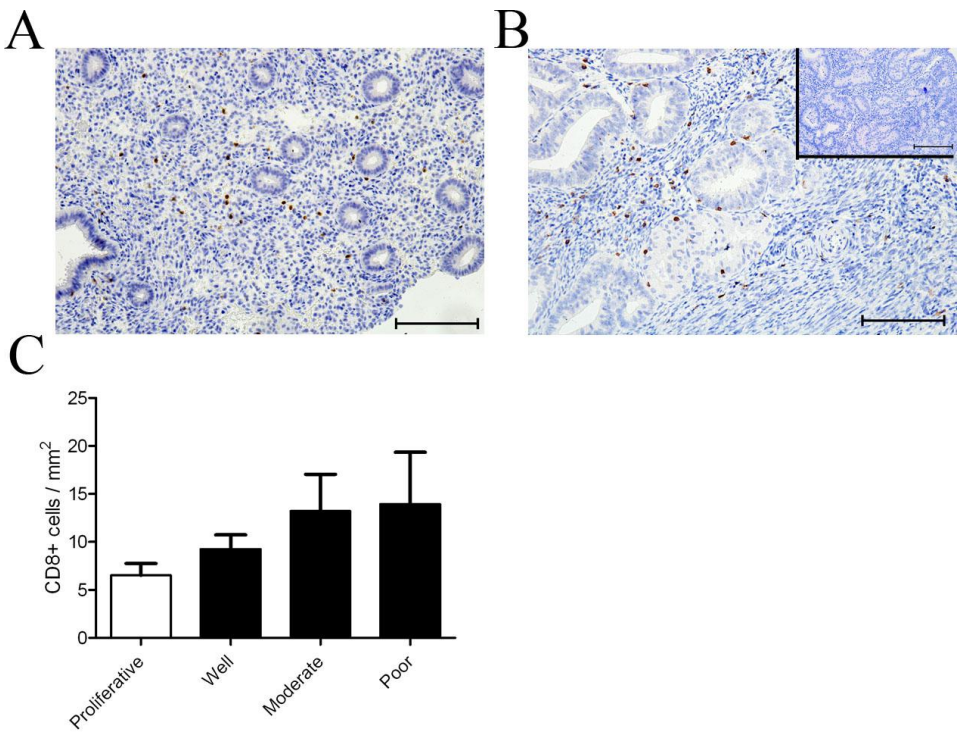

Figure 4.12 Localisation and quantification of CD8+ T cells in normal endometrium and endometrial adenocarcinoma. A: Representative image demonstrating the localisation of CD8+ T cells in normal endometrium from the proliferative phase of the menstrual cycle (n=7). T cells are found scattered throughout the stroma. Scale bar represents 100 µM. **B:** Representative image demonstrating the localisation of CD8+ T cells in a well differentiated endometrial adenocarcinoma. T cells are present throughout the stroma (well differentiated adenocarcinoma n=10, moderately differentiated adenocarcinoma n=10, poorly differentiated adenocarcinoma n=10). Negative control (inset) was incubated with non-immune IgG in place of primary antibody, Scale bar represents 100 µM. **C:** CD8+ T cell numbers did not significantly differ in endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the menstrual cycle. No significant difference was found between different grades of cancer (n= as above). Data are expressed as number of CD8+ T cells cells/mm^2 and are presented as mean ± SEM.

4.3.3.5 B cell infiltration into endometrial adenocarcinoma as compared to normal endometrium

B cells in normal endometrium from the proliferative phase of the menstrual cycle and all grades of endometrial adenocarcinoma were measured by expression of CD20

(representative images shown in Figure 4.13). CD20+ B cells were present in small numbers in the stroma of normal proliferative phase endometrium (Figure 4.13A). In all grades of endometrial adenocarcinoma, B cells were present throughout the stroma, often in small clusters. A representative image of a well differentiated adenocarcinoma is presented in Figure 4.13B. No significant difference was found between infiltrations of B cells in normal endometrium from the proliferative phase of the menstrual cycle as compared to endometrial adenocarcinoma, or between different grades of cancer (Figure 4.13C).

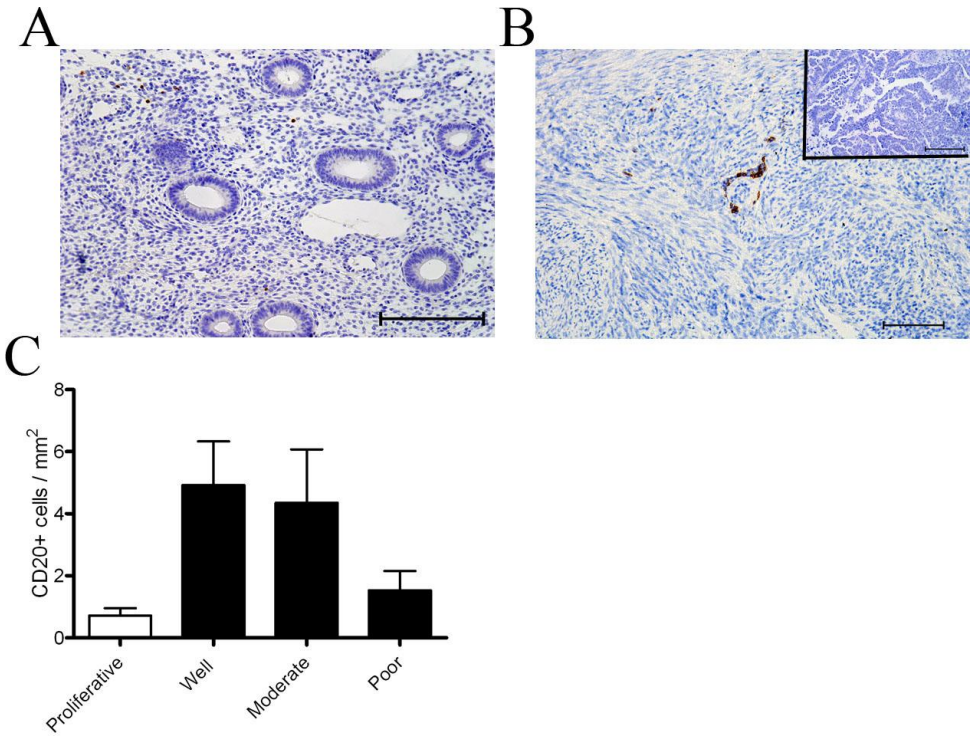

Figure 4.13 Localisation and quantification of B cells in normal endometrium and endometrial adenocarcinoma. A: Representative image demonstrating the localisation of CD20, a B cell marker, in normal endometrium from the proliferative phase of the menstrual cycle (n=7). B cells are found scattered throughout the stroma. Scale bar represents 100 µM. **B:** Representative image demonstrating the localisation of B cells in a well differentiated endometrial adenocarcinoma. B cells are present in small clusters throughout the stroma (well differentiated adenocarcinoma n=10, moderately differentiated adenocarcinoma n=10, poorly differentiated adenocarcinoma n=10). Negative control (inset) was incubated with non-immune IgG in place of primary antibody, Scale bar represents 100 µM. **C:** B cell numbers did not significantly differ in endometrial adenocarcinoma as compared to normal

endometrium from the proliferative phase of the menstrual cycle. No significant difference was found between different grades of cancer (n= as above). Data are expressed as number of CD20 positive cells/mm^2 and are presented as mean ± SEM.

4.3.3.6 NK cell infiltration into endometrial adenocarcinoma as compared to normal endometrium

NK cells in normal endometrium from the proliferative phase of the menstrual cycle and in all grades of endometrial adenocarcinoma were measured by expression of CD56 (representative images shown in Figure 4.14). CD56+ NK cells were localised to the stroma in normal proliferative phase endometrium and were present in high numbers (Figure 4.14A). NK cells were present sparsely in all grades of endometrial adenocarcinoma studied. A representative image of a moderately differentiated adenocarcinoma is shown in Figure 4.14B. No difference in numbers of NK cells between different grades of endometrial adenocarcinoma was found. Hence data were pooled and NK cells were significantly decreased in endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the menstrual cycle ($p<0.001$; Figure 4.14C) by 9.5 ± 2.8 fold.

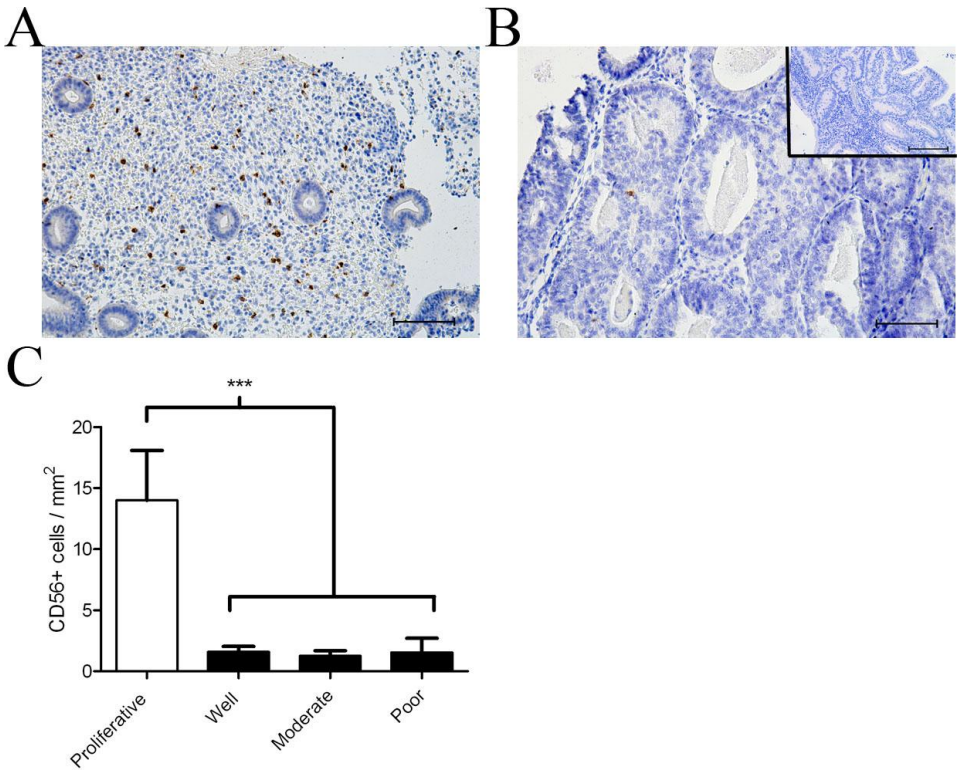

Figure 4.14 Localisation and quantification of NK cells in normal endometrium and endometrial adenocarcinoma. A: Representative image demonstrating the localisation of CD56 expression, a NK cell marker, in normal endometrium from the proliferative phase of the menstrual cycle (n=7). NK cells were observed in high numbers throughout the stroma. Scale bar represents 100 µM. **B:** Representative image demonstrating the localisation of NK cells in a moderately differentiated endometrial adenocarcinoma. NK cells were present rarely throughout the stroma (well differentiated adenocarcinoma n=10, moderately differentiated adenocarcinoma n=10, poorly differentiated adenocarcinoma n=10). Negative control (inset) was incubated with non-immune IgG in place of primary antibody, Scale bar represents 100 µM. **C:** NK cells were significantly decreased in endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the menstrual cycle. No significant difference was found between different grades of cancer (n= as above). Data are expressed as number of neutrophil elastase positive cells/mm^2 and are presented as mean ± SEM. *** denotes $p<0.001$.

4.3.3.7 Dendritic cell infiltration into endometrial adenocarcinoma as compared to normal endometrium

Dendritic cells in normal endometrium from the proliferative phase of the menstrual cycle and in all grades of endometrial adenocarcinoma were localised by CD11c expression (representative images shown in Figure 4.15). Dendritic cells were sparsely localised within the stroma of normal endometrium from the proliferative phase of the menstrual cycle (Figure 4.15A) and were seen more frequently in all grades of endometrial adenocarcinoma (Figure 4.15B). There was no significant difference in dendritic cell number between different grades of endometrial adenocarcinoma, and hence the data were pooled. Dendritic cells were significantly increased in endometrial adenocarcinoma as compared to normal endometrium ($p<0.05$; Figure 4.15C) by 6.8 ± 0.9 fold.

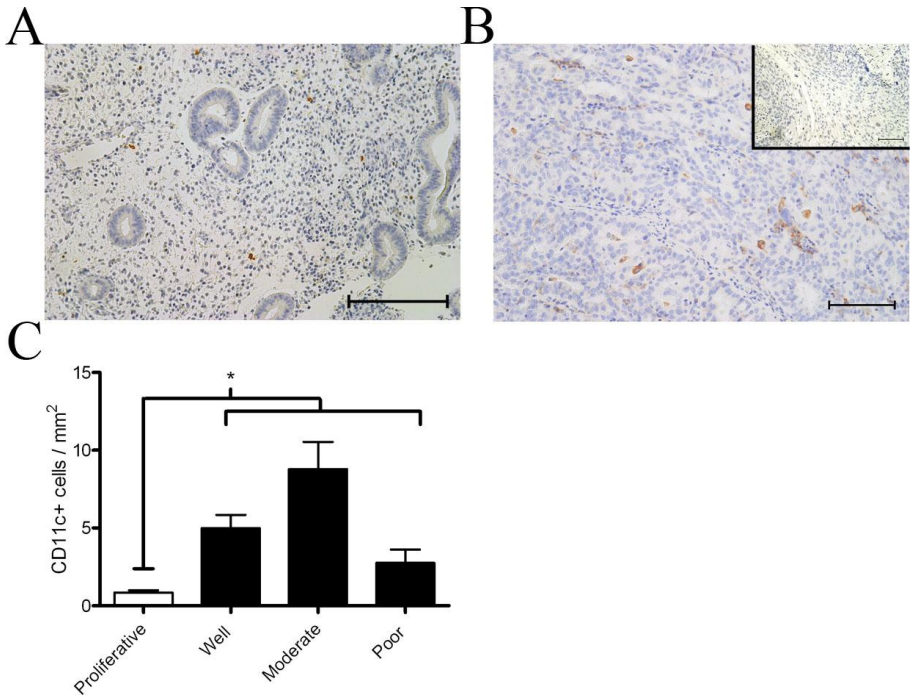

Figure 4.15 Localisation and quantification of dendritic cells in normal endometrium and endometrial adenocarcinoma. A: Representative image demonstrating the localisation of CD11c expression, a dendritic cell marker, in normal endometrium from the proliferative phase of the menstrual cycle (n=7). Dendritic cells were found in low numbers throughout the stroma. Scale bar represents 100 µM. **B:** Representative image demonstrating the localisation of dendritic cells in a poorly differentiated endometrial

adenocarcinoma. Dendrtitic cells were present throughout the stroma (well differentiated adenocarcinoma n=10, moderately differentiated adenocarcinoma n=10, poorly differentiated adenocarcinoma n=10). Negative control (inset) was incubated with non-immune IgG in place of primary antibody, Scale bar represents 100 μM. **C:** Dendritic cells were significantly increased in endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the menstrual cycle. No significant difference was found between different grades of cancer (n = as above). Data are expressed as number of CD11c positive cells/mm^2 and are presented as mean ± SEM. * denotes $p<0.05$.

4.3.4 $PGF_{2\alpha}$-induced CXCL1 expression by FPS cells induces neutrophil migration *in vitro*

As CXCR2 expression was colocalised with neutrophil elastase (Figure 4.2) and neutrophils were present at increased levels in endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the menstrual cycle (Figure 4.9), it was next determined whether the CXCL1 induced in FPS cells via $PGF_{2\alpha}$-FP receptor interaction (as described in Chapter 3) could induce neutrophil chemotaxis *in vitro*. Human neutrophils were purified from peripheral blood and used in a transwell chemotaxis assay (Figure 4.16). A significant increase in neutrophil chemotaxis in response to conditioned media from FPS cells treated with 100 nM $PGF_{2\alpha}$ for 48 hours ($PGF_{2\alpha}$-CM) was demonstrated as compared with media from vehicle-treated cells (V-CM; $p<0.001$). Chemotaxis was significantly inhibited with immunoneutralisation of CXCL1 from the conditioned media before incubation with neutrophils, or with the addition of the CXCR2 antagonist SB225002 to $PGF_{2\alpha}$-CM ($p<0.001$). Incubation of $PGF_{2\alpha}$-CM with goat IgG as a control for antibody absorption did not significantly affect chemotaxis induced by $PGF_{2\alpha}$-CM. Serum free media was used as a negative control for random migration, and data are presented relative to this. Recombinant CXCL1 was used as a positive control for directed migration (data not shown).

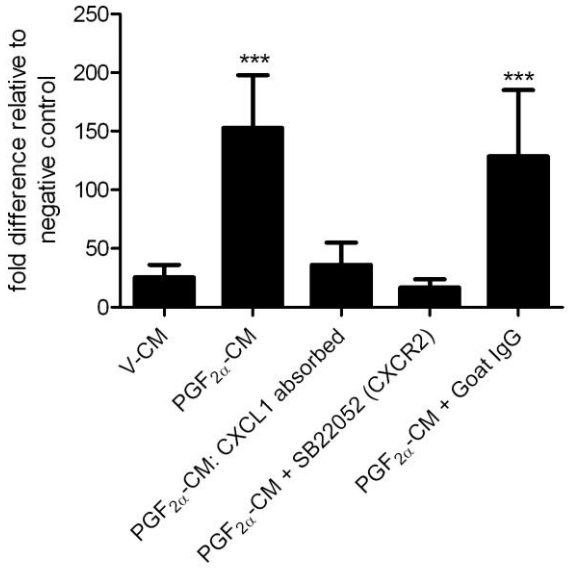

Figure 4.16 $PGF_{2\alpha}$-induced CXCL1 can induce neutrophil chemotaxis in vitro. A transwell assay was used to measure neutrophil chemotaxis (n=4) in response to conditioned media from FPS cells treated with $PGF_{2\alpha}$ for 48 h ($PGF_{2\alpha}$-CM) compared with vehicle-treated cells (V-CM). Chemotaxis was increased by $PGF_{2\alpha}$–CM, and this was significantly reduced with immunoneutralisation of CXCL1 protein from the medium or the addition of 60 nM SB-225002. Control goat IgG immunoneutralisation showed no significant effect. Data shown are mean ± SEM *** denotes $p<0.001$.

4.3.5 $PGF_{2\alpha}$-stimulated CXCL1 induces neutrophil migration *in vivo*

To explore whether FP receptor signalling could promote neutrophil migration *in vivo*, a mouse xenograft model was used. Ishikawa wild-type (WT) cells with low level of FP expression or FPS cells were injected subcutaneously into nude mice (experiment performed by Dr. R Catalano). Mice were then regularly injected with control IgG (WT and FPS xenografts) or CXCL1 antibody (FPS xenografts only), as described in section 4.2.8. Tumours formed from FPS cells demonstrated significantly higher CXCL1 mRNA expression compared with WT tumours ($p<0.05$; Figure 4.17).

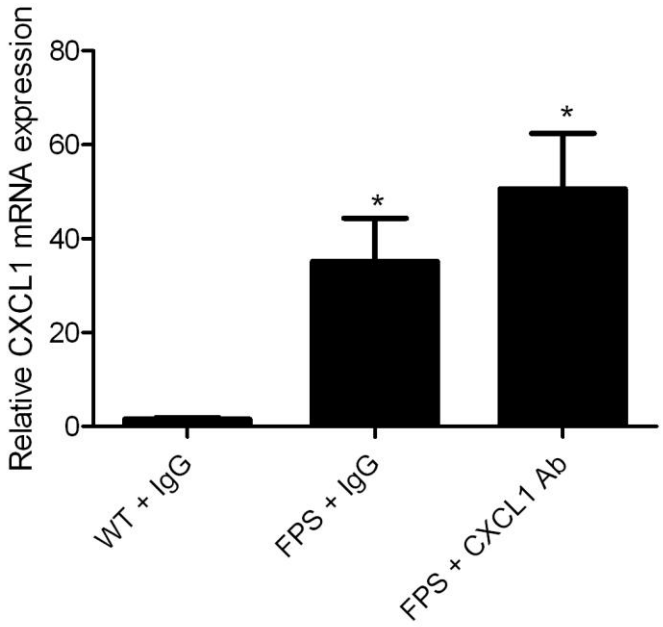

Figure 4.17 CXCL1 mRNA is significantly increased in FPS xenografts. CXCL1 mRNA expression is significantly increased in nude mice xenografts (n=10 each group) formed from FPS cells compared with WT cell xenografts. Injection of CXCL antibody did not significantly alter CXCL1 mRNA expression in FPS tumours as compared to FPS tumours injected with control IgG antibody. Data are presented as mean ± SEM, * denotes $p<0.05$.

Neutrophil infiltration into the xenografts (n=5 tumours from each treatment group) was then analysed by flow cytometry (Figure 4.18A). FPS xenografts displayed increased neutrophil infiltration as compared to WT xenografts ($p<0.001$; Figure 4.18B). This infiltration was significantly decreased in FPS xenografts from mice injected with CXCL1 neutralizing antibody compared to FPS xenografts from mice injected with IgG antibody ($p< 0.001$).

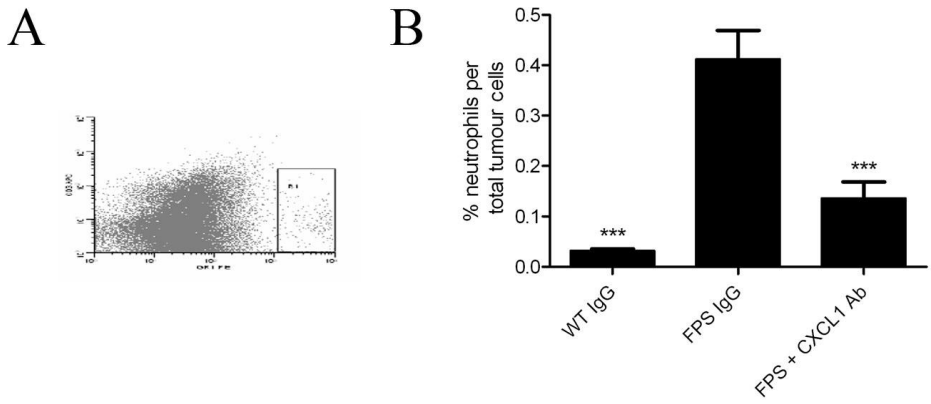

Figure 4.18 Neutrophil infiltration is significantly increased in FPS xenografts. A: Representative image of scatter profile of neutrophils identified in mouse xenografts (inset box) by flow cytometry. **B:** Percentage of neutrophils in total tumour cells from WT and FPS xenografts (n = 5 per treatment group), measured by flow cytometry. Increased neutrophil infiltration is seen in FPS xenografts, which is significantly reduced by injection of CXCL1-neutralising antibody. Data are presented as mean ± SEM, *** denotes $p<0.001$.

To further confirm this analysis, immunohistochemistry of the specific murine neutrophil marker granulocyte receptor -1 (Gr-1) was performed on xenograft tissue (Figure 4.19, neutrophils indicated by arrowheads). Higher numbers of neutrophils were seen distributed throughout FPS xenografts (Figure 4.19B) as compared to WT (Figure 4.19A) or CXCL1 immunoneutralised FPS (Figure 4.19C) xenografts. Neutrophils were commonly localised to the leading edge of the xenografts, but could be seen scattered throughout the tissue in some samples.

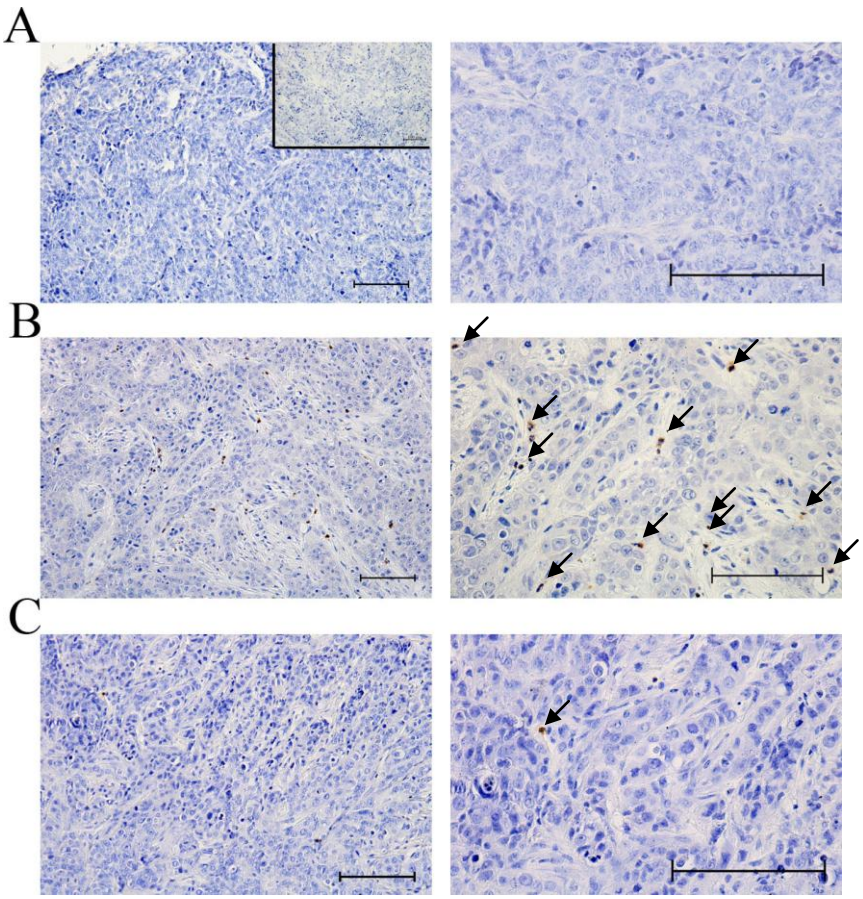

Figure 4.19 Localisation of neutrophils in mouse xenografts by Gr-1 immunohistochemistry. Representative images showing localisation of neutrophils by Gr-1 expression (as indicated by arrowheads) in xenografts. **A:** Xenografts arising from WT cells treated with IgG. **B:** Xenografts arising from FPS cells treated with IgG. **C:** Xenografts arising from FPS cells treated with CXCL1 antibody. Left image is x200 magnification, right image is x400. Negative control (inset) is incubated with non-immune IgG in place of primary antibody. All scale bars represent 100 µm.

4.3.6 Localisation of CXCL1 and CXCR2 to endothelial cells in endometrial adenocarcinoma

CXCL1 binding to CXCR2 has been demonstrated to promote angiogenesis in a number of mouse models (Moore et al. 1999; Haghnegahdar et al. 2000; Barcelos et al. 2004; Wang et al. 2006). As described in Chapter 3, expression of CXCL1 and CXCR2 could be localised to endothelial cells in all grades of endometrial

adenocarcinoma. This localisation was further confirmed using dual immunofluorescence microscopy. Expression of CXCL1 (Figure 4.20A, green channel) and the specific endothelial cell marker CD31 (red channel) was colocalised in all grades of endometrial adenocarcinoma (yellow channel, merge). CXCR2 expression (Figure 4.20B, green channel) also colocalised to that of CD31 (red channel; yellow channel, merge) localising its expression to endothelial cells in all grades of endometrial adenocarcinoma. Representative images of a well differentiated carcinoma (Figure 4.20A) and moderately differentiated adenocarcinoma (Figure 4.20B) are shown.

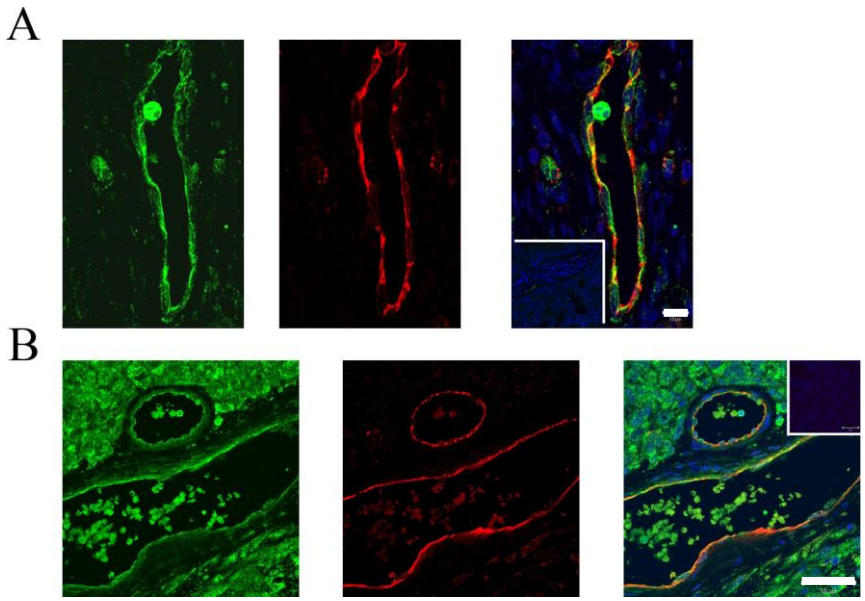

Figure 4.20 Expression of CXCL1 and CXCR2 by CD31 positive vascular endothelial cells. A: Expression of CXCL1 (green channel) and CD31 (red channel) were colocalised (yellow channel, merge), demonstrating the expression of CXCL1 by endothelial cells in a well differentiated endometrial adenocarcinoma. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 10 µm. **B:** Expression of CXCR2 (green channel) and CD31 (red channel) was colocalised (yellow channel, merge), demonstrating the expression of CXCR2 by endothelial cells in a moderately differentiated endometrial adenocarcinoma. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 100 µm.

4.3.7 $PGF_{2\alpha}$-stimulated CXCL1 does not alter microvascular density *in vivo*

As CXCR2 was expressed on endothelial cells in endometrial adenocarcinoma (Figure 4.20B) and its expression has been previously demonstrated on mouse endothelial cells (Devalaraja et al. 2000), angiogenesis in WT, FPS and CXCL1-immunoneutralised FPS xenografts was examined (n=10 each group). The expression of CD31, a vascular marker, was identified by immunohistochemistry and CD31+ cells were counted. There was no significant difference in microvascular density between the three groups examined (Figure 4.21).

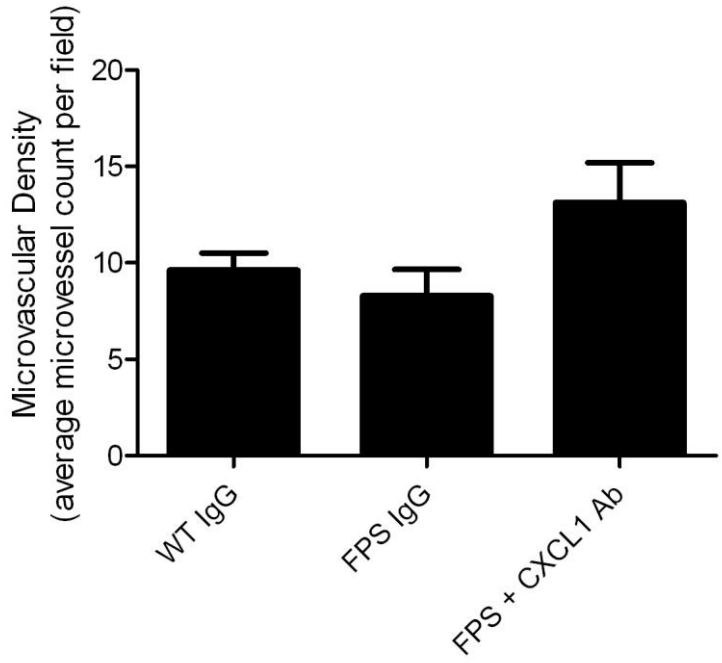

Figure 4.21 Microvascular densities in WT, FPS and FPS xenografts neutralised for CXCL1. CD31 immunohistochemistry was used to examine microvascular density in nude mice xenografts (n=10 each group) arising from injection of WT cells treated with IgG, FPS cells treated with IgG and FPS cells treated with CXCL1 antibody. No significant difference was found between the three groups. Data are presented as mean ± SEM.

4.4 Discussion

Expression of chemokines in cancer contributes to a tumour microenvironment displaying classic features of inflammation, including the recruitment of immune cells and tissue remodelling by angiogenesis. In Chapter 3, the expression of CXCR2 on cells throughout the stroma of endometrial adenocarcinoma and on vascular endothelium was demonstrated. This chapter therefore sought to investigate the identity of the CXCR2 expressing stromal cells, and the potential consequences of $PGF_{2\alpha}$-regulated CXCL1 signalling to CXCR2 on immune cell infiltration and angiogenesis.

CXCR2 is expressed on a number of leukocytes (Murphy et al. 2000). Dual immunofluorescent immunohistochemistry of the pan-leukocyte marker CD45 and CXCR2 in endometrial adenocarcinoma demonstrated both the large number of leukocytes infiltrating the tumours and the expression of CXCR2 on a proportion of these. However, a number of leukocytes did not express CXCR2, and conversely a number of CXCR2-positive cells did not express this leukocyte marker. The identity of these latter cells is still unknown. Cells which were CD45+/CXCR2+ were then further investigated by the use of a number of specific immune cell markers. The majority of CXCR2 expression in the stroma was colocalised to cells expressing neutrophil elastase and to a lesser extent CD68, which are neutrophil and monocyte/macrophage markers respectively. No colocalisation was seen with markers of T or B lymphocytes, NK cells or dendritic cells. Interestingly, CXCR2 expression in some stromal cells also colocalised to CXCL1 expression, indicating that their infiltration could further chemoattract other CXCR2-expressing immune cells.

CXCR2 localization in neutrophils in endometrial adenocarcinoma suggested that CXCL1 signalling via CXCR2 could play a role in immune cell function. A role for CXCL1 in neutrophil influx has been previously shown in an angiogenic sponge model in the mouse, as endogenous CXCL1 expression increased immediately

preceding a neutrophil influx (Barcelos et al. 2004). As neutrophil infiltration has not previously been quantified in endometrial adenocarcinoma, the number of neutrophils and other immune cells in endometrial adenocarcinoma was investigated by immunohistochemistry.

Macrophage infiltration into cancer has been intensely studied in recent years, as tumour associated-macrophages (TAM) are increased in a number of cancer types including breast, colon, bladder and melanoma (Bingle et al. 2006; Hussein 2006; Forssell et al. 2007; Chen et al. 2009). Immunohistochemistry demonstrated increased macrophage infiltration into all grades of endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the menstrual cycle, in agreement with a number of studies where macrophages are increased in cancer as compared to normal tissue (Miyatake et al. 2007). This has been associated with a poor prognosis in cancer patients (Salvesen et al. 1999; Yang et al. 2007). Macrophages are heterogeneous cells, with the ability to alter their phenotype dependent on the environment. This may explain the contrasting reports of roles in tumour progression and destruction (Saccani et al. 2006; Forssell et al. 2007). Typically, the phenotype adopted by TAM is similar to a polarised M2 response, which involves low cytotoxic and antigen presenting functions and the secretion of growth factors promoting metastasis and angiogenesis (Luo et al. 2006).

In other cancer types, macrophages may play a key role in tumour progression. This is demonstrated by a mouse model of breast cancer lacking macrophage infiltration which has a greatly decreased tumour progression and metastasis rate. When macrophages were re-introduced, tumour progression rapidly caught up with control counterparts (Lin et al. 2001). A number of other studies have shown similar results, suggesting macrophages secrete factors such as VEGF and angiopoetins which increase angiogenesis (Bingle et al. 2006; Venneri et al. 2007) and are essential for the angiogenic switch (Lin et al. 2007). Macrophages also secrete proteases which influence metastasis and invasion (Dirkx et al. 2006; Yang et al. 2007). In endometrial cancer, expression of platelet-derived endothelial cell growth factor

(thymidine phosphorylase) by macrophages is correlated with increased microvascular density (Tanaka et al. 2002). Therefore, increased macrophage numbers found in endometrial adenocarcinoma described in this chapter may play a role in cancer progression through one or more of these mechanisms. CXCR2 expression was seen on a small number of macrophages in endometrial adenocarcinoma, and although there is some evidence suggesting CXCL1 can chemoattract monocytes, this is primarily mediated by promoting their adhesion to the endothelium (Weber et al. 1999; Traves et al. 2004; Smith et al. 2005). Therefore the CXCL1 regulated by $PGF_{2\alpha}$-FP signalling in endometrial adenocarcinoma is unlikely to play a major role in their chemoattraction into the tumour, however CXCL1 expression which was demonstrated in the vascular endothelium may affect this.

By immunohistochemical analysis, no significant difference was found between T cell infiltration in normal endometrium and endometrial cancer. However there was a trend towards increased numbers of CD4+ and CD8+ T cells in human endometrial adenocarcinoma. Previous studies have also described increased T cell infiltration in endometrial adenocarcinoma as compared to normal endometrium (Hachisuga et al. 1997).

Most evidence points towards a cytotoxic role for T cells in cancer. CD4+ T cells can recognise tumour antigen and with the aid of CD8+ T cells have been demonstrated to eradicate tumours from mouse models though production of cytokines (Nishimura et al. 1999). The cytokine profile produced by CD4+ cells however appears to be dependent on the environment of the tumour and cancer model used (Ellyard et al. 2007). CD4+ T cells can initiate the CD8+ T cell cytotoxic response and play a central role in maintaining it, however through the production of cytokines may also recruit other cells of the innate immune system such as macrophages (Badoual et al. 2006). The plasticity and complexity of immune cell responses to tumours is demonstrated by CD4+ T cells, as a subset of these are known as regulatory T cells, as indicated by the specific expression of the cell surface antigen forkhead box p3

(FOXP3) which drives the development of this cell type. These cells have a distinct phenotype which can suppress T cell population expansion and cytotoxicity, and are associated with increased microvascular density and angiogenic factors including VEGF in endometrial adenocarcinoma (Giatromanolaki et al. 2008)

The infiltration of CD8+ T cells into endometrial cancer has previously been inversely correlated with high COX-2 expression (Ohno et al. 2005). This low infiltration was associated with poor prognosis, further demonstrating that a high CD8+ infiltration may be destructive to the tumour. Additionally, a high CD8+ cell infiltration in endometrial adenocarcinoma has been correlated with an increased survival rate (Kondratiev et al. 2004). Beyond the expression of CD4 and CD8, the phenotype of T cells was not investigated in this chapter. However the non-significant increase in infiltration in endometrial cancer as compared to normal endometrium may suggest that an inefficient cytotoxic response is being mounted.

B cells were identified by CD20 staining. No significant difference was found between normal endometrium and endometrial adenocarcinoma, however similarly to T cells there was a trend towards increased numbers in cancer tissue. Previously in endometrial adenocarcinoma, B cells have been shown to be significantly increased (Hachisuga et al. 1997). As described earlier in this chapter, a popular model of immune cell infiltration suggests that adaptive immune cells such as B cells are recruited to the tumour and can destroy cancerous cells through mechanisms including antibody-induced complement-mediated lysis. However, evidence also suggests that the serum proteins secreted by B cells which chemoattract innate immune cells may promote chronic inflammation and therefore tumour progression (de Visser et al. 2006). Indeed, elimination of B and T cells in a mouse model reduced innate immune cell infiltration into developing tumours and levels of tumourigenic factors including VEGF and MMP9. This caused a decrease in tumour growth. Re-introduction of B cells restored the infiltration of innate immune cells and angiogenesis to control levels (de Visser et al. 2005). The function of B cells

seen in human endometrial adenocarcinomas is therefore unclear, as both tumour promoting and destructive properties are possible.

The population of uterine NK cells fluctuates in the endometrium throughout the menstrual cycle, and they are most abundant during the secretory phase (King et al. 1989). By examining CD56 expression, NK cells were found to be significantly decreased in endometrial adenocarcinoma as compared to normal endometrium, agreeing with previously published data (Ferguson et al. 1985). NK cells are specialised lymphocytes which can recognise MHC class I molecules. The frequent lack of MHC class I molecules on cancer cells means that NK cells can identify and lyse tumour cells (Zamai et al. 2007). However a subset of NK cells indiscriminately secrete cytokines to kill infected or transformed cells, for example IFN-γ and TNF-α, and therefore NK cells are seen as bridging the innate and adaptive immune systems (Caligiuri 2008). In a subcutaneous tumour mouse model decreased NK cell infiltration is also observed. This is due to secreted factors including granulocyte macrophage colony-stimulating factor (GM-CSF), IL-3 and IL-6 from the tumour impairing the maturation of NK cells (Richards et al. 2008). The cause of decreased NK infiltration into endometrial tumours as compared to normal tissue remains to be elucidated, however the low number of NK cells seen in endometrial adenocarcinoma suggests these tumours may be evading recognition by NK cells.

Dendritic cells are the primary antigen-presenting cells which can activate T and B cell responses and also tolerance. By immunohistochemistry, dendritic cells were shown to be significantly increased in endometrial adenocarcinoma as compared to normal endometrium. The marker CD11c was used, which is expressed highly on immature dendritic cells that are unable to present antigen. Tumours often contain high numbers of these cells as they are recruited by factors secreted by the cancer, including VEGF and other C-C and C-X-C chemokines (Murdoch et al. 2008). A high number of immature dendritic cells can promote immune tolerance to tumours, as has been demonstrated in a mouse model of colon cancer (Bonnotte et al. 2004). Recently, a role for dendritic cells in angiogenesis has been demonstrated. Dendritic

cells have been observed producing VEGF adjacent to vasculature in models of both endometriosis and cancer (Fainaru et al. 2008). These same immature dendritic cells could also increase endothelial cell migration *in vitro*, demonstrating their potential to influence angiogenesis (Fainaru et al. 2008). Finally, in a study by Coneja-Garcia et al (Conejo-Garcia et al. 2005), cells with a dendritic cell phenotype expressing CD11c were able to differentiate into cells expressing endothelial markers and assemble into blood vessels *in vivo.* The increased numbers of immature dendritic cells present in endometrial adenocarcinoma could therefore be promoting both evasion of the adaptive immune system by the tumour and tissue remodelling via production of VEGF.

Neutrophils were significantly increased in human endometrial adenocarcinoma as compared to normal endometrium from the proliferative phase of the cycle, as demonstrated by immunohistochemistry. As neutrophils were the main cell type expressing CXCR2, the ability of $PGF_{2\alpha}$-regulated CXCL1 to chemoattract neutrophils was investigated. CXCL1 was strongly chemotactic to neutrophils as conditioned media from $PGF_{2\alpha}$-stimulated FPS cells induced chemotaxis of peripheral human neutrophils. This chemotaxis was significantly reduced by CXCL1 immunoneutralisation and CXCR2 inhibition using a specific antagonist. To determine a role for CXCL1 induced by $PGF_{2\alpha}$-FP interaction in vivo, nude mice were inoculated with FPS and WT cells. By flow cytometry, it was demonstrated that the increased neutrophils in the resulting FPS tumours compared with WT were significantly reduced by injection of CXCL1 neutralizing antibodies, demonstrating that $PGF_{2\alpha}$ signalling via CXCL1 is influencing neutrophil infiltrate in endometrial adenocarcinomas. Neutrophil infiltration into tumours has also been shown to be dependent on CXC chemokine-CXCR2 signalling in a model of melanoma in a CXCR2 null nude mouse (Singh et al. 2009).

A chemokine-mediated influx of neutrophils is seen in the late secretory phase of the normal endometrium (Critchley et al. 1994). Their role may be dependent on the activating agents and cytokines present, but they are thought to be involved in the

breakdown and repair at menstruation by degranulation and the release of proteases which degrade the extracellular matrix (Kaitu'u-Lino et al. 2007). They have also been implicated in angiogenesis, as neutrophils found close to or associated with endothelial microvessels express VEGF during or coincident with angiogenesis (Gargett et al. 2001).

The role of neutrophils in endometrial adenocarcinoma is unclear, and in our xenograft model, similarly to other reports (Tazawa et al. 2003; Shojaei et al. 2008) neutrophil influx did not affect tumour size (R. Catalano, personal communication). However, considering their profound tissue-remodelling capabilities, which have been shown in a number of animal models of other cancer types, it is possible that they play a similar role in endometrial cancer. For example, neutrophils have been shown to uniquely produce a tissue inhibitor of metalloproteinase (TIMP)–free MMP-9, a key protease involved in extracellular matrix degradation, which may affect the tumour microenvironment by tissue remodelling (Ardi et al. 2007). In addition, the depletion of neutrophils in a mouse model prevented metastasis of fibrosarcoma cells from the primary tumour, suggesting a role for neutrophils in the switch to a metastatic phenotype (Tazawa et al. 2003). In a nude mouse model of breast cancer, over-expression of interleukin-8, a chemokine related to CXCL1, caused an infiltration of neutrophils which increased invasiveness of the tumour, likely due to an increase in protease production (Yao et al. 2007). Similarly, the decrease in neutrophil infiltration caused by an inhibition of CXCL1 expression in a nude mouse model of colon cancer significantly decreased metastasis in these animals (Yamamoto et al. 2008).

Neutrophils may also promote tumourigenesis through means other than tissue remodelling. In an *in vitro* model of colon cancer, neutrophils promote cellular stress by inducing transient errors in DNA replication in epithelial cells (Campregher et al. 2008), which could ultimately lead to carcinogenesis. Neutrophils from ovarian cancer patients released higher levels of reactive oxygen species, which could

potentially lead to cellular changes that support tumour progression (Klink et al. 2008).

As discussed in Chapter 3, as well as being chemotactic for neutrophils CXCL1 is an angiogenic chemokine. Despite this and evidence that neutrophils can contribute to angiogenesis, no difference in microvascular density was observed between WT, FPS or CXCL1-immunoneutralised xenografts. The reasons for this are unclear. However in the xenograft tissue, although significantly different rates of neutrophil infiltration are observed between groups, immunohistochemical staining of Gr-1 reveals that the infiltration is lower than that seen in human tissue. It is possible that this nude mouse model does not replicate closely enough the situation seen in human tumours, and therefore the extent to which neutrophils could be affecting tissue remodelling within the tumour. Furthermore, only microvascular density was closely examined. Blood vessel size and arrangement in FPS xenograft tumours appeared different to that of WT xenografts or FPS xenografts from mice injected with CXCL1 (R. Catalano, personal communication), indicating that alterations in vasculature were occurring which were not purely based on blood vessel number. Other aspects of blood vessel development therefore need to be more closely examined in this model to determine the effects of CXCL1 on angiogenesis.

This chapter provides evidence for a novel $PGF_{2\alpha}$ –FP pathway that can regulate the inflammatory microenvironment in endometrial adenocarcinoma via CXCL1-induced neutrophil chemotaxis. The presence of high numbers of other types of immune cells which do not express CXCR2 suggests other cytokines may play a role in their chemoattraction into the tumour. The role of these immune cells in endometrial adenocarcinoma is unclear, yet their incidence in endometrial tumours reflects the published literature on other cancer types describing the presence of both an infiltration of adaptive, cytotoxic immune cells and a higher infiltration of innate immune cells which can secrete factors influencing tumour progression.

Chapter 5

$PGF_{2\alpha}$-F-prostanoid receptor regulation of chemokine (C-C) motif ligand 20 in endometrial adenocarcinoma

5 $PGF_{2\alpha}$-F-prostanoid receptor regulation of chemokine (C-C) motif ligand 20 expression in endometrial adenocarcinoma

5.1 Introduction

As described in Chapter 3, chemokines are a group of small molecular weight proteins which are members of the cytokine family. They are characterised by their ability to induce chemotaxis and activation of haematopoietic cells at sites of inflammation. Novel actions of chemokines on other cell types including epithelial and endothelial cells have recently been discovered, including promotion of angiogenesis, invasion and proliferation (Slettenaar et al. 2006).

The chemokine (C-C motif) ligand 20 (CCL20; also known as macrophage inflammatory protein-3α) belongs to the CC-family of chemokines, so named for the position of two cysteine molecules at the amino terminal of the proteins. CCL20 binds to the CC-receptor 6 (CCR6), a G-protein coupled receptor that is expressed on dendritic cells, B and T lymphocytes, and tissues including spleen, lymph nodes, appendix and pancreas. Coupling of chemokines to receptors is generally promiscuous between members of the same family, however CCL20 is the only chemokine known to interact with the CCR6 receptor (Schutyser et al. 2003).

CCL20 signalling plays an important role in the chemoattraction of immature dendritic cells to sites of inflammation. Dendritic cells are the main antigen presenting cells of the immune system, which infiltrate tissues as immature cells. Upon the uptake of antigen and in response to inflammatory stimuli they differentiate into mature dendritic cells capable of activating lymphocytes (Schutyser et al. 2003). Upon differentiation, CCR6 expression on dendritic cells and responsiveness to CCL20 decreases (Dieu et al. 1998).

Like many other chemokines, the expression of CCL20 and CCR6 has also been described in several different types of cancer. CCL20 is over-expressed in cancers of the colon (Brand et al. 2006) liver (Rubie et al. 2006), pancreas (Campbell et al. 2005), prostate (Ghadjar et al. 2008) and Hodgkins lymphoma (Baumforth et al. 2008). CCR6 is also upregulated in colon (Brand et al. 2006), liver (Dellacasagrande et al. 2003) and prostate cancer (Ghadjar et al. 2008). Within these cancer types, CCL20-CCR6 signalling has been proposed to influence neoplastic epithelial cell migration or metastasis (Dellacasagrande et al. 2003; Campbell et al. 2005; Rubie et al. 2006) and proliferation (Brand et al. 2006; Beider et al. 2009). Additionally, the influx of immature dendritic cells mediated by CCL20 signalling has been implicated in the promotion of immune tolerance to cancer and therefore tumour growth (Bonnotte et al. 2004; Wang et al. 2008).

The expression of CCL20 has been described in the HHUA endometrial epithelial cell line and more recently primary endometrial epithelial cells (Ghosh et al. 2009), as well as primary endometrial stromal cells (Sun et al. 2002). Its expression in endometrial adenocarcinoma has been identified by a gene array comparing normal endometrium with adenocarcinoma samples (Wong et al. 2007); however protein localisation and physiological effects of CCL20 in endometrial cancer have not previously been examined.

Chapters 3 and 4 of this thesis described a cytokine pathway in endometrial adenocarcinoma promoted by $PGF_{2\alpha}$-FP signalling. A gene array carried out in our laboratory on FPS cells used as an endometrial adenocarcinoma cell model has identified the chemokine CCL20 as a target gene of $PGF_{2\alpha}$-FP signalling. This chapter therefore investigated the expression and localisation of CCL20 and CCR6 in endometrial adenocarcinoma. The role of $PGF_{2\alpha}$-FP signalling in the regulation of CCL20 and potential physiological effects of CCL20 expression in endometrial adenocarcinoma were also examined.

5.2 Materials and Methods

5.2.1 Tissue collection

Normal endometrium and endometrial adenocarcinoma tissue were collected as described in sections 2.1.1 and 2.1.2, for use in RT-PCR and immunohistochemical analysis. Normal endometrial tissue was collected from women undergoing surgery for minor gynaecological procedures with no underlying endometrial pathology and endometrial adenocarcinoma tissue was obtained from women undergoing hysterectomy after diagnosis of adenocarcinoma of the uterus. Stage of cycle and grade of cancer was assessed by a pathologist. Written informed consent was collected from all patients before surgery and ethical approval was obtained from Lothian Research Ethics Committee.

5.2.2 Cell Culture

Ishikawa FPS cells were maintained as described in section 2.2.1. Primary tissue explants were maintained as described in section 2.2.2.

5.2.2.1 Cell and tissue treatments

To determine the pattern of CCL20 expression in response to $PGF_{2\alpha}$, FPS cells were serum starved in media containing 3 µg/ml indomethacin for at least 18 hours prior to stimulation for 2, 4, 6, 8, 12, 18, 24, 48 and 72 hours with fresh serum-free media containing indomethacin and vehicle or 100 nM $PGF_{2\alpha}$ (n=5). Carcinoma tissue was serum starved in media containing 3 µg/ml indomethacin for at least 18 hours prior to treatment for 2, 4, 6, 8, and 24 hours with fresh serum-free media containing indomethacin and vehicle or 100 nM $PGF_{2\alpha}$ (n=8).

To determine signalling pathways leading to CCL20 expression regulated by $PGF_{2\alpha}$, FPS cells or carcinoma tissue were serum starved in media containing 3 µg/ml indomethacin for at least 18 hours and treated for 8 or 24 hours respectively with vehicle, inhibitor alone, 100 nM $PGF_{2\alpha}$ alone or 100 nM $PGF_{2\alpha}$ and a panel of chemical inhibitors indicated in the figure legends (n=7).

5.2.3 Polymerase chain reaction

5.2.3.1 RNA extraction and reverse transcriptase PCR

RNA from cells was extracted as described in section 2.4.1. Briefly, 1 ml TRI-reagent was added to experimental wells and RNA extracted using the phenol method.

RNA from tissue was extracted as described in section 2.4.1 using the Qiagen minikit protocol. Pieces of tissue, in 2 ml microcentrifuge tubes containing a stainless steel ball, were homogenised in 600 µl RLT lysis buffer using a tissue lyser (Qiagen, Crawley, West Sussex, UK) for 2 x 2 minutes at 25 Hz. RNA was then extracted following manufacturers guidelines (Qiagen). cDNA was prepared as described in section 2.4.2.

5.2.3.2 Taqman quantitative RT-PCR

CCL20 and CCR6 mRNA expression in cells and tissues was examined using Taqman quantitative PCR as described in section 2.4.3.2. Briefly, primer and probe sequences were designed to amplify CCL20 and CCR6 (sequences given in Table 2.3). A master mix was made up containing 18S primers and probes and 200ng cDNA added to each sample. Samples were added in duplicate to a 96 well MicroAmp fast optical reaction PCR plate and analysed using an ABI Prism 7900 HT Fast Real Time PCR machine. Expression of analyzed genes was normalised to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed as fold increase above vehicle treated cells or relative to a normal endometrium control.

5.2.4 Adenoviral and lentiviral constructs

5.2.4.1 FP adenovirus

A short hairpin adenovirus to knock down expression of FP was kindly prepared by Dr Pamela Brown (Biomolecular Core Facility, MRC Human Reproductive Sciences Unit, Edinburgh, UK). FP cDNA (ORIGENE, Rockville, MD) was excised with

EcoRI and SMA1 and fused to the pDC316 vector to create pDC316-FP. HEK-293 cells were seeded in 6-well plates in nutrient mixture F-12 with Glutamax-1 and pyridoxine supplemented with 10% FCS and 1% penicillin-streptomycin (500 IU/ml penicillin and 500 µg/ml streptomycin). Once at 50% confluency, cells were co-transfected with 0.75 µg pDC316-FP and 2.25 µg pBGH10 ΔE1,3 Cre adenoviral backbone in Optimem Reduced Serum Media (Invitrogen) using Trans-IT-293 transfection reagent (Mirus Bio Corp, Madison, WI) as per the manufacturer's instructions. Cells were incubated for approximately 2 weeks with regular changes of media until evidence of cytopathic effect (CPE) was seen, which was defined as a rounded appearance to cells and when approximately 70% of cells had lifted from the plate surface. Adenovirus was then harvested and virus released from the cells by 3 freeze-thaw cycles. Serial dilutions of the virus were performed and used to re-infect HEK-293 cells at 75-85% confluence. Wells were overlaid with 0.5% SeaPlaque Agarose (FMC Corp, Rockland, ME) to ensure homogenous plaques were obtained. Plaques were visible within 4-5 days and were picked using a P1000 tip. Plaques were freeze-thawed 3 further times and used to re-infect a further T75 flask which was incubated until CPE was observed. The virus from this flask was harvested and termed the first seed. The adenovirus was then expanded by inoculation of further flasks with the first seed. Adenovirus was purified and concentrated using the Vivapure AdenoPACK 100 purification kit (Sartorius AG, Goettingen, Germany) as per manufacturer's instructions. Virus titres were determined using the AdenoX Rapid titre kit (Clontech, France) as per manufacturer's instructions.

FPS cells were seeded in 6-well plates and incubated for 24 hours. Cells were then infected with 5 viruses / cell in complete medium. After 24 hour incubation, cells were starved in media containing 3 µg/ml indomethacin for 18 hours before treatment with 100 nM $PGF_{2\alpha}$ or vehicle for 8 hours (n=4).

5.2.4.2 RCAN1-4 adenovirus

An adenoviral construct was used to over-express RCAN-1-4, and was kindly prepared by Dr Pamela Brown. cDNA of RCAN-1-4 was prepared as previously

described (Maldonado-Perez et al. 2009) and fused to the pDC316 shuttle vector (Microbix Biopharmaceuticals, Toronto, Canada) to create pDC316-RCAN-1-4. The virus was then propagated and purified as described for the FP adenovirus in section 5.2.4.3. Efficacy of RCAN1-4 over-expression has been previously described (Maldonado-Perez et al. 2009).

FPS cells were seeded in 6-well plates and incubated for 24 hours. Cells were then infected with 5 viruses / cell in complete medium. After 24 hour incubation, cells were starved in media containing 3 µg/ml indomethacin for 18 hours before treatment with 100 nM $PGF_{2\alpha}$ or vehicle for 8 hours (n=4).

5.2.4.3 RCAN1-4 lentivirus

A short hairpin RNA lentivirus was used to knock down expression of RCAN-1-4 as previously described (Bush et al. 2007), and was the kind gift of Professor Aubrey Thompson (Mayo Clinic, Florida, USA). FPS cells were seeded in 6-well plates and incubated for 24 hours. Cells were then infected with 5 viruses / cell in complete medium. After 24 hour incubation, cells were starved in media containing 3 µg/ml indomethacin for 18 hours before treatment with 100 nM $PGF_{2\alpha}$ (n=4).

5.2.5 Immunohistochemistry

5.2.5.1 DAB immunohistochemistry

3,3- diaminobenzidine tetrahydrochlorine (DAB) immunohistochemistry to localise CCL20 and CCR6 was performed as described in section 2.5.2 (for immunolocalisation of CCL20: proliferative phase endometrium n=7, early secretory phase endometrium n=3, mid secretory phase endometrium n=4, late secretory phase endometrium n=5, well differentiated adenocarcinoma n=7, moderately differentiated adenocarcinoma n=5, poorly differentiated adenocarcinoma n=3. For immunolocalisation of CCR6: proliferative phase endometrium n=7, early secretory phase endometrium n=3, mid secretory phase endometrium n=4, late secretory phase endometrium n=5, well differentiated adenocarcinoma n=7, moderately

differentiated adenocarcinoma n=5, poorly differentiated adenocarcinoma n=3). Tissue sections (5 µm) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed, followed by blocking for endogenous peroxidise activity by washing sections in 3% hydrogen peroxidise in methanol. Sections were then blocked in normal serum of rabbit followed by overnight incubation with primary antibody at the concentrations indicated in Table 2.4. Control sections were included with non-immune IgG. Sections were then incubated in rabbit anti-goat biotinylated secondary antibody and subsequently streptavidin-HRP. Sections were then incubated with DAB for 1-5 minutes until positive staining was identified by a brown colour under x10 magnification.

5.2.5.2 Dual immunofluorescence microscopy

To colocalise CCR6 with CD11c, sections (5 µm; well differentiated adenocarcinoma n=3, moderately differentiated adenocarcinoma n=3, poorly differentiated adenocarcinoma n=3) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed and sections were blocked in 5% normal rabbit serum diluted in PBS with 5% BSA. Sections were then incubated with goat anti-CCR6 (1:50) overnight at 4ºC. Rabbit anti-goat biotinylated antibody was then added, diluted in PBS at 1:200 for 30 minutes. Streptavidin AlexaFluor 546 (Molecular Probes) diluted 1:200 in PBS was then added for 30 minutes at room temperature. Sections were then re-blocked in normal goat serum and rabbit anti-CD11c (1:75) overnight at 4°C. Tissues were then incubated with a fluorophore-conjugated antibody diluted 1:200 in PBS (AlexaFluor 488, Molecular Probes, UK) for 1 hour. Nuclei were counterstained in To Pro (Molecular Probes, UK) and then coverslipped and visualised as described in Section 2.5.3 and 2.5.4

5.2.6 CCL20 ELISA

CCL20 protein released into the culture media from treatments described in section 5.2.2.1 was assayed by Human CCL20/MIP-3 alpha DuoSet ELISA (R& D Systems, Abingdon, UK) as described in section 2.6.2.

5.2.7 Chemotaxis assay

FPS cells were serum starved for at least 18 hours prior to harvesting with HyQtase cell detachment solution (Thermo Scientific, Leicestershire, UK). Cells were then seeded (2.5×10^4) in 96-well Transwell inserts (8 µm pore, Corning Costar, Leicestershire, UK). A standard curve of cells was seeded to act as an internal control for random chemotaxis. Either serum free media, complete media containing fetal calf serum (FCS), or serum free media with recombinant CCL20 at concentrations of 10, 100, 200 and 300 ng / ml was added to the bottom chamber. Cells were cultured for 24 hours. Chemotaxis was then assayed by dissociating cells from the underside of the transwell insert using enzyme-free cell dissociation solution (Millipore, Herts, UK) with the addition of calcein acetoxymethylester (AM; BD Biosciences, Oxford, UK). Calcein AM is a non-fluorescent cell permeable compound which is metabolised into the fluoresecent anion calcein in viable cells, and thus can be used for the detection of cells in chemotaxis assays. Fluorescence was subsequently measured on a FLUOstar OPTIMA fluorescent plate reader (BMG Labtech, Aylesbury, UK) at 485 and 520 nm. Data are expressed as fold increase in chemotaxis above vehicle treated control (n=4).

5.2.8 Proliferation assay

FPS cells were seeded in complete medium in 96 well plates at 5×10^3 cells per well. Cells were serum starved for at least 18 hours prior to stimulation for 24 hours with fresh media containing 1% serum and vehicle or recombinant CCL20 at a concentration of 10, 100, 200 or 300 ng/ml.

Viable cells were then measured 24 hours later by CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Southampton, UK). This assay involves

the addition of the tertrazolium compound MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and an electron coupling agent, PES (phenazine ethosulfate). When added to cells, MTS is bioreduced in the mitochondria of cells to form a soluble coloured formazan product. The quantity of formazan product can be measured optically at 490 nm and is directly proportional to the number of viable cells in culture, and therefore is a measure of proliferation. Results are expressed as fold difference in proliferation of cells treated with CCL20 as compared to vehicle treated cells (n=4).

5.2.9 Statistical analysis

Where appropriate, data were analysed by Students t-test and one way ANOVA using GraphPad Prism (GraphPad Software. San Diego, California, USA). Data are presented as mean ± SEM.

5.3 Results

5.3.1 Expression of CCL20 mRNA in normal endometrium across the menstrual cycle and endometrial adenocarcinoma

The up-regulation of CCL20 mRNA expression induced by $PGF_{2\alpha}$ treatment was demonstrated in a gene array study carried out in our laboratory (unpublished results). CCL20 mRNA expression was then examined in normal endometrium (n=29) and endometrial adenocarcinoma tissue (n=29) by quantitative PCR (Figure 5.1). In normal endometrial tissue, CCL20 expression was significantly increased in the mid-late secretory phases as compared to early secretory and proliferative phase endometrium ($p<0.05$; Figure 5.1A). There was no significant difference in CCL20 mRNA expression between different grades of cancer studied, hence data were pooled. CCL20 mRNA expression was significantly increased in endometrial adenocarcinoma by 45.4 ± 11.9 fold as compared to normal endometrium ($p<0.01$; Figure 5.1C).

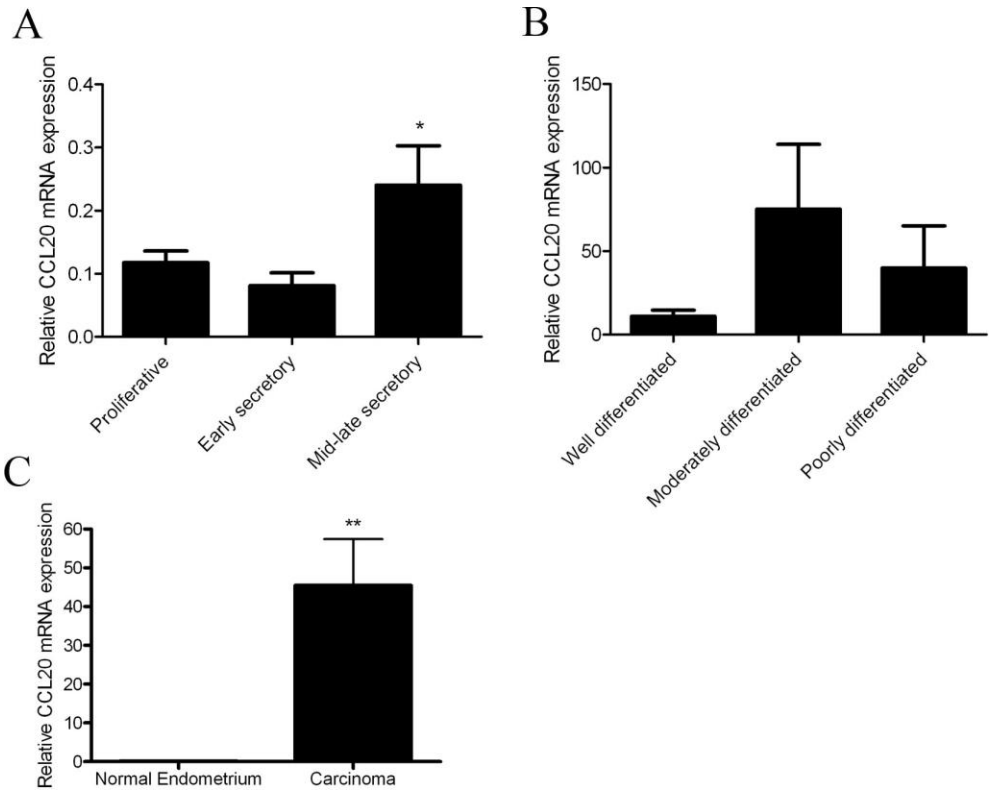

Figure 5.1 Expression of CCL20 mRNA in normal endometrium and endometrial adenocarcinoma. A: CCL20 mRNA was examined by quantitative RT-PCR in proliferative (n=9), early secretory (n=10) and mid-late secretory (n=10) phase endometrium. Expression was significantly increased in the mid-late secretory phase as compared to other stages of the cycle. **B:** CCL20 mRNA expression was examined by quantitative RT-PCR in well (n=9), moderately (n=8) and poorly (n=10) differentiated cancers. No significant difference was demonstrated. **C:** CCL20 mRNA expression was examined by quantitative RT-PCR and found to be significantly increased in pooled endometrial adenocarcinoma tissue as compared to pooled normal endometrial tissue from all stages of the cycle. *denotes $p<0.05$, ** denotes $p<0.01$, data are expressed relative to a normal endometrium control and are presented as mean ± SEM.

5.3.2 Expression of CCR6 mRNA in normal endometrium and endometrial adenocarcinoma

The expression of the receptor for CCL20, CCR6, was then examined in normal endometrium (n=30) and endometrial adenocarcinoma (n=28) by quantitative RT-PCR (Figure 5.2). CCR6 mRNA expression did not significantly vary throughout the

menstrual cycle (Figure 5.2A) or between different grades of cancer (Figure 5.2B). Data were therefore pooled, and CCR6 mRNA expression was found to be significantly increased by 3.4 ± 1.3 fold in cancer as compared to normal endometrium ($p<0.01$, Figure 5.2C).

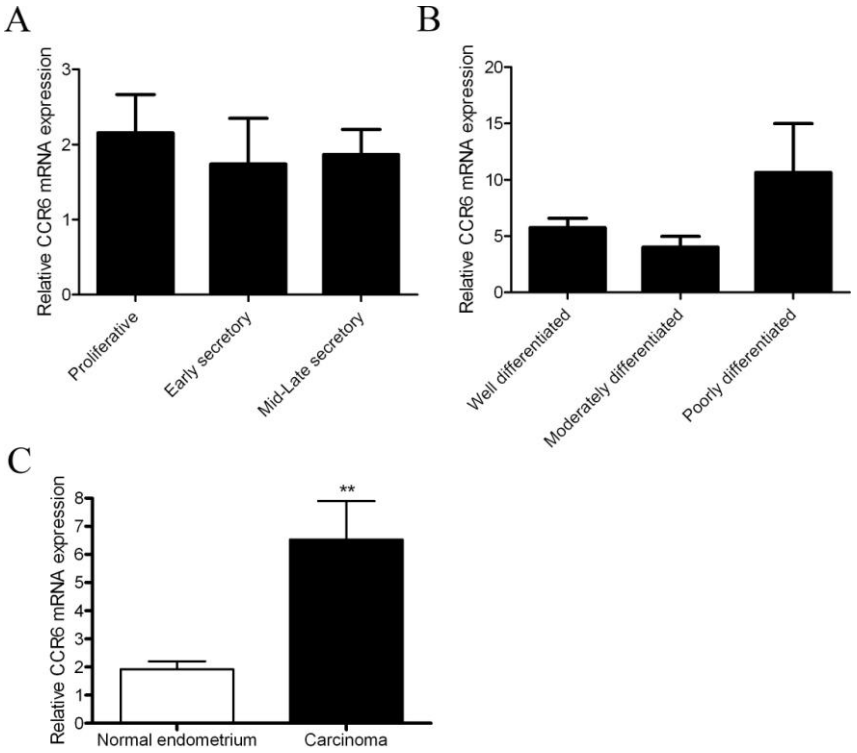

Figure 5.2 Expression of CCR6 mRNA in normal endometrium and endometrial adenocarcinoma. A: CCR6 mRNA was examined by quantitative RT-PCR in proliferative (n=10), early secretory (n=10), and mid to late secretory (n=10) phase endometrium. There was no difference in levels of CCR6 expression at different stages of the menstrual cycle. **B:** CCR6 mRNA was examined by quantitative RT-PCR in well (n=10), moderately (n=10) and poorly (n=8) differentiated cancers. No significant difference was demonstrated between different grades of cancer. **C:** CCR6 mRNA expression was examined by quantitative RT-PCR in pooled normal endometrial tissue from all stages of the cycle and pooled endometrial adenocarcinoma tissue, and demonstrated to be significantly increased in carcinoma tissue. ** denotes $p<0.01$, data are expressed relative to a normal endometrium control and are presented as mean ± SEM.

5.3.3 Expression and localisation of CCL20 in normal endometrium and endometrial adenocarcinoma

As mRNA expression of CCL20 had been demonstrated in endometrial tissue, the protein expression was then examined by immunohistochemistry in normal endometrial tissue from all stages of the cycle (proliferative phase endometrium n=7, early secretory phase endometrium n=3, mid secretory phase endometrium n=4, late secretory phase endometrium n=5). There was little to no immunoreactivity observed in endometrium from the proliferative phase of the menstrual cycle, with some staining seen in stromal cells (S; Figure 5.3A). In early secretory phase endometrium, positive CCL20 staining was observed in the cytoplasm of glandular epithelial cells (GE; Figure 5.3B). This glandular epithelial staining was also observed in mid and late secretory phase endometrium (Figure 5.3C, D). Negative controls were incubated with non-immune IgG in place of primary antibody and displayed no immunoreactivity (inset; Figure 5.3D).

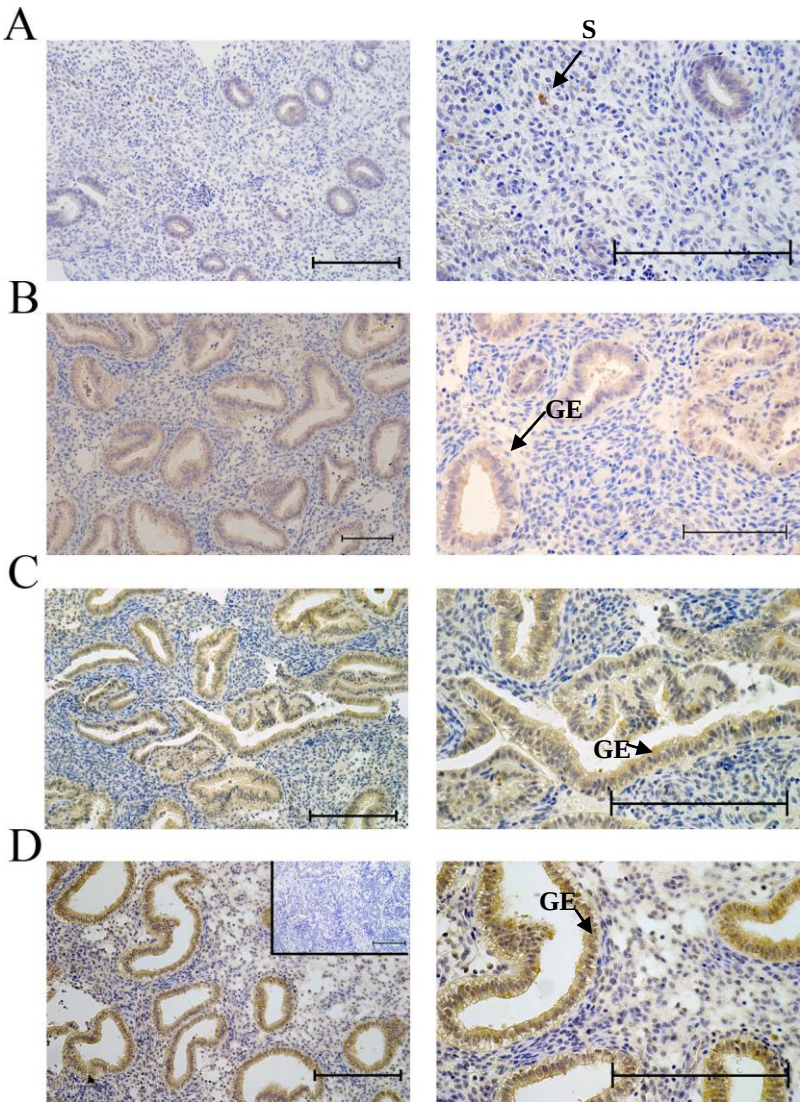

Figure 5.3 Localisation of CCL20 protein in normal endometrium by immunohistochemistry. Representative images demonstrating that **A:** CCL20 localises to the stroma in proliferative phase endometrium (S; left image is x200 magnification, right image is x400). **B:** In early secretory phase endometrium, weak CCL20 immunoreactivity can be seen in the glandular epithelium (GE; left image is x200 magnification, right image is x400). **C:** In mid secretory phase endometrium, CCL20 can be localised to glandular epithelium (left image is x200 magnification, right image is x400). **D:** In late secretory phase endometrium, CCL20 can be localised to glandular epithelium (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune goat IgG in place of primary antibody. All scale bars represent 100 µm.

CCL20 expression was then examined in all grades of endometrial adenocarcinoma by immunohistochemistry (well differentiated n=7, moderately differentiated n=5, poorly differentiated n=3). Staining of neoplastic epithelial cells (E) was observed in all grades of cancer (Figure 5.4A-C). Stromal cells (S) were also observed displaying immunoreactivity in all grades of cancer, and this is demonstrated in Figure 5.4B. Very weak staining of some vascular endothelial cells was observed, as demonstrated in Figure 5.4C (VE). Negative controls were incubated with primary antibody pre-absorbed with blocking peptide (inset; Figure 5.4A).

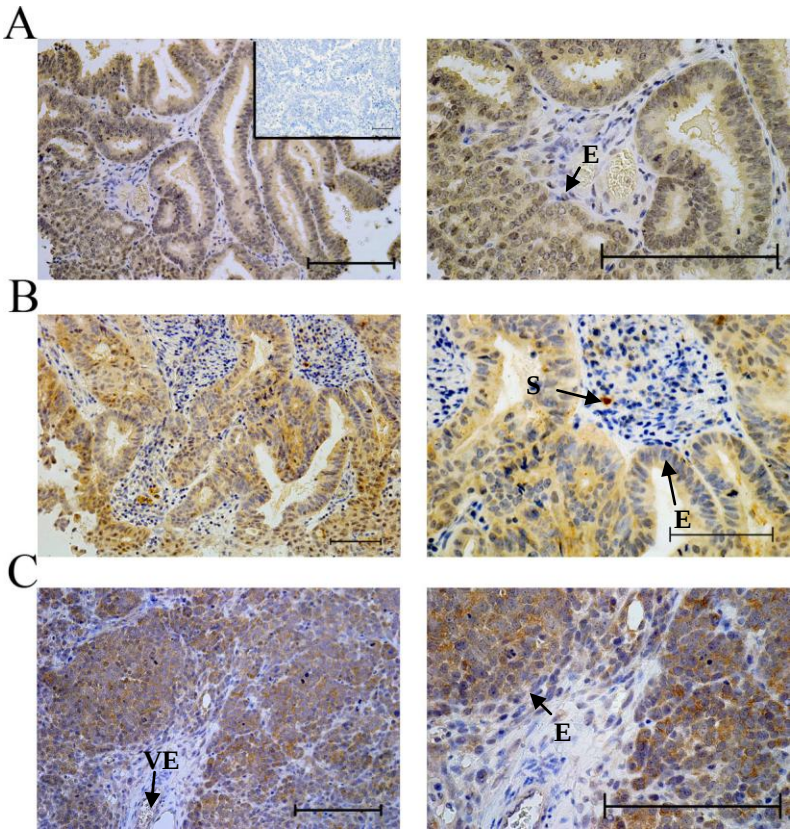

Figure 5.4 Localisation of CCL20 protein in endometrial adenocarcinoma by immunohistochemistry. Representative images demonstrating that CCL20 localises to the epithelium (E) and stroma (S) in **A:** Well differentiated carcinoma (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with primary antibody pre-absorbed with blocking peptide. **B:** Moderately differentiated carcinoma (left image is x200 magnification, right image is x400). **C:** Poorly differentiated carcinoma (left image is x200 magnification, right image is x400). All scale bars represent 100 µm.

5.3.4 Expression and localisation of CCR6 in normal endometrium and endometrial adenocarcinoma

Expression of mRNA of the receptor for CCL20, CCR6, was also demonstrated in normal endometrium and endometrial adenocarcinoma (Figure 5.2). The expression and localisation of the protein was then examined by immunohistochemistry in normal endometrium (proliferative phase endometrium n=7, early secretory phase endometrium n=3, mid secretory phase endometrium n=4, late secretory phase endometrium n=5). Little or no CCR6 immunoreactivity could be seen in the glandular epithelial cells of normal endometrium at all stages of the cycle (Figure 5.5A-C). CCR6 expression was observed in rare cells dispersed throughout the stroma in all stages of the cycle, demonstrated here in late secretory phase endometrium (Figure 5.5C). CCR6 expression was not demonstrated on vascular endothelium, demonstrated here in proliferative phase endometrium (VE; Figure 5.2A). Negative control is incubated with non-immune goat IgG in place of primary antibody (inset, Figure 5.5A).

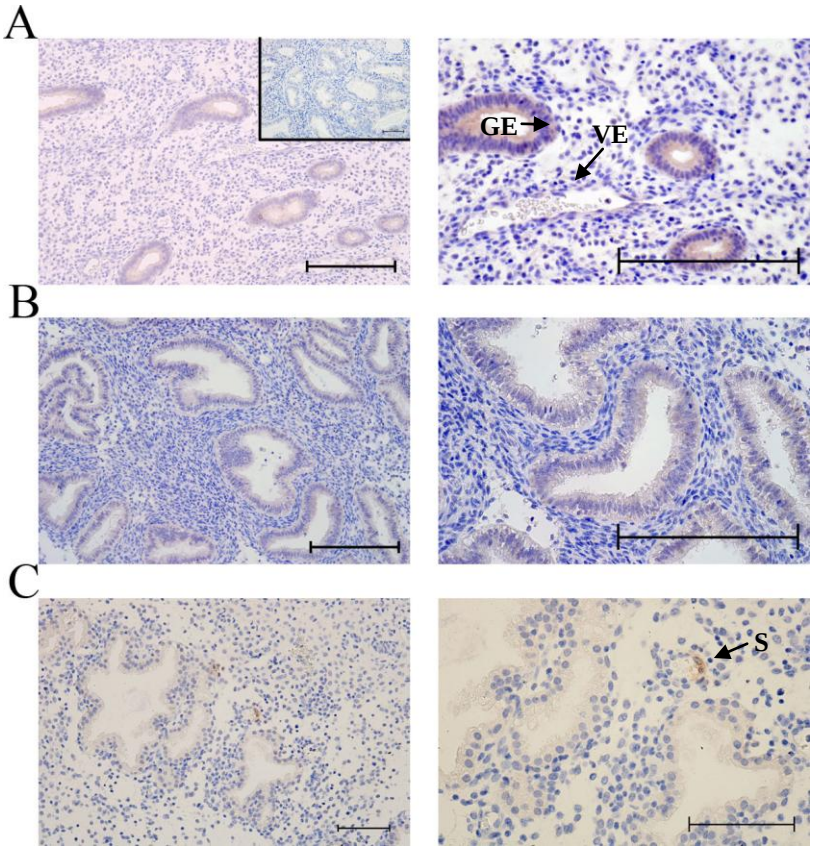

Figure 5.5 Localisation of CCR6 protein in normal endometrium by immunohistochemistry. Representative images demonstrating **A:** Weak immunoreactivity to CCR6 in the glandular epithelium of proliferative phase endometrium (GE; left image is x200 magnification, right image is x400). **B:** In early secretory phase endometrium, no CCR6 immunoreactivity can be seen (left image is x200 magnification, right image is x400). **C:** In late secretory phase endometrium, CCR6 can be localised to some stromal cells (S;left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune goat IgG in place of primary antibody. All scale bars represent 100 µm.

The expression of CCR6 in endometrial adenocarcinoma was then examined by immunohistochemistry (well differentiated n=7, moderately differentiated n=5, poorly differentiated n=3). Expression of CCR6 in neoplastic epithelial cells was observed in every grade of cancer examined (Figure 5.6A-C). No expression was seen in vascular endothelial cells in endometrial adenocarcinoma, as demonstrated in Figure 5.6B and C (VE). Stromal expression was also observed in rare cells in all grades of cancer examined, demonstrated here in moderately differentiated

carcinoma (S; Figure 5.6B). Negative control is incubated with non-immune goat IgG in place of primary antibody (inset, Figure 5.6A).

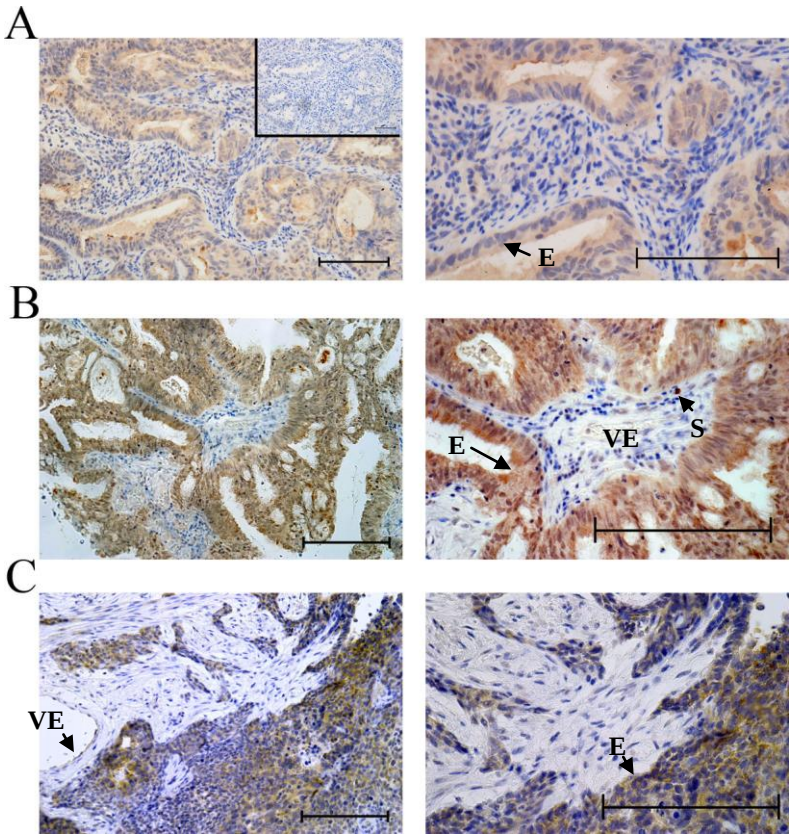

Figure 5.6 Localisation of CCR6 protein in endometrial adenocarcinoma by immunohistochemistry. Representative images demonstrating that CCR6 localises to the epithelium (E) and stroma (S) but not vascular endothelium (VE) in **A:** Well differentiated carcinoma (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune goat IgG in place of primary antibody. **B:** Moderately differentiated carcinoma (left image is x200 magnification, right image is x400). **C:** Poorly differentiated carcinoma (left image is x200 magnification, right image is x400). All scale bars represent 100 µm.

CCR6 is expressed on dendritic cells (Murdoch et al. 2008). To examine if CCR6 positive cells throughout the stroma were dendritic cells, dual immunofluorescence microscopy with the dendritic cell marker CD11c was carried out (well differentiated

n=3, moderately differentiated n=3, poorly differentiated n=3). Expression of CD11c (Figure 5.7A) was observed in the stroma alongside expression of CCR6-positive neoplastic epithelial cells in well differentiated adenocarcinoma (Figure 5.7B), however no colocalisation of CCR6 and CD11c was observed in all grades of endometrial adenocarcinoma examined (Figure 5.7C). Negative control (inset) was incubated with non-immune IgG in place of primary antibody.

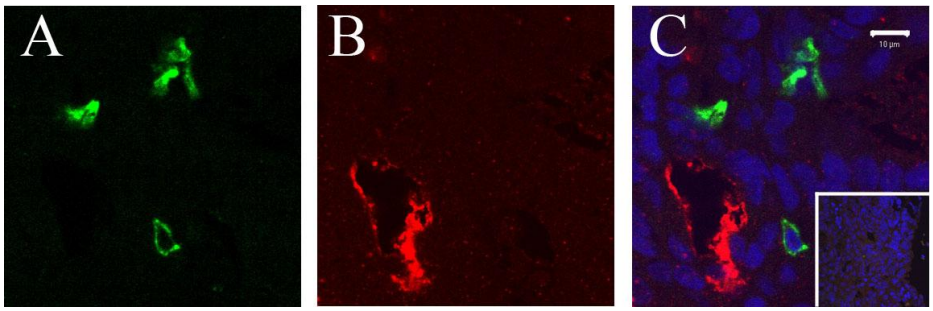

Figure 5.7 Expression of CD11c and CCR6 in endometrial adenocarcinoma by dual immunofluorescence microscopy. CD11c, a dendritic cell marker, **(A)** and CCR6 **(B)** were expressed in endometrial adenocarcinoma but did not colocalise **(C)**. Negative control (inset) was incubated with non-immune IgG in place of primary antibody, scale bar represents 10 µm.

5.3.5 Localisation of CCL20 and CCR6 to the same cells as the FP receptor in endometrial adenocarcinoma

To further support a role for FP in the regulation of CCL20, immunohistochemistry for both proteins was carried out on serial sections of endometrial adenocarcinoma (well differentiated n=2, moderately differentiated n=2, poorly differentiated n=2). Immunohistochemistry for the receptor CCR6 was also carried out on serial sections. In all grades of cancer, FP could be localised to a number of the same epithelial cells as CCL20 and CCR6. Shown are representative images from a moderately differentiated cancer (Figure 5.8). Localisation of FP, CCL20 and CCR6 can be demonstrated in the same epithelial cells and is indicated by arrowheads. FP negative controls were incubated with non-immune IgG, CCL20 and CCR6 negative controls

incubated with blocking peptide and non-immune IgG are shown in Figures 5.4 and 5.6 respectively.

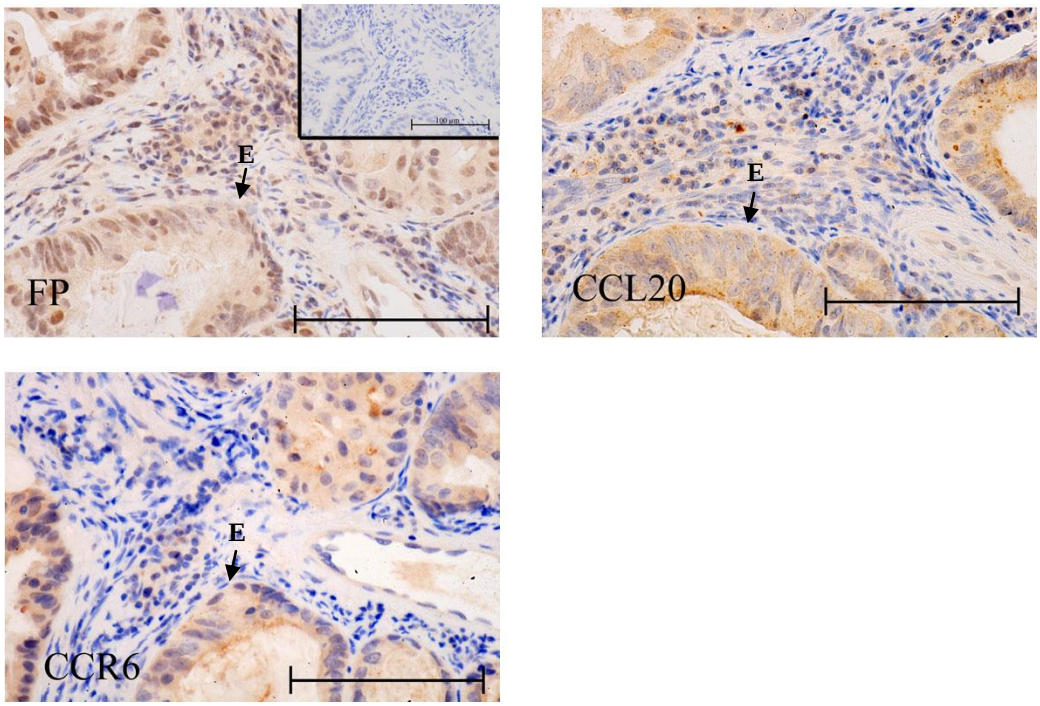

Figure 5.8 CCL20 and CCR6 can be localised to the same cells as the FP receptor by immunohistochemistry of serial sections. Representative images demonstrating that FP, CCL20 and CCR6 can be colocalised to neoplastic epithelium (E; inicated by arrowheads) in moderately differentiated endometrial adenocarcinoma (x 200 magnification. All scale bars represent 100 µm.

5.3.6 CCL20 mRNA expression is regulated by $PGF_{2\alpha}$ in Ishikawa FPS cells

As the regulation of CCL20 by $PGF_{2\alpha}$ had been suggested by gene array, the temporal regulation of CCL20 in FPS cells was investigated by quantitative RT-PCR and ELISA. Cells were treated for 2-72 hours with vehicle or $PGF_{2\alpha}$ (Figure 5.9). After 12 hours of $PGF_{2\alpha}$ treatment, CCL20 mRNA was significantly elevated 609.1 ± 94.7 fold as compared to vehicle treated cells, and was then decreased until 72 hours ($p<0.01$; Figure 5.9A). CCL20 protein was then measured in medium of FPS cells treated with either 100 nM $PGF_{2\alpha}$ or vehicle for 24-72 hours. CCL20 protein was

present in the media from 24 hours of $PGF_{2\alpha}$ treatment, and was significantly increased compared to vehicle treated cells by 55.7 ± 14.6 fold at 48 hours, and remained significantly elevated 93.3 ± 16.4 fold above vehicle treated control at 72 hours ($p<0.01$, Figure 5.9B).

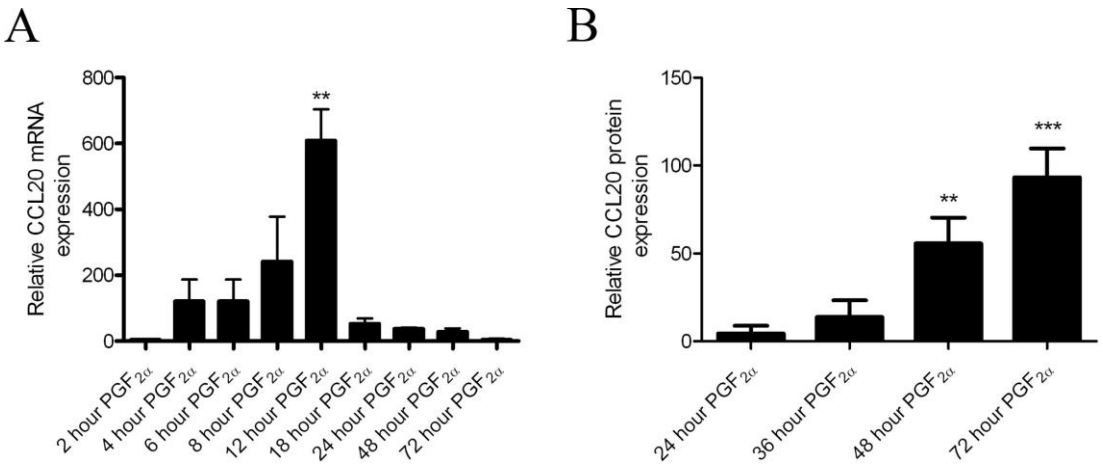

Figure 5.9 Regulation of CCL20 by $PGF_{2\alpha}$ in FPS cells. A: CCL20 mRNA expression in FPS cells was measured by quantitative RT-PCR after stimulation with vehicle or 100 nM $PGF_{2\alpha}$ over a period of 2 to 72 hours. Data are expressed as fold increase over vehicle treated control. **B:** CCL20 protein secreted into media by FPS cells after stimulation with vehicle or 100 nM $PGF_{2\alpha}$ over a period of 24 to 72 hours was measured by ELISA. CCL20 protein production was significantly elevated above vehicle treated control cells after treatment with $PGF_{2\alpha}$ for 48 hours. Data are expressed as fold increase over vehicle treated control cells. All data are presented as mean ± SEM. ** denotes $p< 0.01$, *** denotes $p<0.001$.

5.3.7 CCL20 mRNA expression is regulated by $PGF_{2\alpha}$ in endometrial adenocarcinoma explants

To confirm that $PGF_{2\alpha}$ also regulates CCL20 expression in carcinoma tissue, human endometrial adenocarcinoma explants were used (n=5). Tissue was treated for 2, 4, 6, 8, and 24 hours with vehicle or 100 nM $PGF_{2\alpha}$ and CCL20 mRNA expression measured by quantitative RT-PCR. After 24 hours $PGF_{2\alpha}$ treatment, CCL20 mRNA expression was significantly increased in carcinoma explants 2.5 ± 0.8 fold as compared to vehicle treated control explants (Figure 5.10).

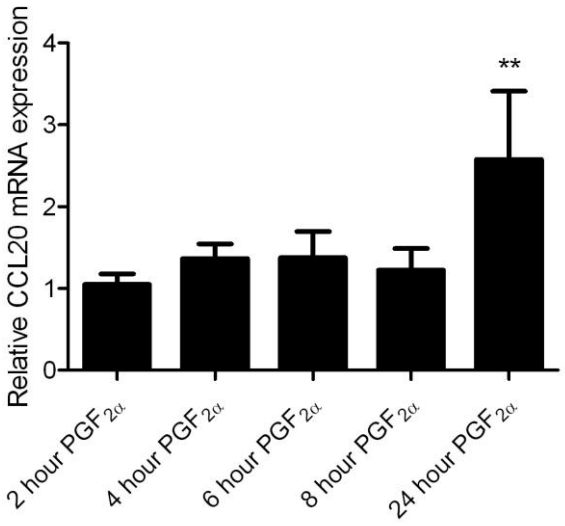

Figure 5.10 $PGF_{2\alpha}$ regulates CCL20 expression in endometrial adenocarcinoma explants. Endometrial adenocarcinoma explants (n=5) were treated for 2 - 24 hours with vehicle or 100 nM $PGF_{2\alpha}$ and CCL20 mRNA was measured by quantitative RT-PCR. CCL20 mRNA was significantly increased after 24 hours of $PGF_{2\alpha}$ treatment. Data are expressed as fold over vehicle treated control cells and are presented as mean ± SEM. ** denotes $p<0.01$.

5.3.8 Intracellular pathways mediating $PGF_{2\alpha}$ regulation of CCL20 expression in FPS cells

In chapter 3, the activation of intracellular signalling pathways by the FP receptor was discussed. The same panel of chemical inhibitors was used to examine signalling pathways regulating CCL20 production. FPS cells were treated with vehicle, 100 nM $PGF_{2\alpha}$ alone or 100 nM $PGF_{2\alpha}$ and a panel of chemical inhibitors (n=7; Figure 5.11). Treatment of FPS cells with $PGF_{2\alpha}$ for 8 hours induced a 341.2 ± 75.2 fold increase in CCL20 mRNA compared to vehicle treated cells. This increase in mRNA expression was abolished by treatment of cells with a selective inhibitor of G_q (YM254890; $p<0.01$) and significantly inhibited with the FP receptor antagonist AL8810 ($p<0.05$) and inhibitors of epidermal growth factor receptor (EGFR, AG1478; $p<0.01$), mitogen activated protein kinase kinase (MEK; PD98059, $p<0.05$), calcineurin (CsA; $p<0.05$) and calcium (EGTA, $p<0.05$). An inhibitor of

protein kinase A (PKA; 43CMQ) did not significantly inhibit $PGF_{2\alpha}$ induced CCL20 mRNA expression.

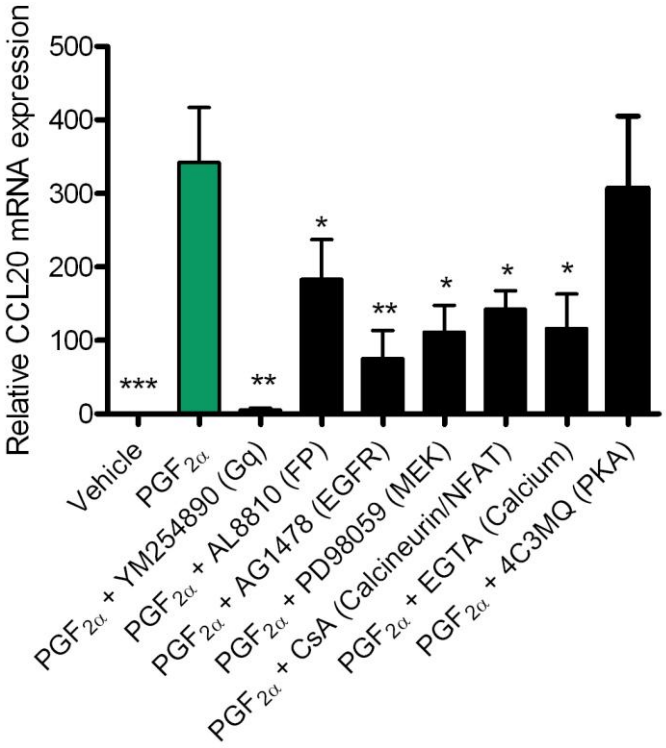

Figure 5.11 $PGF_{2\alpha}$ regulated CCL20 expression is decreased by selected chemical inhibitors. FPS cells were treated for 8 hours in the presence or absence of vehicle or 100 nM $PGF_{2\alpha}$ and chemical inhibitors of G_q, FP, EGFR, MEK, calcineurin, calcium and PKA (n=7). $PGF_{2\alpha}$ regulated CCL20 mRNA expression was examined by quantitative RT-PCR and found to be significantly inhibited by inhibitors of G_q, FP, EGFR, MEK, calcineurin and calcium. Data are expressed as fold over vehicle treated control. * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$.

Adenovirus-mediated delivery of short hairpin mRNA directed against the FP receptor (sh FP) or a scrambled sequence as a control (sh scrambled) was used to confirm the role of the FP receptor in CCL20 regulation by $PGF_{2\alpha}$ (n=4). Twenty-four hours after infection, FPS cells were treated for 8 hours with 100 nM $PGF_{2\alpha}$ or vehicle. Relative CCL20 mRNA expression with the addition of scrambled shRNA was increased 207.3 ± 131.3 fold over vehicle treated cells. This increase was

reduced with the addition of shRNA targeted against the FP receptor to 41 ± 16.4 fold; however this reduction did not reach statistical significance.

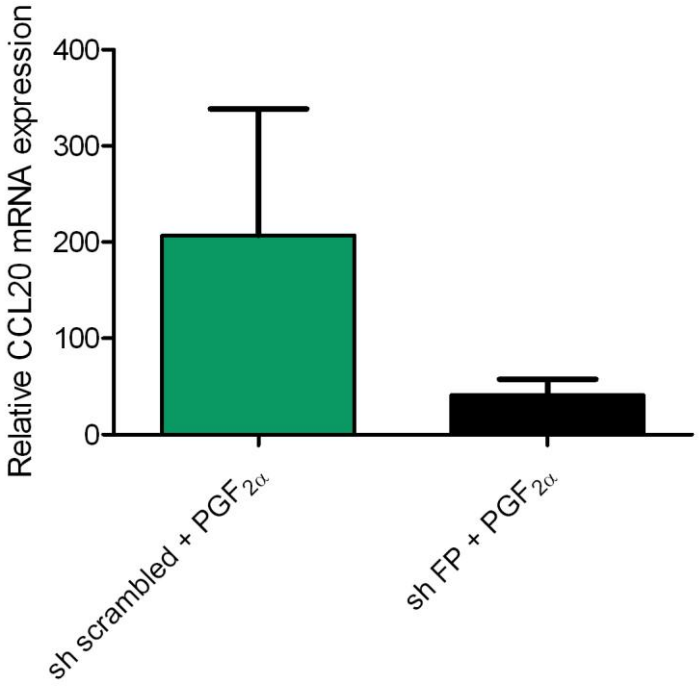

Figure 5.12 $PGF_{2\alpha}$ regulated CCL20 mRNA expression is decreased with shRNA directed against the FP receptor. FPS cells were infected with adenovirus containing scrambled sequence shRNA (sh scrambled) or shRNA directed against the FP receptor (shFP) and treated for 8 hours with vehicle or 100 nM $PGF_{2\alpha}$ (n=4). CCL20 expression was decreased in cells infected with shRNA directed against the FP receptor as compared to scrambled sequence shRNA. Data are expressed as fold over vehicle treated control and are presented as mean ± SEM.

To further confirm the involvement of components of the NFAT signalling pathway in the regulation of CCL20, the role of the protein regulator of calcineurin1-4 (RCAN1-4) was examined. RCAN1-4 binds to calcineurin to inhibit downstream NFAT activity (Maldonado-Perez et al. 2009). RCAN1-4 was overexpressed in FPS cells by adenoviral delivery, and 24 hours after infection, FPS cells were treated for 8 hours with vehicle or $PGF_{2\alpha}$ (n=4). A significant reduction in $PGF_{2\alpha}$-induced CCL20 mRNA expression was seen in cells over-expressing RCAN1-4, as compared to FPS

cells infected with a control adenovirus ($p<0.05$, Figure 5.13A). In order to further demonstrate the role of this signalling pathway in CCL20 production, a shRNA lentiviral construct targeting RCAN1-4 was transfected into FPS cells (n=4). Twenty-four hours after infection, FPS cells were treated with $PGF_{2\alpha}$ for 8 hours, and inhibition of RCAN1-4 caused a significant increase in CCL20 mRNA expression as compared to cells transfected with the control lentivirus ($p<0.05$, Figure 5.13B).

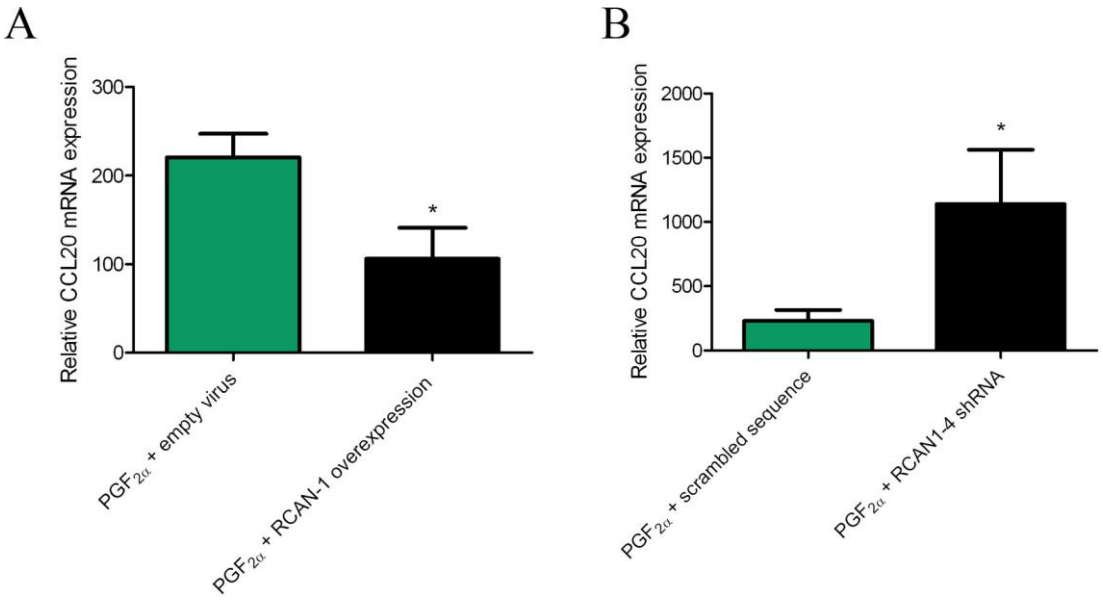

Figure 5.13 RCAN1-4 is involved in the signalling pathway mediating $PGF_{2\alpha}$ regulation of CCL20. A: FPS cells were infected with control empty adenovirus or RCAN1-4 adenovirus and treated for 8 hours with vehicle or $PGF_{2\alpha}$ (n=4). CCL20 expression was significantly decreased in cells treated with the RCAN1-4 adenovirus. **B:** FPS cells were treated for 8 hours with vehicle or $PGF_{2\alpha}$ and lentivirus containing scrambled sequence shRNA or shRNA against RCAN1-4 (n=4). CCL20 expression was significantly increased in cells infected with RCAN1-4 shRNA as compared to scambled sequence shRNA. Data are expressed as fold over vehicle treated control and are presented as mean ± SEM, * denotes $p<0.05$.

5.3.9 Expression of CCR6 in FPS cells

CCR6 expression was detected in endometrial adenocarcinoma epithelial cells by immunohistochemistry (Figure 5.6). As autocrine signalling was therefore possible,

CCR6 expression in FPS cells was then examined by quantitative RT-PCR. CCR6 mRNA expression was detected in FPS cells, however at significantly lower levels than in cancer tissue ($p<0.001$, Figure 5.14).

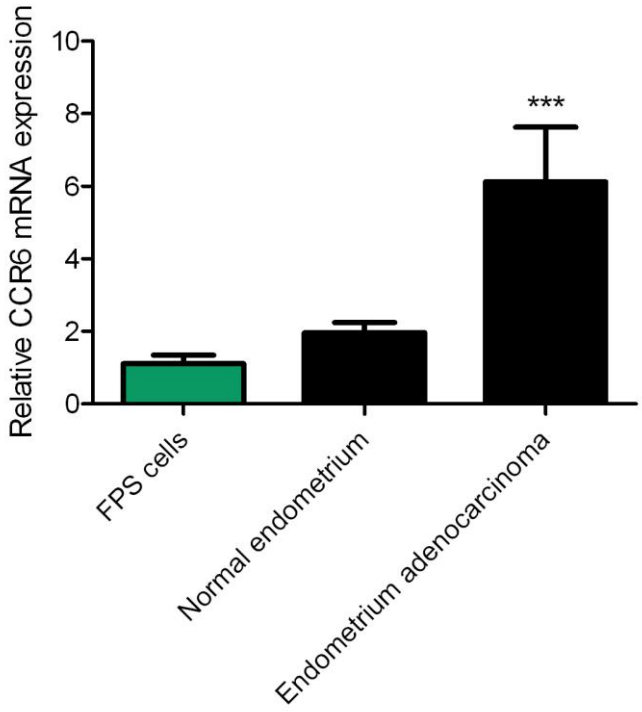

Figure 5.14 Expression of CCR6 mRNA in FPS cells. FPS cells, normal endometrium and endometrial adenocarcinoma tissue was examined by quantitative RT-PCR for CCR6 mRNA expression. CCR6 mRNA expression was significantly higher in endometrial adenocarcinoma tissue. Data are expressed as mean ± SEM, *** denotes $p<0.001$.

5.3.10 CCL20 does not significantly affect chemotaxis of FPS cells

CCL20 promotes cell migration in several cancer types (Kimsey et al. 2004; Brand et al. 2006; Rubie et al. 2006). To examine the physiological effects of CCL20 in endometrial adenocarcinoma, chemotaxis of FPS cells towards recombinant CCL20 was assessed by transwell assay after 24 hours (n=4). The chemotaxis of FPS cells towards the positive control of complete medium containing fetal calf serum was significantly increased as compared to a negative control of serum-free medium

(FCS, $p<0.05$; Figure 5.15). However the addition of recombinant CCL20 from concentrations of 10 ng/ml to 300 ng/ml did not significantly affect FPS cell chemotaxis.

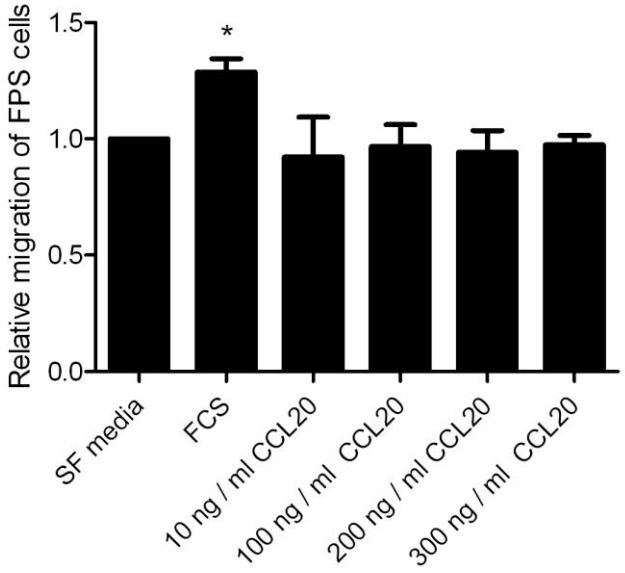

Figure 5.15 CCL20 does not increase migration of FPS cells. Chemotaxis of FPS cells towards recombinant CCL20 after 24 hours was assessed by transwell assay (n=4). Chemotaxis towards complete media containing fetal calf serum (FCS) was significantly increased as compared to serum free (SF) medium. No significant chemotaxis toward recombinant CCL20 was observed. *denotes $p<0.05$.

5.3.11 CCL20 promotes proliferation of FPS cells

CCL20 has also been demonstrated to promote proliferation of colorectal cancer cells (Brand et al. 2006) and leukaemia cells (Beider et al. 2009). To examine if similar events are occurring in endometrial adenocarcinoma, proliferation of FPS cells was examined after treatment with vehicle or recombinant CCL20 (n=5). FPS cells were treated in 1% serum media with vehicle or CCL20 at doses between 10 – 300 ng / ml and proliferation examined by Cell Titer One Solution Assay (Promega). CCL20 treatment resulted in significant increases in proliferation of 1.39 ± 0.07 fold at 10 ng / ml ($p<0.01$), 1.45 ± 0.07 fold at 100 ng / ml ($p<0.01$), 1.40 ± 0.08 fold at 200 ng / ml ($p<0.01$) and 1.36 ± 0.1 fold at 300 ng / ml ($p<0.05$; Figure 5.16).

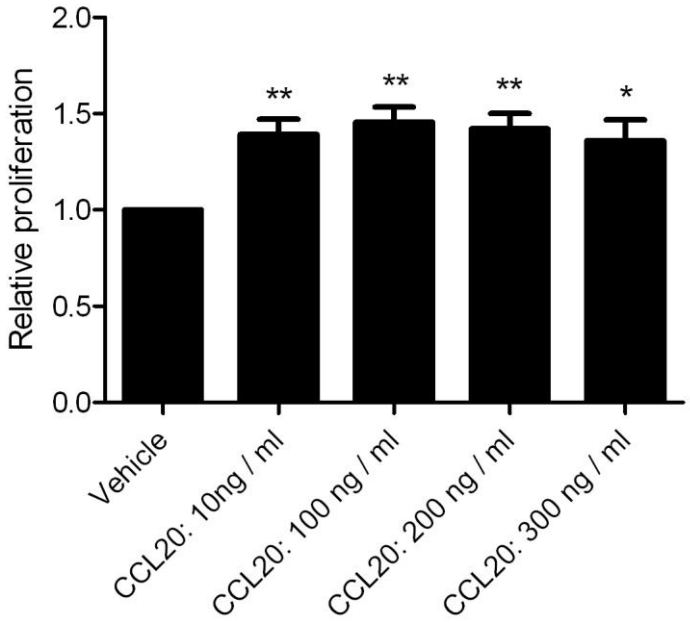

Figure 5.16 CCL20 induces FPS cell proliferation. FPS cells were treated with recombinant CCL20 protein at 10, 100, 200 and 300 ng / ml and proliferation measured using Cell Titer One Solution assay (n=5). FPS cell proliferation was significantly increased with the addition of CCL20 as compared to vehicle treated cells. Data are expressed as mean ± SEM, * denotes $p<0.05$, ** denotes $p<0.01$.

5.4 Discussion

A gene array carried out by our laboratory first identified CCL20 as a target of $PGF_{2\alpha}$-FP signalling (unpublished observations). This chapter confirmed the regulation of CCL20 by $PGF_{2\alpha}$ in FPS cells and endometrial adenocarcinoma tissue explants, and the signalling pathways involved in its production. The localisation of CCL20 protein in endometrial tissue was described, and a role for CCL20-CCR6 signalling in the promotion of epithelial cell proliferation was identified.

The expression of CCL20 mRNA in endometrial tissue was significantly increased in the mid-late secretory phase of the menstrual cycle. A number of cytokines are upregulated at this stage of the cycle in preparation for implantation in order to facilitate complex interactions between the implanting embryo, uterus and other cells

including immune cells, and CCL20 may play a role in one of these processes (Guzeloglu-Kayisli et al. 2009). The expression of CCL20 in glandular epithelial cells in normal endometrium is in agreement with previous studies showing its expression in an endometrial epithelial cell line (Sun et al. 2002) and in primary endometrial epithelial cells (Ghosh et al. 2009). CCL20 mRNA expression has been previously demonstrated in a gene array of endometrial adenocarcinoma (Wong et al. 2007), however no further literature exists describing its localisation or function in endometrial cancer.

CCR6 expression did not significantly vary throughout the stages of the menstrual cycle, however its expression was significantly increased in endometrial adenocarcinoma. CCR6 expression in the human endometrium and endometrial adenocarcinoma has not previously been described. The expression of CCR6 in normal endometrium as demonstrated by immunohistochemistry was present only in rare cells throughout the stroma and was weakly localised to glandular epithelial cells in proliferative phase endometrium. In endometrial adenocarcinoma, CCR6 expression was demonstrated in the neoplastic epithelial cells. This implied potential for autocrine signalling, as CCL20 expression was also present in the neoplastic epithelium in endometrial adenocarcinoma.

A role for $PGF_{2\alpha}$-FP signalling in CCL20 production was confirmed by the demonstration of increased CCL20 mRNA and protein expression in FPS cells. The signalling pathways mediating expression of CCL20 expression were therefore investigated. In Chapter 3, $PGF_{2\alpha}$-FP regulation of the chemokine CXCL1 by an EGFR-Ras-ERK1/2 pathway was described, and therefore components of this MAPK pathway were investigated. CCL20 has been previously demonstrated to be regulated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (Harant et al. 2001) and the related NFAT family of transcription factors (Pietila et al. 2007). As it has been previously demonstrated in our laboratory that NFκB signalling is not activated by $PGF_{2\alpha}$-FP interaction in FPS cells (K Sales, unpublished observations), only the MAPK and NFAT signalling pathways were

investigated. Inhibitors of FP, G_q, EGFR, MEK, calcineurin and calcium significantly decreased CCL20 mRNA expression. A chemical inhibitor of PKA did not decrease CCL20 mRNA. These findings correspond with previous data where CCL20 has been induced via an ERK signalling pathway (Sandri et al. 2008; Kanda et al. 2009), and a calcineurin-mediated NFAT signalling pathway (Pietila et al. 2007).

To further confirm the involvement of the NFAT signalling pathway in the regulation of CCL20, over-expression and knockdown of the calcineurin-binding protein RCAN1-4 was performed using adenoviral and lentiviral constructs. The efficacy of these constructs have been previously demonstrated (Maldonado-Perez et al. 2009). RCAN1-4 binds to calcineurin, preventing the dephosphorylation and activation of NFAT. NFAT translocation to the nucleus is prevented, and therefore NFAT-dependent gene transcription is inhibited. Over-expression of RCAN1-4 caused a significant decrease in CCL20 mRNA, implying NFAT activation is crucial in CCL20 regulation. RCAN1-4 knockdown caused a significant increase in CCL20 mRNA expression indicating that increased availability of calcineurin, and therefore increased translocation of NFAT to the nucleus, led to increased CCL20 mRNA expression. The crosstalk between the ERK and NFAT signalling pathways leading to production of CCL20 is unclear. ERK signalling can activate the DNA binding protein activating-protein complex-1 (AP-1), which is made up of a dimer of the Jun and Fos proteins. AP-1 can act synergistically with NFAT to regulate gene transcription (Macian et al. 2001). A role for AP-1 in CCL20 induction has been previously demonstrated in human keratinocyte cells (Kanda et al. 2009), and therefore this may be occurring in FPS cells. Furthermore, RCAN1-4 can also be directly phosphorylated by MEK, demonstrating a further degree of cross talk that could be acting between these two pathways. Whether the function of this phosphorylation is to inhibit or increase calcineurin binding is unclear (Harris et al. 2005). The interaction occurring between these pathways after $PGF_{2\alpha}$ signalling is therefore unclear but both appear central in regulation of CCL20 expression. The potential signalling pathways leading to CCL20 expression in FP cells is illustrated

in Figure 5.17, with components of the signalling pathways investigated in this chapter highlighted in red.

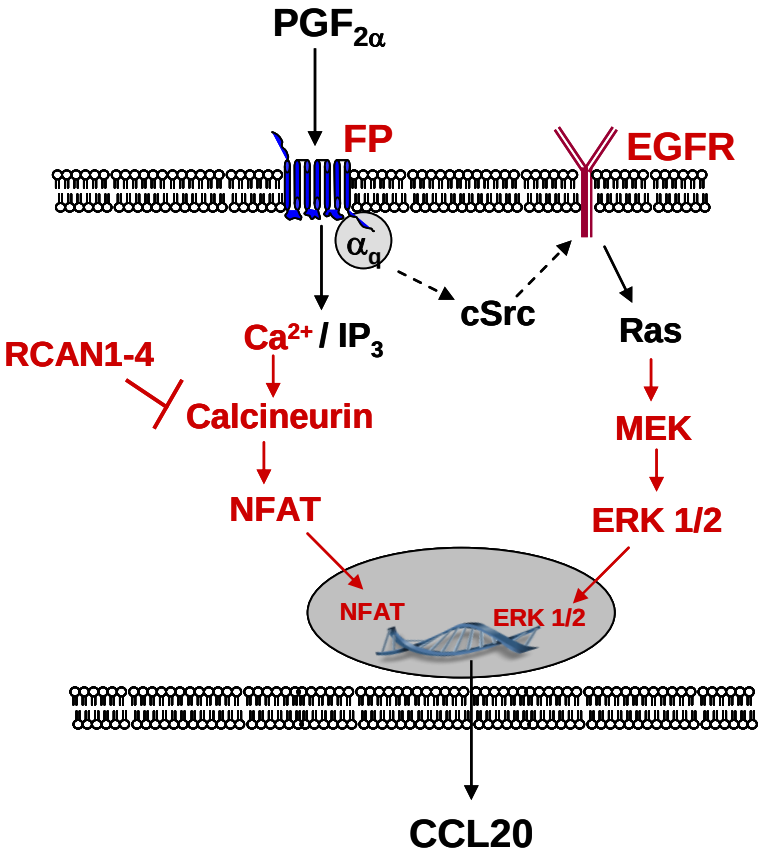

Figure 5.17 PGF$_{2\alpha}$ stimulates CCL20 production in FPS cells via FP-G$_q$-EGFR-MEK and FP-G$_q$-calcium-calcineurin-NFAT dependent pathways. Use of shRNA constructs and chemical inhibitors of cell signalling (highlighted in red) demonstrated that CCL20 production by PGF$_{2\alpha}$-FP signalling is dependent on FP-G$_q$-EGFR-MEK and FP-G$_q$-calcium-calcineurin-NFAT signalling pathways. Mechanisms of crosstalk between the pathways are unknown.

In addition to regulation at the mRNA level by the signalling pathways described, CCL20 is also likely to be regulated post-transcriptionally. CCL20 mRNA contains the destabilisation motif AUUUA, which is associated with rapid mRNA degradation (Harant et al. 2001).

CCL20-CCR6 signalling has an established role in dendritic cell and lymphocyte migration (Schutyser et al. 2003), and has recently been shown to be important in epithelial cell migration. In pancreatic cancer tissue, CCL20 and CCR6 were co-localised to the same cells, and in a pancreatic cancer cell line this autocrine signalling caused a significant increase in cell invasion (Kimsey et al. 2004). This was discovered to be dependent on an upregulation of MMP-9 by CCL20-CCR6 signalling (Campbell et al. 2005). Increased migration was also demonstrated in colorectal cancer cells after autocrine CCL20-CCR6 signalling (Brand et al. 2006). Increased invasiveness and metastasis were also associated with CCL20 or CCR6 expression in nasopharyngeal cancer (Chang et al. 2008), prostate cancer (Ghadjar et al. 2008) and liver cancer (Rubie et al. 2006).

Interestingly, an increase in CCR6 expression can influence metastasis in some cancer types. Increased CCR6 expression in plasmacytoma cells in a mouse model led to increased liver metastasis. This was found to be due to the high levels of CCL20 production by the liver causing migration of cancer cells towards this chemokine gradient, in a similar mechanism to chemoattraction of immune cells (Dellacasagrande et al. 2003). This mechanism has also been proposed as the reason for the high number of patients with colorectal cancer presenting with liver metastasis (Ghadjar et al. 2006). This evidence indicates that CCL20-CCR6 interactions may influence cell migration in endometrial adenocarcinoma.

In this chapter, however, significantly increased proliferation rather than migration was demonstrated after addition of CCL20 to CCR6-expressing endometrial adenocarcinoma cells. Proliferation was significantly increased but not dose-dependently after the addition of recombinant CCL20 from concentrations between 10 – 300 ng / ml, potentially as a result of desensitisation or internalisation of the CCR6 receptor at high chemokine concentrations (Beider et al. 2009). An increase in proliferation with similar concentrations of CCL20 has also been demonstrated in colorectal cancer cells (Brand et al. 2006). In a further study, CCL20 expression was upregulated by signalling at another chemokine receptor, CXCR4. This increase in

CCL20 expression caused an increase in proliferation of prostate, colon and promyelocytic leukaemia cancer cell lines (Beider et al. 2009). Proliferation was shown to be dependent on the ERK and c-Jun N-terminal kinase (JNK; a further mitogen activated protein kinase) signalling pathways in colorectal cancer (Brand et al. 2006). Further work is required in endometrial adenocarcinoma cells to determine the signalling pathways leading to increased cell proliferation. Levels of CCR6 mRNA expression in FPS cells were demonstrated to be significantly lower than those seen in cancer tissue. Therefore effects of CCL20 on FPS cell functions may be more pronounced if expression of CCR6 at similar levels to cancer in FPS cells was achieved.

CCL20 has also previously been demonstrated to contribute to tumour growth by promoting a tumour microenvironment which induces immune tolerance to tumour cells. As described in Chapter 4, dendritic cells present antigen to activate T and B lymphocytes which participate in cell-mediated cytotoxicity. In a previous study in colorectal cancer cells, CCL20 signalling induced chemotaxis of immature dendritic cells. These were defective in tumour antigen presenting and therefore a means to escape immune surveillance (Wang et al. 2008). Increased tumour growth was also demonstrated in a colon cancer cell line stably transfected with CCL20 which displayed increased immature dendritic cell infiltration (Bonnotte et al. 2004). However, further demonstrating the complexity of the role of immune cells within cancer, a similar mouse model of lung cancer led to an increase in infiltrating immature dendritic cells yet a decrease in tumour growth (Zhu et al. 2006). In Chapter 4, significantly increased numbers of dendritic cells as measured by CD11c expression were described in endometrial adenocarcinoma as compared to normal endometrium. Co-localisation of CD11c and CCR6 was not observed in cancer sections studied in this chapter; therefore CCL20 signalling may not play a large role in any induction of immune tolerance in endometrial adenocarcinoma.

This chapter has described a further chemokine regulated by $PGF_{2\alpha}$ in an endometrial adenocarcinoma cell line and endometrial adenocarcinoma explants. The expression

of the chemokine CCL20 and its receptor CCR6 was identified and localised in normal endometrium and endometrial adenocarcinoma tissue, and CCL20 expression induced by $PGF_{2\alpha}$ was found to be regulated by ERK and NFAT dependent signalling pathways. Finally, CCL20 was found to promote proliferation of endometrial adenocarcinoma cells. This chapter therefore highlights a function of the chemokine CCL20 in endometrial adenocarcinoma extending beyond its traditional role as a dendritic cell attractant.

Chapter 6

Interactions between the lysophosphatidic acid (LPA) and $PGF_{2\alpha}$ signalling pathways

6 Interactions between the lysophosphatidic acid (LPA) and $PGF_{2\alpha}$ signalling pathways

6.1 Introduction

The previous chapters in this thesis have examined the signalling pathways and the downstream effects resulting from $PGF_{2\alpha}$-FP receptor signalling in endometrial adenocarcinoma. However, in a physiological setting, the net effect of cell signalling is not only determined by single signalling pathways but also by the integrative effect of all the pathways activated.

Similarly to $PGF_{2\alpha}$, lysophosphatidic acid (LPA) is a membrane derived lipid mediator. The formation of LPA has been reported by different metabolic pathways dependent on the cell type examined; commonly the choline group from lysophosphatidylcholine is removed by the enzyme autotaxin (ATX) or phospholipids are cleaved extracellularly by phospholipase D into phosphatidic acids. A subsequent hydrolysis step by phospholipase A1 and A2 then forms LPA (Ye 2008). LPA acts through G-protein coupled receptors, of which seven have now been identified and named LPAR1-7 (Murph et al. 2008). LPAR1-3 are widely expressed in the body, while LPAR4-7 have limited expression and as yet unknown functions (Murph et al. 2006). Activation of LPAR1-3 leads to several downstream phenotypic effects including increased cell proliferation and migration, yet despite numerous studies, signalling cascades resulting from their activation are still unclear (Ye 2008).

LPA receptors 1-3 (also known as endothelial differentiation genes 2, 4, and 7) share high sequence homology with each other but less than 20% homology with LPAR4-7 (Murph et al. 2008). They have been reported to couple to G_i, G_q or $G_{12/13}$ dependent on the cell type and model used (Ishii et al. 2004). In the female reproductive tract, LPA signalling plays a key role in fertilisation, implantation, decidualisation and parturition (Ye 2008), and has been shown to induce oocyte maturation *in vitro*

(Komatsu et al. 2006). While knock out of LPAR1 and 2 showed no adverse affects on reproduction, LPAR3-knock out mice have impaired implantation and embryo spacing, resulting in embryo death and small litter sizes (Ye et al. 2005). LPAR3 expression in the murine uterus is also upregulated by progesterone and downregulated by oestrogen, further implicating LPA signalling in implantation (Hama et al. 2006). Throughout pregnancy, LPA expression is increased in serum (Tokumura et al. 2002) and expression of LPAR 2 and 3 in the human and mouse placenta has been reported (Ye 2008).

In cancer, LPA signalling can promote cell migration, cell proliferation and tissue remodelling through production of proteases and stimulation of angiogenesis (Murph et al. 2006). Elevated LPA concentrations have been found in the ascites and serum of patients with ovarian (Shen et al. 1998) and endometrial (Tokumura et al. 2007) cancer. Expression of LPAR1-3 has been documented in numerous cancers including breast (Kitayama et al. 2004), colon (Yun et al. 2005), prostate (Guo et al. 2006), gastrointestinal (Shin et al. 2009), and ovarian (Jeong et al. 2008; Yu et al. 2008). The expression of LPAR2 has also been demonstrated in an endometrial adenocarcinoma cell line (Hope et al. 2009). LPA signalling may promote tumour growth through the regulation of other signalling molecules, including angiogenic factors and cytokines. LPA promotes expression of CXCL1 in ovarian cancer via LPAR2 (Lee et al. 2006) as well as the related chemokine IL-8 in endometrial stromal cells via a G_i-MAPK-NFκB pathway (Chen et al. 2008).

The convergence of the COX-2 and LPA signalling pathways has been previously demonstrated, as LPA signalling via LPAR1-3 upregulates COX-2 expression and therefore prostaglandin production (Ye et al. 2005; Liszewska et al. 2009; Woclawek-Potocka et al. 2009; Woclawek-Potocka et al. 2009). Additionally, the LPAR3 knock out mouse displays a similar reproductive phenotype to the COX-2 knock out mouse and mice treated with an inhibitor of prostaglandin formation (Shah et al. 2005; Ye et al. 2005). The phenotype of the LPAR3 knockout mouse can be partly reversed by exogenous prostaglandin treatment, further indicating possible

feedback between these signalling molecules (Ye et al. 2005). This chapter examined a further interaction between the two pathways. As the FP receptor is known to localise to neoplastic epithelial cells in endometrial adenocarcinoma, the localisation of the LPA receptors 1-3 was investigated in endometrial adenocarcinoma to determine if potential cross talk could occur. The effects of LPA signalling on FP receptor expression and on the expression of genes demonstrated to be regulated by FP previously in this thesis were then examined.

6.2 Materials and methods

6.2.1 Tissue collection

Normal endometrium and endometrial adenocarcinoma tissue was collected as described in sections 2.1.1 and 2.1.2. Normal endometrial tissue was collected from women undergoing surgery for minor gynaecological procedures with no underlying endometrial pathology, and endometrial adenocarcinoma tissue was obtained from women undergoing hysterectomy after diagnosis of adenocarcinoma of the uterus. Stage of cycle and grade of cancer was assessed by a pathologist. Written informed consent was collected from all patients before surgery and ethical approval was obtained from Lothian Research Ethics Committee.

6.2.2 Cell culture

Ishikawa FPS cells were maintained as described in section 2.2.1.

6.2.3 Cell treatments

To determine the pattern of FP, CXCL1, CCL20, COX-2 and $PGF_{2\alpha}$ expression in response to LPA, FPS cells were serum starved for at least 18 hours prior to stimulation for 2, 4, 6, 8, 12, 18, 24, 48 and 72 hours with fresh serum-free media containing vehicle or 10 µM LPA (n=5).

To determine if target gene expression occurred independently of activation of the FP receptor, FPS cells were serum starved for at least 18 hours and treated for 4 hours with vehicle, AL8810 alone, 10 µM LPA or 10 µM LPA and AL8810 (n=4).

6.2.4 Polymerase chain reaction

6.2.4.1 RNA extraction and reverse-transcriptase PCR

RNA from cells was extracted as described in section 2.4.1. Briefly, 1 ml TRI-reagent was added to experimental wells and RNA extracted using the phenol method.

RNA from tissue was extracted as described in section 2.4.1 using the Qiagen minikit protocol. Pieces of tissue, in 2 ml microcentrifuge tubes containing a stainless steel ball, were homogenised in 600 µl RLT lysis buffer using a tissue lyser (Qiagen, Crawley, West Sussex, UK) for 2 x 2 minutes at 25 Hz. RNA was then extracted following manufacturers guidelines (Qiagen). cDNA was prepared as described in section 2.4.2.

6.2.4.2 Taqman quantitative RT-PCR

FP, CXCL1, CCL20, COX-2, LPAR1, LPAR2 and LPAR3 mRNA expression in FPS cells (n=6), normal endometrium (n=29) and endometrial adenocarcinoma tissue (n=29) was examined using Taqman quantitative PCR as described in section 2.4.3.2. Briefly, primer and probe sequences were designed to amplify the above genes (sequences given in Table 2.3). A master mix was made up containing 18S primers and probes and 200 ng cDNA was added to each sample. Samples were added in duplicate to a 96 well MicroAmp fast optical reaction PCR plate and analysed using an ABI Prism 7900 HT Fast Real Time PCR machine. Expression of analyzed genes was normalised to RNA loading for each sample using the 18S ribosomal RNA as an internal standard. Results are expressed as fold increase above vehicle treated cells or relative to a normal endometrium control.

6.2.5 Immunohistochemistry

3,3- diaminobenzidine tetrahydrochlorine (DAB) immunohistochemistry to localise LPAR1, LPAR2 and LPAR3 was performed as described in section 2.5.2 (normal endometrium: proliferative phase n=4, early secretory phase n=3, mid secretory phase n=3, late secretory phase n=4; endometrial adenocarcinoma: well differentiated n=4, moderately differentiated n=3, poorly differentiated n=3). Tissue sections (5 µm) were dewaxed in xylene then rehydrated through graded ethanols and water. Antigen retrieval in citrate buffer (0.01M, pH 6) was performed, followed by blocking for endogenous peroxidase activity by washing sections in 3% hydrogen peroxidase in methanol. Sections were then blocked in normal serum of goat followed by overnight incubation with primary antibody at the concentrations indicated in Table 2.4. Control sections were included with non-immune IgG. Sections were then incubated in goat anti-rabbit biotinylated secondary antibody and subsequently streptavidin-HRP. Sections were then incubated with DAB for 1-5 minutes until positive staining was identified by a brown colour under x10 magnification.

6.2.6 $PGF_{2\alpha}$ assay

$PGF_{2\alpha}$ released into the culture medium from treatments described in section 6.2.3 was measured using an assay developed in our laboratory. 96-well plates were coated with donkey anti-rabbit serum diluted 1:200 in PBS overnight. After removal of this, wells were blocked with Dry Coat solution (2% polyvinyl pyrollidine, 1% BSA, 0.05% sodium azide, 5mM EDTA in Tris-buffered saline) for 1 hour and washed in wash buffer (0.05% Tween in PBS, pH 7.2-7.4). A volume of 50 µl of rabbit antiserum, 50 µl of biotin-labelled prostaglandin link and 100 µl of sample or standards were then added to each well. The $PGF_{2\alpha}$ assay works by competitive binding. Any $PGF_{2\alpha}$ present in the sample will displace binding of the link, and therefore at the addition of streptavidin peroxidase and substrate color detection step, a stronger colour indicates a low concentration of $PGF_{2\alpha}$. The link was prepared by ether extraction and purified by reverse phase chromatography using 20mg of synthetic $PGF_{2\alpha}$, 320µL of dry dimethylformamide, 3µl butylchlororomate and

0.05nM biocytin. The link and antisera were diluted 1:1 million and 1:50000 in assay buffer (150 mM NaCl, 100 mM Tris-HCl, 50 mM phenol red 1 mM 2-methylisothiaolone, 1 mM bromonitrodioxane, 2 mM EDTA, 2 mg/ml BSA to a final pH of 7.2) respectively. A control was included containing no antiserum. After overnight incubation at 4 °C plates were washed in wash buffer 4 times. Streptavidin peroxidase (Roche, UK) diluted 1:2000 in assay buffer was added for 20 minutes. Colour change was detected by addition of substrate solution (1:1 mix in 100mM sodium acetate buffer, pH 6, of: 5mg/ml hydrogen peroxide in sodium acetate buffer, pH 6: tetramethylbenzidine 3mg/ml in dimethyl formamide) and the reaction was stopped by addition of 2N sulphuric acid. Optical density of wells was read at 450nM.

6.2.7 Statistical analysis

Where appropriate, statistical analysis was carried out using GraphPad Prism (GraphPad Software. San Diego, California, USA). Data are presented as mean ± SEM.

6.3 Results

6.3.1 Expression of the LPA receptors in normal endometrium and endometrial adenocarcinoma

The mRNA expression of three of the receptors for LPA was examined in normal endometrium and endometrial adenocarcinoma.

6.3.1.1 Expression of LPAR1 mRNA in normal endometrium and endometrial adenocarcinoma

The expression of LPAR1 was examined in normal endometrium (n=29) and endometrial adenocarcinoma (n=29) by quantitative RT-PCR. LPAR1 mRNA expression was significantly lower in the mid secretory phase of the menstrual cycle as compared to the early secretory phase ($p<0.01$; Figure 6.1A). LPAR1 mRNA

expression did not significantly differ between different grades of endometrial adenocarcinoma (Figure 6.1B) and did not significantly differ between pooled normal endometrium samples and pooled endometrial adenocarcinoma samples (Figure 6.1C).

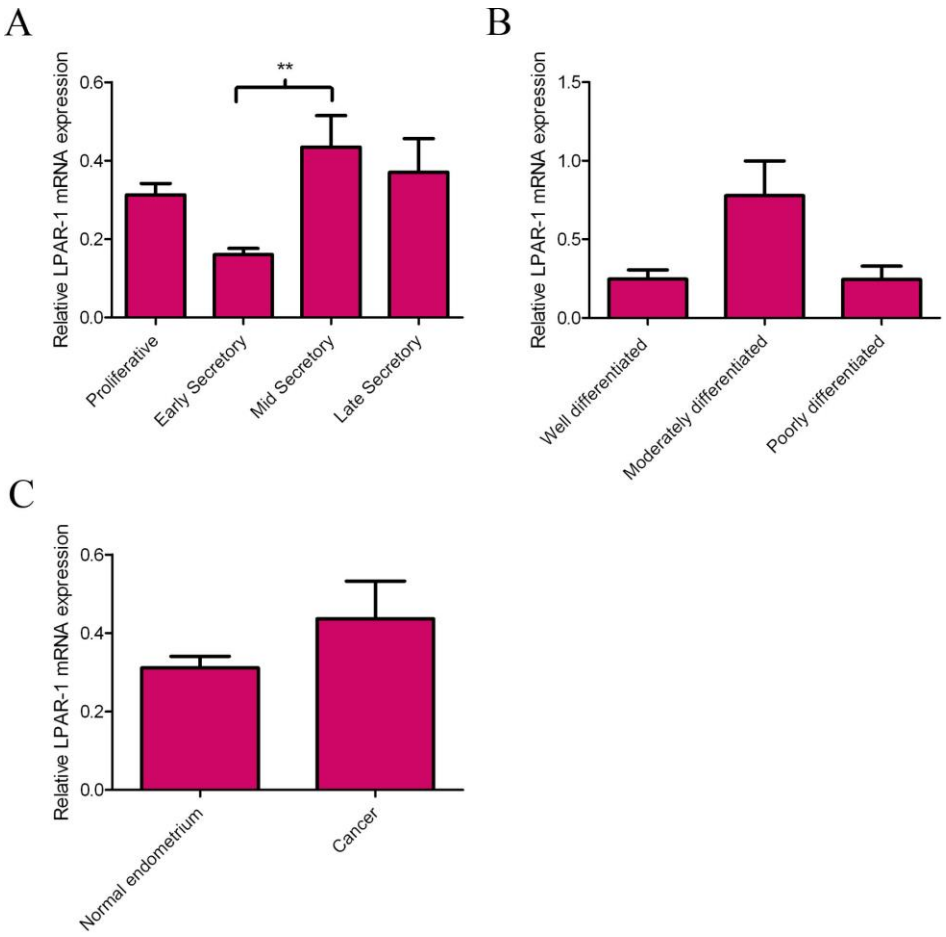

Figure 6.1 Expression of LPAR1 mRNA in normal endometrium and endometrial adenocarcinoma. A: LPAR1 mRNA was examined by quantitative RT-PCR in endometrium from the proliferative (n=11), early secretory (n=7), mid secretory (n=6) and late secretory (n=5) phases of the menstrual cycle. Expression in the early secretory phase was significantly lower than the mid secretory phase. **B:** LPAR1 mRNA was examined by quantitative RT-PCR in well (n=9), moderately (n=10) and poorly (n=10) differentiated endometrial adenocarcinoma. No significant difference was demonstrated. **C:** LPAR1 mRNA expression was examined by quantitative RT-PCR in pooled normal endometrial and endometrial adenocarcinoma tissue. There was no significant difference between normal

endometrium and cancer. ** denotes p<0.01, data are expressed relative to a normal endometrium control and are presented as mean ± SEM.

6.3.1.2 Expression of LPAR2 mRNA in normal endometrium and endometrial adenocarcinoma

Expression of LPAR2 mRNA was also examined in normal endometrium (n=30) and endometrial adenocarcinoma (n=30) by quantitative RT-PCR. LPAR2 mRNA expression was significantly increased in the early secretory phase of the menstrual cycle (p<0.01; Figure 6.2A). No significant difference in LPAR2 mRNA expression was observed between different grades of endometrial cancer (Figure 6.2B), however mRNA expression was significantly higher by 10.9 ± 0.29 fold in pooled endometrial adenocarcinoma as compared to pooled normal endometrium (p<0.001; Figure 6.2C).

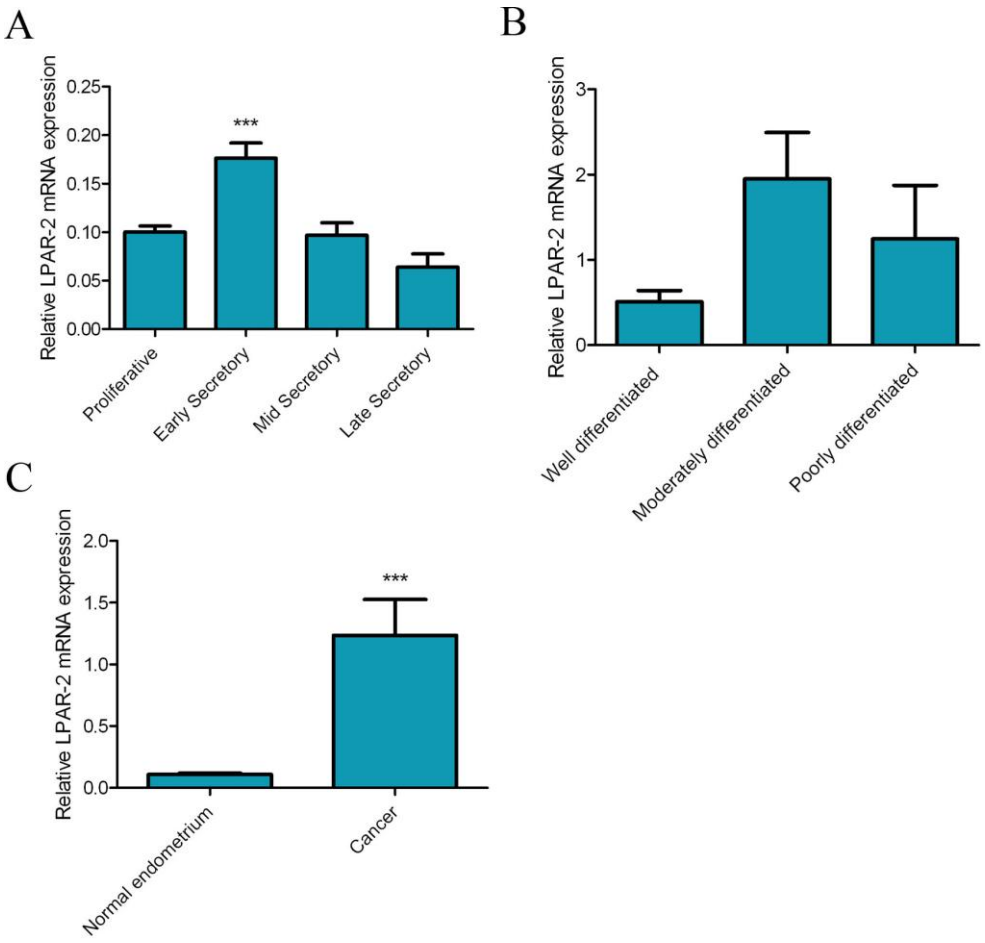

Figure 6.2 Expression of LPAR2 mRNA in normal endometrium and endometrial adenocarcinoma. A: LPAR2 mRNA was examined by quantitative RT-PCR in endometrium from the proliferative (n=11), early secretory (n=7), mid secretory (n=6) and late secretory (n=6) phases of the menstrual cycle. Expression in the early secretory phase was significantly higher as compared to all other stages of the cycle. **B:** LPAR2 mRNA was examined by quantitative RT-PCR in well (n=10), moderately (n=10) and poorly (n=10) differentiated endometrial adenocarcinoma. No significant difference was demonstrated. **C:** LPAR2 mRNA expression was examined by quantitative RT-PCR and found to be significantly higher in pooled endometrial adenocarcinoma tissue as compared to pooled normal endometrial tissue. *** denotes $p<0.001$, data are expressed relative to a normal endometrium control and are presented as mean ± SEM.

6.3.1.3 Expression of LPAR3 mRNA in normal endometrium and endometrial adenocarcinoma

Expression of LPAR3 mRNA was also examined in normal endometrium (n=30) and endometrial adenocarcinoma (n=28) by quantitative RT-PCR. LPAR3 mRNA expression was significantly higher in the early secretory phase of the menstrual cycle ($p<0.001$; Figure 6.3A). No significant difference in LPAR3 mRNA expression was observed between different grades of endometrial cancer (Figure 6.3B). LPAR3 mRNA expression was also significantly higher by 3.2 ± 2.1 fold in pooled endometrial adenocarcinoma as compared to pooled normal endometrium ($p<0.05$; Figure 6.3C).

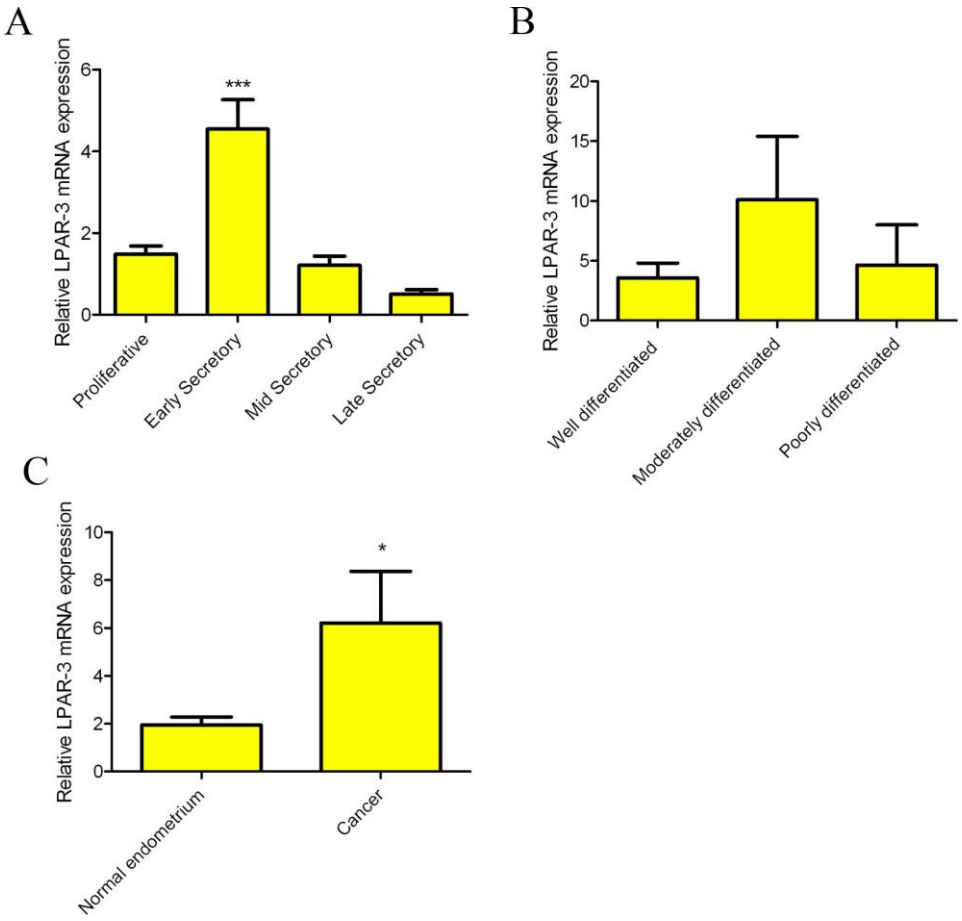

Figure 6.3 Expression of LPAR3 mRNA in normal endometrium and endometrial adenocarcinoma. A: LPAR3 mRNA was examined by quantitative RT-PCR in endometrium from the proliferative (n=11), early secretory (n=7), mid secretory (n=6) and late secretory (n=6) phase of the menstrual cycle. Expression in the early secretory phase was significantly higher as compared to all other stages of the cycle. **B:** LPAR3 mRNA was examined by quantitative RT-PCR in well (n=10), moderately (n=10) and poorly (n=8) differentiated endometrial adenocarcinoma. No significant difference was demonstrated. **C**: LPAR3 mRNA expression was examined by quantitative RT-PCR and found to be significantly higher in pooled endometrial adenocarcinoma tissue as compared to pooled normal endometrial tissue. *denotes $p<0.05$, *** denotes $p<0.001$, data are expressed relative to a normal endometrium control and are presented as mean ± SEM.

6.3.2 Localisation and expression of LPA receptors in normal endometrium and endometrial adenocarcinoma

Expression of the LPA receptors 1, 2 and 3 was then examined by immunohistochemistry in normal endometrium and endometrial adenocarcinoma.

6.3.2.1 Localisation and expression of LPAR1 in normal endometrium and endometrial adenocarcinoma

LPAR1 expression has been previously demonstrated by immunohistochemistry in epithelial, stromal and vascular endothelial cells in normal endometrial tissue (Chen et al. 2008). Expression has also been previously shown in bovine endometrium (Woclawek-Potocka et al. 2009). Normal endometrial tissue from all phases of the menstrual cycle (proliferative n=3, early secretory n =3, mid secretory n=3, late secretory n=4) was examined by immunohistochemistry for LPAR1 protein expression. At all stages of the cycle, LPAR1 expression was present in glandular epithelium, stroma and some vascular endothelial cells (GE, S, VE; Figure 6.4). In the proliferative phase, strong expression can be seen in glandular epithelium and vascular endothelial cells (Figure 6.4A). This expression in glandular epithelium and stroma can also be seen in the early, mid and late secretory phases (Figure 6.4 B, C, D). Negative controls included were incubated with non-immune IgG in place of primary antibody (Figure 6.4D, inset).

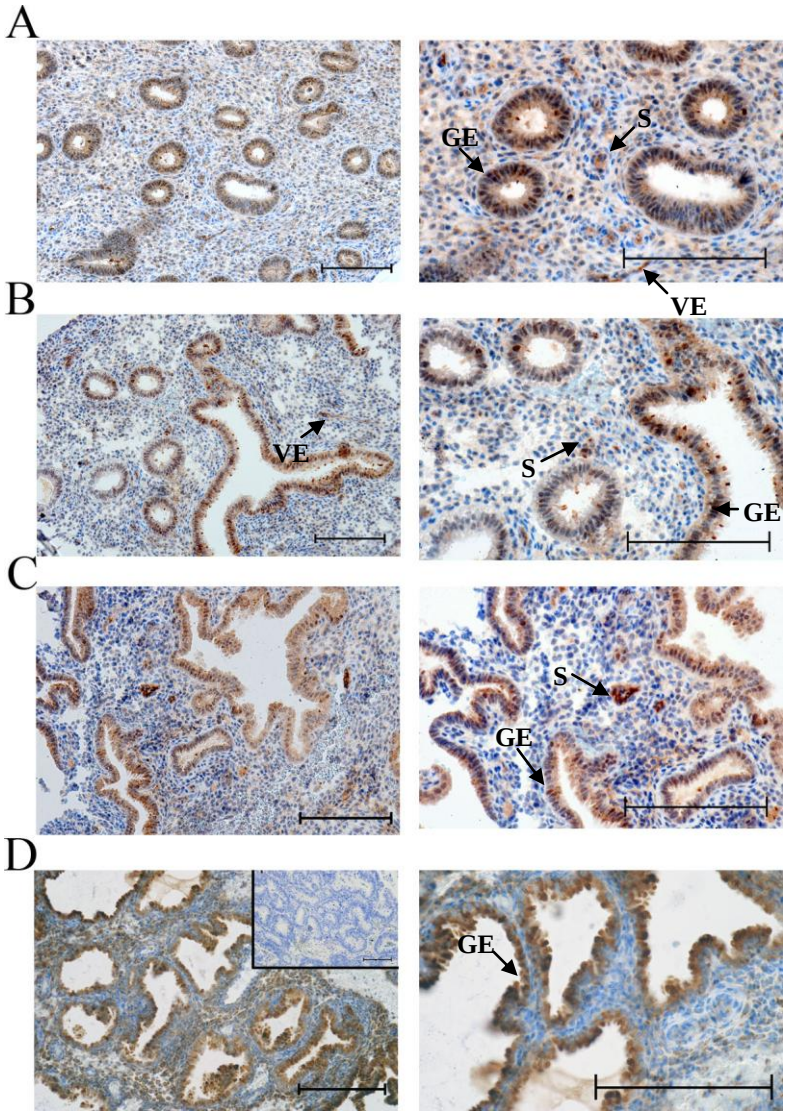

Figure 6.4 Localisation of LPAR1 protein in normal endometrium by immunohistochemistry. Representative images demonstrating that LPAR1 localises to the glandular epithelium, stroma and vascular endothelium in normal endometrium. **A:** LPAR1 localises to the glandular epithelium (GE), stroma (S) and vascular endothelium (VE) in proliferative phase endometrium (left image is x200 magnification, right image is x400). **B:** In early secretory phase endometrium, LPAR1 immunoreactivity can be seen in the glandular epithelium, stroma and vascular endothelial cells (left image is x200 magnification, right image is x400). **C:** In mid secretory phase endometrium, LPAR1 can be localised to glandular epithelium and stroma (left image is x200 magnification, right image is x400). **D:** LPAR1 also localises to glandular epithelium in late secretory phase endometrium. Negative control (inset) is incubated with non-immune IgG in place of primary antibody. All scale bars represent 100 µm.

Expression of LPAR1 was also examined in all grades of endometrial adenocarcinoma by immunohistochemistry (well differentiated n=4, moderately differentiated n=4, poorly differentiated n=4). LPAR1 was localised to epithelial cells (E), stroma (S) and vascular endothelial cells (VE) in all grades of cancer examined. Representative images are shown in Figure 6.5. Negative controls included were incubated with non-immune IgG in place of primary antibody (inset; Figure 6.5A).

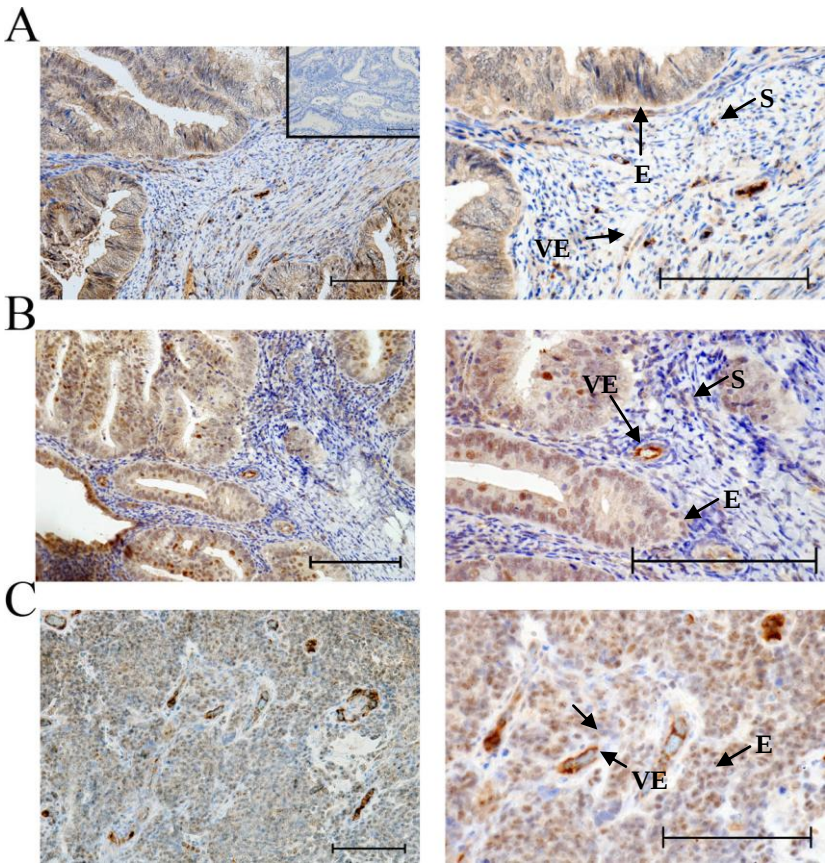

Figure 6.5 Localisation of LPAR1 protein in endometrial adenocarcinoma by immunohistochemistry. Representative images demonstrating that LPAR1 localises to the neoplastic epithelium (E), vascular endothelium (VE) and stroma (S) in **A:** Well differentiated carcinoma (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune IgG in place of primary antibody. **B:** Moderately differentiated carcinoma (left image is x200 magnification, right image is x400). **C:** Poorly differentiated

carcinoma (left image is x200 magnification, right image is x400). All scale bars represent 100 μm.

6.3.2.2 Localisation and expression of LPAR2 in normal endometrium and endometrial adenocarcinoma

The localisation of LPAR2 was then examined by immunohistochemistry in endometrial tissue. Endometrial tissue from all stages of the menstrual cycle was examined (proliferative n=3, early secretory n =3, mid secretory n=3, late secretory n=4). In the proliferative phase of the cycle, LPAR2 expression was found to be weakly localised to glandular epithelial cells (GE) and strongly to cells throughout the stroma (S; Figure 6.6A). Immunoreactivity in the glandular epithelium was increased in the early and mid secretory phases, with decreased stromal expression (Figure 6.6 B, C). In the late secretory phase, epithelial and stromal expression was decreased (Figure 6.6C). Negative controls included were incubated with non-immune IgG in place of primary antibody (inset; Figure 6.6A)

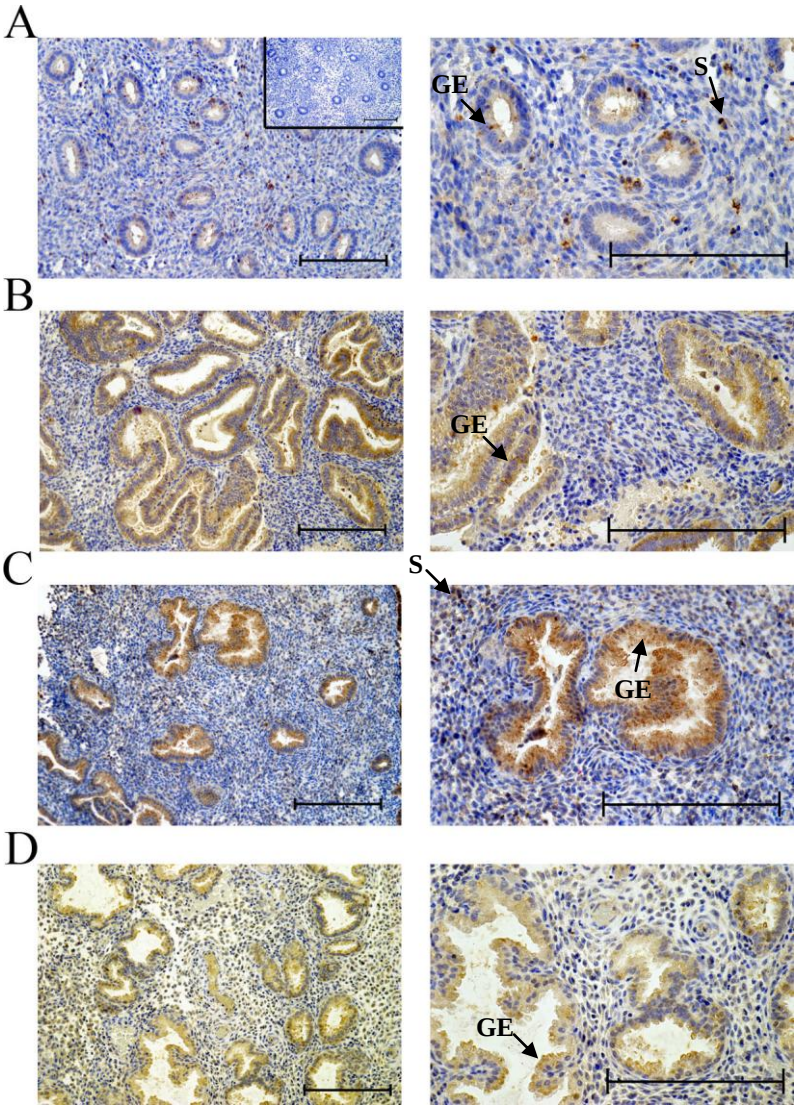

Figure 6.6 Localisation of LPAR2 protein in normal endometrium by immunohistochemistry. Representative images demonstrating that LPAR2 localises to the glandular epithelium and stroma in normal endometrium. **A:** LPAR2 localises weakly to the glandular epithelium (GE) and more strongly in the stroma (S) of proliferative phase endometrium (left image is x200 magnification, right image is x400). **B:** In early secretory phase endometrium, LPAR2 immunoreactivity can be seen in the glandular epithelium (left image is x200 magnification, right image is x400). **C:** In mid secretory phase endometrium, LPAR2 can be localised to glandular epithelium and stroma (left image is x200 magnification, right image is x400). **D:** LPAR2 localises weakly to glandular epithelium in late secretory phase endometrium. Negative control (inset) is incubated with non-immune IgG in place of primary antibody. All scale bars represent 100 µm.

Expression of LPAR2 was also examined in all grades of endometrial adenocarcinoma by immunohistochemistry (well differentiated n=4, moderately differentiated n=4, poorly differentiated n=4). LPAR2 was localised weakly to epithelial cells (E) and stroma (S) in all grades of cancer examined. LPAR2 did not localise to vascular endothelial cells. Representative images are shown in Figure 6.8. Negative controls included were incubated with non-immune IgG in place of primary antibody (inset; Figure 6.7A)

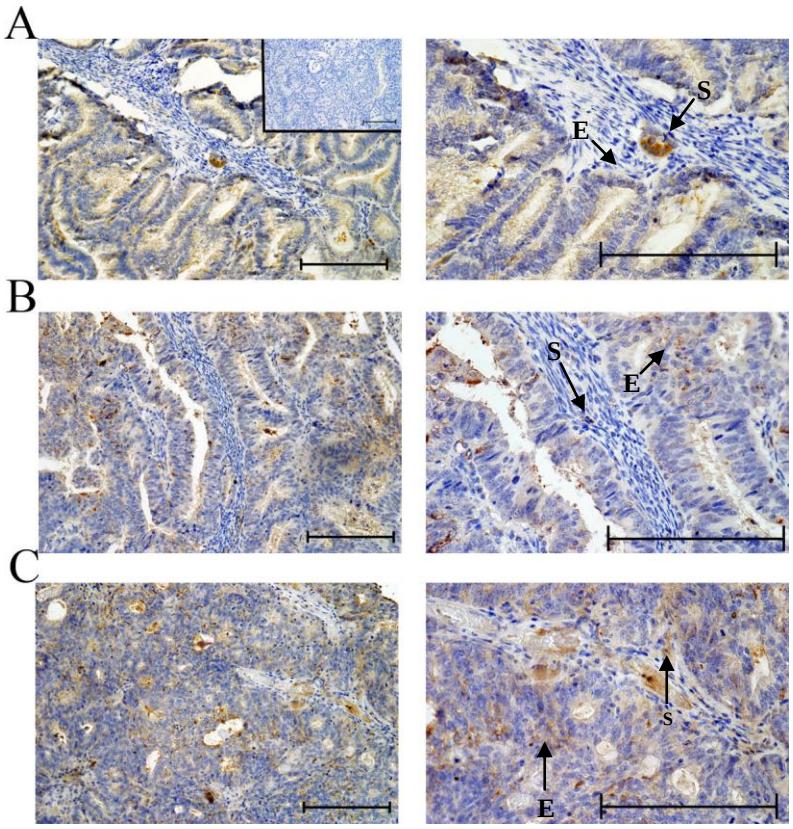

Figure 6.7 Localisation of LPAR2 protein in endometrial adenocarcinoma by immunohistochemistry. Representative images demonstrating that LPAR2 localises to the neoplastic epithelium (E) and stroma (S) in **A:** Well differentiated carcinoma (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune IgG in place of primary antibody. **B:** Moderately differentiated carcinoma (left image is x200 magnification, right image is x400). **C:** Poorly differentiated carcinoma (left image is x200 magnification, right image is x400). All scale bars represent 100 µm.

6.3.2.3 Localisation and expression of LPAR3 in normal endometrium and endometrial adenocarcinoma

LPAR3 expression has been demonstrated in the luminal and glandular epithelium of the porcine endometrium (Seo et al. 2008). LPAR3 expression was localised by immunohistochemistry in endometrial tissue from all phases of the menstrual cycle (proliferative n=3, early secretory n =3, mid secretory n=3, late secretory n=4). In the all phases of the cycle, LPAR3 expression was demonstrated in glandular epithelial cells (GE) and cells throughout the stroma (S; Figure 6.8). Strong immunoreactivity was seen in the glandular epithelium in the proliferative phase of the cycle (Figure 6.8A), and immunoreactivity in the stroma was increased in the mid secretory phase (Figure 6.8C). Expression was not seen in vascular endothelial cells at any stage of the menstrual cycle. Negative controls included were incubated with non-immune IgG in place of primary antibody (inset; Figure 6.8D)

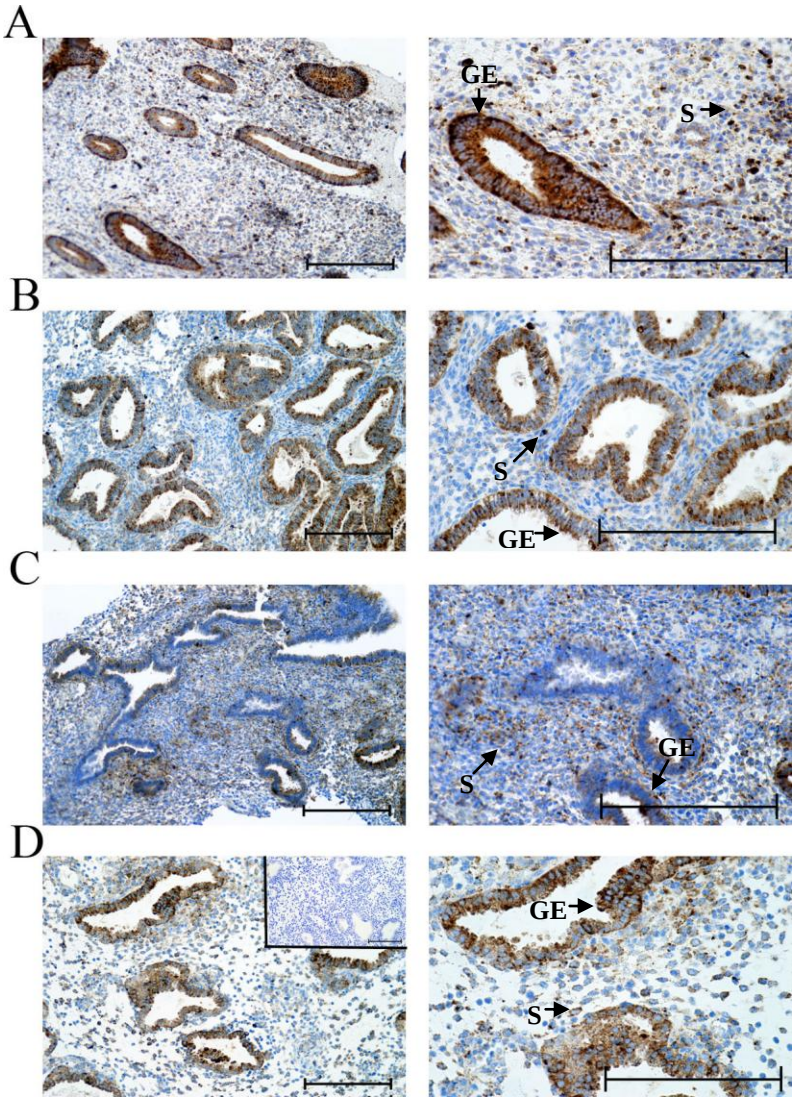

Figure 6.8 Localisation of LPAR3 protein in normal endometrium by immunohistochemistry. Representative images demonstrating that LPAR3 localises to the glandular epithelium and stroma in normal endometrium. **A:** LPAR3 localises to the glandular epithelium (GE) and the stroma (S) of proliferative phase endometrium (left image is x200 magnification, right image is x400). **B:** In early secretory phase endometrium, LPAR3 immunoreactivity can be seen in the glandular epithelium and stroma (left image is x200 magnification, right image is x400). **C:** In mid secretory phase endometrium, LPAR3 can be localised to glandular epithelium and expression is increased in the stroma (left image is x200 magnification, right image is x400). **D:** LPAR3 localises to glandular epithelium and stroma in late secretory phase endometrium. Negative control (inset) is incubated with non-immune IgG in place of primary antibody. All scale bars represent 100 µm.

Expression of LPAR3 was also examined in all grades of endometrial adenocarcinoma by immunohistochemistry (well differentiated n=4, moderately differentiated n=4, poorly differentiated n=4). LPAR3 was localised to epithelial cells (E), stroma (S) and vascular endothelial cells (VE) in all grades of cancer examined. In well differentiated cancer, LPAR3 expression was present in some but not all neoplastic epithelial cells, in cells throughout the stroma and in some endothelial cells (Figure 6.9A). A similar pattern of expression was demonstrated in moderately differentiated cancer and poorly differentiated cancer (Figure 6.9B,C). Negative controls included were incubated with non-immune IgG in place of primary antibody (inset; Figure 6.9A)

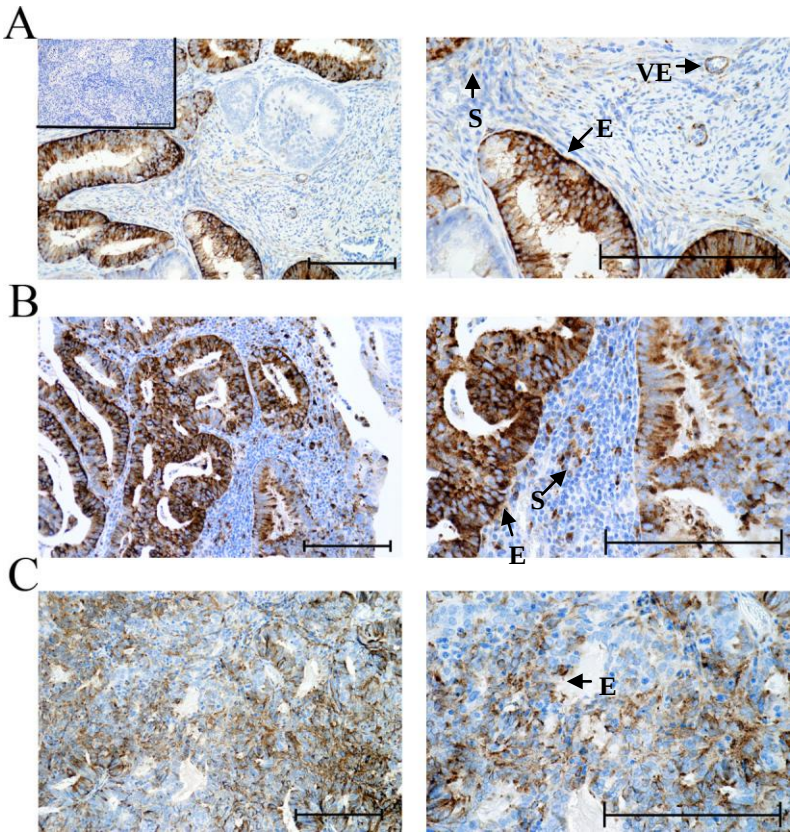

Figure 6.9 Localisation of LPAR3 protein in endometrial adenocarcinoma by immunohistochemistry. Representative images demonstrating that LPAR3 localises to the neoplastic epithelium (E), vascular endothelium (VE) and stroma (S) in **A:** Well differentiated carcinoma (left image is x200 magnification, right image is x400). Negative control (inset) is incubated with non-immune IgG in place of primary antibody. **B:** Moderately differentiated

carcinoma (left image is x200 magnification, right image is x400). **C:** Poorly differentiated carcinoma (left image is x200 magnification, right image is x400). All scale bars represent 100 µm.

6.3.3 Expression of target genes mediated by LPA signalling in FPS cells

Crosstalk between the LPA and prostaglandin signalling pathways has been previously demonstrated. The localisation of LPAR1-3 to the neoplastic epithelium in endometrial adenocarcinoma (Figures 6.5, 6.7 and 6.9) indicated that LPA signalling could interact with the FP receptor signalling pathway, as FP is also expressed on neoplastic epithelial cells. To examine this, the expression of FP, COX-2 and target genes previously demonstrated to be regulated by the FP receptor in this thesis, CXCL1 and CCL20, were examined by RT-PCR. Gene expression was measured in FPS cells which were treated for 2-72 hours with vehicle or 10 µm LPA. After 8 hours of LPA treatment, FP receptor mRNA was significantly elevated 8.6 ± 4.1 fold as compared to vehicle treated cells ($p<0.05$). FP receptor mRNA expression then decreased until 72 hours (Figure 6.10A). COX-2 mRNA expression was slightly but non-significantly increased after 2 hours of LPA treatment to 1.6 ± 0.3 fold over vehicle treated cells (Figure 6.10B). After 4 hours of LPA treatment, CXCL1 mRNA was significantly increased 6.3 ± 2.3 fold above vehicle treated cells ($p<0.05$; Figure 6.10C). CCL20 mRNA expression was also significantly increased after 4 hours of LPA treatment to 9.2 ± 2.5 fold after 4 hours LPA treatment ($p<0.05$; Figure 6.10D).

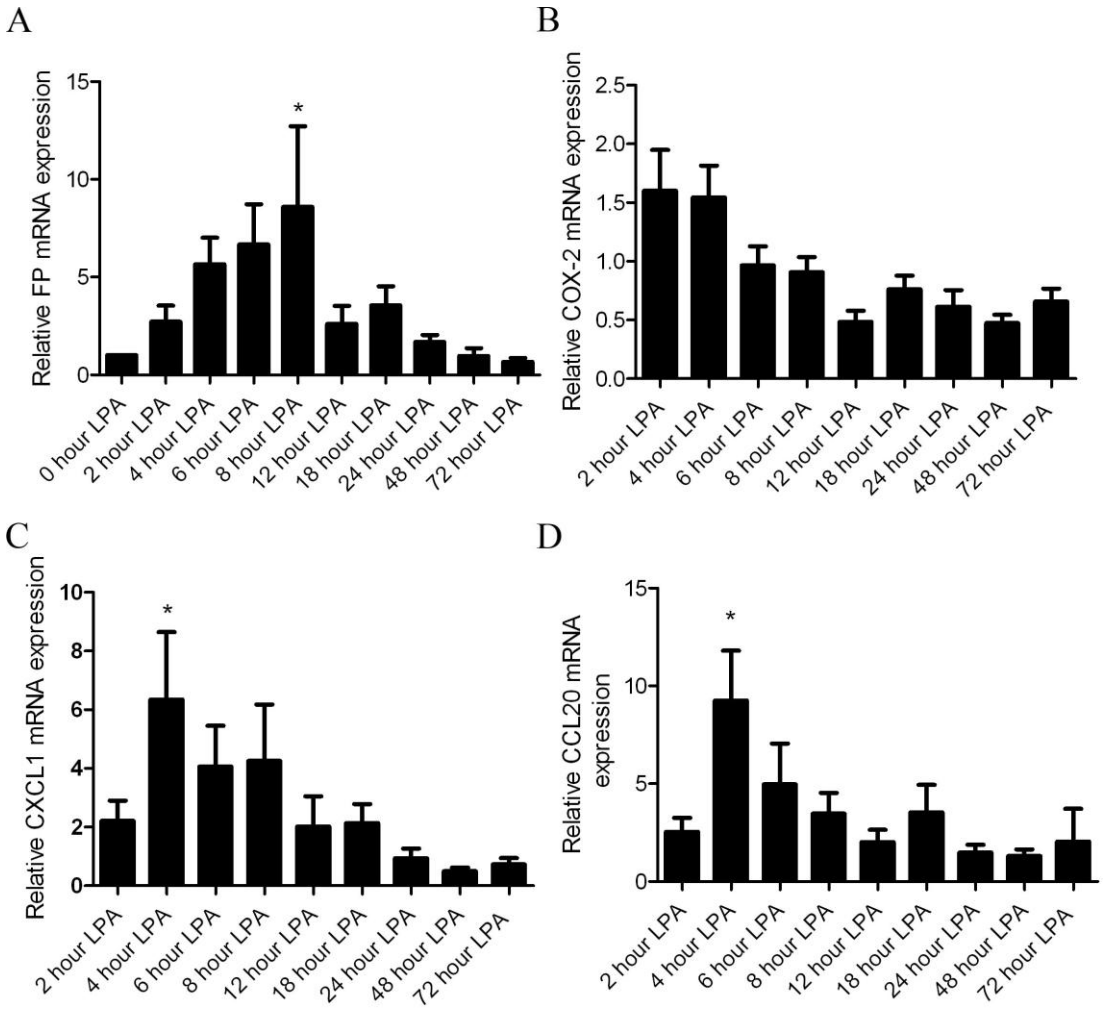

Figure 6.10 Regulation of FP, COX-2, CXCL1 and CCL20 mRNA expression in FPS cells by LPA. A: FP mRNA expression in FPS cells was measured by quantitative RT-PCR after stimulation with vehicle or 10 µM LPA over a period of 2 to 72 hours, and was significantly increased at 8 hours. **B:** COX-2 mRNA expression in FPS cells was measured by quantitative RT-PCR after stimulation with vehicle or 10 µM LPA over a period of 2 to 72 hours. **C:** CXCL1 mRNA expression in FPS cells was measured by quantitative RT-PCR after stimulation with vehicle or 10 µM LPA over a period of 2 to 72 hours, and was significantly increased at 4 hours. **D:** CCL20 mRNA expression in FPS cells was measured by quantitative RT-PCR after stimulation with vehicle or 10 µM LPA over a period of 2 to 72 hours, and was significantly increased at 4 hours. All data are expressed as fold increase over vehicle treated control and are presented as mean ± SEM. * denotes $p< 0.05$.

6.3.4 Regulation of target genes by LPA is independent of $PGF_{2\alpha}$

LPA signaling has been previously shown to upregulate COX-2 and prostaglandin expression (Jeong et al. 2008; Woclawek-Potocka et al. 2009; Woclawek-Potocka et al. 2009) and a small increase in COX-2 was shown after LPA treatment of FPS cells (Figure 6.10B). To confirm that the upregulation of target genes shown in Figure 6.10 was mediated by LPA independently of $PGF_{2\alpha,}$ $PGF_{2\alpha}$ synthesis was measured by prostaglandin assay. After 2 hours LPA treatment, $PGF_{2\alpha}$ synthesis was slightly but non-significantly increased (Figure 6.11A). FPS cells were also treated with 10 µm LPA and the specific FP antagonist AL8810 for 4 hours. LPA treatment resulted in increases in FP, CXCL1 and CCL20 mRNA expression. Addition of the FP antagonist AL8810 made no significant difference to LPA induced FP, CXCL1 and CCL20 mRNA expression (Figure 6.11 B, C, D).

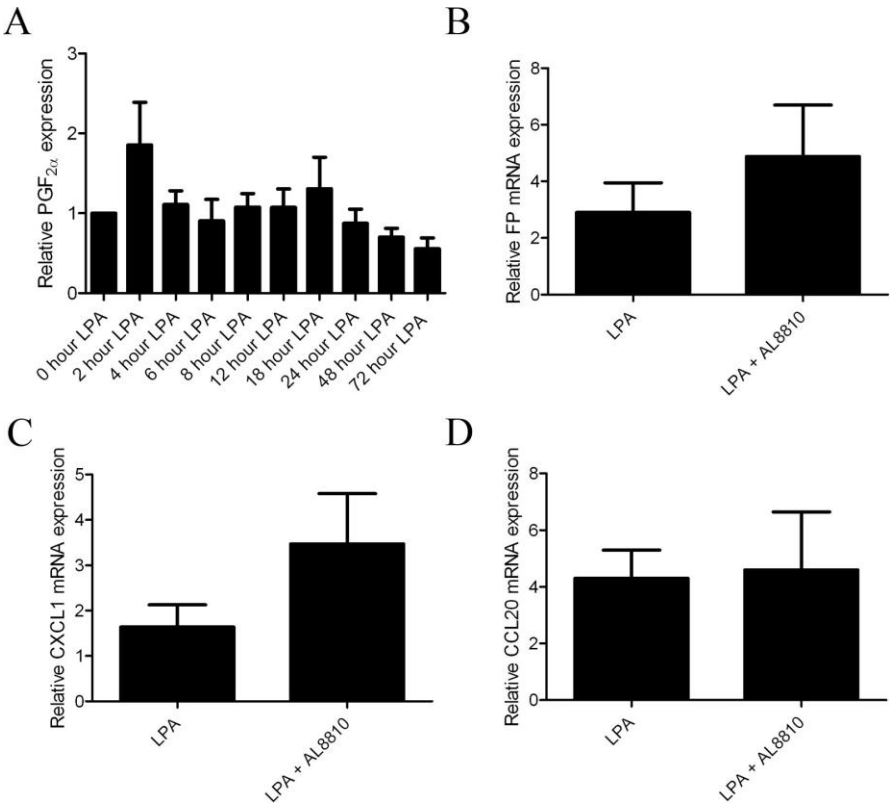

Figure 6.11 Increased FP, CXCL1 and CCL20 mRNA expression is independent of $PGF_{2\alpha}$ – FP signaling. ***A:*** $PGF_{2\alpha}$ secretion into the media was measured by prostaglandin after treatment of FPS cells with 10 µm LPA for 2-72 hours. No significant increase over

vehicle treated cells was shown. **B:** FP mRNA expression in FPS cells was measured by quantitative RT-PCR after treatment with vehicle control, 10 µM LPA or 10 µM LPA and AL8810 for 4 hours. No significant difference was demonstrated between treatments. **C:** CXCL1 mRNA expression in FPS cells was measured by quantitative RT-PCR after treatment with vehicle control, 10 µM LPA or 10 µM LPA and AL8810 for 4 hours. No significant difference was demonstrated between treatments **D:** CCL20 mRNA expression in FPS cells was measured by quantitative RT-PCR after treatment with vehicle control, 10 µM LPA or 10 µM LPA and AL8810 for 4 hours. No significant difference was demonstrated between treatments. All data are expressed as fold increase over vehicle treated control and are presented as mean ± SEM.

6.4 Discussion

Over-expression of the LPA receptors in cancer has been associated with increased cell proliferation, migration and neovascularisation (Murph et al. 2006). This chapter identified the temporal expression and localisation of LPAR1-3 in normal endometrium, and the increased expression of LPAR2 and 3 in endometrial adenocarcinoma. Crosstalk between the LPA and $PGF_{2\alpha}$ signalling pathways was then identified in endometrial adenocarcinoma cells.

LPAR1 expression varied little throughout the normal menstrual cycle however was significantly lower in the early secretory phase. It could be localised to the glandular epithelium and stromal cells, in agreement with a previous study describing LPAR1 localisation in late secretory phase endometrium (Chen et al. 2008). LPAR1 signalling has been proposed to play a role in angiogenesis in the normal endometrium, as endometrial stromal cells stimulated with LPA produced the angiogenic chemokine IL-8 via a LPAR1 and NFκB pathway (Chen et al. 2008). Increased expression of LPAR1 has also been described in the bovine endometrium during the estrous cycle, however in this animal model it is thought to contribute to $PGF_{2\alpha}$ induced luteolysis (Woclawek-Potocka et al. 2009). Nonetheless knock-down of LPAR1 in a mouse model shows no adverse effects on reproduction (Ye et al. 2005), and as its expression did not vary a large amount between stages of the

menstrual cycle, it is possible that it plays a largely redundant role in the normal endometrium.

As expression of LPAR1,2 and 3 has been documented in many different cancer types including breast and ovarian (Nakamoto et al. 2005; Chen et al. 2007; Yu et al. 2008), the expression of these LPA receptors was examined in endometrial adenocarcinoma. No difference in LPAR1 expression was detected between normal endometrium and endometrial adenocarcinoma. In endometrial adenocarcinoma, LPAR1 expression could be localised to the neoplastic epithelial cells, in the stroma and to what appears to be endothelial cells. LPAR1 expression has been previously demonstrated on cultured vascular endothelial cells (Lee et al. 2000). In the stroma, the cell types expressing LPAR1 are unclear however expression of the receptor has been documented on a number of cell types which make up the stroma of endometrial adenocarcinoma including macrophages, neutrophils and lymphocytes (Zheng et al. 2000; Hornuss et al. 2001; Graler et al. 2002; Tou et al. 2005). LPAR1 expression in other cancer types promotes a variety of effects contributing toward tumour progression. In gastrointestinal cancer, LPAR1 was shown to be the main LPA receptor involved in mediating LPA-induced cell migration via an ERK 1/2 signalling pathway (Shin et al. 2009). In prostate cancer, LPAR1 over-expression resulted in increased cell proliferation *in vitro* and increased tumour growth *in vivo*. This occurred independently of ERK 1/2 signalling, demonstrating the complex downstream signalling of LPA receptors (Guo et al. 2006). In breast cancer cell lines, LPA treatment can also increase migration through LPAR1 (Chen et al. 2007).

Expression of LPAR2 and LPAR3 in normal endometrium was significantly increased in the early secretory phase of the menstrual cycle, just before the window of implantation at the mid-secretory phase of the cycle. Immunohistochemical analysis of LPAR2 also reflected the mRNA data. The involvement of LPAR3 in implantation has been previously suggested as it is upregulated by progesterone and down-regulated by oestrogen in the mouse (Hama et al. 2006), and the deletion of LPAR3 in mice led to aberrant embryo implantation (Ye et al. 2005).

In endometrial adenocarcinoma, LPAR2 expression was predominantly expressed in the stroma, with weak localisation to the epithelial cells. LPAR2 mRNA has been previously found to be increased in endometrial cancer tissue (Yun et al. 2005), and in a moderately differentiated endometrial adenocarcinoma cell line (Hope et al. 2009). This supports the finding in this chapter that LPAR2 mRNA is increased in moderately differentiated cancer as compared to well or poorly differentiated cancer, although this difference was not statistically significant. In the study by Hope et al (Hope et al. 2009), LPA signalling through LPAR2 significantly increased endometrial adenocarcinoma cell invasion, which was dependent on an up-regulation of matrix metalloprotease (MMP)-2 and MMP-7 expression (Hope et al. 2009). In other cancer types, LPAR2 has been shown to promote tumour progression through other mechanisms. In colon cancer for example, over-expression of LPAR2 increased in ERK1/2 phosphorylation, leading to an up-regulation of the angiogenic chemokine IL-8 (Yun et al. 2005).

Localisation of LPAR3 in endometrial adenocarcinoma followed a similar expression pattern to that of LPAR1 and 2, with strong expression seen in the neoplastic epithelial cells, stroma and endothelial cells. One of the cancer types studied extensively in relation to LPA signalling is ovarian cancer, due to the high concentration of LPA found in the ascites of ovarian cancer patients (Shen et al. 1998). LPAR2 and LPAR3 both display increased expression in ovarian cancer (Nakamoto et al. 2005; Jeong et al. 2008). LPA treatment increased ovarian cancer cell line migration and invasion, an effect that could be abolished with siRNA against LPAR2 (Jeong et al. 2008). LPA signalling also promotes VEGF expression in ovarian cancer cells (Hu et al. 2001). The knockdown of LPAR2 or LPAR3 in ovarian cancer cells has also been reported to decrease LPA-induced expression of angiogenic factors including VEGF, and over-expression of these same two receptors increased invasiveness of ovarian cancer xenografts in mice (Yu et al. 2008). Interestingly, a link between LPA signalling and CXCL1 expression has been

previously described in this cancer type, as LPA signalling via LPAR2 increased CXCL1 expression in ovarian cancer cell lines (Lee et al. 2006).

Collectively, LPAR1-3 mediate effects in cancer ranging from promotion of cell proliferation and migration to production of angiogenic factors. However the LPA receptors which are over-expressed and mediate LPA signalling differ dependent on the cancer type. A significant increase in LPAR2 and 3 mRNA expression was seen in endometrial adenocarcinoma. Therefore these receptors may play a role in LPA signalling in this cancer type. Enzymes in the LPA-producing pathway, including ATX, are elevated in the ascites of patients with endometrial cancer (Tokumura et al. 2007), and have been shown to promote tumour progression in other models of cancer (Nam et al. 2001). Therefore this indicates that the availability of LPA in endometrial adenocarcinoma may lead to increased signalling, and the up-regulation of genes targeted by LPA.

In our Ishikawa endometrial adenocarcinoma cell line model (FPS cells), LPA treatment upregulated FP receptor mRNA expression. LPA signalling has been previously demonstrated to promote COX-2 upregulation and prostaglandin synthesis (Ye et al. 2005; Jeong et al. 2008; Liszewska et al. 2009; Woclawek-Potocka et al. 2009; Woclawek-Potocka et al. 2009). However a direct effect of LPA signalling on prostaglandin receptor expression has not previously been described. It remains to be determined if an up-regulation in FP receptor protein is seen in this cell type after LPA treatment. In addition, treatment with LPA resulted in a significant increase in CXCL1 and CCL20 expression. However, no significant increase in COX-2 or $PGF_{2\alpha}$ synthesis was observed in response to LPA treatment. This suggests that in our cell line model LPA induction of FP receptor, CXCL1 and CCL20 expression was independent of $PGF_{2\alpha}$ secretion or FP receptor activation. Indeed, cells treated with LPA and the FP antagonist AL8810 did not alter LPA mediated gene expression.

Therefore, in a physiological or pathological setting *in vivo*, LPA may increase expression of the FP receptor which can be activated by endogenous enhanced synthesis of $PGF_{2\alpha}$. Concomitant co-activation of the LPA and FP receptors (by LPA and $PGF_{2\alpha}$ respectively) may enhance the expression of common chemokines such as CXCL1 and CCL20. LPAR1-3 activation leading to increased cytokine expression has been previously described for the chemokines IL-6, IL-8 and CXCL1 (Lee et al. 2006; Chen et al. 2008). This would thereby lead to an exacerbated inflammatory phenotype. A schematic representation of this is shown in Figure 6.12, with pathways regulated by $PGF_{2\alpha}$ displayed in blue and those regulated by LPA displayed in green.

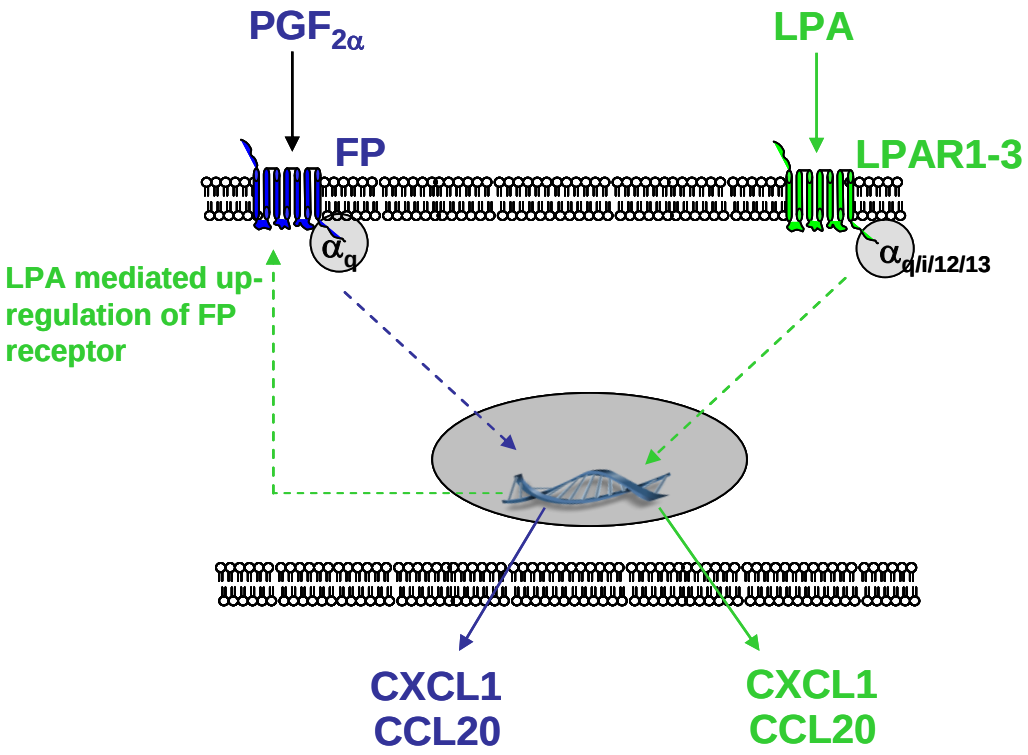

Figure 6.12 Schematic representation of possible $PGF_{2\alpha}$ and LPA signalling interactions in endometrial adenocarcinoma. $PGF_{2\alpha}$ signalling (shown in blue) activates the FP receptor to up-regulate expression of the chemokines CXCL1 and CCL20. LPA signalling (shown in green) activates the LPA receptors to also up-regulate expression of the chemokines CXCL1 and CCL20. LPA signalling additionally up-regulates expression of the FP receptor, further promoting inflammatory pathways in endometrial adenocarcinoma.

Data presented in this chapter have described a novel pathway mediated by LPA which may amplify $PGF_{2\alpha}$-FP receptor signalling in endometrial adenocarcinoma. LPAR1-3 are expressed in endometrial adenocarcinoma and both LPAR2 and 3 are upregulated as compared to normal endometrium. LPA may therefore stimulate tumour progression in endometrial adenocarcinoma by two mechanisms; firstly by an increase in FP mRNA expression which may increase FP signalling, and secondly by acting directly to up-regulate expression of the chemokines CXCL1 and CCL20, which have been previously discussed in this thesis. As both FP and LPA signalling pathways can converge on these chemokines, future studies are required to determine if a synergistic effect of these two lipid mediators can promote progression of endometrial adenocarcinoma.

Chapter 7

General Discussion

7 General Discussion

Elevated expression of the prostaglandin-forming enzyme COX-2 has been demonstrated in several epithelial cell cancer types including breast, stomach, bladder and lung (Cha et al. 2007). It is also elevated in endometrial adenocarcinoma (Tong et al. 2000), as is expression of the FP receptor (Sales et al. 2004). A role for the FP receptor and prostaglandins in the progression of endometrial adenocarcinoma has been hypothesised, as $PGF_{2\alpha}$–FP receptor signalling up-regulates expression of angiogenic genes (Sales et al. 2005; Sales et al. 2007). It also promotes cell migration and proliferation (Milne et al. 2003; Sales et al. 2008). The work described in this thesis investigated the contribution of the FP receptor to inflammatory pathways in endometrial adenocarcinoma.

Data from epidemiological studies propose that chronic inflammation pre-disposes to cancer development. For example inflammatory bowel disease has been associated with an increased risk of developing colorectal cancer (Flossmann et al. 2007), and persistent inflammation of the prostate has been associated with an increased risk of prostate cancer (Sandhu 2008). Chronic inflammation can therefore drive tumour initiation, and inflammation also promotes tumour progression through the up-regulation of chemokines and the presence of leukocytes (Mantovani et al. 2008). Inflammatory conditions also lead to tissue remodelling within the tumour microenvironment. For instance a number of chemokines have angiogenic properties (Strieter et al. 2006), and leukocytes have been demonstrated to produce proteases which can increase cancer cell invasion and metastasis (Ardi et al. 2007; Yang et al. 2007).

Many of the risk factors for development of endometrial adenocarcinoma promote prolonged exposure of the endometrium to oestrogen, which is an established cause of endometrial adenocarcinoma (Modugno et al. 2005). However these risk factors are also known to be linked to inflammatory pathways. For example, obesity is one of the major risk factors for endometrial adenocarcinoma (Reeves et al. 2007), and

may increase likelihood of cancer development due to high rates of oestrogen formation in excess adipose tissue (Akhmedkhanov et al. 2001). Excess adipose tissue also leads to increased levels of pro-inflammatory cytokines such as IL-6 and TNFα (Bastard et al. 2000). These pro-inflammatory cytokines have been shown to activate signalling pathways mediating angiogenesis and invasion in endometrial adenocarcinoma (Seo et al. 2004; Choi et al. 2009), and elevated levels of these cytokines have been associated with a poor prognosis (Bellone et al. 2005). Therefore inflammatory signalling pathways may be acting in conjunction with oestrogen exposure to increase risk of endometrial adenocarcinoma. Non-steroidal anti-inflammatory drug (NSAID) intake in patients at risk of developing cancers significantly reduces cancer occurrence (Dannenberg et al. 2003). These drugs inhibit the cyclo-oxygenase (COX) enzymes, preventing prostaglandin synthesis and signalling. The role of the receptor for $PGF_{2\alpha}$, FP, in cancer has not previously been extensively studied.

In this chapter, the main findings of this thesis are summarised. Future research directions that could further investigate the mechanisms by which the $PGF_{2\alpha}$ and LPA signalling pathways contribute to inflammation in endometrial adenocarcinoma are also considered.

7.1 $PGF_{2\alpha}$ regulates CXCL1 expression in endometrial adenocarcinoma

A cytokine antibody array was used initially in this thesis to investigate cytokines up-regulated by the $PGF_{2\alpha}$-FP receptor signalling pathway. The chemokine CXCL1 displayed increased expression in an endometrial adenocarcinoma cell line model after $PGF_{2\alpha}$ treatment, and this signalling pathway was confirmed in endometrial adenocarcinoma tissue explants. CXCL1 expression was also found to be elevated in endometrial adenocarcinoma as compared to normal endometrium.

The elevation of CXCL1 expression in cancer as compared to normal tissue has also been demonstrated in melanoma, colorectal and prostate cancer (Haghnegahdar et al.

2000; Wang et al. 2006; Wen et al. 2006; Kawanishi et al. 2008; Rubie et al. 2008). CXCL1 expression was regulated via an ERK dependent signalling pathway. This is similar to a study of colorectal cancer demonstrating CXCL1 expression mediated by the prostaglandin PGE_2 and ERK activation (Wang et al. 2006). Inhibition of components of the ERK signalling pathway is effective in reducing cancer growth in *in vivo* models of cancer (Iverson et al. 2009). Indeed, a specific inhibitor of MEK, the protein directly upstream of ERK, has entered clinical trials in late-stage cancer patients (Iverson et al. 2009). In endometrial adenocarcinoma, the receptor for CXCL1, CXCR2, was localised to neoplastic epithelial cells. Therefore autocrine signalling is possible. In colorectal cancer and melanoma, autocrine CXCL1-CXCR2 signalling increases cell proliferation and migration (Li et al. 2004; Singh et al. 2009). Thus investigating this further in endometrial adenocarcinoma could determine if this is one mechanism by which CXCL1 promotes tumour progression.

7.1.1 Impact of CXCL1 expression in endometrial adenocarcinoma on angiogenesis

The up-regulation of chemokines in cancer is believed to drive leukocyte influx and tissue remodelling. In other cancer types, CXCL1 has been shown to promote tissue remodelling by increasing angiogenesis. This is achieved by signalling directly to its receptor, CXCR2, which is expressed on endothelial cells. CXCL1 induced angiogenesis and increased tumour growth in mouse models of melanoma (Moore et al. 1999), lung cancer (Keane et al. 2004) and prostate cancer (Singh et al. 2009). In our study, CXCR2 expression was localised to endothelial cells in endometrial cancer. Therefore the effects of FP-mediated CXCL1 expression on angiogenesis were examined in this thesis by the measurement of microvascular density (MVD) in xenografts arising from the inoculation of nude mice with FPS cells. These were compared to xenografts arising from WT cells, or xenografts arising from FPS cells in mice treated with CXCL1 antibody. No significant difference in MVD was shown between the three groups.

Measurement of MVD in tumours is frequently used to investigate angiogenesis. In numerous immunohistochemical studies of tumours, an increase in MVD has been associated with increased severity of disease (Weidner et al. 1993; Weidner 1995; Mentzel et al. 2001). However, evidence has shown that measuring MVD to establish the angiogenic capability of one tumour-derived factor is not effective (Hlatky et al. 2002). This may be because tumour angiogenesis is the net product of the balance of pro and anti-angiogenic factors. For example, although VEGF and FGF are known to be key factors contributing to tumour angiogenesis, many studies have not found an association between expression levels of these proteins and MVD (Terris et al. 1998; Smith et al. 1999). A recent study found no association between VEGF expression and MVD in endometrial adenocarcinoma (Erdem et al. 2007). The vasculature present in tumours develops rapidly and is therefore frequently abnormal in structure, and can be ineffective in oxygen transportation and lead to hypoxia (Stefansson et al. 2006). Therefore MVD may not always be an accurate measure of rates and efficacy of tumour angiogenesis. In endometrial adenocarcinoma, measurement of vascular proliferation was in fact shown to be more indicative of aggressiveness of tumour and adverse prognosis than assessment of MVD (Stefansson et al. 2006).

In the xenograft study described in Chapter 4 of this thesis, the structure of vessels was altered between FPS, WT and CXCL1-neutralised groups (R Catalano, personal communication). Any alterations in vessel structure and integrity were not analysed within this thesis. Therefore future research should examine the impact of FP receptor expression on changes to the structure of blood vessels, which may be indicative of an alteration in angiogenesis. This could be examined by analysing the density of surrounding perivascular cells. A loose arrangement of perivascular cells is associated with rapid angiogenesis, and has also been described as indicative of integrity and maturity of vessels (Bergers et al. 2003). Vascular endothelial cell proliferation could also be measured as this has previously been correlated with both increased levels of angiogenic factors and a poor prognosis in endometrial adenocarcinoma (Hlatky et al. 2002; Stefansson et al. 2006). These studies would

assist in elucidation of the angiogenic activities of $PGF_{2\alpha}$ induced CXCL1 in endometrial adenocarcinoma.

7.1.2 Impact of CXCL1 and cytokine expression in endometrial adenocarcinoma on immune cell infiltration

CXCL1 is a potent neutrophil chemoattractant (Moser et al. 1990). Studies presented in Chapter 4 of this thesis demonstrated that CXCL1 expression mediated by $PGF_{2\alpha}$-FP receptor signalling increased neutrophil chemotaxis and infiltration into endometrial cancer xenografts. Neutrophils were also increased in human endometrial adenocarcinoma tissue as compared to normal endometrium. In cancer, the up-regulation of chemokine expression by neoplastic cells is proposed to chemoattract cells of the innate immune system (de Visser et al. 2006). These cells have been demonstrated to contribute to tumour progression. Neutrophils promote cancer growth by producing angiogenic factors such as VEGF and C-X-C chemokines (Scapini et al. 2004; Ai et al. 2007). They also secrete proteases which may aid tissue remodelling and metastasis (Ardi et al. 2007). Despite an increase of neutrophil infiltration into tumour xenografts mediated by FP receptor signalling, no significant difference in tumour size was observed between the different xenograft groups. Furthermore, in human endometrial adenocarcinoma tissue, increased neutrophil numbers did not correlate with grade of cancer. Thus, although the role of the FP receptor in mediating neutrophil infiltration has been demonstrated, it is impossible to tell from data presented within this thesis if neutrophils are performing pro or anti-tumourigenic functions in endometrial adenocarcinoma.

Therefore, future research could be directed into understanding the role of neutrophils in endometrial adenocarcinoma. Neutrophils have been demonstrated to promote angiogenesis in normal tissues and pathologies, and the production of VEGF by neutrophils has been described in the normal endometrium (Gargett et al. 2001). Increased neutrophil numbers have been correlated with increased vasculature and grade of tumour in myxofibrosarcoma (Mentzel et al. 2001), and a number of experimental *in vitro* models have also demonstrated the angiogenic capability of

neutrophils. For example, pro-inflammatory cytokines often elevated in cancers such as TNF-α and IL-6 induce VEGF release from neutrophils (McCourt et al. 1999). A more complex interaction has been described in breast cancer. Cytokines secreted by breast cancer cells have been demonstrated to induce production of the cytokine oncostatin M from neutrophils, which reciprocally induces VEGF expression by the neoplastic epithelial cells (Queen et al. 2005). Furthermore, the production of MMP-9 by neutrophils is well described (Coussens et al. 2000; Ardi et al. 2007) and in the chick angiogenesis assay this MMP-9 was demonstrated to be crucial in the induction of angiogenesis (Ardi et al. 2007).

As discussed above, no difference in MVD was observed in our xenograft endometrial adenocarcinoma model, indicating that in this model neutrophils may not be contributing to angiogenesis. However, future research into the angiogenic properties of neutrophils in endometrial adenocarcinoma is warranted due to the body of evidence demonstrating neutrophil mediated angiogenesis in other cancer types. In the normal endometrium, an immunohistochemical study has correlated neutrophils expressing VEGF to areas of proliferating endothelial cells (Gargett et al. 2001). Similar assessments of the physical relationship between neutrophils, VEGF expression and the vasculature in endometrial adenocarcinoma could indicate the contribution of neutrophils to angiogenesis. Subsequently, the functions of neutrophils in endometrial adenocarcinoma should be examined *in vitro*. To investigate this, flow cytometry could be used to isolate these cells from human endometrial tissue and analysis of gene and protein expression could then indicate if factors produced by neutrophils are pro or anti-angiogenic. It is also possible that interactions between the neoplastic epithelial cells and neutrophils could synergistically promote tumour growth, as has been demonstrated by interactions between macrophages and lung carcinoma cells (Kimura et al. 2007) and between neutrophils and breast cancer cells discussed previously (Queen et al. 2005). To investigate this, co-cultures of neutrophils with our endometrial adenocarcinoma cell line model could be used.

Neutrophils also influence tumour progression through the promotion of invasion and metastases. This has been demonstrated in *in vivo* models of cancer. The depletion of neutrophils in a mouse model of fibrosarcoma prevented metastasis of cells from the primary tumour, suggesting a role for neutrophils in the switch to a metastatic phenotype (Tazawa et al. 2003). Equally, an increase in invasion of breast cancer xenografts was associated with an increased infiltration of neutrophils mediated by IL-8 over-expression. Invasion was increased due to elevated protease production by neutrophils (Yao et al. 2007). Therefore, future research into the role of neutrophils in endometrial adenocarcinoma could also be directed toward their role in cancer cell migration. To investigate this, it would be valuable to use an alternative xenograft mouse model of endometrial adenocarcinoma in which metastases can be measured (Vollmer 2003). The metastatic ability of endometrial adenocarcinoma cells could then be investigated with the depletion of neutrophils from the mouse, for example, as described in the paper by Kaitu'u-Lino et al (2007).

Several other cytokines were shown to be upregulated by the $PGF_{2\alpha}$-FP signalling pathway in the antibody array used in Chapter 1 of this thesis, including the chemokine IL-8. IL8 is an ELR+ C-X-C chemokine which acts as a potent neutrophil chemoattractant by binding to CXCR2 and the structurally related CXCR1. Therefore IL-8 may play a similar role in endometrial adenocarcinoma to that described in this thesis for CXCL1. In the normal endometrium, IL-8 is thought to recruit neutrophils during the menstrual cycle (Critchley et al. 1994). It has also been demonstrated to promote angiogenesis in breast cancer (Yao et al. 2007). Dependent on the cancer type, the relative role that different C-X-C chemokines play in tumour development differs (Moore et al. 1999). Therefore it would be interesting to determine if IL-8 is produced by a similar signalling mechanism to that of CXCL1 in endometrial cancer cells. As neutralisation of CXCL1 in FPS xenografts did not bring neutrophil infiltration down to the basal levels seen in the WT xenografts, it may be that this extra neutrophil infiltration was dependent on IL-8. Hence, studies with neutralisation of both CXCL1 and IL-8 may be informative about the

contribution of these two chemokines to the inflammatory environment in endometrial adenocarcinoma.

Additional cytokines which were elevated after activation of the FP receptor in our endometrial adenocarcinoma cell line model were IL-6, IL-7, CXCL2, CXCL3 and leptin. These cytokines are also likely to play a role in inflammation in endometrial adenocarcinoma. For example, the receptor for IL-6 is expressed on macrophages and T cells. Activation of this receptor can cause cytokine secretion by these cells, which may be pro or anti-tumorigenic. IL-6 signalling can also promote apoptosis of cancer cells (Rose-John et al. 2007); however elevated expression has been linked with increased aggressiveness of endometrial adenocarcinoma (Bellone et al. 2005). Leptin has been shown to regulate expression of angiogenic factors including VEGF in endometrial adenocarcinoma cell lines, indicating it may contribute to tissue remodelling of the microenvironment (Carino et al. 2008). Therefore, the regulation of these cytokines by the $PGF_{2\alpha}$-FP signalling pathway warrants further investigation, to determine if all cytokines regulated by this pathway contribute to tumour progression.

Furthermore, a result of this cytokine secretion may be increased immune cell influx, as de Visser et al (2006) described the influx of innate immune cells into a tumour to be driven by cytokine secretion. Immune cell infiltration into endometrial adenocarcinoma was examined in this thesis. In addition to neutrophils, significantly elevated numbers of macrophages and dendritic cells were observed. These innate immune cells have all been demonstrated to promote angiogenesis in different cancer types (Scapini et al. 2004; Lin et al. 2007; Fainaru et al. 2008), and therefore may be promoting tumour growth in endometrial adenocarcinoma.

NK cells were observed at significantly lower numbers in endometrial adenocarcinoma as compared to normal endometrium. These cells can secrete cytokines which lyse tumour cells (Zamai et al. 2007), therefore the low infiltration observed in endometrial adenocarcinoma may imply low levels of leukocyte-

mediated tumour cell death. No significant difference was observed in influx of CD4+ T cells, CD8+ T cells or B cells between normal and cancerous endometrial tissue. T cells have been frequently reported to be cytotoxic to tumour cells, as a high T cell infiltration has been associated with a good prognosis (Kondratiev et al. 2004). This is because T cells are able to recognise antigens present on cancer cells in order to destroy them (Nishimura et al. 1999). The role of B cells in cancer is unclear, as they have been demonstrated to lead to tumour cell destruction (de Visser et al. 2006) but also tumour promotion by the chemoattraction of innate immune cells such as macrophages and neutrophils (de Visser et al. 2005). The comparable levels of these cells in endometrial adenocarcinoma to normal endometrium suggest an ineffective cytotoxic immune response in endometrial adenocarcinoma. This would enable tumour growth. However, in this thesis these cells have been compared in endometrial cancer to endometrial tissue of a normal cycling uterus. Most patients with endometrial adenocarcinoma are post-menopausal. It would therefore be worthwhile examining immune cell numbers by flow cytometry in endometrial tissue adjacent to the tumour in the same patient, to determine more accurately the levels of immune cells in normal and cancerous tissue. In this situation, after isolation these immune cells could be cultured *in vitro* to investigate cell function, to determine pro or anti-tumourigenic functions. Leukocytes can make up to 50% of the tumour microenvironment (Colotta et al. 2009). Therefore determining their function in endometrial adenocarcinoma would be valuable to investigate downstream consequences of inhibiting inflammatory cytokine pathways mediated by FP.

7.2 $PGF_{2\alpha}$ regulates CCL20 expression in endometrial adenocarcinoma

The roles of chemokines in cancer beyond their ability to chemoattract immune cells has been expanded with evidence that they promote tissue remodelling by influencing cell proliferation and migration (Slettenaar et al. 2006). In this thesis, the regulation of expression of the chemokine CCL20 by $PGF_{2\alpha}$–FP receptor signalling was described in an endometrial adenocarcinoma cell line and confirmed in endometrial adenocarcinoma explants. The elevated expression of CCL20 and its

receptor CCR6 in endometrial adenocarcinoma as compared to normal endometrium was described, and activation of the CCR6 receptor on endometrial epithelial cells was found to promote cell proliferation.

CCL20 signalling plays an important role in the chemoattraction of immature dendritic cells to sites of inflammation (Schutyser et al. 2003). However, as CCR6 expression was not demonstrated on dendritic cells in endometrial adenocarcinoma the chemoattraction of dendritic cells by CCL20 may not be impacting on tumour growth. Like many other chemokines, the expression of CCL20 and CCR6 has also been described in several different types of cancer. CCL20 is over-expressed in cancers of the colon (Brand et al. 2006) liver (Rubie et al. 2006), pancreas (Campbell et al. 2005), prostate (Ghadjar et al. 2008) and Hodgkins lymphoma (Baumforth et al. 2008). CCR6 is also upregulated in colon (Brand et al. 2006), liver (Dellacasagrande et al. 2003) and prostate cancer (Ghadjar et al. 2008). In these cancer types, CCL20 expression promotes both cell migration (Dellacasagrande et al. 2003; Campbell et al. 2005; Rubie et al. 2006) and proliferation (Brand et al. 2006; Beider et al. 2009). CCL20 caused a significant increase in proliferation of endometrial adenocarcinoma cells. This is similar to published literature describing autocrine CCL20-CCR6 signalling of colorectal and prostate cancer cell lines which promoted cell proliferation (Brand et al. 2006; Beider et al. 2009). Cell migration was not affected in endometrial adenocarcinoma cells by CCR6 activation.

These data demonstrate the pleiotropic nature of chemokines in tumour progression, as CXCL1 increased immune cell infiltration while CCL20 is able to signal directly to neoplastic epithelial cells to promote proliferation. The regulation of CCL20 by $PGF_{2\alpha}$ was also mediated through an ERK dependent signalling pathway, similarly to CXCL1. Therefore, the effects of inhibiting FP activation may impact on many facets of inflammation. In addition to an inhibition of chemokine expression and leukocyte infiltration, a decrease in tissue remodelling mediated by angiogenesis and cell proliferation may be observed with decreased $PGF_{2\alpha}$-FP signalling.

7.3 The role of LPA signalling in endometrial adenocarcinoma

Data presented in this thesis describes the signalling pathways activated by $PGF_{2\alpha}$-FP receptor interactions, and their downstream consequences in endometrial adenocarcinoma. In a physiological setting, activation of other signalling cascades will influence the overall signalling outcome in a cell. LPA is a membrane derived lipid mediator, which has been proposed to play an important role in implantation in the normal endometrium (Ye et al. 2005). It has also been demonstrated to contribute to tumour progression in numerous cancers including breast (Kitayama et al. 2004), colon (Yun et al. 2005) and ovarian (Yu et al. 2008). In Chapter six, it was demonstrated that the receptors for LPA, LPAR1, 2 and 3, are expressed at all stages of the menstrual cycle in the endometrium. The expression of LPAR2 and LPAR3 was elevated in endometrial adenocarcinoma as compared to normal endometrium. LPA signalling increased expression of the chemokines CXCL1 and CCL20 in endometrial adenocarcinoma cells. An elevation of FP receptor mRNA expression was also mediated by LPA signalling, independently of $PGF_{2\alpha}$ signalling. These data demonstrate a convergence of the $PGF_{2\alpha}$ and LPA signalling pathways.

LPA signalling contributes to cancer progression. Activation of the LPA receptors promotes cell proliferation in ovarian, prostate and breast cancer (Shida et al. 2005; Guo et al. 2006; Yu et al. 2008), and migration in gastrointestinal and ovarian cancer (Jeong et al. 2008; Shin et al. 2009). In addition to tissue remodelling of the tumour microenvironment, a number of factors regulated by LPA signalling are inflammatory. For example, LPA signalling promotes the release of chemokines including IL-8 (Chen et al. 2008) and CXCL1 in ovarian cancer (Lee et al. 2006). LPA signalling also interacts with the COX inflammatory pathway by elevating prostaglandin and COX-2 synthesis (Liszewska et al. 2009; Woclawek-Potocka et al. 2009; Woclawek-Potocka et al. 2009).

The preliminary study of LPA signalling in endometrial adenocarcinoma presented in this thesis therefore brings up numerous questions. Firstly, the receptor type mediating LPA signalling in endometrial adenocarcinoma cells is unclear. LPAR3

signalling is important in reproduction (Ye et al. 2005), however mRNA and protein expression of all three receptors was found in endometrial adenocarcinoma. Expression of both LPAR2 and 3 was significantly increased in endometrial adenocarcinoma as compared to normal tissue, indicating that these receptors may be involved in mediating signalling pathways activated by LPA. The receptor which mediates the effects of LPA may determine the functional outcome. For example LPAR3 does not couple to the G-protein $G_{12/13}$, which mediates movement of cells through activation of the protein Rho. Thus LPA mediated promotion of migration is achieved through activation of LPAR1 or 2 (Ishii et al. 2000). Invasion of endometrial cancer cells was previously shown to be increased by LPAR2 activation (Hope et al. 2009); therefore it is likely this receptor plays an important role in mediating the effects of LPA signalling in endometrial cancer. Future research into the role of LPA in endometrial adenocarcinoma should examine the importance of each of the LPA receptors in LPA signalling. To achieve this, silencing of each receptor independently would need to be achieved. Some LPAR antagonists exist, including pan-LPAR antagonists (Jan et al. 2003; Ohta et al. 2003) and some newly discovered antagonists selective for LPAR2 and LPAR3 (Fischer et al. 2001; Beck et al. 2008; Fells et al. 2008).

The outcome of LPA signalling is mediated by diverse signalling pathways downstream of receptor activation. For example, NFκB activation was essential for LPA-mediated IL-8 production in endometrial stromal cells (Chen et al. 2008), and in ovarian cancer cells ERK signalling was essential for LPA-driven COX-2 production and cell migration (Jeong et al. 2008). In this thesis, LPA signalling was demonstrated to up-regulate expression of the chemokines CXCL1 and CCL20. Further research could therefore determine if the regulation of these chemokines converges on the same signalling pathways as those mediated by the FP receptor. This could be achieved by use of chemical inhibitors to identify other components of the signalling pathway. If LPA and $PGF_{2\alpha}$ signal via the same pathway in endometrial adenocarcinoma cells, it would be valuable to determine if $PGF_{2\alpha}$ and LPA signalling display an additive effect on chemokine production. Inhibition of

both of these pathways may be required to significantly decrease inflammation in endometrial adenocarcinoma.

Furthermore, the downstream consequences of LPA signalling in endometrial adenocarcinoma should be investigated. As discussed in Chapter 6, LPA signalling inreases cell proliferation and migration in many cancer types. In addition to *in vitro* studies on endometrial adenocarcinoma cell lines, the nude mouse xenograft model used in this thesis could be employed to examine tumour growth regulated by LPA signalling. By xenografting cells with depleted LPA receptors the downstream consequences of signalling through each receptor may be ascertained. Interactions with the FP receptor signalling pathway could be further investigated by simultaneously silencing FP receptor expression. Finally, immunohistochemistry identified expression of LPAR on stromal cells which may be leukocytes. Previous evidence suggests that LPA signalling to its receptor on leukocytes causes release of inflammatory mediators rather than the chemoattraction of leukocytes (Zheng et al. 2000; Hornuss et al. 2001; Tou et al. 2005). Therefore this could be another mechanism by which LPA promotes inflammation in endometrial cancer, and warrants further investigation.

7.4 Conclusions: The FP receptor and inflammation in endometrial adenocarcinoma

Evidence presented in this thesis suggests that signalling via the FP receptor in endometrial adenocarcinoma promotes inflammation by the increase of chemokine expression, the infiltration of immune cells, and tissue remodelling in the form of cell proliferation. The up-regulation of FP receptor mRNA expression mediated by LPA and the effects of LPA signalling on chemokine expression serve to demonstrate that other factors may amplify these signalling pathways in endometrial adenocarcinoma, further contributing to an inflammatory environment.

As described in Chapter 1, inflammation in cancer is proposed to function by two pathways (Colotta et al. 2009). In the extrinsic pathway, local inflammation

promotes malignant transformation of the tissue (Flossmann et al. 2007; Sandhu 2008). In the intrinsic pathway, genetic changes give rise to cancerous cells which leads to the up-regulation of inflammatory pathways promoting tumour progression and growth. In the endometrium, the insertion of contraceptive intra-uterine devices promotes a local environment of inflammation, with an infiltration of leukocytes and increase in prostaglandin expression (Srivastava et al. 1989). However, users of this contraceptive method do not display a higher incidence of endometrial adenocarcinoma (Beining et al. 2008). Additionally, prospective studies have now been carried out examining the effects of NSAID use on development of endometrial adenocarcinoma. In general, these have shown that use of NSAIDs does not significantly influence risk of developing endometrial adenocarcinoma (Viswanathan et al. 2008; Danforth et al. 2009) except in some small subgroups of very obese women (Viswanathan et al. 2008; Fortuny et al. 2009). A retrospective study also displayed these results (Moysich et al. 2005).

These epidemiological data suggest that in endometrial cancer, it may generally be the intrinsic pathway which is important in initiation of tumour development. This implies that cancer development is initiated by genetic mutations, possibly in stem cells (Rutella et al. 2009), causing uncontrolled proliferation and subsequently cellular changes such as elevated FP receptor expression. These genetic changes may be a result of increased proliferation driven by an increased oestrogen to progesterone ratio in endometrial adenocarcinoma (Jazaeri et al. 2001). In further support of the intrinsic pathway, a number of genetic mutations such as those of PTEN and K-Ras have been associated with the development of endometrial adenocarcinoma (Enomoto et al. 1990; Tashiro et al. 1997). The deletion of PTEN in the endometrium of a mouse model rapidly leads to endometrial adenocarcinoma development. In the initial stages of hyperplasia and tumour development in this model, COX-2 was highly upregulated, implicating this pathway in enhancing tumour growth (Daikoku et al. 2008). Therefore cancer will develop by mutation, and the COX-2 signalling pathway, and those described in this thesis driven by the FP receptor will enhance and promote inflammation. This further facilitates cancer

growth and the acquisition of more hallmarks of cancer by the cells. This is consistent with the theory that low level chronic inflammation, or "smouldering" inflammation, can promote tumour growth more than acute "polarised" inflammation which may be self-limiting (Balkwill et al. 2005).

Endometrial adenocarcinoma at advanced stages has a poor prognosis (Fleming et al. 2004). By reducing inflammation, it is possible that late-stage tumour growth or further metastases may be prevented. The use of specific COX-2 inhibitors in cancer trials has been halted since the discovery of cardiovascular side effects after repeated intake (Cha et al. 2007). Therefore there has been increased interest in inhibiting other targets in the COX-2-prostaglandin signalling pathway by targeting the prostaglandin receptors (Rocca 2006). Currently, it is thought that cancer treatment will necessitate targeting numerous pathways in the tumour, to treat all the hallmarks of cancer (Hanahan et al. 2000). Therefore, antagonists of the FP receptor may be a further target to reduce inflammation and consequent processes driving tumour progression in endometrial adenocarcinoma.

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Appendix 1: Publications

A selection of the work from Chapters 3 and 4 has been published in a paper in Cancer Research, and has been included in this thesis with permission.

Wallace,A.E, Sales, K.J, et al. (2009). "Prostaglandin $F_{2\alpha}$-F-prostanoid receptor signalling promotes neutrophil chemotaxis via chemokine (C-X-C motif) ligand 1 in endometrial adenocarcinoma." *Cancer Res* **69** (14): 5726-33