Investigation of uterine blood vessel development in Heavy Menstrual Bleeding

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Ma your courage, hard work and perseverance and Baba your persistence, determination and devotion, I shall always endeavour to equal.

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Abstract

Heavy menstrual bleeding (HMB) affects 30% of women of reproductive age, accounting for two-thirds of all hysterectomies. 50% HMB cases are unexplained and current treatment options often compromise fertility. Understanding the mechanisms underlying HMB is therefore critical to delineate potential pathways for therapeutic intervention. Previous studies suggested structural and functional roles for endometrial blood vessels in the pathogenesis of HMB. Endothelial cells (ECs) are associated with growth and formation of blood vessels; extracellular matrix (ECM) provides the framework for maintaining vascular structure, while vascular smooth muscle cell (VSMC) differentiation regulates blood flow and pressure. Altered endometrial vascular maturation is seen in recurrent miscarriage in association with increased uterine natural killer (uNK) cell number and variation in endometrial angiogenic growth factor (AGF) expression, but these processes have not been extensively studied in HMB. This study aimed to assess vascular maturation and AGF expression in HMB and investigate some of their effects *in vitro*.

A study of VSMC differentiation revealed reduced endometrial calponin expression, suggesting a dysfunctional vascular contraction mechanism in HMB. This was associated with increased osteopontin expression, supporting previous evidence of regulation of vascular calponin expression by osteopontin. Endometrial collagen IV expression was lower in HMB, reflecting weaker vascular structure and definition. An altered pattern of uNK cell density in HMB may reflect altered *in situ* differentiation and or proliferation, which could impact vascular development and/or endometrial preparation for menstruation. VSMCs showed decreased endometrial expression of PDGFRα and TGFβRI, highlighting altered state of VSMC differentiation and potentially dysregulated endometrial vascular development in HMB. Furthermore, preliminary results indicated that PDGFBB helped to maintain EC ‘honeycomb’ structures, while anti-PDGFBB or TGFβ1 treatment decreased their numbers *in vitro*.

Collectively, this study has highlighted key alterations in blood vessels in HMB, and established an *in vitro* model for future functional studies of blood vessel development.

Publications

Manuscripts

- *Endometrial vascular development in heavy menstrual bleeding: altered spatio-temporal expression of endothelial cell markers and extracellular matrix components*, S. Biswas Shivhare *et al.*, Human Reproduction, (submitted).
- *Menstrual cycle distribution of uterine natural killer cells is altered in menorrhagia*, S. Biswas Shivhare *et al.*, Modern Pathology, (submitted).
- *Altered vascular smooth muscle cell differentiation in the endometrial vasculature in menorrhagia*, S. Biswas Shivhare *et al.*, Human Reproduction, 2014; 29: 1884-1894.

Published abstracts

- *Spatio-temporal pattern of endometrial vascular extracellular matrix expression is dysregulated in women with menorrhagia*, S. Biswas Shivhare *et al.*, ESHRE Conference (p202), Munich, 2014.
- *Menstrual cycle distribution of uterine natural killer cells is altered in menorrhagia*, S. Biswas Shivhare *et al.*, ASRM Conference (pS125), Boston, 2013.
- *New insights into the mechanisms underlying menorrhagia: roles of smoothelin and calponin*, S. Biswas Shivhare *et al.*, ESHRE Conference (p263), London, 2013.

Book chapter

- *Immunity at the maternal-fetal interface*, S. Biswas Shivhare, *et al.*, (2015), in Mestecky, J., Strober, W., Russell, M. W., Kelsall, B. B., Cheroutre, H. and Lambrecht, B. N. (eds.) Mucosal Immunology. 4th edn. Oxford: Elsevier.

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Abbreviations

αSMA	Alpha smooth muscle actin
AGFs	Angiogenic growth factors
AMH	Anti-Müllerian Hormone
Ang	Angiopoietin
AUB	Abnormal uterine bleeding
BTB	Breakthrough bleeding
CNN	Calponin
CREG	Cellular repressor of E1A-stimulated genes
DCs	Dendritic cells
DAB	3,3'-Diaminobenzidine
ECs	Endothelial cells
ECM	Extracellular matrix
EGF	Epidermal growth factor
EPCs	Endothelial progenitor cells
ER	Oestrogen receptor
Erk	Extracellular regulated kinase
EVT	Extravillous trophoblast
FAK	Focal-adhesion kinase
FGF	Fibroblast growth factor
FIGO	International Federation of Gynaecology and Obstetrics
Flk	Fetal liver kinase
FSH	Follicle stimulating hormone
GAPDH	Glyceraldehyde-3-phosphatedehydrogenase
GnRH	Gonadotropin releasing hormone
H-Cal	H-Caldesmon
HMB	Heavy menstrual bleeding
HSPGs	Heparan sulphate proteoglycans
IL	Interleukin
IUGR	Intrauterine growth restriction
IVF	*In vitro* fertilisation
KDR	Kinase insert domain receptor
LAP	Latency-associated peptide
LH	Luteinizing hormone
llc	Large latent complex
LNG	Levonorgestrel
LTBP	Large latent TGFβ-binding protein
MAPK	Mitogen activated protein kinase
MBL	Menstrual blood loss
MC	Mast cell
MCt	Mast cell expressing tryptase
MCtc	Mast cell expressing chymase
MIS	Müllerian-inhibiting substance

MMPs	Matrix metalloproteinases
MyHC	Myosin heavy chain
Myocd	Myocardin
NCS	Nuclear channel systems
NRP	Neuropilin
PCNA	Proliferating cell nuclear antigen
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PE	Pre-eclampsia
PECAM	Platelet endothelial cell adhesion molecule
PGs	Prostaglandins
PI	Pulsatility index
PlGF	Placenta growth factor
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
pNK cells	Peripheral blood natural killer cells
PR	Progesterone receptor
RM	Recurrent miscarriage
RTKs	Receptor tyrosine kinases
SLC	Small latent complex
SMCs	Smooth muscle cells
SMTN	Smoothelin
SRF	Serum response factor
STAT	Signal transducer and activator of transcription
SVs	Saphenous veins
TGF	Transforming growth factor
TGFβR	Transforming growth factor beta receptor
TIMP	Tissue inhibitor of metalloproteinases
tPA	Tissue plasminogen activator
Tregs	Regulatory T cells
TSAd	T-cell specific adaptor
Tsp	Thrombospondin
uNK cells	Uterine natural killer cells
uPA	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VPF	Vascular permeability factor
VRAP	VEGFR-associated protein
VSMCs	Vascular smooth muscle cells

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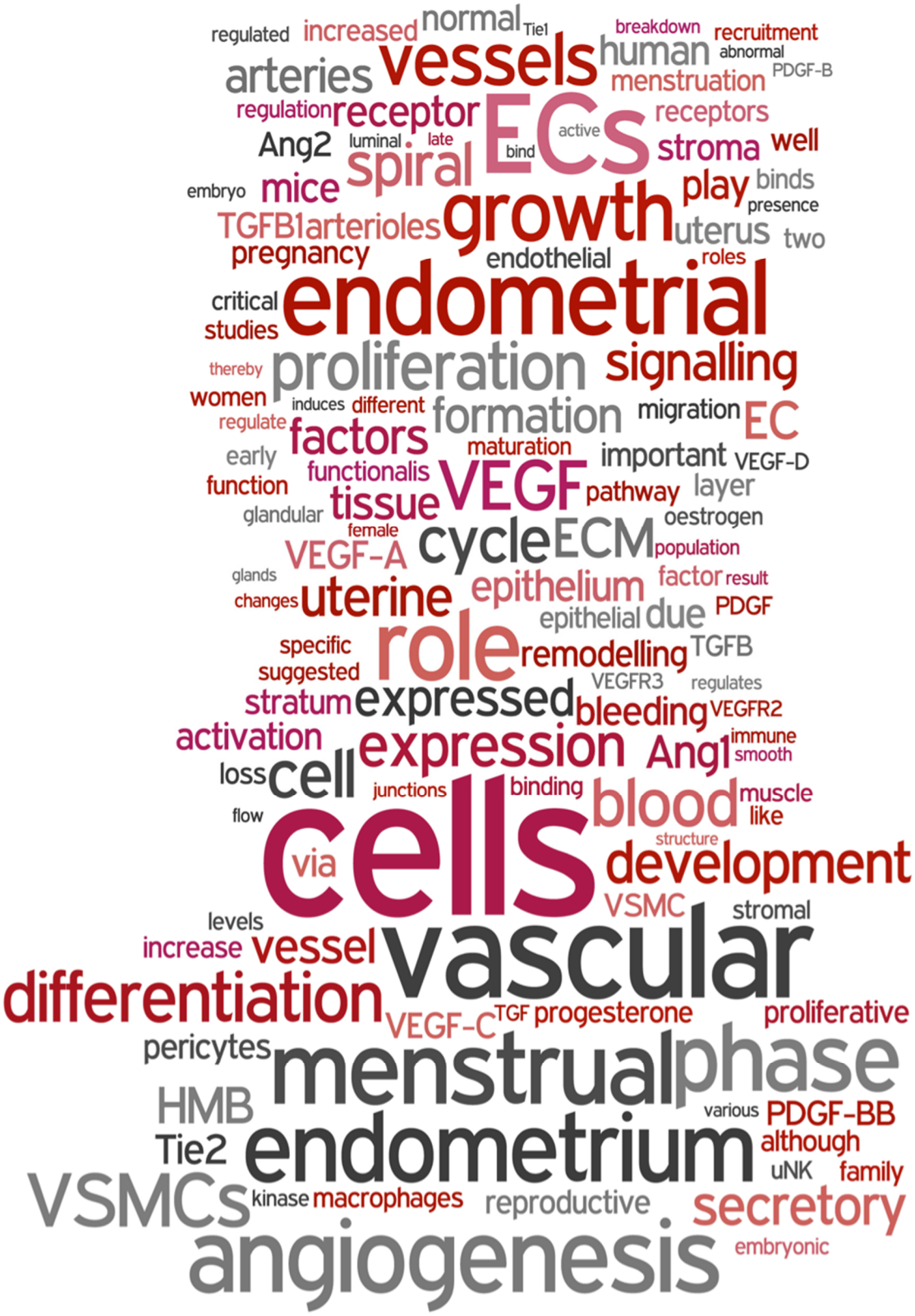
CHAPTER 1

INTRODUCTION



1 INTRODUCTION

Procreation is central to all living organisms and is indispensible to the continuation of a species and its evolution. In many organisms, including humans, the main purpose of the male reproductive system may be simplified as the mechanism of delivery of the paternal genetic material into the female reproductive tract, while that of the female reproductive system is much more complicated. In mammals, the female bears an enormous reproductive burden since she is not only responsible for producing an egg carrying the maternal genetic content (ovulation), but also has to provide a suitable environment for the journey of the sperm towards the egg, fertilization of the egg, sustaining pregnancy via implantation, accommodating the subsequent growth of the embryo and fetus during the nine months of gestation, successful delivery of the child and finally lactation for healthy maintenance of the new-born (Figure 1.1). Most of these early stages of development are accommodated in the uterus of the mother. Uterine anomalies can therefore have diverse effects and may lead to developmental inaccuracies, infertility and even death during pregnancy or childbirth. In humans, abnormal uterine-tract formation is estimated to occur in 0.1-4.0% of live births (Gidwani, 1999; Grimbizis *et al.*, 2001). Although the percentage may vary depending on population and diagnostic technique, uterine anomalies are associated with adverse reproductive outcomes and thus pose a significant concern (Venetis *et al.*, 2014). Understanding the underlying mechanisms of development and normal function of the uterus becomes essential, since it not only enhances our understanding of basic reproductive biology but also more importantly opens the door for practical applications including new less invasive and more effective treatments, regenerative medicine and infertility treatment through *in vitro* fertilisation (IVF).

1.1 THE UTERUS

The uterus, which sustains the very beginning of life, forms the central part of the female reproductive system. It is a hormone-responsive reproductive organ, present in most mammals, including humans. The uterus originates from a pair of paramesonephric ducts, first observed in 1825, by the German physiologist, Johannes Müller (Figure 1.2). Before sexual differentiation,

Figure 1.1: Early stages of development in the female reproductive tract. (A) The journey of the sperm through the uterus; (B) Fertilisation, stages of early embryonic development and implantation; and (C) Fetus *in utero*. Diagrams A and B are drawn by the author. Diagram C has been modified from birthinjuryjustice.org.

Figure 1.2: The origin of the male and female reproductive systems. The Müllerian duct is formed during early fetal development. In males, Müllerian-inhibiting substance (MIS) leads to the regression of these ducts, while in the absence of this hormone in females the ducts give rise to the oviduct and the uterus. Diagram adapted from Kobayashi and Behringer (2003).

embryo possesses both the Wolffian duct (mesonephric) and the Müllerian duct (paramesonephric). However, during differentiation, in males the presence of Müllerian-inhibiting substance (MIS) or anti-Müllerian Hormone (AMH) leads to regression of the Müllerian duct along with formation of the male reproductive tract. In the female the Wolffian duct degenerates and the Müllerian duct differentiates anteriorly into the oviducts, the uterus and posteriorly into the neck of the uterus called the cervix, which opens into the vagina (Aplin *et al.*, 2008). The uterus broadly consists of two layers; the myometrium is made up of layers of smooth muscle, while the uterine mucosa or the endometrium forms the innermost layer lining the uterine cavity and is made up of glands lined by columnar epithelium and a supporting stroma. The endometrium forms the site of implantation and therefore accommodates the development of the fetoplacental unit.

1.1.1 Types of uteri

Across the vast mammalian species uteri can be divided into four types depending on the extent of lateral fusion of the two paramesonephric ducts (Figure 1.3). A duplex uterus is defined by two entirely separate uteri with one fallopian tube each as found in kangaroos, mice and rabbits, a bipartite uterus consists of two uteri which share a single cervix as seen in cats, dogs and pigs. Cows and horses have a bicornuate uterus, which consist of two uterine horns; one cervix and the lower part remaining fused forming a larger uterine body. Higher primates including human and chimpanzee have a simplex uterus with a single uterine body and cervix, which is formed as a result of complete fusion of the paramesonephric ducts in the uterine segment (Aplin *et al.*, 2008).

1.1.2 Uterine vasculature

The uterine arteries form the main blood supply to the uterus and also supply branches to the cervix and the vagina. This blood supply to the uterus forms a branched structure with a gradual decrease in vessel diameter during the course of its journey through the myometrium and endometrium. The arcuate arteries originating from the uterine and ovarian arteries, supply blood to the myometrium. They give rise to the radial arteries, which in turn branch into basal and spiral arteries. While basal arteries supply the basal layer, spiral

Figure 1.3: Types of uteri amongst different mammalian species. Basic anatomical features of the female reproductive tract (a) and amongst different mammalian species (b-g). Diagram adapted from Kobayashi and Behringer (2003). US-urogential sinus

arteries supply the functional layer of the endometrium and the decidua during pregnancy (Figure 1.4). During pregnancy the spiral arteries undergo substantial remodelling and are transformed from high-resistance, low-flow vessels into large dilated vessels with increased blood flow velocity and reduced pressure (Burton *et al.*, 2009; Whitley and Cartwright, 2010). In the absence of fertilisation the spiral arteries regress and are eventually lost during menstrual shedding of the endometrium.

Figure 1.4: Uterine vasculature. Arcuate arteries form the radial arteries, which in turn branch into basal and spiral arteries. While basal arteries supply the basal layer, spiral arteries supply the functional layer of the endometrium.

1.2 THE ENDOMETRIUM

The endometrium, which is one of the fastest growing tissues in the human, forms the inner lining of the uterus. This complex mucosal lining consists of two major layers: a germinal layer or stratum basalis, which persists from one menstrual cycle to the next and the transient superficial layer, the stratum functionalis. It is the germinal basal layer, which regenerates the stratum functionalis after menstruation (Figure 1.5). While the former is not hormone-responsive, the stratum functionalis undergoes dynamic cyclical changes such as proliferation, maintenance, differentiation and loss in response to specific hormonal signalling (Heller, 1994; Jabbour *et al.*, 2006). While in humans the endometrium goes through the complete cycle of proliferation through to decidualisation or shedding in the absence of pregnancy, in rodents decidualisation occurs only in the presence of a blastocyst (Finn, 1974). Normal functioning of the endometrium is integral to reproductive health, with endometrial pathologies ranging from abnormal uterine bleeding, pregnancy related anomalies such as infertility and recurrent miscarriage (RM). Study of the underlying physiological and molecular mechanisms therefore remains of utmost importance and requires our understanding of the structural and temporal framework of the endometrium.

The cellular structure of the endometrium broadly comprises of a lining surface or luminal epithelium, associated glands and glandular epithelium, a connective tissue bed termed stroma, blood vasculature and extracellular matrix (ECM) (Figure 1.5). The glands and stroma, along with the endometrial vasculature in the stratum functionalis undergo dramatic morphological changes during the menstrual cycle.

1.2.1 The menstrual cycle

The human menstrual cycle encompasses structural and functional remodelling of the endometrium and its blood vasculature under the dynamic influence of the hormones oestrogen and progesterone. The menstrual cycle is divided into three phases: menstrual (days 1-4), proliferative (or follicular, days 5-14) and secretory (or luteal, days 14-28) phases.

Figure 1.5: Structure of the endometrium. (**A**) The blood supply to the endometrium through the spiral arteries and capillary bed. (**B**) The cellular components of the stratum functionalis and stratum basalis of the endometrium.

The standard menstrual cycle length is 28 days; the first day of menstrual bleeding is day 1 and ovulation occurs approximately on day 14 under the surge of luteinizing hormone (LH) (Figure 1.6). However, there is variability between women from different regional, ethnic and socioeconomic backgrounds (Harlow, 2000), mainly due to variability in length of the follicular phase (Waller *et al.*, 1998; Mihm *et al.*, 2011). The menstrual phase involves breakdown and shedding of the stratum functionalis and initiation of re-epithelialisation (Ludwig and Spornitz, 1991). In the proliferative phase oestrogen leads to an increased proliferation of all cell types. This post-menstrual re-epithelialisation together with growth of stroma and glands leads to an increase in endometrial thickness from 1mm to 3-4mm at ovulation.

Ovulation in mid-cycle marks the switch of hormonal control to progesterone produced by the corpus luteum, which regulates endometrial functional differentiation to accommodate implantation. In the absence of pregnancy, a reduction in levels of oestrogen and progesterone leads to endometrial regression, exposing open blood vessels and glands and resulting in menstrual bleeding (Aplin *et al.*, 2008). This event is associated with local presence of proinflammatory cells such as neutrophils, macrophages and uterine natural killer cells, vasoactive mediators such as prostaglandins (PGs) and matrix metalloproteinases (MMPs) such as MMP-2, MMP-9 (King *et al.*, 1998a; Salamonsen and Woolley, 1999). Although this decrease in levels of oestrogen and progesterone is common to all mammals, it is only in humans, some old-world primates and bats that menstrual bleeding occurs. Figure 1.7 outlines the probable mechanisms of ECM breakdown leading to tissue destruction at menstruation. The histological changes in the endometrium during the menstrual cycle are summarised in Table 1.1.

Thrombin production in the basal layer allows for homeostasis, while regeneration from the basal stumps appears in the superficial layer, and extends through the entire stratum functionalis (Ludwig and Spornitz, 1991; Jabbour *et al.*, 2006). This remodelling is essential since the endometrium irreversibly differentiates during the menstrual cycle and therefore needs to be discarded in order to re-proliferate and maintain its reproductive function (Finn, 1998). Interestingly a stem cell population located in the stratum basalis of the

Figure 1.6: Hormonal control of the menstrual cycle with relation to ovulation and the changes in the endometrium. Diagram compiled and modified by the author from lpch.org; SBayram. GnRH-gonadotropin releasing hormone; LH-luteinising hormone; FSH-follicle stimulating hormone.

Figure 1.7: Tissue destruction at menstruation. Progesterone withdrawal affects epithelial cells to stimulate chemokine production, which activates leucocytes. Leucocytes produce cytokines, which stimulate production of latent (pro) matrix metalloproteinases (MMPs), chemokines and other mediators. Leucocytes also produce proteases that start the activation cascade of MMPs. Activation of pro-MMPs can also be triggered by other proteases or by the plasmin system (uPA/tPA) released either by stromal cells or by inflammatory cells. An increase in MMPs leads to an imbalance between the activators and inhibitors of tissue breakdown, leading to breakdown of extracellular matrix (ECM).

Table 1.1: Histological differences and leukocyte population in the human endometrium during the menstrual cycle

MENSTRUAL PHASE	PROLIFERATIVE PHASE	EARLY SECRETORY PHASE	MID SECRETORY PHASE	LATE SECRETORY PHASE
• Loss of epithelium and underlying tissue. • Loss of stratum functionalis containing blood clots, fragments of disintegrated stroma, uterine glands. • The uterine glands in the functionalis layer are infiltrated by blood. • Stratum basalis remains unaffected and contains intact bases of the uterine glands. • The distal parts of the coiled spiral arteries become necrotic, while the deeper parts remain intact. • The stroma contains erythrocytes (extruded from disintegrating blood vessels), moderate numbers of lymphocytes and neutrophils.	• The endometrium is lined by columnar epithelium, which extends down to the stroma to form numerous long, straight, tubular uterine glands. • Cellular proliferation and differentiation occur, marked by the presence of mitotic figures in the glandular epithelium and stroma. • Angiogenesis occurs and the arteries remain relatively straight. • Extracellular remodelling occurs in the stroma. • Stroma is infiltrated with low to moderate numbers of macrophages, mast cells and T-lymphocytes. B-lymphocytes are very few in number in stratum functionalis but more in stratum basalis.	• The endometrium thickens due to increased glandular secretions and stromal oedema, resulting from accumulation of tissue fluid. • The uterine glands become tortuous. • The glandular epithelial cells stop dividing; hence mitotic figures are absent. • The presence of vacuoles and the upward movement of nuclei mark the glandular epithelial cells. • The arteries in the stratum functionalis become increasingly coiled and termed as spiral arteries.	• The endometrium thickens further. • Glands are increasingly tortuous and their secretions are elevated. • Stromal oedema persists and the cells undergo differentiation. • Nuclei travel downwards in the glandular epithelial cells and the subnuclear vacuoles are lost. • Stroma is infiltrated with moderate to high numbers of macrophages and uNK cells.	• The endometrium remains thickened. • Glands remain tortuous. • Stromal oedema persists, but there are signs of apoptosis and extracellular matrix degradation. • Stroma is infiltrated with moderate to high numbers of macrophages, uNK cells, mast cells and T-lymphocytes.

endometrium may play a role in the self-renewal of the human endometrium (Prianishnikov, 1978; Schwab *et al.*, 2005; Gargett, 2007).

1.2.2 The stroma

The stroma of the endometrium is a dynamic connective tissue comprised of specific cells and an ECM. The cells of the stroma include fibroblast-like reticular cells, lymphocytes, macrophages and uterine natural killer (uNK) cells. The reticular cells of the stroma produce most of the components of the ECM. These cells are dynamic in nature with a changing morphology, which leads to 'decidualisation' during pregnancy (Aplin *et al.*, 2008). In the early proliferative phase endometrial stromal cells are more like undifferentiated fibroblast cells with mesenchymal features. Their morphology begins to change around ovulation. The mid secretory phase is marked by stromal cell oedema, with an increase in nuclear diameter and rounding up of nucleoli, suggesting increased transcriptional activity. In the late secretory phase, the stromal cells undergo irreversible differentiation or decidualisation, critical for preparation of menstruation or pregnancy. The initial stages of menstruation are mediated by progesterone expressing stromal cells, which also involve prostaglandin E2 and F2α synthesis, growth factor expression and influx of leukocytes. However, later, in the progesterone-independent stage, menstruation is mediated by leukocytes secreting MMPs, which allow endometrial ECM breakdown (Irwin *et al.*, 1996).

The stromal cells are involved in ECM remodelling during the menstrual cycle (More *et al.*, 1974). The endometrium contains both stromal interstitial matrix as well as basal lamina underlying endometrial glands and vasculature (Evans and Salamonsen, 2012). The ECM provides a structural framework for the stroma; it contains cytokines and growth factors and interacts with the stromal cells via various receptors such as integrins. The stromal matrix is composed of microfibrils, which may be made up of bundles of collagens and fibrillin. Fibulin 1, an ECM protein, interacts with elastic fibres of blood vessels containing elastin. Other factors in the stroma include dermatopontin, which control collagen fibrillogenesis, tenacin, which associates with smooth muscle cells of the spiral arteries and thrombospondin (Tsp-1), which has an inhibitory effect on

angiogenesis (Iruela-Arispe *et al.*, 1996; Haendler *et al.*, 2004; Aplin *et al.*, 2008). MMPs and tissue inhibitors of MMPs (TIMPs), play a critical role in ECM breakdown during menstruation. In addition to endometrial stromal or epithelial cells, which release MMPs (in particular MMP1, MMP3, MMP8, MMP9-11), leukocytes such as neutrophils when activated may also release MMP8, MMP9 (Evans and Salamonsen, 2012; Gaide Chevronnay *et al.*, 2012).

1.2.3 Endometrial leukocytes

Menstruation, although triggered by down regulation of hormones, is considered to be an inflammatory process (Finn, 1986) maintained by the presence of proinflammatory cells such as neutrophils, macrophages and uNK cells (Salamonsen and Woolley, 1999; R. L. Jones *et al.*, 2004; Berbic and Fraser, 2013). The endometrial immune cell populations undergo dynamic changes during the different phases of the menstrual cycle, depending on the underlying need to provide protection to the uterine mucosal layer, facilitate endometrial remodelling and the process of menstruation. Human endometrial leukocyte populations have been widely studied (Bulmer and Sunderland, 1984; Bulmer and Johnson, 1985; Morris *et al.*, 1985; Bulmer *et al.*, 1987; Kamat and Isaacson, 1987; Bulmer *et al.*, 1988; Marshall and Jones, 1988; King *et al.*, 1998a; Salamonsen *et al.*, 2002; Evans and Salamonsen, 2012; Berbic and Fraser, 2013) (Table 1.1). Depending on their state of activation, these cells exhibit phenotypic differences and function differently. Cytolytic activity of $CD3^+$ T cells is present during the proliferative phase (White *et al.*, 1997), while increased numbers of macrophages, mast cells and T cells are reported in the late secretory phase (Poropatich *et al.*, 1987; Salamonsen and Woolley, 1999; Berbic and Fraser, 2013). While uNK cells are scarce in proliferative endometrium, their numbers increase in the mid and late secretory phase of the menstrual cycle (Bulmer *et al.*, 1991). Finally, in the menstrual phase of the cycle there is increased activity of mast cells, matrix metalloproteinases (Zhang and Salamonsen, 2002) and antigen presenting cells such as macrophages and dendritic cells, suggesting a role in tissue breakdown and clearance (Schulke *et al.*, 2008; Berbic *et al.*, 2009; Berbic *et al.*, 2010). The endometrial immune response during the cyclic shedding, tissue remodelling and angiogenesis helps

in maintaining a healthy functioning endometrium, which may otherwise be linked with abnormal uterine bleeding disorders such as menorrhagia.

1.2.3.1 uNK cells

During the progesterone dominated mid and late secretory phases of the menstrual cycle in non-pregnant endometrium (in the stratum functionalis, around arteries and arterioles) and in the first trimester of pregnancy, uNK cells are the most prevalent leukocyte population in the endometrial stroma, although they are present in low numbers in proliferative endometrium (Hamperl and Hellweg, 1958; King *et al.*, 1989; Pace *et al.*, 1989; Bulmer *et al.*, 1991). It has been suggested that the PR expressing endometrial stromal cells promote uNK proliferation and differentiation via secretion of the cytokine interleukin-15 (IL-15) and the differentiated uNK cells modify medial VSMC resulting in vascular stability (Wilkens *et al.*, 2013). uNK cells have been implicated in a wide range of functions; cytokine and growth factor secretion, immune function, regulation of trophoblast invasion in early pregnancy and also in the initial stages of spiral artery remodelling (Hiby *et al.*, 2004; Bulmer and Lash, 2005; Hanna *et al.*, 2006; De Oliveira *et al.*, 2010; Lash *et al.*, 2011b; Robson *et al.*, 2012). They secrete angiogenic growth factors including angiopoietin-1 (Ang1), Ang2, vascular endothelial growth factor C (VEGF-C), placenta growth factor (PIGF) and transforming growth factor β1 (TGF-β1) (Li *et al.*, 2001; Lash *et al.*, 2006). uNK cells assist trophoblast invasion and tissue and spiral artery remodelling by secreting proteolytic enzymes such as MMP2, MMP9, and TIMP1, TIMP2, TIMP3, which help in the breakdown of the ECM (Smith *et al.*, 2009). uNK cells have been implicated in various female reproductive disorders (reviewed by Zhang and Tian, 2007; Lash and Bulmer, 2011). Previous studies in the laboratory have reported a positive association between uNK cell density and vascular maturation in women with RM and recurrent implantation failure (Quenby *et al.*, 2009). However, their role in the non-pregnant cycling endometrium remains unclear. Therefore, given their possible role in endometrial vascular maturation (Wilkens *et al.*, 2013), it is important to understand how they affect the normal as well as pathological states of menstrual bleeding.

1.2.3.2 Eosinophils

Eosinophils, detected by the eosinophil cationic proteins (ECP1 and ECP2), are present in aggregates in the endometrium immediately before the onset of menstruation (Jeziorska *et al.*, 1995; Evans and Salamonsen, 2012). Although IL-5 is known to primarily stimulate development and function of eosinophils, it has not been examined in premenstrual endometrium. Some endometrial eosinophils secrete MMP9 and therefore may play a role in tissue breakdown and menstruation (Jeziorska *et al.*, 1995; Evans and Salamonsen, 2012). However, due to overlapping functions, elucidating their specific roles in the endometrium remains challenging.

1.2.3.3 Macrophages

Amongst the diverse population of immune cells in the endometrium are the macrophages (Bulmer *et al.*, 1988), which have been suggested to play a critical role in endometrial decidualisation and receptivity for the implanting blastocyst (Lea and Clark, 1991; Kats *et al.*, 2005; Thiruchelvam *et al.*, 2013). Following differentiation of peripheral blood monocytes or *in situ* proliferation into tissue specific macrophages, these cells may perform a wide variety of functions including phagocytosis, maintaining tissue homeostasis, regulation of wound healing or other immune cell populations, depending on the requirement of their tissue microenvironment (Thiruchelvam *et al.*, 2013). Hence macrophages may perform different roles in the endometrium depending on the menstrual cycle phase. In the proliferative phase, macrophages make up 1-2% of endometrial cells and have been suggested to be involved in the proliferation and regeneration of stratum functionalis, since they express adhesion and activation markers such as CD71, CD69 and CD54 (Salamonsen *et al.*, 2002; Eidukaite and Tamosiunas, 2004). During the secretory phase macrophage numbers rise making up 3-5% of all endometrial cells and their role is thought to become more focused on maintaining a receptive endometrium and regulating fertility (Salamonsen *et al.*, 2002; Aghajanova, 2004; Kats *et al.*, 2005; Thiruchelvam *et al.*, 2013). Moreover, endometrial macrophages express a variety of chemoattractants during the secretory phase (Jones *et al.*, 2004). Endometrial macrophage numbers reach their maximum in the menstrual phase

when they make up 6-15% of endometrial cells and they express various MMPs (latent MMP9, MMP12, MMP14) and other molecules that regulate MMPs suggesting their role in breakdown of the stratum functionalis marking the initiation of menstruation (Zhang *et al.*, 2000; Salamonsen *et al.*, 2002).

Endometrial macrophages secrete AGFs such as VEGF-A, which is reduced in women suffering from heavy menstrual bleeding (Malik *et al.*, 2006; Berbic *et al.*, 2014). They also act as potent vasodilators (Hickey and Fraser, 2000) and unscheduled endometrial bleeding in women using Levonorgestrel (LNG) contraceptive has been shown to correlate with the increase in endometrial macrophages and cytokines (Clark *et al.*, 1996) suggesting a critical role for macrophages in abnormal menstrual bleeding.

1.2.3.4 Neutrophils

The observation that decidualisation and chemokine secretion in the endometrium occurs first around the endometrial vessels led to a belief that neutrophils transmigrate from peripheral blood into the endometrium via the spiral arterioles (Jones *et al.*, 2004). The function of neutrophils is specific for their tissue microenvironment and they interact with both the endometrial vessel walls as well as the tissue ECM (Soehnlein *et al.*, 2009). They secrete MMPs and other degradative enzymes supporting their role in the menstrual phase. In a mouse model their numbers increased during repair, and depletion of neutrophils delayed endometrial repair (Kaitu'u-Lino *et al.*, 2007). However, whether they play a role in endometrial repair as well as menstrual breakdown in human remains to be investigated.

1.2.3.5 Dendritic cells

Dendritic cells (DCs) are a unique population of leukocytes, which play a critical role in initiation and modulation of immune responses in the entire body, as well as serving as a link between innate and adaptive immunity. DCs communicate with other immune cell populations such as NK cells, macrophages, T-lymphocytes and B-lymphocytes (Banchereau and Steinman, 1998). Moreover, several chemokines and cytokines secreted by DCs have been implicated in angiogenesis, tissue repair, menstruation and blastocyst implantation (Kayisli *et al.*, 2002; Barrientos *et al.*, 2009). Compared with macrophages and uNK cells,

the dendritic cell population in the cyclic endometrium is much lower. Moreover, studies using early DC markers showed few to no DCs in the endometrium (Kamat and Isaacson, 1987; Bulmer *et al.*, 1988), while a more recent study using a combination of immature (CD1a) and mature DC (CD83) marker reported an increase in $CD1a^+$ DCs in stratum basalis and a relatively unaltered mature $CD83^+$ DCs throughout the menstrual cycle (Schulke *et al.*, 2008). This may suggest that although influx of DCs into the uterus may be hormonally regulated, their maturation is not. The specific role of DCs in the menstrual cycle remains unknown. However DCs produce MMPs, cytokines and chemokines, which may directly influence the uterine microenvironment or regulate other immune cell populations and their functions (Kayisli *et al.*, 2002; Schulke *et al.*, 2008).

1.2.3.6 T-lymphocytes

T lymphocytes play critical roles in adaptive immune responses and account for 45% of endometrial leukocytes in the proliferative phase their proportion decreasing gradually in the secretory phase (Vassiliadou and Bulmer, 1996). Two-thirds of this leukocyte population are $CD8^+$ cytotoxic T cells (Yeaman *et al.*, 2001). They target foreign antigens by release of cytotoxins. Moreover, they retain information about the invading antigen, such that of any subsequent invasion by the same antigen, an appropriate immune response is more actively available (Berbic *et al.*, 2014). Others include CD4+ T cells which can either be T helper 1(Th1) and Th2 depending on their respective cytokine production (Vassiliadou and Bulmer, 1996); and forkhead box transcription factor, $FOXP3^+$ regulatory T cells (Tregs), which supress T cell proliferation and activation, macrophage, B lymphocyte, dendritic cell and NK cell function and thus may play a role in immunological tolerance during pregnancy (Campbell and Koch, 2011; Nevers *et al.*, 2011; Berbic *et al.*, 2014). Decidual and circulating Tregs peak in the first trimester of pregnancy (Somerset *et al.*, 2004) coinciding with high levels of vascular development and maturation, trophoblast invasion and spiral artery remodelling, suggesting a possible role in vascular development. In the cyclic non-pregnant endometrium, Tregs increase in number during the proliferative phase peaking before ovulation, but decrease again in the luteal phase (Arruvito *et al.*, 2007). Although important for immune tolerance and

vascular remodelling homeostasis (via IL-10 in pregnancy) (Nevers *et al.*, 2011), the specific roles of Tregs in non-pregnant endometrium are unclear, especially in regulation of menstrual bleeding.

1.2.3.7 Mast cells

Mast cell (MC) populations do not alter during the menstrual cycle, but become highly active immediately before and during the menstrual phase (Jeziorska *et al.*, 1995). On maturation, mast cells (MC) differentiate into two populations; MCt expressing the protease tryptase (t) and mainly seen in the stratum functionalis and MCtc expressing chymase (c) found in stratum basalis only (Rogers *et al.*, 1993; Evans and Salamonsen, 2012). On activation MCs release proteases, which regulate cytokine activity and activate other MMPs. MCs also secrete MMP9, which may reflect their potential role in tissue breakdown and menstruation (Salamonsen and Lathbury, 2000; Baram *et al.*, 2001). However, specific molecular events leading to endometrial MC activation remain unclear.

Alterations in leukocytes populations in particular macrophages, uNK cells, dendritic cells, Tregs and T cells in the endometrium may be reflective of altered vascular development leading to menstrual disturbances, infertility or other endometrial pathologies; therefore inviting further research in both normal and pathological states of the endometrium

1.2.4 Endometrial blood vessel development

The endometrial vasculature primarily consists of the basal arteries supplying the stratum basalis and the spiral arteries supplying the functionalis layer. The coiled shape of the spiral arteries helps to reduce blood pressure and rate of flow along its length, maintaining a steady blood flow to the non-pregnant stratum functionalis and the pregnant decidua. Additionally, the spiral arteries give rise to capillaries forming the sub-epithelial plexus, which empty into the venous plexus and subsequently drain into the inner myometrium (Aplin *et al.*, 2008). In humans, this endometrial vasculature goes through cycles of growth, disruption, remodelling and repair under the universal control of the ovarian steroid hormones oestrogen and progesterone, during the monthly menstrual

cycle. Understanding the menstrual cycle (section 1.2.1) requires knowledge of the structure of blood vessels and the process of angiogenesis including elongation, muscularisation and expansion of the capillary network required for growth and maintenance of the blood supply.

1.2.4.1 Angiogenesis

The structure of a blood vessel broadly consists of the innermost endothelial cell (EC) layer, basement membrane or vascular ECM and an outer covering composed of pericytes or vascular smooth muscle cells (VSMCs). Angiogenesis is the process of development of new vessels from pre-existing capillary network. It broadly consists of an activation phase involving EC activation and degradation of vascular ECM, followed by a resolution phase involving EC proliferation and migration, tube formation and stabilisation. Four different processes underlie angiogenesis: sprouting, intussusception, elongation/widening and incorporation of circulating endothelial progenitor cells (EPC) into growing vessels (Figure 1.8). Sprouting is a multistep process involving EC activation, degradation of vascular ECM, EC proliferation and migration, tube formation and stabilisation (Risau, 1997). Intussusception occurs by virtue of proliferation of ECs and ECM formation, internally dividing the vessel into two. This results in a network of smaller interlocking vessels, which may remodel or enlarge during the formation of a capillary plexus (Peirce and Skalak, 2003). Elongation or widening does not involve formation of additional vascular junctions and occurs by lengthways growth of a vessel. Finally, incorporation of EPCs into the development of a new growing vessel may be another means of angiogenesis (Asahara *et al.*, 1999; Khakoo and Finkel, 2005). However, the timing and role of circulating EPCs in angiogenesis remains to be elucidated. Thus angiogenesis results in formation of new capillary networks, which are primarily EC tubes lacking wall structure including VSMCs. Following this, these vessels undergo arteriogenesis involving enlargement of collateral vessels (Peirce and Skalak, 2003). During this process the capillaries gain a layer of VSMCs and become capable of blood flow regulation by means of rapid internal diameter alteration (Figure 1.8). VSMCs and pericytes belong to the same cell lineage; while VSMCs coat venules and arterioles, pericytes support

Figure 1.8: Mechanisms of angiogenesis and arteriogenesis. While angiogenesis involves sprouting, intussusception or elongation, arteriogenesis involves *de-novo* arteriole formation from new or pre-existing capillaries by recruitment of pericytes or vascular smooth muscle cells (mural cells) and by collateral enlargement. Elongative angiogenesis occurs in the mid-late proliferative phase while branching angiogenesis is common in the early-mid secretory phase of the menstrual cycle. Diagram re-drawn by author with reference to Aplin *et al.* (2008a).

capillaries (Jain, 2003). In this study the process of blood vessel development and functional maturation will be termed vascular maturation. The primate endometrium comprises two different types of arteries: the straight and spiral arteries, both of which originate from the radial arteries. The spiral arteries develop during the progesterone influenced secretory phase and are unique to menstruating primates (Kaiserman-Abramof and Padykula, 1989). While the straight arteries are seen both in the stratum functionalis and stratum basalis, the spiral arteries are almost exclusive to the stratum functionalis. Their heavily coiled structure, larger VSMCs and in human, absence of internal elastic lamina, make them distinct from the straight arterioles (Rogers and Abberton, 2003). The spiral arteries are vital for normal menstruation, implantation and formation of the placenta.

In the menstrual cycle (section 1.2.1) vascular development and maturation occurs in all three phases: during menstruation when repair of vascular bed occurs, during the proliferative phase, marked by rapid endometrial growth and also during the secretory phase with growth and coiling of spiral arteries. While intussusception is the major mechanism underlying angiogenesis in the early-mid proliferative phase, it is vessel elongation or widening that prevails during the oestrogen driven mid-late proliferative phase (Gambino *et al.*, 2002). During the early secretory phase, the increase in endometrial thickness results in increased vessel density and vessel segment length. In the early-mid secretory phase vessel lengths remain elevated, but an increase in vessel junctions leads to a decreased segment length. Finally, in the late secretory phase vascular length density falls to the levels during proliferative phase suggesting vascular regression prior to menstruation. Therefore, branching is key to secretory phase angiogenesis (Gambino *et al.*, 2002).

Angiogenesis is principally regulated by a network of hormones, growth factors and cytokines, along with adhesion, cytoskeletal and extracellular proteins (Figure 1.9). In the endometrium, oestrogen and progesterone are the primary hormonal regulators of angiogenesis. While oestrogen receptor α (ERα) is only present in the VSMCs, oestrogen receptor β (ERβ) is expressed in both ECs and VSMCs (Critchley *et al.*, 2001; Lecce *et al.*, 2001; Hapangama *et al.*, 2015). Oestrogen has been implicated as a proangiogenic factor by several studies in

Figure 1.9: Major pathways involved in angiogenesis and arteriogenesis. Angiogenic factors bind to their receptors and activate the endothelial cells (ECs). EC activation releases proteases, which dissolve basement membrane and start the process of EC assembly via proliferation and migration. While the VEGF receptors maintain normal vasculature, platelet derived growth factor (PDGF) helps to recruit smooth muscle cells (SMCs). Following this transforming growth factor (TGF-β) mediates proliferation and migration of VSMCs to form layers around the EC tube. Once a vessel is mature, a network is formed via destabilization, EC proliferation and migration under actions of VEGF, matrix metalloproteinases (MMPs), and adhesion molecules.

both humans and other primates. Studies have also suggested that increased vascular permeability, dilation of vessels and increased blood flow may be due to the opening of tight junctions between ECs (Albrecht *et al.*, 2003; Kayisli *et al.*, 2004). The role of progesterone in regulation of angiogenesis remains inconsistent and controversial. While some studies have reported the presence of progesterone receptors (PRs) in human and non-human ECs and VSMCs, others have suggested their presence only in some arteries (Press *et al.*, 1988; Rogers *et al.*, 1996). Moreover, several studies showed an inhibitory effect of PR on endometrial angiogenesis (Iruela-Arispe *et al.*, 1999); these were mostly *in vitro* studies and progesterone has been observed to increase *in vivo* angiogenesis in primates without causing vasodilation (Gambino *et al.*, 2002). To summarise, under the action of oestrogen, rapid tissue growth and elongation of vessels occurs during the proliferative phase. In the secretory phase progesterone induces growth and coiling of spiral arteries. Following this, in the late secretory phase a decrease in levels of oestrogen and progesterone leads to regression and breakdown of endometrium. The action of oestrogen and progesterone on endometrial angiogenesis and vascular maturation is mediated by various growth factors such as TGFβ, epidermal growth factor (EGF) and VEGF (Figure 1.9).

1.2.4.2 Vascular smooth muscle cells, endothelial cells and extracellular matrix

Role of VSMCs: VSMC proliferation and differentiation is key to vascular maturation during the menstrual cycle. Alpha smooth muscle actin (αSMA), a contractile cytoskeletal protein is the earliest marker of VSMC differentiation and is located in both small and large vessels. During differentiation VSMCs express a series of markers including αSMA, calponin, H-caldesmon (H-cal), myosin heavy chain (MyHC), desmin and smoothelin amongst others. Changes in the state of VSMC differentiation have been implicated in vascular diseases, while the co-operative signal transduction pathways underlying VSMC differentiation and proliferation have been widely studied (Owens, 1995; Owens *et al.*, 2004; Kawai-Kowase and Owens, 2007). Mature VSMCs have the capacity to de-differentiate in order to fulfil immediate needs for growth and

remodelling throughout the menstrual cycle. Mature VSMCs may therefore alternate between a contractile state expressing a high level of contractile proteins and a secretory state, which involves proliferation and secretion of basement membrane proteins (Owens, 1995; Rogers and Abberton, 2003).

Following recruitment and differentiation, proliferation of VSMCs forms the key to maturation of an arteriole. Studies using proliferating cell nuclear antigen (PCNA) have shown that VSMC proliferation in spiral arteries remained consistently low during the early menstrual cycle and increased during the mid-late secretory phase, explaining the growth and coiling of spiral arteries. In contrast, VSMC proliferation remained constant for straight arterioles (Abberton *et al.*, 1999b). In women with heavy menstrual bleeding (HMB; menorrhagia) VSMC proliferation was significantly reduced in the mid and late secretory phase in both the spiral and straight arterioles (Abberton *et al.*, 1999b). This suggests that the spiral arterioles have an altered growth pattern in HMB, since it is in the secretory phase that spiral arterioles grow and gain their distinctive coiled structure. Interestingly, the total number of VSMCs in spiral arterioles was similar between control and menorrhagic tissue and consequently absolute wall thickness and αSMA actin staining pattern was similar. In addition, staining of MyHC, a late marker of VSMC differentiation was lower in the early secretory phase in women with HMB, suggesting that VSMCs mature at different rates in the spiral arterioles in HMB compared with control endometrium (Abberton *et al.*, 1999b).

Role of ECs: Endometrial ECs play a central role in the menstrual cycle; they regulate cyclic blood vessel maturation in association with VSMC recruitment under the action of angiogenic growth factors (AGFs). Vascular EC-VSMC signalling is key to maintaining normal blood flow by regulation of vascular tone (Feletou, 2011). ECs are also associated with degradation of vascular and stromal ECM by protease production, thereby allowing co-ordinated EC migration and proliferation (Findlay, 1986; Zetter, 1988; Goodger and Rogers, 1994). Moreover, in a study which used EC proliferation as a measure of angiogenesis, patients with HMB had high endometrial EC proliferation compared with controls, indicating altered blood vessel development (Kooy *et al.*, 1996). During the menstrual cycle, angiogenesis is spatially and temporally

regulated: in the menstrual phase there is vascular repair in the stratum basalis; in the stratum functionalis angiogenesis in the proliferative phase supports endometrial growth, while in the secretory phase there is growth and coiling of the spiral arterioles (Gargett and Rogers, 2001). Thus vascular EC differentiation/maturation may also be spatio-temporally regulated during the menstrual cycle and dysregulation of this process may in turn reflect altered vascular function/development, contributing to bleeding disorders such as HMB.

Vascular ECs express a number of molecules, including Factor VIII related antigen (F8RA), CD34 and CD31 and EC specific glycoproteins, which selectively bind the lectin Ulex Europaeus-Agglutinin I (UEA-1). F8RA and CD34 are glycosylated transmembrane proteins, whereas CD31 is an endothelial cell adhesion glycoprotein molecule present at intercellular junctions. During studies of vessel development in secretory phase of the endometrium from women suffering recurrent reproductive failure, heterogeneous staining of different EC markers was noted (Quenby *et al.*, 2009). While there have been several studies of expression of different EC markers in endometrium and myometrium during the menstrual cycle (Rees *et al.*, 1993; Rogers *et al.*, 1993; Tawia *et al.*, 1993; Nikitenko *et al.*, 2000; Zhang *et al.*, 2002), a detailed analysis of ECs in the different layers of the endometrium (luminal region, stratum functionalis, stratum basalis) and superficial myometrium throughout the menstrual cycle and in endometrial bleeding disorders such as HMB has not been reported.

Role of ECM: As a major component of blood vasculature, the ECM is integral to angiogenesis, tissue stability, regulation of cell growth and differentiation and provides mechanical properties maintaining vascular tone. Moreover, ECs and synthetic VSMCs are capable of synthesizing most of the ECM constitutent proteins. Vascular ECM (Paulsson, 1992; Timpl, 1996; Timpl and Brown, 1996) consist of anchoring fibrils and proteins, including type IV collagen (Paulsson, 1992; Schmidt *et al.*, 1992; Poschl *et al.*, 2004), fibronectin (Clark *et al.*, 1982; Risau and Lemmon, 1988; Francis *et al.*, 2002; Astrof *et al.*, 2007; Hynes, 2007; Astrof and Hynes, 2009), laminin (Cheng *et al.*, 1997; Miner *et al.*, 1998; Thyboll *et al.*, 2002; Miner and Yurchenco, 2004; Scheele *et al.*, 2007; Yurchenco and Patton, 2009) and osteopontin (Chen *et al.*, 1992; Denhardt and Guo, 1993;

Giachelli *et al.*, 1993; Liaw *et al.*, 1994; Giachelli *et al.*, 1995; Apparao *et al.*, 2001; Kang *et al.*, 2014). The ECM is located radially outward from the EC layer. The main constituents of the human endometrial vascular ECM have been identified (Faber *et al.*, 1986; Aplin *et al.*, 1988) and shown to be expressed even during the menstrual phase (Kelly *et al.*, 1995). Since the pattern of vascular growth and development differs between the layers and phases of the menstrual cycle, this may also accompany an altered spatio-temporal pattern of ECM expression. Although staining of the ECM components have been studied to some extent in the menstrual cycle (Aplin *et al.*, 1988; Kelly *et al.*, 1995; Oefner *et al.*, 2015), osteopontin, laminin, collagen IV and fibronectin have not been studied in detail in endometrial vasculature and in the context of endometrial bleeding disorders such as HMB.

1.2.4.3 Angiogenic growth factors

Angiogenesis involves various molecular pathways involving growth factors such as PDGF-B and its receptor PDGFR-β. PDGF-B, secreted by ECs probably in response to VEGF-A, is important in recruiting VSMCs and pericytes (Jain, 2003) (Figure 1.9). *In vitro* studies have shown that PDGF-BB is capable of inducing tube formation, sprouting and proliferation by stimulating ECs. PDGF-BB also acts as a mitogen for VSMCs and pericytes leading to cell proliferation (Battegay *et al.*, 1994). Another growth factor, TGF-β1 is involved in a plethora of events including proliferation of ECs, ECM stimulation and production of proteases, and differentiation of ECs and VSMCs (Distler *et al.*, 2003). TGF-β1 performs a dual role; acting both as an angiostatic and as an angiogenic factor. *In vitro* studies have shown that while low doses of TGF-β1 induce EC proliferation and tube formation, higher doses have a negative effect. Alongside its roles in angiogenesis, TGF-β1 is also involved in ECM degradation (Pepper, 1997). Tyrosine kinase receptor Tie-2 and its ligands, Ang1 and Ang2 also play a role. Ang1, which is present throughout the menstrual cycle but in reduced levels in the endometrial stromal fibroblasts, regulates vessel maturation through VSMC recruitment and EC-VSMC structural association (Jain, 2003). Ang2 is mostly present in the secretory phase in the luminal epithelial and is detected in uNK cells around VSMCs. In contrast to the role of Ang1, Ang2 takes part in vessel degradation and

remodelling, destabilising vessels via apoptosis of ECs, which in the absence of VEGF degenerate (Jain, 2003; Rogers and Abberton, 2003).

It is important to note that the development of the endometrial vascular bed results from a strict balance between inducers and inhibitors. Inhibition of vessel formation can occur via various mechanisms: blocked production, downregulation or neutralization of an inducer or its receptor, direct inhibition of migration or proliferation of ECs or VSMCs. Inhibitors of angiogenesis include endostatin, tumstatin, angiostatin, Tsp-1 and tissue inhibitor of MMP-2 (TIMP-2). As an example, Tsp-1 produced by the endometrial stromal cells inhibits angiogenesis via downregulation of platelet endothelial cell adhesion molecule 1 (PECAM1) and by binding to FGF2 and endothelial cell glycoprotein CD36. It also promotes apoptosis of ECs either by upregulating the proapoptotic factor BAX or by downregulating the antiapoptotic factor BCL2 (Smith, 2001). It is clear that the mechanism of endometrial blood vessel development involves a complex network of interactions between several AGFs, the understanding of which requires further knowledge of the key AGFs involved.

1.2.5 The TGFβ family

The evolutionarily conserved TGFβ superfamily, the largest family of secreted cytokines or growth factors in mammals, play key roles in a number of diverse physiological and pathological process (Massague *et al.*, 2000; Chang *et al.*, 2002; Juengel and McNatty, 2005; Padua and Massague, 2009; Ikushima and Miyazono, 2010) from embryo development to tissue homeostasis (Knight and Glister, 2006; Gordon and Blobe, 2008; Wu and Hill, 2009). TGFβs are secreted in latent forms, and their bioavailability is a strictly regulated multistep process including secretion, storage and interaction with ECM components, release from ECM and finally activation prior to receptor binding (ten Dijke and Arthur, 2007). The inactive TGFβ peptide consists of a TGFβ dimer covalently bound to its latency-associated peptide (LAP) forming the small latent complex (SLC), which can subsequently covalently attach to the large latent TGFβ-binding protein (LTBP) to form the large latent complex (LLC) (Kanzaki *et al.*, 1990; Saharinen *et al.*, 1996; Maroni and Davis, 2011). The LLC binds to microfibrils and ECM via the LTBP. The degradation of these microfibrils via inflammatory proteolytic

enzymes like elastase displaces the LLC from ECM, prior to TGFβ activation (Chaudhry *et al.*, 2007; ten Dijke and Arthur, 2007). Following displacement from ECM, the mature TGFβ dimer is released from the LAP and activated by proteases, thrombospondin-1 (THBS1) or integrins in a cell-specific manner (Figure 1.10A), prior to binding to their signalling receptors (Crawford *et al.*, 1998; Annes *et al.*, 2003; Sheppard, 2005; ten Dijke and Arthur, 2007). Active TGFβ dimer signalling is mediated via two pairs of transmembrane receptor serine/threonine kinases knows as the TGFβ type I (TGFβRI) and TGFβ type II (TGFβRII) and/or accessory receptors TGFβRIII (betaglycan) and endoglin (on ECs). One TGFβRII and two distinct TGFβRI receptors; endothelium restricted activin receptor-like kinase (ALK)1 and widely expressed ALK5 are indicated in the TGFβ signalling. Active TGFβ binds to TGFβRII, which recruits, phosphorylates and activates TGFβRI (ALK5), which in turn phosphorylates SMAD transcription factors SMAD 2 and 3. Subsequently, SMAD2 and SMAD3 proteins interact either with SMAD4 to regulate targeted gene expression or with inhibitory SMAD7 resulting in inhibition of this cascade (ten Dijke and Arthur, 2007; Massague, 2008).

TGFβ exists in three isoforms; TGFβ1, TGFβ2 and TGFβ3, which show some overlap as well as distinct functions (Lebrin *et al.*, 2005). While active TGFβ2 binds to TGFβRIII, on ECs TGFβ1 and TGFβ3 bind to endoglin (Omwandho *et al.*, 2010). TGFβ1 is expressed in ECs during embryogenesis (Akhurst *et al.*, 1990). Mice lacking Tgfb1 either present with abnormal angiogenesis and die at embryonic day (E)10.5 or develop a normal cardiovascular system but die neonatally from an uncontrolled autoimmune disorder (Goumans and Mummery, 2000). In contrast, mice lacking either Tgfb2 or Tgfb3 show relatively normal angiogenesis (Kaartinen *et al.*, 1995; Bartram *et al.*, 2001). These studies suggest that TGFβ1 is more important than TGFβ2 and TGFβ3 for angiogenesis (ten Dijke and Arthur, 2007).

1.2.5.1 TGFβ1 and vascular development

The TGFβ1 signalling pathway is critical for angiogenesis (Figure 1.10B). In the activation phase it regulates cell proliferation, adhesion, differentiation and migration through the TGFβ/ALK1 pathway (Goumans *et al.*, 2002). However, in

Figure 1.10: The TGFβ regulatory pathway. (**A**) Regulation of TGFβ bioavailability and (**B**) the TGFβ signal transduction pathway. Diagram adapted from ten Dijke and Arthur (2007).

the resolution phase, it inhibits EC migration and proliferation through the TGFβ/ALK5 pathway (Goumans *et al.*, 2002), imparts stability to the blood vessels (Pardali and ten Dijke, 2009) through recruitment and differentiation of VSMCs as well as by facilitating changes in the ECM (Hanahan and Folkman, 1996; Massague, 1998; Smith, 1998; Lebrin *et al.*, 2005). Not surprisingly, various cardiovascular diseases in humans result from dysregulated TGFβ signalling (Gordon and Blobe, 2008). Moreover, mice deficient in Tgfb1 die *in utero* due to abnormal vasculogenesis (Dickson *et al.*, 1995).

Depending on the experimental setup, TGFβ1 can either stimulate or inhibit angiogenesis *in vivo* and *in vitro* (Goumans *et al.*, 2009). This variation in action is due to three fundamental features of the TGFβ signalling pathway; pleiotropy (based on a large set of transcription factors), co-ordination (due to the presence of a common enhancer element) and context dependence (due to cell or environment dependant SMAD expression) (Massague, 2008). *In vitro* studies have shown that while TGFβ1 disrupts growth in microvascular EC in bovine corpus luteum (Maroni and Davis, 2011), induces apoptosis in bovine aortic ECs (Pollman *et al.*, 1999b) and rat pulmonary microvascular ECs (Lu *et al.*, 2009), it also protects against EC apoptosis induced by serum deprivation in rat pulmonary arteries (Q. Lu, 2008). In comparison, *in vivo*, TGFβ1 induced angiogenesis via VEGF mediated EC apoptosis, followed by increased EC proliferation (Ferrari *et al.*, 2009). However, it is important to remember that, VEGF mediated apoptosis of ECs via TGFβ1 is essential during angiogenesis and its inhibition results in abnormal vessels (Pollman *et al.*, 1999a; Pollman *et al.*, 1999b; Ferrari *et al.*, 2009).

Both VEGF and TGFβ1 have also been shown to increase EC differentiation (Ferrari *et al.*, 2009), although some discrepancy exists in the context of their collaborative role (Mallet *et al.*, 2006). Interaction between ECs and ECM is crucial for angiogenesis, vascular stabilisation, storage and presentation of a variety of growth factors and cytokines which regulate vascular remodelling (Davis and Senger, 2008). One set of molecules key to mediating this interaction is the integrins; ECs interact with ECM components such as collagen, laminin and fibronectin through integrins (Weis, 2007). TGFβ1 also regulates the expression of various integrin molecules in ECs (Margadant and

Sonnenberg, 2010), thus allowing EC-ECM interactions thereby co-ordinating vascular stabilisation and development. Once vascular stability is achieved, ECs induce paracrine TGFβ1 signalling on neighbouring mesenchymal cells to promote pericyte or VSMC differentiation (Hirschi *et al.*, 1998; Mao *et al.*, 2012; Shi and Chen, 2014; Shi *et al.*, 2014). Consequently, these cells cover the new vessels achieving muscularisation and vascular maturation (ten Dijke and Arthur, 2007). The importance of TGFβ1 signalling in VSMCs is further highlighted by the study, which showed that loss of TGFβ receptors TGFβRII or ALK5 in VSMCs results in vascular defects and embryonic lethality (Jiao *et al.*, 2006). The fact that TGFβ1 signalling critically affects ECs prior to vascular maturation as well as VSMCs signifies its critical role in both early and late stages of angiogenesis.

1.2.5.2 TGFβ1 in endometrium

Amongst other reproductive events, the TGFβ1 signalling pathway has been implicated in uterine decidualisation and embryo implantation in mice (Roelen *et al.*, 1994; Lee *et al.*, 2007). Although understanding the specific functions of this pathway *in vivo* remains poorly understood due to receptor redundancy and/or embryonic lethality of Tgfb null mice, Li *et al.* (2011) showed the importance of Tgfbr1 in the function of the female reproductive tract by developing a conditional knockout (cKO) Tgfbr1 mouse model; these cKO female mice were sterile, had abnormal embryo development and defective SMC development in the uteri. In the endometrium TGFβ1 is expressed abundantly and is secreted by several different cell types including stromal cells, glandular epithelium, ECs, VSMCs and the immune cell population (Omwandho *et al.*, 2010; Lash *et al.*, 2012). Since TGFβ1 is involved in wound repair, it is also expressed by neutrophils, macrophages, NK cells and fibroblasts (Margadant and Sonnenberg, 2010). TGFβ1 is known to play a role in tumour promotion and even initial steps of endometrial carcinoma invasion (Muinelo-Romay *et al.*, 2011). Again, transcriptional downregulation of TGFβ1 has been found to correlate with transformation of the endometrium in malignancy (Soufla *et al.*, 2013). Interestingly, TGFβ1 transcript levels correlate with those of VEGF in endometrial tissue (Soufla *et al.*, 2013) and TGFβ1 has been shown to regulate VEGF production in breast cancer cell models (Donovan *et al.*, 1997). Although,

the presence of TGFβ1 and its receptors TGFβRI and TGFβRII in normal human endometrium, endometrial cancer and RM have been reported (Abberton *et al.*, 1999b; von Wolff *et al.*, 2000; Piestrzeniewicz-Ulanska *et al.*, 2002; Omwandho *et al.*, 2010; Lash *et al.*, 2012), to the best of our knowledge their presence in the context of heavy menstrual bleeding remains unknown.

1.2.6 The PDGF family

PDGF, discovered in the 1970s, is a serum growth factor, which stimulates growth and migration in glial cells (Westermark and Wasteson, 1976), fibroblasts (Kohler and Lipton, 1974) and vascular smooth muscle cells (Ross *et al.*, 1974). This highly conserved heparin-binding family is structurally and functionally related to the VEGF family of growth factors and can even stimulate VEGF secretion (Paez-Ribes *et al.*, 2009). The PDGF family consists of five disulphide bonded homo or heterodimer compositions; PDGF-AA, PDGF-BB, PDGF-AB resulting from PDGF-A and PDGF-B genes and the more recently described PDGF-CC and PDGF-DD, encoded by PDGF-C and PDGF-D genes respectively (Heldin and Westermark, 1999; Li *et al.*, 2000; Bergsten *et al.*, 2001; Fredriksson *et al.*, 2004). The A- and B- precursors are synthesised intracellularly as cleaved polypeptide chains, while the C- and D- precursors are not processed intracellularly and contain an N-terminal CUB domain, cleavage of which by tissue-type plasminogen activator (tPA) or plasmin for PDGF-C, or by urokinase-type PA (uPA) or matriptase (MT-Sp1) for PDGF-D, is essential for activation and receptor binding (Heldin, 2014). Spatial diffusion of the PDGFs in the tissue is also regulated by ECM binding or cleavage and release from the ECM by endogenous proteases (Figure 1.11A). Although such endogenous proteases are yet to be identified, Kelly *et al.* (1993) identified thrombin as a potential candidate (Kelly *et al.*, 1993; Andrae *et al.*, 2008). Once cleaved from ECM, the PDGFs can bind to their respective receptors for downstream signal transduction (Fredriksson *et al.*, 2004; Ustach and Kim, 2005; Ustach *et al.*, 2010).

The PDGFs act via two receptor tyrosine kinases (RTKs), PDGFRα and PDGFRβ. PDGFRα binds to all PDGF isoforms except PDGF-DD, while PDGFRβ binds to PDGF-BB and PDGF-DD. Additionally a heterodimeric

Figure 1.11: The PDGF regulatory pathway. (**A**) Regulation of PDGF bioavailability and (**B**) the PDGF signal transduction pathway. Diagrams (**A**, **B**) adapted from Demoulin and Essaghir (2014), Heldin (2014) respectively.

PDGFRαβ receptor exists which binds to PDGF-AB, BB and possibly even to PDGF-CC and DD (Demoulin and Essaghir, 2014). Ligand binding leads to autophosphorylation of the receptor, which activates its tyrosine kinase activity, which in turn allows docking sites for downstream signalling molecules containing Src homology 2 (SH2) domains. Subsequently, activation of the PDGFR and downstream signalling results in cell proliferation, survival, actin re-organisation and cell migration (Andrae *et al.*, 2008; Heldin, 2014), which may be enhanced by the interaction of PDGFR with integrins (Frisch and Ruoslahti, 1997). Factors that have been shown to induce PDGFR expression, include TGFβ, FGF-2 and oestrogen (Heldin and Westermark, 1999).

The PDGF signalling pathway is critical to normal embryo development since mice lacking PDGF ligands and receptors die shortly after birth. Importantly, mice lacking PDGFRα showed severe multi-organ defects, which included those observed in mice deficient in PDGF-A and –C (Soriano, 1997; Ding *et al.*, 2004), while those lacking PDGF-B and PDGFRβ were deficient in VSMCs and died at birth. Although PDGFRβ also binds PDGF-D, further studies did not indicate any major role for PDGF-D (Lindahl *et al.*, 1997). PDGF also plays an important role in wound healing in relation to ECM components such as collagen and heparin (Sun *et al.*, 2009).

Amongst the PDGF isoforms, PDGF-D expression is the least characterised, although it has been shown to be present in fibroblasts and smooth muscle cells. While PDGF-A and –C are expressed in epithelial cells, muscle and neuron progenitor cells, PDGF-B is primarily expressed in vascular ECs, megakaryocytes and neurons (Andrae *et al.*, 2008). In contrast to PDGF-A, PDGF-B can induce proliferation and migration of ECs and tube formation (Gacche and Meshram, 2014). Amongst the receptors, PDGFRα is expressed in mesenchymal progenitors in lung, skin and intestine, while PDGFRβ is particularly expressed in VSMCs and pericytes. Current evidence suggests a model such that vascular ECs secrete PDGF-BB, which interact with heparan sulphate (HS) at the EC surface or in the periendothelial matrix (Abramsson *et al.*, 2003; Lindblom *et al.*, 2003; Abramsson *et al.*, 2007). HS is suggested to play two roles at this site: it interacts with and results in local deposits of PDGF-BB, which are critical for appropriate recruitment of pericytes or VSMCs to the

vessel wall, while HS on the EC surface may enhance PDGF-BB mediated PDGFRβ signalling in the neighbouring pericytes (Andrae *et al.*, 2008). Thus, PDGF promotes maturation of blood vessels through VSMC recruitment, consequently supporting the structure of blood vessels especially through the PDGF-BB/PDGFRβ pathway and hence playing an important role in arteriogenesis (Betsholtz, 2004).

1.2.6.1 PDGF-BB and vascular development

All members of the PDGF and PDGFR family display angiogenic activity *in vivo* and are essential for normal vascular development (Figure 1.11B). PDGF-BB and its receptor PDGFRβ are the most widely evaluated (Leveen *et al.*, 1994; Soriano, 1994), although PDGFRα is also being considered for different types of mesenchymal/fibroblast derived pathologies (Andrae *et al.*, 2008). PDGF-BB and PDGFRβ are primarily expressed in developing vasculature, where quiescent ECs produce PDGF-BB and both perivascular cells and ECs produce PDGFRβ. Although initially PDGF-BB is widely expressed by the vascular ECs, with time it is restricted to the leading ECs at the sprouting tip, which remain in close contact with pericytes or VSMCs, thereby facilitating the release of PDGF-BB (Gerhardt and Betsholtz, 2003). Stimulation of ECs with PDGF-BB induces sprouting, proliferation and tube formation *in vitro* (Battegay *et al.*, 1994). Disruption of this signalling has been shown to result in irregular EC proliferation and near complete loss of pericytes, resulting in weakened vascular walls, dilated vessels and haemorrhage (Lindahl *et al.*, 1997; Shih and Holland, 2006; Andrae *et al.*, 2008), although the loss of pericytes in PDGF-BB and PDGFRβ knockouts was less severe in placenta than in kidney (Ohlsson *et al.*, 1999). Therefore, while TGFβ is indicated in both recruitment and differentiation of VSMCs during angiogenesis, recruitment of pericytes or VSMCs appears to be the primary function of PDGF-BB (Lindahl *et al.*, 1999).

1.2.6.2 PDGF-BB in endometrium

During the oestrogen dependant proliferative phase of the menstrual cycle, PDGF promotes endometrial stromal proliferation via PDGFRβ (Chegini *et al.*, 1992; Gargett *et al.*, 2008). PDGF also stimulates VEGF, which may play a role in vascular growth and remodelling during pregnancy (Taniguchi *et al.*, 2001).

Dysregulated PDGF expression has also been implicated in RM (Lash *et al.*, 2012). Moreover the importance of PDGF-BB in vascular development is further highlighted by the fact that dysregulated expression of PDGF is reported in tumorigenesis and vascular disorders such as atherosclerosis, restenosis and pulmonary hypertension (Andrae *et al.*, 2008; Heldin, 2013; Heldin, 2014). Strikingly, although processes such as altered vascular development, EC proliferation and VSMC differentiation, shown in heavy menstrual bleeding, are directly or indirectly regulated by the PDGF-BB signalling pathway; the role of PDGF-BB and its receptors PDGFRα and PDGFRβ remains unknown in this context, inviting further research.

1.2.7 The VEGF family

The VEGF family of growth factors are potent angiogenic and EC specific mitogenic factors critical to regulation of angiogenesis and lymphangiogenesis (Figure 1.12). VEGF was first identified as a vascular permeability factor (VPF) in the 1980s (Senger *et al.*, 1983) and sequenced later (Leung *et al.*, 1989) VEGF is a heparin-binding glycoprotein and in mammals its family comprises five homologous factors including VEGF-A (also known as VEGF), VEGF-B, VEGF-C, VEGF-D and PlGF (Gacche and Meshram, 2014). In normal tissues, VEGF-A is present at highest levels in adult kidney, lung, heart and adrenal gland, although lower levels are also detectable in other tissues (Hoeben *et al.*, 2004). As a result of alternative splicing, VEGF-A has five different isoforms including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (Hoeben *et al.*, 2004; Gacche and Meshram, 2014). VEGF-A binds to its receptors VEGFR1 and VEGFR2 and is perhaps the most widely studied member with important roles in blood vessel development, especially during embryogenesis. Studies in mice showed that VEGF-A homozygous as well as heterozygous knockout mice are embryonic lethal due to immature blood vessel formation (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996).

In adults, VEGF-B is abundantly expressed in myocardium, skeletal muscle and pancreas, while in mouse embryonic tissues VEGF-B is found in the developing heart, arterial smooth muscle and in the spinal cord (Hoeben *et al.*, 2004). It has

Figure 1.12: The VEGF family. (**A**) The VEGF family of receptors and ligands and (**B**) co-receptors involved in VEGF pathway. Diagram adapted from Olsson *et al.* (2006).

two isoforms generated through alternative splicing; VEGF-B_{167} VEGF-B_{186}, which are differentially expressed. Both VEGF-B and PlGF act via VEGFR1; while VEGF-B has been suggested to play significant roles in stimulation and maintenance of the coronary artery system (Aase *et al.*, 2001), PlGF promotes angiogenesis and stimulates EC survival (Luttun *et al.*, 2002). VEGF-C is predominantly expressed in the adult heart, placenta, ovary, small intestine and thyroid gland, and in sprouting lymphatic vessels in embryonic tissues. VEGF-C homozygous knockout mice are embryonic lethal due to fluid accumulation from dysregulated lymphangiogenesis (Karkkainen *et al.*, 2004). VEGF-C has also been shown to regulate differential expression of VEGF-A in cancer by promoting angiogenesis (Gacche and Meshram, 2014). It is secreted as a precursor and proteolytically processed to an active state, which acts via VEGFR3 (Hoeben *et al.*, 2004). Both VEGF-C and VEGF-D play an important role in lymphagiogenesis. VEGF-D is present in adult lung, heart, skeletal muscle, colon and small intestine, and in the developing lung in the embryo. Similar to VEGF-C, VEGF-D is secreted as a precursor, which is proteolytically processed for activation and binds to VEGFR3. VEGF-C and VEGF-D may also bind to VEGFR2, but with lower affinity than with VEGFR3 (Hoeben *et al.*, 2004; Olsson *et al.*, 2006).

In mammals, the five VEGF ligands bind to three RTKs VEGFR1-3 and co-receptors such as heparin sulphate proteoglycans (HSPGs) and neuropilins (NRP) (Olsson *et al.*, 2006). VEGFR1 binds VEGF-A, VEGF-B and PlGF. The VEGF signalling pathway is illustrated in Figure 1.12. VEGFR1 exists either in full length or soluble form (sFlt1), both of which bind VEGF-A with greater affinity than VEGFR2, and may thus negatively influence VEGFR2-mediated EC proliferation function. sFlt1 has been suggested to negatively regulate angiogenesis (Koch *et al.*, 2011). Other than ECs, VEGFR1 is expressed on monocytes and macrophages, where it regulates migration of these cells in response to VEGF-A (Koch *et al.*, 2011). $Flt1^{-/-}$ ($Vegfr1^{-/-}$) is embryonic lethal due to an increase in EC progenitors and formation of vessels without lumens (Fong *et al.*, 1995). This may suggest that VEGFR1 plays a negative role in angiogenesis, but deletion of VEGFR1 tyrosine kinase domain in mice [$Vegfr1(TK)^{-/-}$] show normal vascular development (Hiratsuka *et al.*, 1998). The

primary role of VEGFR1 may therefore be to capture excess VEGF and spatially regulate VEGFR2 signalling, rather than as a signalling receptor on ECs (Kappas *et al.*, 2008). VEGFR1 contains several tyrosine phosphorylation sites, which interact with p85/phosphatidylinositol-3-kinase (PI3K), phospholipase C-γ (PLCγ), SHP2, growth-factor-receptor bound 2(Grb2) protein and Nck, although downstream signal transduction remains unclear (Olsson *et al.*, 2006).

VEGFR2 is the principal receptor on ECs and is critical both during development and in adult physiology and pathology. It is suggested to be the main transducer for VEGF-A regulating EC differentiation, proliferation, migration and tubule formation (Olsson *et al.*, 2006; Koch *et al.*, 2011). In human VEGFR2 is also known as kinase insert domain receptor (KDR) and in mouse as fetal liver kinase (Flk1). It binds to proteolytically processed VEGF-C, VEGF-D and also VEGF-A with a 10-fold lower affinity than VEGFR1. VEGFR2 also exits in an alternatively spliced soluble form (sVEGFR2), which binds VEGF-C and prevents VEGFR3 binding, thereby inhibiting EC proliferation (Koch *et al.*, 2011). VEGFR2 is primarily expressed in vascular ECs or their precursors during embryogenesis. Vegfr2$^{-/-}$ mice are embryonic lethal (E8.5-E9.5) due to abnormal EC development (Shalaby *et al.*, 1995).

VEGFR3 has a high affinity for VEGF-C and VEGF-D. VEGF-C/VEGFR3 critically regulates lymphangiogenesis. However, VEGFR3 is also expressed in vascular ECs and is upregulated during angiogenesis (Koch *et al.*, 2011). Moreover, Vegfr3 gene targeted mice are embryonic lethal due to cardiovascular defects. Although later in development VEGF-C/VEGFR3 expression becomes restricted to lymphatic ECs, ECs at active angiogenesis sites such as tumor vasculature and tip cells of angiogenic sprouts in retina express VEGFR3 (Tammela *et al.*, 2008). Similar to the other receptors, VEGF-C and VEGF-D binding to VEGFR3 leads to kinase activation and phosphorylation at specific tyrosine sites, which induces association with proteins like Shc–Grb2 complex, resulting in activation of downstream pathways such as PI3/AKT, which are critical for lymphendothelial cell migration (Koch *et al.*, 2011).

1.2.7.1 VEGF and vascular development

VEGFs are crucial regulators of vasculogenesis in the developing embryo as well as angiogenesis in the adult (Figure 1.13). Hence, they are key target for drug therapy in pathological angiogenesis (Shibuya, 2013; Gacche and Meshram, 2014). In adults, most blood vessels remain quiescent, but in the presence of a physiological stimulus, vascular ECs can rapidly divide and activate angiogenesis under the regulation of VEGFs (Karamysheva, 2008). VEGF acts on ECs originating from arteries, veins or lymphatic vessels and induces angiogenesis or lymphangiogenesis. VEGF also induces expression of Bcl-2 and A1 in ECs, which are antiapoptotic proteins, and it has also been shown to act as a survival factor for ECs (Gerber *et al.*, 1998). In new-born mice, inhibition of VEGF resulted in extreme apoptotic changes, while mice older than four weeks showed no effect, suggesting that only newly formed ECs and not mature ECs are VEGF dependant (Karamysheva, 2008). Immature vessels lacking periendothelial cells remain dependant on VEGF, which is required for maintaining vascular homeostasis in adults (Benjamin et al., 1999; Lee et al., 2007). As vessels mature, pericytes or VSMCs are recruited which form a layer over the ECs. VEGFs are also involved in regulating vascular permeability (Karamysheva, 2008).

1.2.7.2 VEGF in endometrium

VEGF is expressed in a variety of tissues, including primate and rodent endometrium (Girling and Rogers, 2005). Apart from the ovary, endometrium is the only other tissue where physiological angiogenesis takes place in adulthood. Given the importance of the VEGF family in angiogenesis, it is unsurprising that VEGF is expressed in the normal human endometrium and is implicated in pathological states (Charnock-Jones *et al.*, 1993; Shifren *et al.*, 1996; Donnez *et al.*, 1998; Malik *et al.*, 2006; Girling and Rogers, 2009; Lash *et al.*, 2012; Li *et al.*, 2013). Human endometrium expresses VEGF-A isoforms, with cyclic variability, although VEGF-A_{165} is suggested to be the dominant isoform (Girling and Rogers, 2009). VEGF-A mRNA was shown to localise to the stroma, luminal and glandular epithelium in the proliferative phase, with expression remaining in the epithelial cells but not in the stroma in the secretory

Figure 1.13: The VEGF regulatory pathway via (**A**) VEGFR1, (**B**) VEGFR2 and (**C**) VEGFR3. Diagram adapted from Olsson *et al.* (2006).

phase. Moreover, its expression increases in the secretory phase and is at its highest in the menstrual phase (Charnock-Jones *et al.*, 1993; Shifren *et al.*, 1996; Sharkey *et al.*, 2000). VEGF-C and VEGF-D are also expressed in human endometrial stroma, glands and blood vessels (Moller *et al.*, 2002; Lash *et al.*, 2012). VEGF-C mRNA has been detected in endometrial biopsies throughout the menstrual cycle, with highest levels in glandular epithelial cells, vascular endothelium and a population of stromal cells, but there were no differences between the stratum functionalis and stratum basalis (Girling and Rogers, 2009). VEGF-D has been reported in low or moderate levels in both endometrium and myometrium throughout the menstrual cycle, without any significant differences between the epithelium, stroma, stratum basalis or functionalis or between the phases of the cycle (Girling and Rogers, 2009; Lash *et al.*, 2012).

1.2.8 The angiopoietin family

Angiopoietins are a family of secreted glycoproteins with major roles in regulation of blood vessel development and stability (Figure 1.14). They consist of four distinct members: Ang1, Ang2, Ang3 and Ang4, where Ang4 and Ang3 are human and mouse orthologues respectively (Singh *et al.*, 2011). Ang1 and Ang2 are the most widely studied members especially in vascular development. Ang1 binds to the RTK Tie2 (Tek) and is expressed in perivascular cells such as pericytes and VSMCs, where presence is induced by hypoxia and other growth factors such as VEGF and PDGF-B (Park *et al.*, 2003; Nishishita and Lin, 2004).

Ang2 acts as an antagonist to Ang1 and suppresses its effects on vascular remodelling and stability (Maisonpierre *et al.*, 1997). It is expressed by ECs and expression is transcriptionally regulated by factors such as hypoxia and VEGF (Oh *et al.*, 1999). Ang2 is stored within the endothelium in Weibel-Palade bodies but can be actively released in response to thrombin, histamine or pharmacological activators such as phorbol ester (Fiedler *et al.*, 2004). Similar to Ang1, Ang2 also binds to Tie2, although with much lower affinity than Ang1 and may also act as an antagonist to Ang1, although the mechanism is unclear (Singh *et al.*, 2011). Ang3 is expressed in various mouse tissues, while Ang4 is

present specifically in human lungs. Ang4 phosphorylates Tie2, and both Ang3 and Ang4 act as agonists of species specific Tie2 (Fagiani and Christofori, 2013).

Angiopoietins primarily bind to endothelial Tie2 and indirectly to Tie1 in Tie1/Tie2 heterodimers for downstream signal transduction (Davis *et al.*, 1996). Tie1 and Tie2 are expressed by both vascular and lymphatic ECs (Thomas and Augustin, 2009). On Ang1 binding, Tie2 receptors cluster to bring their kinase domains in close proximity to allow transphosphorylation and receptor activation, stimulating intracellular signalling pathways such as PI3K/Akt, mitogen activated protein kinase (MAPK) and Dok-R pathways (Singh *et al.*, 2011). Tie2 has been proposed to be primarily important for vascular EC formation rather than proliferation and maintenance (Dumont *et al.*, 1994). ECs also express Tie1, and although Tie1 is similar in structure to Tie2, it is unable to bind angiopoietins, but instead interacts with Tie2 on ECs (Singh *et al.*, 2011). The Tie1-Tie2 interaction limits Ang1 signalling through Tie2. However, AGFs such as VEGF promote Tie1 cleavage and can therefore regulate Ang1 responsiveness (Singh *et al.*, 2011). Tie1 null mice are also embryonic lethal, due not to perturbed angiogenesis but to loss of vascular integrity, which results in oedema and haemorrhage (Puri *et al.*, 1995). Angiopoietins may also bind to integrins α2β1, α5β1, αvβ3 and αvβ5, which allow it to transduce signal in non-endothelial cells lacking Tie2 receptor, such as cardiac and skeletal myocytes (Dallabrida *et al.*, 2005).

1.2.8.1 Angiopoietin and vascular development

The Ang/Tie signalling pathway is crucial for vascular remodelling and maturation by recruitment of perivascular cells during embryogenesis and adult vascular homeostasis (Figure 1.14). Transgenic mice with an inactive Tie2 gene are embryonic lethal due to defective maturation, stabilisation and the inability to transform a primary capillary plexus to a complex branched vascular network (Dumont *et al.*, 1994; Karamysheva, 2008). Ang1 mediates vascular remodelling, maturation, and perivascular cell association and lowers vascular permeability. Ang1 signalling is strictly monitored by regulation of its ability to

Figure 1.14: The angiopoietin regulatory pathway. Angiopoietin signaling pathways involved in (**A**) cell proliferation and (**B**) cell migration, vascular leakage and inflammation. Diagram adapted from Thomas and Augustin (2009).

bind to its receptor rather than by levels in the tissue microenvironment (Singh *et al.*, 2011). In the presence of VEGF, its competitive inhibitor Ang2 regulates EC migration, vascular sprouting and growth (Thurston *et al.*, 1999), while in the absence of VEGF it contributes to vascular regression (Maisonpierre *et al.*, 1997; Sheppard, 2005; Karamysheva, 2008). Interestingly, subcellular localisation of Tie2 in sparse or confluent ECs is determined by angiopoietins, thus affecting the outcome of downstream signalling. ECM bound Ang1 associates, activates and localises Tie2 to cell-matrix contacts in angiogenic ECs, thereby regulating cell migration and matrix adhesion (Fukuhara *et al.*, 2008; Saharinen *et al.*, 2008). However, in quiescent ECs, Ang1 promotes cell survival through association with Tie2 at inter-endothelial cell-cell junctions, which mediates further downstream signalling such as Dll/Notch4, which is known to regulate vascular quiescence (Zhang *et al.*, 2011). Tie2 is exclusively expressed in ECs (stalk cells), which follow the leading 'tip cells' during angiogenic sprouting where the Ang1/Tie2 pathway induces extracellular collagen IV deposition. Ang1/Tie2 may regulate Dll expression in stalk cells, thereby affecting vascular maturation in newly formed vessels (Zhang *et al.*, 2011; Fagiani and Christofori, 2013). Collectively the Ang1/Tie2 system is critical for the newly forming vascular network and its stabilisation.

However, vascular destabilisation occurs when in response to other AGFs, Ang2 is released. Increased Ang2 decreases the Ang1/Ang2 ratio, which suppresses Ang1 signalling, thereby leading to vascular destabilisation and active angiogenesis (Fagiani and Christofori, 2013). Additionally, Tie1 phosphorylation and heterodimerisation with Tie2 inhibits Tie2 activation and clustering, thus acting as another mechanism for regulation of Ang1 (Fagiani and Christofori, 2013). Ang1 associates with and activates Tie2 in the absence of Tie1 and therefore its action is increased with diminished levels of Tie1. Collectively, it is this balance of Ang1 to Ang2 or Tie2 to Tie1, which critically regulates and determines the transition between the quiescent and activated status of ECs (Fagiani and Christofori, 2013).

At sites of active angiogenesis, Ang2 promotes pericyte dissociation and vascular permeability. This facilitates cytokines and proteases to penetrate into the vascular bed preparing it to respond effectively to AGFs (Hammes *et al.*,

2004). As a result active angiogenesis by sprouting takes place where VEGF-A acts via VEGFR2 to induce Dll-4 expression in tip cells, which then induces Notch-1 expression in neighbouring ECs (stalk cells). The tip cells migrate towards the VEGF gradient followed by stalk cells (Lobov *et al.*, 2002; Bentley *et al.*, 2009), where high levels of Ang2 prevent pericyte recruitment to the newly forming vessels, thus helping them to continue elongation and remain in an active, unstable state (Fagiani and Christofori, 2013). Proliferation and elongation continues until lumen formation in these tubes, which allows blood flow (Fagiani and Christofori, 2013). This process of lumen formation and pericyte recruitment is repressed by TGFβ1, where Ang2 promotes this inhibitory effect, although Ang1 signalling can counteract this (Armulik *et al.*, 2005; Fagiani and Christofori, 2013). Therefore, again, it is the balance between factors such as Ang1-2, VEGF-A, PDGF-B and TGFβ that ultimately determines spatio-temporal angiogenesis and the fate of active ECs as angiogenic sprouts or mature vessels.

1.2.8.2 Angiopoietin in endometrium

Members of the angiopoietin family play an important role in angiogenesis and vascular remodelling throughout the body and are expressed in the human endometrium, although results vary (Hirchenhain *et al.*, 2003; Rogers and Abberton, 2003; Lee *et al.*, 2008; Lash *et al.*, 2012). Nevertheless, Ang1 immunostaining was observed in the ECs, VSMCs, stroma, luminal and glandular epithelium with Ang1 mRNA peaking in the mid secretory phase of the menstrual cycle. Ang2 was also detected in the ECs, VSMCs, stroma and glandular epithelium, mRNA peaking in the late secretory phase. Tie2 staining was also observed in VSMCs, stroma, glandular epithelium, with strongest staining on ECs (Akhurst *et al.*, 1990; Hirchenhain *et al.*, 2003; Rogers and Abberton, 2003; Girling and Rogers, 2009; Lash *et al.*, 2012).

1.3 HEAVY MENSTRUAL BLEEDING DISORDERS

Abnormalities in menstruation are the leading cause of gynaecological morbidity in the USA and UK, constituting 12% of all gynaecological referrals. Although abnormal uterine bleeding (AUB) disorders are not life threatening, they can have a major impact on woman's quality of life, posing a crucial health and economic problem in the UK and worldwide (Palep-Singh and Prentice, 2007; Shankar *et al.*, 2008).

Until recently, AUB disorders were classified as heavy menstrual bleeding (HMB or menorrhagia; blood loss of >80ml per cycle, flooding), metrorrhagia (prolonged, irregular periods), polymenorrhoea (frequent periods), oligomenorrhoea (scanty and infrequent periods), amenorrhoea (absent menstrual periods), intermenstrual bleeding and postcoital bleeding (Palep-Singh and Prentice, 2007). However, confusion and inconsistency in this nomenclature hampered investigation and management of AUB and there is now a universally accepted classification and nomenclature (Munro *et al.*, 2011; Munro *et al.*, 2012). Consistent with the International Federation of Gynaecology and Obstetrics (FIGO), AUB, is defined as any alteration from the normal menstrual cycle including variations in the regularity and frequency of menses, duration of flow or amount of blood loss (Singh *et al.*, 2013). Table 1.2 shows the normal limits of menstrual parameters suggested by FIGO, while Table 1.3 lists previously used terminologies that are now made redundant. Further definitions of terminologies in the FIGO classification of AUB are listed in Table 1.4.

AUB can result from a wide range of conditions such as endometrial or endocervical polyps (AUB-P), adenomyosis (AUB-A), leiomyoma (AUB-L) and hyperplasia or malignancy (AUB-M) (Munro *et al.*, 2011). AUB due to hormonal imbalances affecting the menstrual cycle such as ovulatory dysfunction (AUB-O), endometrial (AUB-E) or due to acquired or inherited bleeding disorders termed 'coagulopathy' (AUB-C). However, causes which cannot be demonstrated conclusively fall under the 'not yet classified' group (AUB-N) (Munro *et al.*, 2011; Ray and Ray, 2014).

Table 1.2: Normal limits of menstrual parameters (FIGO)

CLINICAL DIMENSIONS OF MENSTRUATION AND MENSTRUAL CYCLE	DESCRIPTIVE TERM	NORMAL LIMITS (90% CI)
Frequency of menses (*d*)	Frequent	<24
	Normal	24-38
	Infrequent	>38
Regularity of menses: cycle-cycle variation over 12 months (*d*)	Absent	No bleeding
	Regular	Variation ±2-20
	Irregular	Variation>20
Duration of flow (*d*)	Prolonged	>8.0
	Normal	4.5-8.0
	Shortened	<4.5
Volume of monthly blood loss (*ml*)	Heavy	>80
	Normal	5-80
	Light	<5

d-days; *ml*-milliliters; Table adapted from Munro *et al*. (2012).

Table 1.3: Discarded terminologies and accepted abbreviations (FIGO)

DISCARDED TERMINOLOGIES	ACCEPTED ABBREVIATIONS	
	Abbreviation	Full terminology
Dysfunctional uterine bleeding	AUB	Abnormal uterine bleeding
Epimenorrhagia	HMB	Heavy menstrual bleeding
Epimenorrhea	HPMB	Heavy and prolonged menstrual bleeding
Functional uterine bleeding		
Hypermenorrhea	IMB	Intermenstrual bleeding
Hypomenorrhea	PMB	Postmenopausal bleeding
Menometrorrhagia		
Menorrhagia		
Metrorrhagia		
Metropathica hemorrhagica		
Oligomenorrhea		
Polymenorrhagia		
Polymenorrhea		
Uterine hemorrhage		

Table adapted from Garza-Cavazos and de Mola (2012); Munro *et al*. (2012).

Table 1.4: Definitions of terms for uterine bleeding (FIGO)

CHARACTERISTICS	TERMINOLOGY	DESCRIPTION
Volume	Heavy menstrual bleeding	Excessive menstrual blood loss which interferes with the women's physical, emotional, social and material quality of life, which can occur alone or in combination with other symptoms
Regularity	Irregular menstrual bleeding	A range of varying lengths of bleeding-free intervals exceeding 20 days within one 90-day reference period
	Absent menstrual bleeding	No bleeding in a 90-day reference period
Frequency	Infrequent menstrual bleeding	Bleeding at intervals >38 days apart (1 or 2 episodes in a 90-day period)
	Frequent menstrual bleeding	Bleeding at intervals <24 days apart (>4 episodes in a 90-day period)
Duration	Prolonged menstrual bleeding	Menstrual blood loss which exceeds 8 days in duration
	Shortened menstrual bleeding	Menstrual bleeding less than 3 days in duration
Non-menstrual bleeding	Intermenstrual	Irregular episodes of bleeding, often light and short, occurring between otherwise fairly normal menstrual periods
	Post-coital	Bleeding post-intercourse
	Premenstrual, post-menstrual spotting	Bleeding that may occur on a regular basis for one or more days before or after the recognized menstrual period
Bleeding outside reproductive age	Post-menstrual bleeding	Bleeding occurring more than one year after the acknowledged menopause
	Precocious menstruation	Bleeding occurring before the age of 9 years
Acute or chronic AUB	Acute AUB	An episode of bleeding in a not pregnant woman of reproductive age, that is of sufficient quantity to require immediate intervention to prevent further blood loss
	Chronic AUB	Bleeding that is abnormal in duration, volume, and/or frequency and has been present for most of the last 6 months

Table adapted from Munro *et al.* (2012); S. Singh *et al.* (2013).

The most common AUB is HMB or menorrhagia. While HMB can be the presenting symptom of an underlying bleeding disorder, women with bleeding disorders commonly suffer from HMB (Kadir and Davies, 2013). Table 1.5 lists the prevalence of bleeding disorders in women with HMB. Therefore menstrual history, clinical impression and laboratory tests are utilised often alongside other objective questionnaires to differentiate between them (James, 2010).

Table 1.5: Prevalence of bleeding disorders in women with HMB

BLEEDING DISORDER	PREVALENCE
von Willebrand disease (vWD)	5-20% (Overall estimate 13%)
Platelet dysfunction	<1-47%
Factor XI deficiency	<1-4%
Haemophilia carriage	<1-4%
Rare factor deficiencies	<1%

Table adapted from James (2010).

1.4 HEAVY MENSTRUAL BLEEDING (MENORRHAGIA)

HMB can be defined as excessive menstrual blood loss which interferes with the woman's physical, emotional, social and material quality of life, and which can occur alone or in combination with other symptoms (NICE, 2007). 30% of reproductive age women are affected by HMB, with 1 in 20 women consulting a general practitioner (Garside *et al.*, 2004; Collins and Crosignani, 2007). By the age of 60 years, 20% of women in the UK have had a hysterectomy, HMB being the underlying cause in at least 50-70% (El-Hemaidi *et al.*, 2007). This results in an enormous economic burden on the National Health Service (NHS), with annual treatment costs of > £65 million in the UK. HMB significantly affects the well being of otherwise healthy women with 3.5 million working days lost annually (Collins and Crosignani, 2007). While HMB may result from clinical conditions (Hapangama and Bulmer, 2015), approximately 50% of cases remain unexplained and current treatment options such as oral contraceptives, intrauterine systems, endometrial ablation, hysterectomy, etc. affect fertility. Studies of the mechanisms that underlie HMB are therefore central not only to the understanding of this common condition but also to the possible development of new, less invasive and more effective treatments.

1.4.1 Mechanisms and regulation

One or more of the following processes may play a role in HMB: elevation in or premature breakdown of endometrial tissue and vessels at the beginning of menstruation; increased blood flow rate through structurally impaired, aberrant blood vessels; and failure of repair at the end of menstruation. Previous studies have shown that women with HMB bleed longer but, more critically, exhibit a significantly increased (3-4 fold increase) rate of blood loss (flow) (Abberton *et al.*, 1999b). Consistent with this Hurskainen et al. (1999) showed that women with HMB had a lower uterine artery pulsatility index (PI) than normal, since a higher mean velocity of blood flow results in decreased PI. Correlation between MBL and PI suggests that abnormal structure and or function of the uterine spiral arteries, including inefficient vasodilation or vasoconstriction may contribute to HMB.

The apparent role of spiral arteries in HMB raises questions regarding the underlying mechanisms. Failure of spiral arteries to control the rate of blood loss could be due to a failure in normal vasoconstriction, which in turn may be explained by abnormal spiral arteriogenesis, together with impaired VSMC maturation and function. Further evidence of the crucial role of spiral arterioles and VSMCs comes from a report that in women with HMB, VSMC proliferation in the spiral and straight arterioles is significantly reduced in the mid and late secretory phase (Abberton *et al.*, 1999b). This suggests that the spiral arterioles in HMB may have an altered growth pattern, since it is in the secretory phase that spiral arterioles grow and gain their distinctive coiled structure. Interestingly, the absolute wall thickness and αSMA staining pattern did not differ between control and HMB groups. However, it is possible that differentiation of VSMCs in the endometrial spiral arterioles is altered in HMB. Abberton *et al*. (1999a) reported that MyHC, which is a later marker for VSMC differentiation, was expressed at significantly lower levels in the early secretory phase in women with HMB, suggesting that VSMCs mature at different rates in the spiral arterioles of HMB endometrium compared with control endometrium. It is therefore of interest to determine whether differences in temporal and spatial patterns of staining of VSMC differentiation markers underlie HMB.

Given the vital role of various growth factors in the initiation and formation of endothelial tubes, recruitment of cells to the developing vessel and differentiation into smooth muscle cells (Smith, 1998; Lash *et al.*, 2012), it is probable that they may play a role in HMB. Of particular interest is the TGF-β1 family and its receptors, which stabilize newly formed vessels and also play a role in VSMC differentiation. Additionally, the PDGF family and their receptors also play a significant role in vascular maturation. Amongst these growth factors which form homo and heterodimers, PDGF-BB is of particular interest since it has been shown to have a role in vessel dilation in mice (Lindahl *et al.*, 1997; Betsholtz, 2004). In terms of endometrial vessel formation an important question in this context is how these factors link to exert an overall effect on angiogenesis and how these may be altered in HMB.

In the endometrium, angiogenesis involves tightly regulated interactions between the ECs and VSMCs, an alteration in which may result in HMB. It has

been reported that ECs proliferate more in women with HMB than in controls and endometrial spiral arterioles remain less covered by VSMCs (Kooy *et al.*, 1996; Smith, 2001). However, our understanding of the underlying mechanisms remains unclear. With the advent of techniques such as live-cell imaging it is now possible to study interactions even at a single cell or molecule level. It would therefore be of immense significance to study interactions between ECs and VSMCs *in vitro*, and importantly in the presence of factors that may play a potential role in vascular maturation or whose absence may have been implicated in HMB. For example, how do ECs interact with VSMCs and does exposure to proangiogenic factors such as VEGF-C, PDGF-BB or TGFβ1 affect their normal interaction?

1.5 HYPOTHESES AND AIMS

1.5.1 Hypotheses

Given the important role of uterine blood vasculature and the potential effects of endometrial leukocytes and angiogenic growth factors in HMB, we hypothesised that (illustrated in Figure 1.15):

1. Endometrial blood vessel structure and development is altered in women with HMB

2. Altered endometrial leukocytes and/or staining for angiogenic growth factors in the uterine tissue layers may underlie HMB

3. Use of an *in vitro* assay will aid investigation of the roles of different angiogenic growth factors in EC-VSMC association and tubule formation and possible future testing of potential interventions

1.5.2 Aims

To test the above hypotheses the aim was to:

1. Determine any impairment in uterine blood vessel development with respect to its constituents (ECs, VSMCs and ECM) in women with HMB

2. Determine whether the distribution of endometrial leukocyte populations and angiogenic growth factors PDGF-BB and TGF-β1 and their receptors are altered in the uterine tissue layers of women with HMB

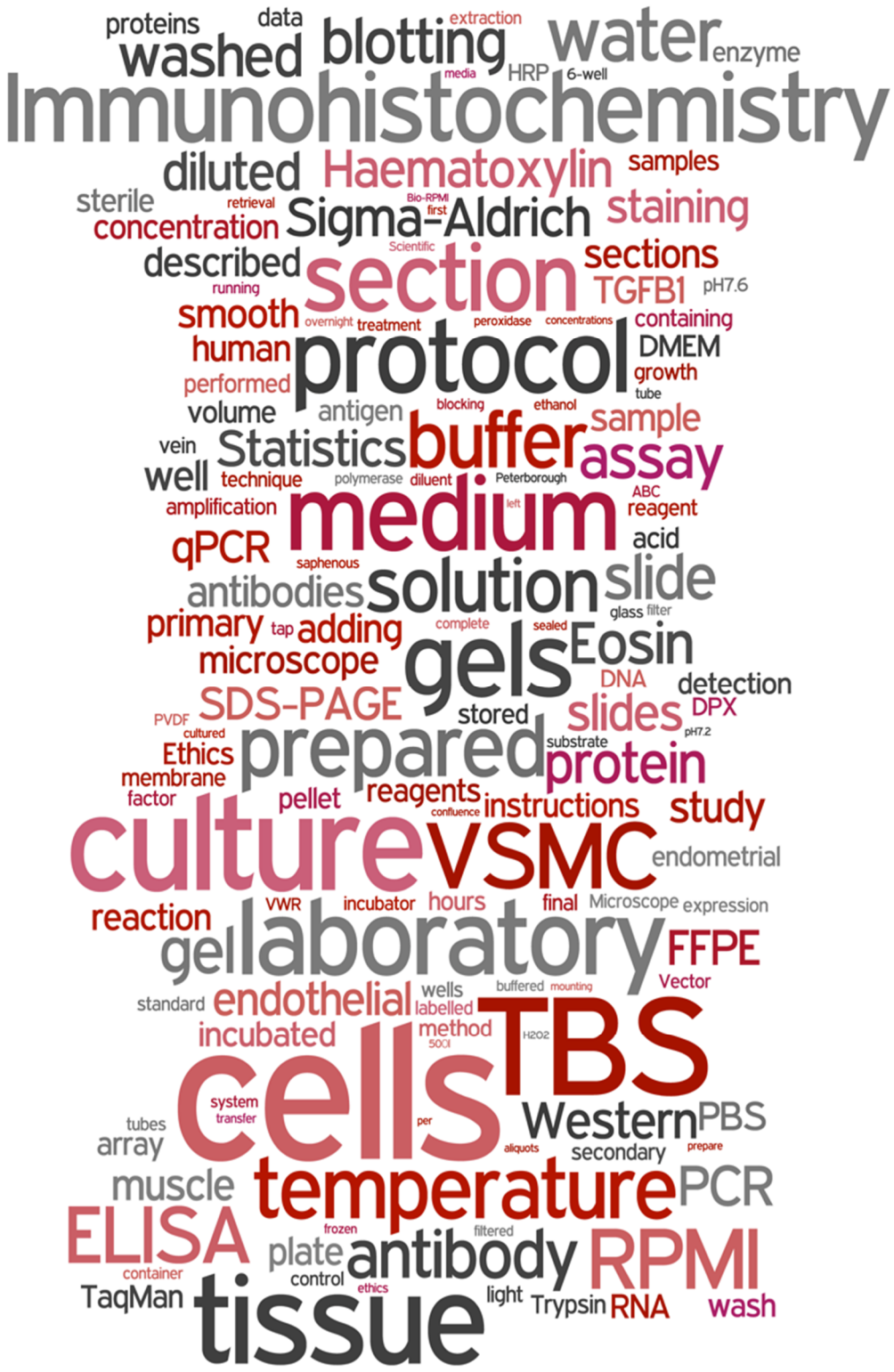
3. Develop an *in vitro* assay to investigate endothelial and vascular smooth muscle cell association and tubule formation

4. Determine the roles of PDGF-BB and TGF-β1 in endothelial and vascular smooth muscle cell association *in vitro*

Figure 1.15: Factors involved in dysregulation of angiogenesis underlying HMB. This study aims to investigate the factors highlighted in orange. ESP-early secretory phase; MSP-mid secretory phase; LSP-late secretory phase; PI- pulsatility index.

CHAPTER 2

MATERIALS AND METHODS



2 MATERIALS AND METHODS

This chapter specifies the general materials and methods used. The detailed description of methods for specific studies is discussed in appropriate chapters (sections 3.3, 4.3, 5.3, 6.3).

2.1 MATERIALS

2.1.1 Tissue materials and clinical data

2.1.1.1 Ethical considerations

Tissues and anonymised clinical data used in this study were obtained from patients at the Royal Victoria Infirmary, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne or at the Liverpool Women's Hospital, Liverpool with written informed consent (Appendix A1) in compliance with the Helsinki Declaration. In Newcastle, research nurses or midwives from the Reproductive Health and Childbirth Research team selected eligible patients on the gynaecological operating list and obtained their written informed consent. Collection of the endometrial samples was approved by Newcastle and North Tyneside Research Ethics Committee (Ref:10/H0906/71) and Liverpool Adult Ethics committee (Ref:09/H1005/55).

2.1.1.2 Endometrium for immunohistochemistry or explant culture

Endometrial biopsies were obtained from 106 women undergoing hysterectomy at the Royal Victoria Infirmary, Newcastle upon Tyne or at the Liverpool Women's Hospital, Liverpool. Following surgery the hysterectomy specimen was collected from the operating theatre by a member of the research team and transported to the Pathology Department. Here, a histopathologist inspected the specimen, excluded any suspicion of significant endometrial or cervical pathology and excised a block of endometrium or myometrium with overlying endometrium measuring approximately 1x1cm. The biopsy was collected in a sterile container, transported to the research laboratory and immediately fixed in 10% neutral buffered formalin or further dissected into smaller sections used for explant cultures. For some cases where only a small endometrial research sample was obtained, archival paraffin blocks were retrieved and used for the

respective immunohistochemical studies. The control group included fertile women without any uterine pathology potentially associated with endometrial abnormality (e.g. endometriosis, adenomyosis, intramural leiomyomata >2cm, any submucosal leiomyomata, history of heavy irregular bleeding or HMB) undergoing hysterectomy usually for prolapse, cystocele, rectocele, urinary or stress incontinence. The experimental group consisted of women with a history of HMB with unknown aetiology, defined as 'excessive menstrual blood loss which interferes with the woman's physical, emotional, social and material quality of life, and which can occur alone or in combination with other symptoms' (NICE, 2007). Any women who had received hormone treatment within 3 months prior to the operation were excluded from the study. Demographics of patients in the control and HMB groups as obtained from sample information form (Appendix A2) are shown in Figure 2.1 and medications taken by some patients are detailed in Appendices A3 and A4.

2.1.1.3 Saphenous veins for *in vitro* cell association study

Saphenous veins (SVs) were obtained from 9 patients undergoing coronary artery bypass graft surgery at the James Cook University Hospital, Middlesbrough. Demographic information obtained on these patients is shown in Table 2.1. Following surgery any surplus SVs were collected in a sterile container and transported to the research laboratory on ice. They were processed for endothelial and vascular smooth muscle cell extraction (section 2.2.6.7).

Table 2.1: Demographic details of patients used in the cell association study

CHARACTERISTICS	PATIENT (N=9)
Age (Years) Mean (±SEM)	67 ±3
Sex (%)	Male: 89 Female: 11
Smoking History (%)	No: 33 Yes: 22 Ex-smoker: 45
Diabetes (%)	No: 78 Yes: 22
Hypertension (%)	No: 44 Yes: 56

Figure 2.1: Demographic details of control and menorrhagia groups. In **A** and **B** the stacked bars represent number of patients in each of control and heavy menstrual bleeding (HMB) groups, while the different colours represent their ethnic origin or smoking history, respectively. In **C**, **D** and **E** the box plots (median indicated with black line, within each box) compares the age range, body mass index (BMI) and parity between control and HMB groups, respectively. Chi-square test (ethnicity, smoking history), Fisher's test followed by unpaired Student's t-test (age and BMI) and Mann-Whitney test (parity) indicated no statistical significance ($P>0.05$). Variation in patient number (N) in (**A**-**E**) is due to availability of different information.

2.2 METHODS

2.2.1 General

All chemicals, reagents, plasticware and glassware used for tissue culture were sterile unless stated otherwise.

2.2.1.1 Sterilization

Sterilization was carried out in an autoclave (Priorclave, London, UK) with moist heat at 121°C for 40 min to sterilize liquid or in a sterilizing oven at 185°C for 90 min for glassware.

2.2.1.2 Water

All tissue culture media and solutions were prepared using double distilled, sterilised water. Non tissue culture reagents and solutions were prepared in distilled water. PCR reagents were diluted using nuclease-free water (♯AM9932, Life Technologies, Paisley, UK).

2.2.1.3 Surface decontamination

Work surfaces were decontaminated before and after experiments with 70% ethanol or RNaseZap (♯AM9780, Life Technologies, Paisley, UK) and wiped dry with paper towel as appropriate.

2.2.1.4 Incubation

All cell and tissue culture incubations were carried out in a moisture enhanced incubator, supplemented with 5% CO_2 at 37°C.

2.2.2 Human tissue preservation

Preservation of cells and tissues in their natural state is central to all histological techniques. To this end, tissue blocks or sections are immersed in a fixative fluid (e.g. formalin), which prevents autolysis, necrosis of the excised tissue and makes macromolecules resistant to disintegration by water and other liquids, thereby stabilising both intra and inter-cellular structures within the tissue. Moreover, fixative fluids act as anti bacterial agents.

2.2.2.1 Preparation of formalin fixed paraffin embedded (FFPE) tissue

Following tissue collection, endometrial or myometrial biopsies were transported to the laboratory and immediately fixed in 10% neutral buffered formalin (NBF), which is the most common fixative used for histopathology. It fixes tissue by the addition of crosslinking methylene bridges to the side chains of basic amino acids. This results in low macromolecule permeability, while largely not affecting structures of protein molecules. Additionally formaldehyde molecules are small in size resulting in faster penetration. This makes it applicable for fixation of both small and large tissues. Although NBF penetrates quickly, the cross-linking reaction is a slow process. Formalin fixation of tissue was therefore performed at room temperature for 24-48 hr following which the tissue was routinely processed, embedded in molten paraffin wax and allowed to set in a plastic cassette. 3µm tissue sections were cut using a microtome, mounted on APES-coated glass slides (Appendix B), dried overnight in an oven at 37℃, labelled and stored appropriately in a slide storage unit for a short time until use.

2.2.2.2 Haematoxylin and Eosin (H&E) staining

Haematoxylin and eosin (H&E) staining is the commonest staining system used in the histology laboratory. When haematoxylin dissociates in water, its positive ion binds to negatively charged (basophilic) phosphate groups of nucleic acids (DNA and RNA) in the nuclei of cells, colouring them blue. On the other hand, when eosin dissociates in water, its negative ion combines non-specifically with positively charged (acidophilic) proteins in the cell cytoplasm and extracellular matrix, colouring them pink.

H&E staining was used for initial assessment and staging of FFPE endometrial or myometrial tissue. FFPE sections on APES-coated glass slides were dewaxed in xylene for 5 min, rehydrated through descending concentrations of ethanol (99%, 95%, 70%) and washed in running tap water. Slides were then immersed in Mayer's haematoxylin for approximately 90 sec to stain the cell nuclei blue and then washed in running tap water. Slides were placed for 1 min in Scott's tap water substitute for further development of the blue stain. After washing in running tap water, the slides were placed for 2 min in eosin to stain the cytoplasm pink. The slides were washed further in running tap water,

dehydrated sequentially through ascending concentrations of ethanol (70%, 95%, 99%), cleared in xylene and mounted using DPX synthetic mounting resin and glass cover slips. Figure 2.2 shows the human endometrium at the different stages of the menstrual cycle as observed using H&E staining.

2.2.2.3 Preparation of frozen tissue samples

Myometrial tissue to be frozen was placed on small pieces of filter paper. 2-methylbutane also called Isopentane (♯10093, AnalaR, VWR International) was carefully poured into a metal beaker, which was then lowered into a flask containing liquid nitrogen for 1-2 min to cool the isopentane. The tissue on filter paper was carefully lowered and held in the cooled isopentane for approximately 2-3 min, carefully using forceps. The frozen tissue was placed into labelled freezer bags or cryotubes (♯377267, Fisher Scientific) and immediately stored at -80℃ until use.

2.2.3 Immunohistochemistry

Immunohistochemistry (IHC), first reported in 1941 (Coons *et al.*, 1941), is a technique used to detect antigens or proteins with specific primary or secondary antibodies tagged with a visible label. It is an extremely useful technique, which allows detection and localisation of cellular components within tissue. In this study IHC was performed on FFPE samples as described in section 2.2.2.1. Although various labelling and detection systems are used in IHC, the system used in this study was the avidin-biotin (ABC) technique as described below.

2.2.3.1 Avidin-biotin complex (ABC) method

3µm sections of FFPE tissue mounted on APES-coated glass slides were dewaxed for 5 min in xylene, rehydrated sequentially in ethanol (99%, 95%, 70%) and washed in running tap water. Following this the slides were immersed into 1.5% H_2O_2 in distilled water for 10 min to block any endogenous peroxidase activity.

The slides were further washed in running tap water prior to the antigen retrieval process. Antigen retrieval is essential to retrieve epitopes masked by protein crosslinking caused by fixation. In this study either an enzyme induced epitope retrieval (using Trypsin-$CaCl_2$ working solution, Appendix B) or a Heat-Induced

Figure 2.2: Haematoxylin and eosin stained human endometrium (**A**, **C**, **E**, **G**; 100x original magnification) and endometrial glands (**B**, **D**, **F**, **H**; 400x original magnification). Arrows indicate respective changes at the different stages of the menstrual cycle.

Epitope Retrieval (HIER) method utilising various sources of heat (pressure cooker, microwave) was applied for antigen retrieval. The detailed antigen retrieval conditions used in this study are listed in Table 2.2.

Following antigen retrieval the slides were once again washed in TBS (pH7.6) for 5 min. When using fixed frozen samples, antigen retrieval was not required. Slides were retrieved from -20°C, brought to room temperature, washed in TBS (pH 7.6) and IHC was carried out as for FFPE sections as follows.

The sections were immunolabelled using an avidin-biotin (ABC) technique. This technique depends on secondary antibodies conjugated to biotin, which link tissue-bound primary antibody to an avidin-biotin-reporter enzyme complex. Avidin forms large complexes containing multiple copies of the biotinylated reporter enzyme, horseradish peroxidase (HRP). An HRP substrate is then added, which in the presence of H_2O_2 is converted into a coloured insoluble reaction product detectable in the tissue using light microscopy. The principle of the streptavidin-biotin complex (ABC) method is outlined in Figure 2.3A.

The tissue sections were outlined using a hydrophobic barrier pen (♯H-4000, Vector Laboratories, Peterborough, UK) and overlaid with blocking serum for 10 min at room temperature, to block non-specific binding sites in the tissue sections. Following this the blocking serum was tipped off and appropriately diluted primary antibody was applied. The incubation time and temperature used was antibody dependant. Details of the primary antibodies used (dilution, incubation times, temperatures) in this study are listed in Table 2.2. The slides were washed 2 x 5 min in TBS (pH7.6) on a rocking platform and secondary antibody was applied to the sections and incubated at room temperature for 30 min. Following this the slides were washed 2 x 5 min in TBS (pH7.6) and Vectastain ABC reagent was applied and incubated at room temperature for 30 min. Vectastain Elite secondary antibody and ABC reagents are detailed in Table B5 and Table B6. The slides were washed once again 2 x 5 min in TBS (pH7.6) and the reaction was developed using 3,3'-Diaminobenzidine (DAB) solution (Appendix B) for approximately 1 min. The slides were then washed in running tap water, counterstained with Mayer's haematoxylin for 1 min, followed by further development of the blue stain in Scott's tap water substitute for 30

Figure 2.3: (**A**) The avidin-biotin complex (ABC) method for immunohistochemical staining. (**B**) Formalin fixed paraffin embedded 3µm section of human kidney positively stained for collagen-IV using ABC method (200x original magnification).

sec. Finally, the slides were dehydrated in ascending concentrations of ethanol (70%, 95%, 99%), cleared in xylene and mounted using DPX synthetic mounting resin and glass cover slips. Figure 2.3B shows collagen-IV staining in human kidney as observed using the ABC immunolabeling technique.

2.2.3.2 Double immunohistochemical labelling

Double IHC was performed sequentially using the ABC method and two different primary antibodies. Two different combinations of visualization was used; either the first immunostain was visualized using DAB solution containing 0.01% H_2O_2 (Appendix B) to give a brown reaction product and the second with Vector SG substrate kit for peroxidase to give a silver-grey reaction product (Appendix B, Table B8), or the first using Vector SG substrate kit for peroxidase (Appendix B, Table B8), and the second with Vector NovaRED substrate kit for peroxidase (Appendix B, Table B7). Appropriate positive and no primary negative controls were performed for each antibody run, including single immunohistochemistry labelling with each antibody used. Negative controls showed no immunostaining for any of the protocols used.

2.2.3.3 Optimisation of antibodies

All antibodies were optimised using appropriate positive and negative controls prior to staining the tissue of interest using different combinations of antibody dilutions and antigen retrieval methods. The final parameters were selected on the basis of strongest specific reactivity with the least background for each antibody used.

2.2.3.4 Quantification of immunohistochemistry

Immunostained slides were blinded independently before assessment. Tissue sections were examined using a Nikon Eclipse 80i microscope with a 20x or 40x objective and 10x eyepiece, as appropriate. Depending on the study question, images were quantified either by counting positively stained cells, a modified quick score method or measurement of the area of positive staining using ImageJ (Version 1.46, NIH, Maryland, USA), or NIS Elements Viewer, NIS Elements Ar Viewer (NIKON Instruments Inc., Surrey, UK). The modified 'Quickscore' method (Schiessl *et al.*, 2009) took into account both

Table 2.2: Primary antibodies used for immunohistochemistry

ANTIBODY	CATALOGUE NO:	CLONE	HOST	ANTIGEN RETRIEVAL	DILUTION	INCUBATION
αSMA[1]	M0851	1A4	Mouse	None	1:200	RT, 60 min
Caldesmon[1]	M3557	h-CD	Mouse	PC in citrate buffer pH 6.0, 1 min	1:100	RT, 60 min
Calponin[1]	M3556	CALP	Mouse	Trypsin buffer pH7.8, 37℃, 10 min	1:80	RT, 30 min
CD3[2]	NCL-L-CD3-565	LN10	Mouse	PC in citrate buffer pH 6.0, 1 min	1:100	RT, 60 min
CD8[2]	NCL-CD8-295	1A5	Mouse	PC in EDTA buffer pH 8.0, 1 min	1:100	RT, 60 min
CD14[2]	NCL-CD14-223	7	Mouse	PC in citrate buffer pH 6.0, 1 min	1:20	RT, 60 min
CD31[2]	NCL-CD31-1A10	1A10	Mouse	PC in citrate buffer pH 6.0, 1 min	1:20	RT, 60 min
CD34[1]	M7165	QBEnd-10	Mouse	PC in citrate buffer pH 6.0, 1 min	1:250	RT, 60 min
CD56[2]	NCL-CD56-1B6	1B6	Mouse	PC in citrate buffer pH 6.0, 1 min	1:50	RT, 60 min
CD83[2]	NCL-CD83	1H4b	Mouse	PC in citrate buffer pH 6.0, 1 min	1:40	4℃, ON
Desmin[2]	NCL-DES-DERII	DE-R-11	Mouse	Trypsin buffer pH7.8, 37℃, 10 min	1:50	RT, 60 min
Collagen-IV[2]	COLL-IV	PHM-12	Mouse	Trypsin buffer pH7.8, 37℃, 10 min	1:60	RT, 60 min
F8RA[1]	M0616	F8/86	Mouse	Trypsin buffer pH7.8, 37℃, 10 min	1:50	RT, 60 min
Fibronectin[2]	NCL-FIB	568	Mouse	Trypsin buffer pH7.8, 37℃, 10 min	1:100	RT, 60 min

[1]Dako Cytomation, Cambridgeshire, UK; [2]Leica Laboratories, Newcastle, UK; [3]Abcam Cambridgeshire, UK; [4]Sigma Chemical Co. Dorset, UK; pd-prediluted, RT-Room temperature; PC-Pressure cook, ON-overnight, MW-microwave.

Table 2.2 (continued): Primary antibodies used for immunohistochemistry

ANTIBODY	CATALOGUE NO:	CLONE	HOST	ANTIGEN RETRIEVAL	DILUTION	INCUBATION
FOXP3[3]	ab20034	236A/E7	Mouse	PC in EDTA buffer pH 8.0, 1 min	1:50	RT, 60 min
Ki67[2]	NCL-Ki67-MM1	MM1	Mouse	PC in citrate buffer pH 6.0, 1 min	1:50	RT, 60 min
Laminin[2]	NCL-LAMININ	LAM-89	Mouse	Trypsin buffer pH7.8, 37°C, 10 min	1:200	RT, 60 min
Myosin heavy chain[4]	M7786	hSM-V	Mouse	PC in citrate buffer pH 6.0, 1 min	1:600	RT, 60 min
Osteopontin[3]	ab8448	N/A	Rabbit	None	1:100	RT, 60 min
PDGF-BB[3]	ab9704	N/A	Rabbit	MW in citrate buffer pH 6.0, 2 x 5 min	1:20	RT, 60 min
PDGFRα[3]	ab61219	N/A	Rabbit	PC in citrate buffer pH 6.0, 1 min	1:100	RT, 30 min
PDGFRβ[3]	ab15502	N/A	Rabbit	PC in citrate buffer pH 6.0, 1 min	pd	RT, 60 min
Smoothelin[3]	ab8969	R4A	Mouse	PC in citrate buffer pH 6.0, 1 min	1:100	RT, 60 min
TGFβ1[3]	ab92486	N/A	Rabbit	PC in citrate buffer pH 6.0, 1 min	1:50	RT, 60 min
TGFβRI[3]	ab31013	N/A	Rabbit	PC in EDTA buffer pH 8.0, 1 min	1:50	RT, 60 min
TGFβRII[3]	ab78419	MM0056-4F14	Mouse	None	1:75	RT, 60 min

[1]Dako Cytomation, Cambridgeshire, UK; [2]Leica Laboratories, Newcastle, UK; [3]Abcam Cambridgeshire, UK; [4]Sigma Chemical Co. Dorset, UK; pd-prediluted, RT-Room temperature; PC-Pressure cook, ON-overnight, MW-microwave, N/A- not applicable.

percentages of vessels stained with a given antibody (1=0-25%, 2>25-50%, 3>50-75%, 4>75-100%) and the staining intensity for each percentage (0=negative, 1=weak, 2=moderate, 3=strong). The percentage and intensity scores were then multiplied and summed to give a range of possible scores between 0-12. For example: negative staining in 25% of a given cell type (0×1=0), weak staining in 50% (1×2=2) and moderate staining in 25% (2×1=2), would give a total score of (0+2+2)=4.

2.2.4 Lectin histochemistry

FFPE sections (3µm) were dewaxed in xylene, rehydrated through descending concentrations of alcohol (99%, 90%, 70%) and incubated in 1% H_2O_2 in water for 10 min to block endogenous peroxidase activity. Enzyme induced antigen retrieval was carried out at 37°C in Trypsin-$CaCl_2$ working solution for 15 min (Appendix B). All washes and incubation were performed in TBS (pH 7.6) at room temperature. Sections were treated with rabbit serum (♯PK-6100, Vectastain Elite ABC kit; Vector Laboratories) for 10 min to block non-specific binding. Following this, the sections were incubated with biotinylated Europaeus-Agglutinin I (UEA-I) lectin diluted 1:20 in TBS (pH 7.6) for 60 min (♯L8262, Sigma-Aldrich Co.). The sections were further washed and incubated for 30 min with streptavidin horseradish peroxidase (HRP) diluted 1:100 in TBS, pH7.6 (♯P0397, Dako Cytomation, Cambridgeshire, UK). The sections were then washed in TBS (pH7.6) and the reaction was developed using DAB solution (Appendix B) for approximately 1 min. The sections were then lightly counterstained with Mayer's haematoxylin for 30 sec, dehydrated in ascending concentrations of ethanol (70%, 95%, 99%), cleared in xylene and mounted using DPX synthetic mounting resin and glass cover slips.

2.2.5 Immunofluorescence

Immunofluorescence (IF), based on the works of Coons and Kaplan (Coons *et al.*, 1941; Coons and Kaplan, 1950), is a common laboratory technique used to investigate both levels and localisation of proteins within cells or tissue. This technique is applicable to both fresh and fixed samples. Immunofluorescent labelling can be either direct, where the primary antibody against the molecule

of interest is directly conjugated to a fluorescent dye, or indirect, where the primary antibody is unlabelled and requires a fluorescently tagged second anti-immunoglobulin antibody directed towards the primary antibody. Fluorescent dyes are molecules with luminescent properties, which absorb and emit light at different wavelengths. Commonly used dyes are Fluorescein isothiocyanate (FITC), sulforhodamine 101 acid chloride (Texas Red), Tetramethylrhodamin (TRITC), 4', 6-diamidino-2-phenylindole (DAPI), etc.

In this study, indirect IF was used to determine proportion of cells expressing different proteins on 8-well BD Falcon chamber slides (#354108, SLS Ltd., Yorkshire, UK) containing vascular smooth muscle cells grown overnight on matrigel. Following 24 hr culture, medium from the chamber slides was discarded and the cells were washed in PBS (pH7.2), fixed in 99% ethanol for 10 min at room temperature, washed in PBS (pH7.2), air dried for approximately 10 min, wrapped in foil and frozen at -20°C until IF was performed. Prior to immunolabeling, the slides were removed from the -20°C freezer and brought to room temperature. They were washed three times in PBS (pH7.2) for 5 min each, overlaid with the diluted primary antibody of interest (Table 2.3) and incubated for 30 min at room temperature. Following this the slides were again washed three times in PBS (pH7.2) for 5 min each, overlaid with the diluted secondary antibody, conjugated with fluorescent dye FITC (#F1-2000, Vector Laboratories) or Texas Red (#T1-2000, Vector Laboratories) and incubated for 30 min at room temperature. The slides were washed twice in PBS (pH7.2) for 5 min, following which the chamber was removed from the slide using the chamber removal device provided, washed for a further 5 min in PBS (pH7.2) and mounted using Vectashield ultramount aqueous permanent mounting medium with DAPI (#H1200, Vector Laboratories) and glass cover slips. The slides were left to dry wrapped in foil at 4°C in the dark, until imaging was performed. Figure 2.4 illustrates the basic steps in immunofluorescent staining.

Table 2.3: Primary antibodies used for immunofluorescence

	ANTIBODY	CATALOGUE NO:	CLONE	HOST	DILUTION
1	Caldesmon[1]	M3557	h-CD	Mouse	1:100
2	Calponin[1]	M3556	CALP	Mouse	1:100

[1]Dako Cytomation, Cambridgeshire, UK

2.2.6 Tissue and cell culture

All tissue and cell culture was performed in a BioMAT2 Class-II Microbiological Safety Cabinet (CAS, Manchester, UK) using aseptic technique. Cells and tissues were cultured under normal growth conditions in a humidified incubator at 37℃, supplemented with 5% CO_2 (inCu saFe IR Sensor CO2 incubator, Panasonic, Leicestershire, UK).

2.2.6.1 Cell count

Cell concentration was determined using a Neubauer haemocytometer (VWR International, Leicestershire, UK) and an inverted light microscope (Olympus CK2, Olympus, Southend-on-Sea, UK) at 200x magnification. Trypsinised cells were suspended in 1ml appropriate culture medium. 10µl cell suspension was pipetted onto the counting grid of the haemocytometer. The total number of cells (α) was counted by adding the number of cells in the outer four squares and the centre square. Cell concentration was determined by the following equation:

$$\text{Cell concentration } (\text{ml}^{-1}) = \frac{\alpha}{20} \times 10^6$$

2.2.6.2 Cell culture

The routine cell culture method described in this section is applicable to all cells used in this study. Cells were seeded into 25cm^2 T25 plastic cell culture flasks with filtered caps (♯690175, Greiner Bio-One, Gloucestershire, UK) and allowed to reach 90% confluence as assessed by an inverted light microscope (Olympus CK2, Olympus, Southend-on-Sea, UK) using 10x objective and 10x eyepiece. Once confluent, adherent cells in the T25 flasks were washed with sterile PBS (pH7.4), followed by a 3-5 min incubation in 1x Trypsin-EDTA solution containing 0.5g/l porcine trypsin and 0.2g/l EDTA (♯T3924, Sigma-Aldrich Co.) at 37℃, until cells were detached. The trypsinised cells were decanted into a 15ml universal container and cell culture medium was added to inactivate the trypsinisation reaction. The cell suspension was centrifuged at 200g for 5 min (MSE Mistral2000 centrifuge, MSE Ltd., London, UK) to separate the cells from suspension. The supernatant was discarded and the cell

Figure 2.4: (**A**) Immunofluorescent staining of 8-well chamber slides. (**B**) Ethanol fixed, frozen human saphenous vein derived vascular smooth muscle cells positively stained for αSMA (FITC-green) and nuclei (DAPI-blue), 200x original magnification.

pellet was re-suspended in the desired volume of appropriate culture medium before being passaged into a 75cm^2 T75 plastic cell culture flask with filtered caps (♯658175, Greiner Bio-One). Serial passages were carried out when cells were 90% confluent and media was replaced every 2-3 days. In order to assess the purity of each cell type, cell characterisation (section 2.2.6.3) was performed after every two passages.

2.2.6.3 Cell characterisation

Primary cell cultures are mortal and therefore can be passaged a finite number of times before entering senescence. Moreover, these cells may de-differentiate and lose their inherent characteristics during serial passaging. The number of times the cells can be passaged depends on various factors such as nutrients, culture conditions and manipulation during passaging. Therefore it is key to assess the purity of the cells during sequential passaging by cellular characterisation.

Cells to be characterised were added to approximately 4 wells of an 8-well BD Falcon chamber slide. They were allowed to settle down for at least 5 hr, following which medium from the chamber slides was discarded and the cells were fixed in 99% ethanol for 10 min at room temperature, air-dried for approximately 10 min, and immunostained using the ABC method (section 2.2.3.1).

Endothelial cells were characterised for Factor VIII related antigen (F8RA), CD31 and CD34, while vascular smooth muscle cells were characterised for alpha-smooth muscle actin (αSMA), h-Caldesmon, and myosin heavy chain. In this study, cells were included only if characterisation resulted in >90% positive cell population for specific primary markers. Details of primary antibodies and dilutions used are listed in Table 2.4. Figure 2.5 demonstrates human SV derived ECs and VSMCs were characterised at a specific passage.

Table 2.4: Primary antibodies used for immunohistochemistry on fixed frozen cells

ANTIBODY	CATALOGUE NO:	CLONE	HOST	DILUTION
αSMA[1]	M0851	1A4	Mouse	1:200
Caldesmon[1]	M3557	h-CD	Mouse	1:100
CD31[2]	NCL-CD31-1A10	1A10	Mouse	1:200
CD34[1]	M7165	QBEnd-10	Mouse	1:250
F8RA	M0616	F8/86	Mouse	1:800
Myosin heavy chain[3]	M7786	hSM-V	Mouse	1:600

[1]Dako Cytomation, Cambridgeshire, UK; [2]Leica Laboratories, Newcastle, UK; [3]Sigma Chemical Co. Dorset, UK

2.2.6.4 Cryopreservation of cells

Endothelial and vascular smooth muscle cells from SVs and cell lines were trypsinised using Trypsin-EDTA solution, centrifuged and the cell pellet was re-suspended in 1ml RPMI 1640 complete medium (Appendix B). The cell suspension was added to a cryotube containing 1ml thawed cell freezing medium (Appendix B). The cryotube was slowly frozen in a Mr. Frosty freezing container (♯5100-0001, Fisher Scientific,) to prevent temperature shock, while achieving a very slow and optimal rate of cooling at -1°C/min and maintained for 24 hr at -80°C. The cryotube was then transferred to a liquid nitrogen container for storage.

Frozen cells were rapidly thawed at room temperature with gentle shaking. The cell suspension in freezing medium was added to 2ml appropriate culture medium in a 15ml universal container. This suspension was directly seeded into a T25 cell culture flask or centrifuged to collect the cell pellet, re-suspended in 2ml appropriate culture medium and then seeded into a T25 cell culture flask. Culture medium was changed after 24 hr and cell culture was carried out as described in section 2.2.6.2.

2.2.6.5 Human aortic endothelial cell line

The human aortic endothelial cell line (PCS-100-011) was obtained from LGC Standards Ltd, Teddington, UK. This primary cell line isolated from human aorta has been tested for bacteria, yeast, mycoplasma Hepatitis B, Hepatitis C and

Figure 2.5: Characterisation of saphenous vein derived cells. Ethanol fixed, frozen human saphenous vein derived endothelial cells (**A**-**D**) and vascular smooth muscle cells (**E**-**H**) characterised for their respective markers at passage 4 (200x original magnification).

HIV. This cell line demonstrates ≥50% viability when thawed from cryopreservation.

2.2.6.6 Human aortic vascular smooth muscle cell line

The human aortic vascular smooth muscle cell line (T/G HA-VSMC CRL-1999) was obtained from LGC Standards Ltd. This primary cell line isolated from a Caucasian female aorta, has been tested for bacteria, yeast, mycoplasma Hepatitis B, Hepatitis C and HIV.

2.2.6.7 Enzymatic disaggregation of human saphenous veins

Segments from SVs, obtained during coronary bypass operation, were transported to the laboratory as described previously in section 2.1.1.3. Vein segments that were ≥ 2cm were selected as suitable for endothelial and vascular smooth muscle cell extraction. The principle steps used for isolation of endothelial and vascular smooth muscle cells are illustrated in Figure 2.6.

Isolation of endothelial and vascular smooth muscle cells

Once a vein was assessed as suitable for the study, it was removed from the container in a sterile Class II cabinet. The vein was flushed thoroughly three times with 500µl sterile PBS (pH 7.4) to remove red blood cells, placed in a petri dish (#639160, Greiner Bio-One, Gloucestershire, UK) and constricted at one end using a sterile bulldog clip prior to enzymatic disaggregation.

Endothelial cells form the inner lining of the veins; hence, the vein segment was first filled to slight distension with warmed endothelial cell digestion medium (Appendix B), constricted at both ends using sterile bulldog clips and incubated for 15 min at 37℃ in the incubator. After incubation the bulldog clips were removed from the vein and the segment was flushed with 3-4ml sterile PBS (pH 7.4) and the medium collected in a 15ml universal container. Pellets of enzymatically dislodged cells were collected by centrifugation of the flushed medium at 200g for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 2ml endothelial cell culture medium (Appendix B), plated onto one well of a 6-well plate (#657160, Greiner Bio-One) and cultured at 37℃.

Figure 2.6: Enzymatic disaggregation for ECs or VSMCs from human saphenous veins. The incubation and separation and culture and characterisation steps are repeated two additional times for collection of VSMCs.

Once the vein was flushed with PBS, it was sealed at one end with a bulldog clip, filled again with endothelial cell digestion medium, sealed at both ends and incubated for a further 40 min at 37°C. The vein was then flushed with 3-4ml PBS and the medium was collected in a 15ml universal container. The cell pellet was collected by centrifugation at 200g for 5 min, re-suspended in 2ml endothelial cell culture medium, plated onto the next well of the 6-well plate and cultured at 37°C.

Finally, the vein segment was sealed at one end with a bulldog clip, filled with vascular smooth muscle cell digestion medium (Appendix B), sealed at both ends and incubated for 50 min at 37°C in the incubator. The vein was then flushed with PBS and medium was collected into a 15ml universal container. The cell pellet was collected after centrifugation at 200g for 5 min, re-suspended in 2ml vascular smooth muscle cell culture medium (Appendix B), plated onto the third well of the 6-well plate and cultured at 37°C.

After 24 hr, medium in each well was replaced with 2ml appropriate EC or VSMC medium and incubated at 37°C until 90% confluence was reached.

EC and VSMC culture and characterisation

Once the cells were confluent in their individual wells, they were passaged into a T25 cell culture flask until confluent and further subculture and characterisation were carried out as described in section 2.2.6.

Preparation of EC and VSMC cell treatments

The human SV derived endothelial and vascular smooth muscle cells used in this study were treated with VEGFC, anti-VEGFC antibody, TGFβ1, anti-TGFβ1, PDGF-BB or anti-PDGFBB antibody as described in Appendix B.

Preparation of EC and VSMC cell culture supernatants

SV derived endothelial and vascular smooth muscle cells were seeded onto 6-well plates and cultured until confluence at 37°C in the incubator. Once confluent, the culture medium in each well was replaced with 2ml VEGFC, anti-VEGFC, TGFβ1, anti-TGFβ1, PDGF-BB or anti-PDGFBB treatment medium and the cells were cultured for a further 24 hr at 37°C. Supernatants from each well were then collected into labelled sterile Eppendorf tubes and micro-

centrifuged at 14.1rcf for 1 min. The supernatant was collected into 100µl aliquots, placed into labelled freezer bags and stored at -80℃ until required, while the cell pellet was discarded.

2.2.6.8 Endometrium and myometrium explant culture and treatment

Endometrial biopsies with superficial myometrium obtained from women undergoing hysterectomy were transported to the laboratory (section 2.1.1.2). The excised tissue was further dissected into 5 approximately equal fragments using a scalpel (♯INS4684, Merck Millipore, Nottingham, UK) in a glass petri dish in a sterile Class II cabinet. 1-2 ml control medium, TGFβ1, anti-TGFβ1, PDGF-BB or anti-PDGFBB tissue treatment medium (Appendix B) was added to five separate wells of a 48-well plate (♯677180, Greiner Bio-One). Using a pair of forceps the tissue fragments were placed into the 5 wells and cultured for 72 hr. Individual treatment media were changed every 24 hr. After 72 hr the tissue fragments were removed from the plate, fixed immediately in neutral buffered formalin in five separately labelled containers and 3µm FFPE sections were prepared for immunohistochemistry (section 2.2.2.1). The basic steps used for endometrial explant culture are illustrated in Figure 2.7.

2.2.7 Polymerase chain reaction

Polymerase chain reaction (PCR), developed by Kary Mullis in 1986 (Mullis *et al.*, 1986), is a revolutionary technique, which allows scientists to create billions of copies of a specific segment of DNA of interest (amplicons) *in vitro*.

The ingenuity of this method lies in the fact that the key enzyme, DNA polymerase, is primer based and that the primers are heat resistant short pieces of single stranded DNA, which can be custom built to contain any sequence of nucleotides of interest in the laboratory. In a PCR reaction, two primers are designed such that they are complementary to the sequence of interest on both the coding and complementary DNA strands. The DNA polymerase attaches itself near one end of the primer and extends these primers by adding complementary nucleotides.

Essentially a PCR experiment consists of three stages; (i) a denaturation step, where heating the reaction denatures double stranded DNA to form single

Figure 2.7: Human endometrial explant culture.

stranded DNA; (ii) an annealing step, where cooling the reaction leads to primers annealing to the single stranded DNA; and finally (iii) an extension or elongation step, where heating the reaction once again to a temperature optimal to the polymerase enzyme leads to synthesis of a new DNA strand complementary to the target sequence. Under optimal conditions, at each extension step, the amount of target DNA is doubled, resulting in an exponential rate of amplification of the target DNA sequence.

The most commonly used DNA polymerase in PCR reactions is *Taq* polymerase, which is isolated from a strain of bacterium, *Thermus aquaticus*. The *Taq* polymerase is thermo stable and works optimally at 72℃. However, the *Taq* polymerase used in the PCR reaction is a modified monoclonal antibody attached 'hot start' enzyme, which avoids non-specific amplification, by inactivating *Taq* at lower temperatures; only when the amplification temperature reaches 95℃, the specific antibody detaches to release *Taq* polymerase leading to amplification with greater specificity. Therefore, an initial step of heating the reaction to 95℃ is essential to start specific amplification.

2.2.7.1 Total RNA extraction from cells

Total RNA was extracted from endothelial and vascular smooth muscle cells from cell lines and human SVs using the TRIZOL method, according to the manufacturer's instructions. All RNA work was carried out in a ribonuclease and contaminant free surface and apparatus (sections 2.2.1.2, 2.2.1.3).

Cells grown to confluence on 6-well plates were stored at -80℃ until RNA isolation. The frozen 6-well plate was brought to room temperature and 500µl TRI reagent (♯T9424) was added to each well of the 6-well plate and allowed to stand for 5 min at room temperature, prior to RNA isolation.

The plate was then tilted to one side, the TRI reagent collected using a pipette and the cells were scraped from the bottom of the wells by gentle sideways movement of the pipette tip, while slowly releasing the reagent back into the well. This step was repeated three times for each well and finally the cell suspension was collected into six labelled 1.5ml Eppendorf tubes. 100µl chloroform was added to each tube and the mixture was shaken vigorously by inverting the tubes continuously for 5 sec. The tubes were then left to stand at

room temperature for 5 min prior to micro-centrifugation at 14rcf for 15min. Centrifugation yielded three distinct visible layers in each tube; (i) upper aqueous phase containing isolated RNA, (ii) an interphase containing a high amount of DNA and (iii) a lower pink phase. The upper colourless aqueous phase was collected into clean tubes and RNA was precipitated by adding 250µl or an equal volume of isopropanol (♯I9516) and incubating at -20℃ overnight.

The following day, the tubes were retrieved and micro-centrifuged at 14rcf for 10 min. The supernatant was discarded and the RNA pellet in each tube was washed in 500µl 75% ethanol. The tubes were further micro-centrifuged at 14rcf for 10 min and the RNA pellet was left to air dry for 10-15 min at room temperature. Once air dried, the RNA pellet was re-suspended in 30µl nuclease free water, left to stand at room temperature for 30 min and stored at -80℃ until the concentration and quality of the RNA was assessed.

2.2.7.2 Nucleic acid quantification

The concentration and quality of total RNA extracted was determined using a nanodrop spectrophotometer (ND-1000 V3, Labtech Int., UK). RNA has its maximum absorption at 260nm wavelength, such that A260 readout of 1.0 is equivalent to approximately 40µg/ml RNA. The purity of RNA is assessed by the ratio of absorbance at 260nm and 280nm such that pure RNA has A260/A280 ratio of 2.1. Low A260/A280 ratios may indicate the presence of phenol, protein or other contaminants, which absorb strongly at around 280nm. Therefore, only total RNA extractions with A260/A280 ratio ≥1.8 were used in this study.

2.2.7.3 cDNA synthesis

Once the concentration and quality of total RNA was assessed, RNA was reverse transcribed using Superscript III (♯18080-093). All reagents used for cDNA synthesis were obtained from Life Technologies. Up to 5µg RNA was diluted with nuclease free water to bring the volume up to 11µl, to which 1µl 10mM dNTP (♯18427-013) and 1µl 0.5µg/µl Oligo $(dT)_{12\text{-}18}$ (♯18418-012) were added. The reaction tubes were placed in an Eppendorf Mastercycler Gradient PCR system and run at 65℃ for 5 min followed by cooling at 4℃, during which

time a master mix was prepared. The master mix consisted of 5x RT buffer (♯18080-093), 0.1M DTT (♯18080-093), RNaseOUT (♯10777-019) and Superscript III in a 4:1:1:1 ratio by vol/vol respectively. 7µl master mix was added to each of the reaction tubes and run on the PCR system at 50℃ for 30-60 min. The reaction was terminated at 70℃ for 20 min and the newly synthesised cDNA were stored at -20℃ prior to PCR amplification.

2.2.7.4 Quantitative reverse transcription PCR (qRT-PCR)

Quantitative reverse transcription PCR is a modified form of the conventional PCR, which allows for the detection and quantification of gene expression through formation of complimentary strand of DNA (cDNA). In the presence of the specific mRNA, reverse transcriptase and primer anneals to the mRNA sequence to transcribe a cDNA strand, which is consequently replicated as in the conventional PCR in the presence of primers and *Taq* polymerase (section 2.2.7). Moreover, while the conventional PCR allows detection of PCR amplification only at the final phase or endpoint of the reaction, the qRT-PCR allows the reliable detection and measurement of products of PCR amplification, which are directly proportional to the amount of the target template at the start of the PCR process, as the reaction progresses. This is particularly advantageous because a basic PCR consists of three phases; exponential, linear and plateau and the exponential phase is the optimal point for analysing data. Alongside various other applications, qRT-PCR can be used for quantitation of gene expression, when compared with expression of an endogenous control.

In this study real-time RT-PCR was performed on a StepOnePlus™ PCR machine (Life Technologies) using TaqMan® primers/probes (with TAMRA™ dyes as the quencher) and reagents (Life Technologies). Table 2.5 lists the TaqMan® primers/probes used in this study.

A 20µl base reaction mixture was used containing 2µl sample cDNA, 10µl TaqMan® Universal PCR Master Mix (♯4304437), 1µl TaqMan® primers/probes and 7µl nuclease free water. Each run included GAPDH housekeeping reference gene (for normalisation), decidual cDNA positive control (to verify negative amplification) and cDNA negative control (to detect genomic DNA

contamination) and reactions were carried out on MicroAmp® Optical 96-well plates (♯NB10560) in triplicate. All samples were amplified using the following parameters: initial holding for 2 min at 50℃ and denaturation at 95℃ for 10 min, followed by 40 cycles of denaturation at 95℃ for 15 sec and annealing and extension at 60℃ for 1 min.

2.2.7.5 TaqMan® chemistry

TaqMan® real-time PCR probes are fluorogenic-labelled probes, which allow detection of specific PCR products as they accumulate during the PCR cycles.

The TaqMan® oligonucleotide probe consists of a covalently bonded reporter fluorescent dye, 6-carboxyfluorescein (FAM) on the 5' end and a quencher dye tetramethylrhodamine (TAMRA) on the 3' end. As long as the probe is intact, due to the proximity of the reporter and quencher, fluorescence emission does not occur. During PCR amplification; (i) the probe anneals to the complementary target DNA strand, (ii) the *Taq* polymerase enzyme cleaves the 5' end of the probe, separating the fluorescent dye from the quencher resulting in an increase in reporter dye signal and (iii) removes the probe from the target strand leading to primer extension. With every cycle more reporter dyes are cleaved resulting in an increase in fluorescent intensity, which is directly proportional to the amount of PCR amplification product. TaqMan® chemistry depends on both probe hybridisation and PCR amplification for fluorescence emission, reducing false positives and background fluorescence, therefore making it highly specific. Figure 2.8 is a schematic representation of the principle behind TaqMan® chemistry.

Table 2.5: TaqMan® probes used in real-time PCR

NAME	TAQMAN® ASSAY ID	AMPLICON SIZE (bp)
Ang1 (ANGPT1)	Hs00919202_m1	86
Ang2 (ANGPT2)	Hs01048043_m1	113
B-Actin (ACTB)	Hs99999903_m1	171
GAPDH	Hs99999905_m1	122
H-Caldesmon (CALD1)	Hs00263998_m1	71
PDGF-BB (PDGF-B)	Hs00234042_m1	80
PDGF-RA (PDGF-R)	Hs00998027_m1	84
PDGF-RB	Hs00387364_m1	100
Tie2 (TEK)	Hs00176096_m1	82
TGF-B1	Hs00998129_m1	89
TGF-BR1	Hs00610318_m1	92
TGF-BR2	Hs00559661_m1	69
VEGF-A	Hs00900057_m1	81
VEGF-C	Hs01099206_m1	93
VEGF-R1 (FLT1)	Hs01052936_m1	72
VEGF-R2 (KDR)	Hs00911705_g1	84
VEGF-R3 (FLT4)	Hs01047677_m1	111
MYOCD	Hs00538071_m1	63
SRF	Hs00182371_m1	67
CREG1	Hs00355412_m1	84
CREG2	Hs00944324_m1	75
CNN1 (Calponin 1)	Hs00154543_m1	93
CNN2 (Calponin 2)	Hs00854264_s1	79
SMTN (Smoothelin)	Hs00199489_m1	52

Figure 2.8: Principle behind TaqMan® chemistry. During PCR amplification; (**i**) the TaqMan® probe anneals to the complementary target DNA strand, (**ii**) *Taq* polymerase enzyme cleaves the 5' end of the probe, separating the fluorescent dye from the quencher resulting in an increase in reporter dye signal and (**iii**) removes the probe from the target strand leading to primer extension.

2.2.7.6 Relative quantification of gene expression

Prior to running the PCR reactions, primer efficiency was assessed using serial dilutions (1:10 to 1:100000) of decidual cDNA and determining the variation in Ct with each dilution. Following this, a standard curve was generated for each TaqMan® probe and the slope of the curve were used to determine exponential amplification and efficiency of the qPCR reaction by the following reaction (Tichopad *et al.*, 2003):

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

Low efficiency can result from poor quality of cDNA or primers or the length of the amplicon. Therefore, only primers with ≥98% efficiency were used in the study. Fold changes in gene expression from the real-time quantitative PCR were measured relative to a normal housekeeping reference gene (HK) GAPDH (section 5.4.1) or by utilising a second relative parameter comparing the level of expression of the gene of interest (GOI) to a non-treated control (sections 5.4.1.3, 6.4.2), using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Briefly, ΔCt of each sample was calculated by the following equation:

$$\Delta\text{Ct} = \text{Ct of GOI in sample of interest} - \text{Ct of HK in each sample}$$

The ΔΔCt of each sample was calculated by either of the following equations:

$$\Delta\Delta\text{Ct} = \Delta\text{Ct of GOI in sample of interest} - \Delta\text{Ct of GOI in the normalised sample}$$

$$\Delta\Delta\text{Ct} = \Delta\text{Ct of GOI in sample of interest} - \Delta\text{Ct of GOI in sample to be compared}$$

The final step involved determining the comparative fold change in expression level of the GOI in the sample compared with a normalised sample. This was calculated by the following equation:

$$\text{Comparative fold change in level of expression} = 2^{-\Delta\Delta\text{Ct}}$$

2.2.8 Western blotting

Western blotting or protein immunoblotting, developed in 1979, is a common technique (Towbin *et al.*, 1979) for analysis of a specific protein of interest in a

sample, which can be cell lysate or tissue homogenate. Western blotting uses gel electrophoresis to separate proteins based on the isoelectric point, molecular weight, electric charge or a combination of the three. Following this separated protein molecules are transferred onto a suitable membrane such as nitrocellulose or polyvinylidene difluoride (PVDF). The membrane transfer allows detection of the specific protein of interest either using a primary antibody directly conjugated to an enzyme label or using a tagged secondary antibody directed towards a primary antibody. An appropriate chemiluminescent substrate is added to the membrane for chemiluminescence detection. Horseradish peroxidase (HRP) is a common enzyme conjugate, which oxidises luminol-based substrates resulting in emission of light. The emitted light is captured either by a CCD camera or by exposure to X-ray film.

In this study western blotting was performed to investigate the effect of angiogenic growth factors on the expression of smoothelin, calponin and caldesmon in vascular smooth muscle cells (section 6.4.3).

2.2.8.1 Protein extraction from cell lysate

Total protein was extracted from vascular smooth muscle cells grown to confluence on 6-well plates and stored at -80°C. The 6-well plate was brought to room temperature and 200µl 1x protein extraction buffer (Appendix B) was added to each well of the 6-well plate, which was then allowed to stand for 5 min at room temperature prior to protein isolation.

Following this the plate was tilted to one side, the extraction buffer was collected using a pipette and the cells were scraped from the bottom of the wells by gentle sideways movement of the pipette tip, while slowly releasing the buffer back into the well. This step was repeated three times for each well and finally the cell suspension was collected into six labelled 1.5ml Eppendorf tubes. The cell suspensions were sonicated (Soniprep 150 sonicator, MSE Ltd., London, UK), heated at 100°C for 5 min followed by micro-centrifugation at 14rcf for 5 min. The supernatant was collected into fresh labelled Eppendorf tubes and stored at -80°C until required for protein assay.

2.2.8.2 Protein extraction from myometrial tissue homogenate

Total protein was extracted from myometrial tissue, which was stored at -80°C (section 2.2.2.3). Myometrial tissue was removed from the -80°C freezer and weighed. Using a pair of forceps up to 200mg of tissue was transferred into one 2ml Precellys Ceramic Kit tube (♯91-PCS-CKM, VWR International). 1x extraction buffer was added to the tube at 10µl/mg of tissue. The 2ml tube was placed into the tube holder of a MINILYS benchtop homogeniser (PEQLAB, VWR International, Leicestershire, UK) and homogenised at 5000rpm for 25 sec, followed by 30 sec on ice. This step was repeated five times, following which the tube was centrifuged at 14rcf for 10 min at 4°C. Supernatant was collected into a fresh labelled Eppendorf tube and stored at -80°C until required for protein assay.

2.2.8.3 Protein quantification

The total protein concentration of each sample was determined colorimetrically using a BioRad DC protein assay (♯500-0112, Hertfordshire, UK) in accordance with the manufacturer's instructions. Essentially the BioRad DC protein assay involves two steps: (i) a reaction between copper and protein in an alkaline condition and (ii) the reduction of Folin reagent by the copper-treated protein (Lowry *et al.*, 1951). The assay is based on the 'biuret reaction', where peptides containing greater than three amino acid residues form a light blue coloured chelate complex with copper ions (Cu2+) in an alkaline condition containing copper tartrate, with minimum and maximum absorbance at 405nm and 750nm respectively (Peterson, 1979). The extent of blue colouration is proportional to the amount of protein present in the sample.

A bovine serum albumin (BSA) standard curve was prepared by serially diluting BSA (♯441555J, VWR International) between 0mg/ml to 10mg/ml in 1x protein extraction buffer (Appendix B) and by plating 5µl/well in duplicates into a 96-well plate (♯351172, SLS Ltd., Yorkshire, UK). The protein concentration of test samples was determined by adding 5µl/well of sample in duplicate into appropriate wells of the 96-well plate along with 25µl working reagent A (containing 20µl reagent S in 1ml reagent A) and 200µl reagent B. The plate was incubated for 15 min at room temperature. Following this absorbance of

each well was measured at 750nm on a SpectraMax190 Absorbance Microplate Reader (Molecular Devices, CA, U.S.A) and the concentration of test samples was determined from the BSA standard curve (Figure 2.9) using the Softmax® Pro (v4.7.1) software package.

2.2.8.4 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) allows the separation of proteins according to their size. This consists of two essential steps: (i) SDS, an anionic detergent, denatures proteins by breaking hydrogen bonds and confers negative charge proportional to their length; and (ii) the SDS-denatured and reduced proteins are then separated according to their sizes by placing them in a polyacrylamide gel and using electricity to pull the proteins through the gel. The polyacrylamide gel contains a meshwork of pores and the size of the pores is determined by the percentage of acrylamide in the gel, such that the higher the percentage, the smaller the pore size and the smaller the size of proteins that can be resolved.

In this project a 10% polyacrylamide gel (Appendix B) was used to separate the proteins of interest. To catalyse polymerisation, 60µl ammonium persulphate (0.6mg/ml, ♯A3678) and 6µl tetramethylethylenediamine (0.5mg/ml, ♯T9281) were added to 10ml of the 10% gel. The gel solution was poured into a Mini-PROTEAN casting module (♯165-8008, BioRad) and left to polymerise for 30 min at room temperature after insertion of a 1.5mm thick, 10-well spacer plate (♯170-4326, BioRad). Once polymerised, the gel cassette was transferred into a Mini-PROTEAN electrophoresis system and the tank was filled with 1x running buffer (Appendix B). 100µg protein isolate was combined with 5µl protein loading buffer, containing reducing agent (Appendix B) to make up to a maximum of 40µl sample per well, which was then heated at 95℃ for 5 min. The reducing agent β-mercaptoethanol in the protein loading buffer cleaves di-sulphide bonds, thereby linearising proteins. The reduced samples were loaded into respective wells of the gel along with 10µl protein Novex® Sharp Standard (♯LC5800, Life Technologies, Paisley, UK) using a Hamilton syringe. The Mini-PROTEAN electrophoresis system was set to 100V and run for 1-2 hr at room temperature for protein separation.

Figure 2.9: Representative standard curve to determine protein concentration. The standard curve was prepared by serially diluting BSA between 0mg/ml and 10mg/ml. The protein concentration in test samples was determined from the standard curve. Error bars are representative of mean ± standard deviation (N=2).

2.2.8.5 Membrane transfer

After proteins were separated, they were electrophoretically transferred into a 0.45µm PVDF membrane (Immobilon-P Membrane, ♯IPVH00010, Merck Millipore). PVDF is a hydrophobic membrane, which immobilizes the separated proteins and allows for immunodetection of the protein of interest in a sample.

Following electrophoresis, the gel cassettes were removed and placed in a tray with cold transfer buffer (Appendix) for 5 min. Meanwhile, the PVDF membrane was placed in 99% methanol for 15 min for activation followed by equilibration in cold transfer buffer for 5 min. The transfer electroblotting cassette was prepared in cold transfer buffer by placing the protein containing gel and PVDF membrane together, with Whatman filter paper and sponges on either side, between two electrodes, such that the gel was on the side of the negative electrode and the PVDF membrane on the side of the positive electrode. The cassette was then suspended into the Mini-PROTEAN electrophoresis system and the tank was filled with transfer buffer (Appendix B). Protein transfer was performed at 100V for 2 hr on ice.

2.2.8.6 Immunodetection

Immunodetection allows both qualitative and quantitative analysis of proteins in samples. Following electrophoretic transfer of proteins, the PVDF membrane was blocked for 1 hr at room temperature in blocking buffer (Appendix B). The membrane was then probed with primary antibody, diluted in blocking buffer, overnight on a shaker at 4℃. The primary antibodies used for immunodetection in western blotting are listed in Table 2.6. The following day the membrane was washed three times in TBST (Appendix B) for 10 min on a shaker at room temperature, prior to incubating for 2hrs with an HRP-conjugated rabbit anti mouse secondary antibody (♯170-6516, BioRad) diluted 1:3000 in blocking buffer. The membrane was again washed three times in TBST for 10 min on a shaker at room temperature before chemical detection.

Table 2.6: Primary antibodies used for western blotting

	ANTIBODY	CATALOGUE NO:	CLONE	HOST	DILUTION
1	Caldesmon[1]	M3557	h-CD	Mouse	1:100
2	Calponin[1]	M3556	CALP	Mouse	1:1000
3	Smoothelin[2]	ab8969	R4A	Mouse	1:100

[1]Dako Cytomation, Cambridgeshire, UK; [2]Abcam Cambridgeshire, UK

2.2.8.7 Chemical detection of protein and developing signal

After probing the PVDF membrane with appropriate primary and HRP-conjugated secondary antibody, peroxidase activity was detected using Pierce enhanced chemiluminescent (ECL) substrate (♯32106, Fisher Scientific) for 5 min or Amersham ECL Select (♯RPN2235, VWR International) for 1-2 min. This sensitive detection system is based on the principle of oxidation of luminol present in the ECL substrate by the peroxidase activity of the HRP conjugated to the secondary antibody resulting in the emission of light, such that the light emitted is proportional to the quantity of protein present. This resulting light emitted was visualised by the exposure of the PVDF membrane to Kodak® BioMax® light film (♯819-4540) using ready to use developer and fixer (Tetenal, Leicestershire, UK). Developing times ranged from 2-25 min depending on the expression of the protein of interest. Once developed the films were scanned using a desktop scanner and the image analysed qualitatively for the presence or absence of a band suggesting the presence or absence of a protein, respectively, in the sample of interest.

Equal loading was assessed by washing the PVDF membrane once with TBST for 10 min followed by a 25 min incubation in 1% napthol blue solution, subsequent washing for 5 min and air-drying at room temperature.

2.2.9 Enzyme-linked immunosorbent assay (ELISA)

Enzyme linked immunosorbent assay (ELISA), developed by Perlman, Engvall and van Weemen in 1971 (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971), is an analytical technique, which uses solid-phase enzyme immunoassay for the detection and quantification of peptides, proteins, antibodies and hormones in a liquid sample. This assay is based on the following principal steps: (i) immobilization of an antigen onto a solid surface;

followed by (ii) formation of an antibody-antigen complex, where the antibody is directly or indirectly linked to an enzyme; and finally (iii) detection and quantification by incubation with a substrate to produce a measurable product, proportional to the level of antigen present in the sample. The strength of this technique lies in the fact that the antigen is immobilised onto a solid surface, making it possible to wash away any non-specific binding. HRP is the most commonly used enzyme conjugate, which in the presence of an appropriate substrate results in a coloured by-product or the emission of light.

Depending on suitability to the study question, ELISAs can be performed in different ways, which are modifications of the basic principle: direct ELISA, indirect ELISA, competitive ELISA and capture or sandwich ELISA. While direct ELISA utilises a primary antibody directly labelled to an enzyme, in an indirect ELISA an enzyme-conjugated secondary antibody is used directed towards the unlabelled primary antibody is used for antigen detection and quantification. Direct ELISA is quick and minimises cross-reactivity with secondary antibody, although labelling primary antibody can be expensive. In comparison, indirect ELISA offers the option of a variety of secondary antibodies to be used even with the same primary antibody and allows signal amplification through utilisation of multiple epitopes on the primary antibody.

Competitive ELISA involves formation of a primary antibody-antigen of interest complex, which is immobilised on a solid surface, pre-coated with the same antigen. Following this an enzyme conjugated secondary antibody along with an appropriate substrate allows detection and quantification such that the higher the sample antigen, the weaker is the eventual signal, rendering this method highly specific. Some competitive ELISAs may use an enzyme-linked antigen rather than an enzyme linked antibody.

Sandwich ELISA involves attachment of a ‘capture’ antibody onto a solid surface, to which the samples containing the antigen of interest are overlaid. Following this an enzyme conjugated secondary antibody is added, which in the presence of an appropriate enzyme substrate allows the detection and quantification of an antigen of interest in a sample. Since the two antibodies are directed towards two different epitopes of the same antigen, this prevents binding interference and non-specificity, thus making the sandwich ELISA a

highly specific and robust method. The essential steps involved in a sandwich ELISA are illustrated in Figure 2.10.

In this study, the sandwich DuoSet® ELISA development systems (R&D Systems Ltd.) for VEGFC (♯DY752B), Ang1 (♯DY923) and TGFβ1 (♯DY240) were used to investigate (i) the effect of matrigel on angiogenic growth factor expression profile of human SV derived endothelial cells (section 5.4.1) and (ii) the effect of PDGF-BB and TGFβ1 on angiogenic growth factor expression profile of human SV derived endothelial and vascular smooth muscle cells (section 6.4.2).

2.2.9.1 Sandwich ELISA protocol

A sandwich ELISA was used to assess supernatants collected as described in section 2.2.6.7. The protocol for the sandwich DuoSet® ELISA development system was performed in accordance with manufacturer's instructions.

The capture antibody was diluted to working concentration in PBS (pH7.6) (Appendix B) and 50µl was used to coat an appropriate number of wells of a 96-well half-area microplate (♯675001, Greiner Bio-One). The plate was sealed with an adhesive film (♯AB0558, Fisher Scientific) and incubated at room temperature overnight. The following morning, each well was aspirated and washed two times with approximately 250µl/well of wash buffer (Appendix B). The process was repeated three times, after which any wash buffer was thoroughly removed by inverting the plate and blotting against clean paper towels. To minimise non-specific binding, the wells were coated with 150µl reagent diluent or TGFβ1 blocking solution (Appendix B) and incubated at room temperature for 1 hr. Meanwhile, in order to produce a seven point standard curve, a two-fold serial dilution was prepared in reagent diluent or TGFβ1 reagent diluent (Appendix B). The samples along with the standards were first plated according to the study design into a u-bottom 96-well plate (♯675180, Greiner Bio-One). To activate latent TGFβ1, 10µl TGFβ1 acid activation solution (Appendix B) per 50µl sample was added, mixed well and incubated for 10 min at room temperature, following which the acidified sample was neutralised by adding 10µl TGFβ1 acid neutralisation solution (Appendix B) and mixing well prior to the assay.

Figure 2.10: Principle behind DuoSet® Sandwich ELISA development system.

Following blocking, the 96-well plate was washed and aspirated three times with wash buffer as described above. 50µl undiluted or acid activated samples (for TGFβ1), along with 100µl serially diluted standards were separately added in duplicate to the assay plate, sealed with adhesive film and incubated at room temperature for 2 hr. The plate was aspirated, washed three times in wash buffer and 50µl appropriately diluted detection antibody was then added to each well. The sealed plate was then incubated for 2 hr at room temperature. The assay plate was aspirated, washed three times in wash buffer and 50µl diluted (1:200 in reagent diluent) streptavidin-HRP conjugate was added to the wells before incubating for 20 min in the dark at room temperature. The assay plate was again aspirated and washed three times in wash buffer and 50µl substrate solution (Appendix B) was added to each well followed by 20 min incubation in the dark at room temperature, after which light blue colouration was observed. Finally, 25µl stop solution (Appendix B) was added to each well and tapped gently to ensure thorough mixing, resulting in a yellow colouration, prior to determining optical density of each well. The extent of colouration was proportional to the amount of protein of interest present in each sample.

Absorbance of each well was measured at 450nm (corrected at 540nm) on a SpectraMax190 Absorbance Microplate Reader (Molecular Devices, CA, U.S.A) and the concentration of growth factor or cytokine in samples was determined from the standard curve, using the mean value of the duplicates (Figure 2.11) using the Softmax® Pro (v4.7.1) software package. For TGFβ1 the concentration read off the standard curve was multiplied by the dilution factor of 1.4 (as recommended), since the samples were acid diluted and neutralised prior to the assay. Table 2.7 details the concentration of the capture antibodies, detection antibodies and standards used in the different assays.

Table 2.7: Concentration details of antibodies and standards used for ELISA

ASSAY	CAPTURE ANTIBODY	DETECTION ANTIBODY	HIGH STANDARD
VEGFC	4.0µg/ml	100.0ng/ml	4000.0pg/ml
Ang1	4.0µg/ml	200.0ng/ml	10000.0pg/ml
TGFβ1	2.0µg/ml	300.0ng/ml	2000.0pg/ml

Figure 2.11: Representative standard curve to determine protein concentration using the R&D DuoSet® ELISA kit. The standard curve was prepared for human TGFβ1 ranging from 0pg/ml-2000pg/ml. Protein concentrations in test samples were determined from the standard curve.

2.2.10 Quantibody Human Angiogenesis Array

The Quantibody® human angiogenesis array platform (RayBiotech, Norcross, U.S.A) is a multiplexed sandwich ELISA-based technology, which allows simultaneous detection and quantification of 10 proteins or cytokines of interest with high sensitivity and specificity comparable with traditional ELISAs, but combining the high throughput of arrays. The assay follows the basic principles of a sandwich ELISA (section 2.2.9.1), except for the final detection step. The antigen-antibody complex is visualised with streptavidin labelled Cy3 equivalent dye using a laser scanner, such that one array slide spotted with 16 wells of identical cytokine antibodies and appropriate positive controls in quadruplicate can be processed simultaneously.

In this study, the Quantibody® human angiogenesis array platform was used to investigate (i) the effect of matrigel on the angiogenic growth factor secretion profile of human SV derived endothelial cells (section 5.4.1) and (ii) the effect of PDGF-BB and TGFβ1 on angiogenic growth factor secretion profile of human SV derived endothelial and vascular smooth muscle cells (section 6.4.2). Supernatants were collected as described in section 2.2.6.7.

2.2.10.1 Array protocol

The protocol was performed in accordance with the manufacturer's instructions. The glass slides were removed from -20℃ and equilibrated to room temperature inside sealed plastic bags for 20-30 min, after which the plastic bag was removed and the slides were air-dried for 2 hr. Meanwhile, a five point standard dilution was prepared using lyophilized cytokine standard mix diluted in the diluent provided (Appendix B). The serial concentrations for each cytokine varied due to the fact that the starting concentration of each cytokine was different; details are listed in Table 2.8. In order to minimise non-specific binding, the wells were coated with 100µl sample diluent and incubated at room temperature for 30 min. Blocking solution was then decanted from each well and 50µl undiluted sample or the serially diluted standard cytokines were added and incubated overnight at 4℃.

Table 2.8: Standard concentrations used for the Quantibody® human array

(pg/ml)	Std1	Std2	Std3	Std4	Std5
Angiogenin	4000	444	148	49	16
ANG2	8000	889	296	99	33
EGF	200	22	7	2	1
bFGF	8000	889	296	99	33
HB-EGF	8000	889	296	99	33
HGF	8000	889	296	99	33
Leptin	8000	889	296	99	33
PDGF-BB	2000	222	74	25	8
PIGF	8000	889	296	99	33
VEGF	8000	889	296	99	33

The next day, samples and standards were decanted, and the plates were washed five times (5 min each) with 150µl 1x wash buffer I (20x wash buffer I diluted in distilled water) on a shaker at room temperature with complete removal of wash buffer in each step. After washing with wash buffer I, the slides were washed twice (5 min each) with 150µl 1x wash buffer II (20x wash buffer II diluted in distilled water). 80µl detection antibody cocktail (diluted with 1.4ml sample diluent) was added to each well and incubated for 2 hr at room temperature. The slides were decanted and washed five times in wash buffer I and twice in wash buffer II as described previously. 80µl Cy3 equivalent dye-conjugated streptavidin (diluted with 1.4ml sample diluent) was added to each well and incubated in the dark for 1 hr at room temperature. The slides were decanted and washed five times in wash buffer I followed by complete removal of the wash buffer. Finally the slides were disassembled and washed in 1x wash buffer I in a slide washer on a shaker for 30 min at room temperature followed by another 5 min wash in 1x wash buffer II. Wash buffer was completely removed by gentle suction with a pipette.

Integrated density values (IDV) were calculated for each spot (area times relative intensity) in each well at 532nm on a GenePix 4000B Microarray Scanner (Molecular Devices, CA, U.S.A) and the concentration of each cytokine/ growth factor in samples was determined using the mean value of the

quadruplicates from the five point standard curves (Figure 2.12) using ArrayVision FAST® (v8.0) software package. The maximum possible unsaturated reading was obtained for the highest standard for each cytokine.

2.2.11 Statistical analysis of results

Data generated in this study were statistically analysed. Data were organised and analysed first using Microsoft Office Excel 2011 and statistical analysis was carried out in either Prism (GraphPad Software, San Diego, CA, U.S.A) or SPSS (IBM, Hampshire, UK) software packages. Quantitative data were broadly classified as either count (e.g. quickscore method used for IHC) or continuous data (e.g. area of muscle coverage in a tissue, concentration of protein, etc.). Analysis of differences between control and experimental groups was performed based on whether the data followed a normal distribution. A normality test was carried out for continuous data where $N \geq 10$ and if normally distributed, parametric tests such as Students *t*-test, analysis of variance (ANOVA) were applied for further analysis. However, for count (non-continuous) data with $N < 10$, normality could not be reliably tested and therefore non-parametric tests such as Fisher's exact test, Mann-Whitney U or Kruskal Wallis were applied in association with Bonferroni correction for multiple comparisons, where applicable, to derive final conclusions. The significance of the difference was calculated as a probability (*P* value) of ≤ 0.05 suggesting that the result could have only occurred by chance in 1 in 20 cases.

Figure 2.12: Representative standard curves to determine protein concentration using the Quantibody® human angiogenesis array kit. Protein concentrations in test samples were determined from the standard curve.

CHAPTER 3

HISTOCHEMICAL CHANGES IN THE UTERINE VASCULATURE OF WOMEN WITH HEAVY MENSTRUAL BLEEDING

3 HISTOCHEMICAL CHANGES IN THE UTERINE VASCULATURE OF WOMEN WITH HEAVY MENSTRUAL BLEEDING

3.1 INTRODUCTION

Human menstruation is a highly regulated physiological process involving cross communication between the vascular and immune systems, under the control of the endocrine system. The endometrial vasculature includes the basal arteries supplying the stratum basalis and the spiral arteries supplying the stratum functionalis. During the menstrual cycle, angiogenesis is spatially and temporally regulated, occurring primarily during three phases: vascular repair in stratum basalis in the menstrual phase; support growth in stratum functionalis in the proliferative phase, and finally growth and coiling of spiral arterioles in the secretory phase (Gargett and Rogers, 2001). The blood vessels comprise multiple layers: the innermost EC layer and the radially outward ECM and VSMCs. The coiled shape of the spiral arteries and arterioles helps to reduce blood pressure and rate of flow along its length, maintaining a steady blood flow to the stratum functionalis (Aplin *et al.*, 2008). Studies in the past have implicated endometrial vasculature in the pathogenesis of heavy menstrual bleeding (Abberton *et al.*, 1999a; Abberton *et al.*, 1999b; Hurskainen *et al.*, 1999; Rogers and Abberton, 2003; Kawai-Kowase and Owens, 2007).

VSMCs are a major constituent of the endometrial blood vessels and their stage of differentiation contributes to normal blood flow and pressure. Failure of these vessels to control the rate of blood loss could be attributed to a failure in normal vasoconstriction, which in turn may be explained by abnormal arteriogenesis together with impaired VSMC maturation and function. Vascular ECs express F8RA, CD34, CD31 and EC specific glycoproteins, which selectively bind the lectin UEA-1 and play an important role in the menstrual process. They regulate angiogenesis through the production of PDGF-BB and TGFβ1, which aid VSMC recruitment and this EC-VSMC signalling is key in maintaining normal blood flow through regulation of vascular tone (Feletou, 2011). ECs are also associated with degradation of vascular and stromal ECM by protease production, thereby allowing for co-ordinated EC migration and proliferation (Findlay, 1986; Zetter, 1988; Goodger and Rogers, 1994). Moreover, in a study

utilising EC proliferation as a measurement of angiogenesis, patients with HMB had high endometrial EC proliferation compared with controls, indicating altered blood vessel development (Kooy *et al.*, 1996). Additionally, heterogeneous staining between the different EC markers were noted in luteal phase endometrium from women suffering recurrent reproductive failure (Quenby *et al.*, 2009).

ECM is a major component of endometrial blood vasculature consisting of anchoring fibrils and proteins, including type IV collagen, fibronectin, laminin and osteopontin. It plays an integral role in angiogenesis by stabilising tissue structure, regulating cell growth and differentiation, and provides mechanical properties maintaining vascular tone. Both ECs and synthetic VSMCs are capable of synthesizing most of these proteins. Therefore any implication of dysregulated vascular structure or function in HMB may also underlie altered composition of ECM components. However, while vessel number and morphology (Abberton *et al.*, 1999a; Abberton *et al.*, 1999b; Rogers and Abberton, 2003) and staining pattern of the different EC and ECM markers in endometrium during the menstrual cycle (Aplin *et al.*, 1988; Rees *et al.*, 1993; Rogers *et al.*, 1993; Tawia *et al.*, 1993; Kelly *et al.*, 1995; Bilalis *et al.*, 1996; Nikitenko *et al.*, 2000; Zhang *et al.*, 2002) have been studied previously, a detailed analysis in the different layers of the endometrium and superficial myometrium throughout the menstrual cycle and a comparison with HMB has not been reported.

There is evidence for a role of endometrial immune cells in HMB disorders. Endometrial macrophages and uNK cells are a key source of angiogenic growth factors (Saito *et al.*, 1993; Li *et al.*, 2001; Hanna *et al.*, 2006; Lash *et al.*, 2006; Lash *et al.*, 2010) such as vascular endothelial growth factor (VEGF-A) (Ulukus *et al.*, 2006) which has been shown to be reduced in women with HMB (Malik *et al.*, 2006). Since VEGF-A plays a role in vascular development, its reduction in women with HMB may indicate aberrant endometrial vascular development. This exemplifies how the endometrial immune response may affect vascular development in the endometrium. Additionally it has been suggested that macrophages, uNK cells and other migratory leukocytes may directly influence vascular permeability (Hickey and Fraser, 2000). Moreover, macrophages and

uNK cells are often shown to form aggregates around the spiral arteries/arterioles in the stratum functionalis of the late secretory phase endometrium (Bulmer *et al.*, 1991) and altered uNK cell numbers have been associated with several reproductive disorders such as recurrent implantation failure and RM (Clifford *et al.*, 1999; Quenby *et al.*, 1999; Tuckerman *et al.*, 2007; Lash and Bulmer, 2011). Importantly, advanced development of endometrial blood vessels in women with a history of RM was correlated with an increased percentage of endometrial stromal uNK cells in the mid secretory phase (Quenby *et al.*, 2009). Furthermore prednisolone treatment reduced both endometrial uNK cells and angiogenic growth factor expression and vascular maturation in these women (Quenby *et al.*, 2005; Lash *et al.*, 2011a). Taken together these data suggest a role for uNK cells in advanced blood vessel development, although their functional role remains unknown. Recently uNK cells have also been shown to regulate endometrial bleeding by maintaining arterial stability (Wilkens *et al.*, 2013). Dysregulation of leukocyte cell numbers may therefore be linked with abnormal uterine bleeding disorders, although these cell populations have not been investigated in the endometrium of women with HMB.

3.2 HYPOTHESES AND AIMS

3.2.1 Hypotheses

Taking into consideration the important roles of uterine blood vasculature, and the potential effects of endometrial leukocytes in HMB, we hypothesised that:

1. Blood vessel structure (VSMC, EC and ECM) is altered in the endometrium of women with HMB.

2. Stromal, glandular and vascular proliferation across the phases of the menstrual cycle in the normal cycling uterus is spatially altered between the layers of the endometrium and superficial myometrium.

3. Endometrial leukocyte numbers are altered in HMB contributing to abnormal vascular development.

3.2.2 Aims

To test the above hypotheses we aimed to:

1. Examine the thickness of the blood vessel wall and the differentiation status of the VSMCs in endometrium across the menstrual cycle in controls and women with HMB.

2. Compare the pattern of various EC markers in the endometrium and superficial myometrium across the menstrual cycle in controls and women with HMB. Furthermore, we aimed to look at proliferation in vessels expressing each of the EC markers across the menstrual cycle in controls.

3. Compare the distribution of ECM components in the endometrium and superficial myometrium across the menstrual cycle in controls and women with HMB.

4. Assess stromal, glandular and vascular proliferation in the phases of the menstrual cycle with the aim of comparing between the layers of the endometrium and superficial myometrium.

5. Compare endometrial leukocytes across the different phases of the menstrual cycle in controls with those in women with HMB.

3.3 EXPERIMENTAL DESIGN

The experimental design has been illustrated in Figure 3.1 and specific materials and methods used are described below.

3.3.1 Human Tissue

Endometrial biopsies were obtained with informed consent from women undergoing hysterectomy as described in section 2.1.1.2.

3.3.2 Histochemistry and antibodies used

In general, immunohistochemistry, double-labelling immunohistochemistry and lectin histochemistry were performed as described in sections 2.2.3.1, 2.2.3.2 and 2.2.4, respectively. Details of source, pretreatment, dilution and incubation times for all primary antibodies used in immunohistochemistry and double-labelling immunohistochemistry are provided in Table 2.2. Additionally, details of double-labelling immunohistochemistry used to achieve specific aims are described below.

3.3.2.1 Thickness of endometrial blood vessel wall and the differentiation status of VSMCs

Double-labelling immunohistochemistry was used to identify the proportion of vessels identified using endothelial cell markers (CD31 or biotinylated UEA-1 lectin) that were immunopositive for the smooth muscle markers (αSMA, smoothelin or calponin). The antibody combinations for double labelling were selected based on pre-treatment compatibility as follows: CD31/αSMA, calponin/UEA-1 and smoothelin/CD31. The first immunostain was visualized using DAB solution containing 0.01% H_2O_2 (Appendix B) to give a brown reaction product and the second with Vector SG substrate kit for peroxidase to give a silver reaction product (Appendix B).

3.3.2.2 Endometrial leukocyte population in the endometrium

Detection of $CD3^+$ cells in paraffin embedded sections relied on antibodies that detect the epsilon (ε) chain of human CD3. Uterine $CD56^+$ NK cells may also express cytoplasmic CD3ε and CD3ζ (King *et al.*, 1998b). Therefore double-labelling immunohistochemistry was used to identify the proportion of $CD3^+$

Figure 3.1: Experimental design to investigate histochemical changes in the uterine vasculature of women with HMB.

cells that were $CD56^{+}CD3^{+}$ double positive. The first immunostain was visualised using Vector SG substrate kit for peroxidase (Appendix B) and the second with Vector NovaRED substrate kit for peroxidase (Appendix B).

3.3.3 Quantitative image analysis

3.3.3.1 Thickness of endometrial blood vessel wall and differentiation status of VSMCs

Sections from endometrial biopsies (N=7) in each of proliferative phase (PP), early secretory phase (ESP), mid secretory phase (MSP) and late secretory phase (LSP) from both controls and women with HMB were examined. Since tissue sections varied in size, in order to standardise the analysis keeping sample size consistent between control and HMB, images were captured of randomly chosen fields of view, capturing n=25 αSMA positively immunostained vessel cross-sections of similar shape (shape factor ≤2e-316) for each tissue section. Images were captured of the same fields of view in the corresponding serial sections immunostained with different VSMC differentiation markers.

The thickness of the vessel wall muscle was assessed in sections immunostained for αSMA. Measurements were taken of the stained muscular area and the inner luminal cross-sectional area using NIS Elements Ar (Nikon Instruments Inc.). Vessel muscularity was expressed as the ratio of the outer stained muscular area to the total area (stained area + inner luminal area) of the vascular cross-section (Figure 3.2). For each individual cross-section, measurements were obtained in triplicate and the mean was used for the final calculations. The number of vessels expressing αSMA was also quantified as a proportion of the total number of vessels as determined by double immunostaining with CD31 in the stratum functionalis of the endometrium in both controls and women with HMB during the menstrual cycle.

VSMC differentiation markers were analysed by their absence or presence in vascular cross-sections in each field of view, corresponding with the αSMA^{+} vascular cross sections. Vessels expressing smoothelin and calponin were also measured as a proportion of total number of vessels as determined by double immunostaining with CD31 and UEA-1, respectively, in the stratum functionalis.

Figure 3.2: Measurement of endometrial vascular wall thickness. Vessel thickness was expressed as the ratio of the outer stained muscular area (x-y) to the total area (x= stained area + inner luminal area) of the vascular cross-section.

3.3.3.2 Stromal, glandular and vascular proliferation and staining for EC marker and ECM component during the menstrual cycle

Sections were assessed in the entire tissue biopsies (N=3 or N=5 for EC and ECM, respectively) in each of PP, ESP, MSP and LSP from controls and women with HMB by one observer, who was blinded to the origin of the sample. Only muscular vessels with surrounding layer(s) of smooth muscle cells and a visible lumen, which were positively stained for MyHC, were included in the analysis. Entire full thickness immunostained tissue sections were analysed semi-quantitatively for Ki67 using a modified 'Quickscore' method (section 2.2.3.4). The superficial myometrium, stratum basalis (within one 200x field adjacent to the myometrium), stratum functionalis and luminal region (within one 200x field from the surface epithelium) were all scored separately. Proliferative vessel numbers were counted manually as the percentage of $Ki67^+$ vessels compared with the total number of vessels positively stained for UEA-1, F8RA, CD34 or CD31 in 3 randomly selected fields of view (200x original magnification) within the luminal region and stratum functionalis in each control sample (N=3, in each phase).

3.3.3.3 Endometrial leukocyte populations

Sections from endometrial biopsies (N=5), in each of PP, ESP, MSP and LSP from both controls and women with HMB were examined. Leukocyte numbers were counted using the open-source image analysis software package, ImageJ (Version 1.46, NIH, Maryland, USA), in combination with the 'cell counter' plugin as the percentage of positively stained cells for each leukocyte marker compared with the total number of stromal cells in 5 randomly selected fields of view (200x magnification) within the stratum functionalis in each sample.

The proportion of $CD3^+$ cells that were $CD3^+CD56^+$ double positive was assessed in mid and late secretory phase samples from both control and HMB groups (N=3 each group) that were double immunostained for CD3 and CD56. Single and double positive cells were counted in 5 randomly selected fields of view (x400 magnification) in the stratum functionalis in each sample using ImageJ. The number of $CD3^+CD56^+$ double positive cells was quantified both

as a proportion of the total number of $CD3^+$ cells and as a proportion of the total number of $CD56^+$ cells.

3.3.4 Statistical analysis

Statistical analyses were performed using either the Prism software package (GraphPad Software, San Diego, CA, USA) or Statistical Package for Social Sciences version 15.0 (SPSS Inc., Chicago, IL, USA). Data are presented as means ± standard error of mean (SEM) and differences are considered statistically significant at $P \leq 0.05$. Specific tests used are given in the results.

3.4 RESULTS

3.4.1 Altered VSMC differentiation in the endometrial vasculature in HMB

3.4.1.1 Vascular smooth muscle content remained unchanged during the control menstrual cycle and in HMB

Endometrial samples (N=4 each menstrual cycle phase) were double immunostained for αSMA and an endothelial cell marker (CD31) (Figure 3.3A-H). During the normal menstrual cycle 63% of the CD31-positive vessels in the stratum functionalis stained with αSMA in the proliferative phase, increasing to 81% in the late secretory phase (P=0.002) (Figure 3.3I). During the menstrual cycle of women with HMB 63% of vessels in the stratum functionalis stained with smooth muscle marker αSMA in proliferative endometrium, increasing to 79% in the late secretory phase (P=0.02) (Figure 3.3I). There was no difference in this percentage between the two groups at any stage of the menstrual cycle.

Endometrial vascular muscularity was analysed in serial sections from endometrial biopsies, collected from both control and HMB groups (N=7 for each phase of menstrual cycle) (Figure 3.4A-J). The overall vascular smooth muscle cell content (positive αSMA staining) in both groups remained relatively stable, accounting for 78%-81% of the total vascular cross-sectional area at all cycle phases (Figure 3.4K). There was no difference in the muscle content of endometrial vessels between control and HMB groups during the proliferative, early, mid or late secretory phases of the menstrual cycle (Figure 3.4K).

3.4.1.2 Differentiation status of VSMCs in endometrial vasculature is altered in HMB compared with controls

Serial tissue sections were immunostained for the different VSMC differentiation markers; H-cal, calponin, MyHC, desmin and smoothelin (Figure 3.5). Tissue sections, corresponding with those used for the measurement of thickness (αSMA), were imaged in ≥5 fields of views per section. Differentiation markers were analysed by means of ‘presence’ or ‘absence’ in the corresponding areas of the sections. Desmin immunostaining was absent from all tissues (Figure 3.5). Amongst the VSMC differentiation markers, MyHC was the most commonly present (>84% of all vascular sections) in both control and HMB

Figure 3.3: The proportion of vessels stained with αSMA in the control menstrual cycle and in heavy menstrual bleeding (HMB). **A-H** shows representative double immunostaining CD31/αSMA (brown/silver grey) of blood vessels in control (**A-D**) and HMB (**E-H**). The red and black arrows indicate CD31+/αSMA+ (brown/silver) vessels and CD31+/αSMA- vessels, respectively. Images were captured at 200x magnification. Bar chart with a standard error of mean (SEM) represents the percentage of αSMA+ vessels in each phase of the menstrual cycle for control and HMB groups (**I**). Fisher's test followed by unpaired Student's t-test was used (denoted by '*').

Figure 3.4: Vascular smooth muscle content did not differ between control and heavy menstrual bleeding (HMB) endometrium. **A-J** show vessels (arrowed) immunopositive for αSMA in control (**A-D**) and HMB (**F-J**) endometrium in the proliferative, early secretory, mid secretory and late secretory phases of the menstrual cycle. Images were captured at 200x magnification. Bar chart with a standard error of mean (SEM) represents the mean muscle content in each phase of the menstrual cycle for both control and HMB groups (**K**). Fisher's test followed by unpaired multiple Student's t-test, with Holm-Sidak correction method to account for multiple comparisons (P>0.05).

Figure 3.5: VSMC differentiation markers during the menstrual cycle in heavy menstrual bleeding (HMB). Arrows indicate vessels immunopositive for (**A-D**) H-Caldesmon, (**E-H**) calponin, **(I-L)** MyHC, (**M-P**) desmin and (**Q-T**) smoothelin. Images were captured at 200x original magnification.

tissue (Figure 3.6A). H-cal, was present in >26% of all vascular sections in both groups (Figure 3.6B). The staining for MyHC was relatively consistent (85%-96%) across the four menstrual cycle phases, while that of H-cal varied considerably, (26%-50% in control; 35-61% in HMB) during proliferative and early secretory phases (Figure 3.6A-B). None of these variations were significantly different between control and HMB groups.

In contrast to MyHC and H-cal, smoothelin and calponin staining differed markedly between the control and HMB tissues and also varied between the different phases of the menstrual cycle in both groups. In the control group calponin was present in 11-18% of vessels in the proliferative and early secretory phases, before increasing to be expressed in 37% vessels in the late secretory phase. In comparison, in the HMB group, calponin was expressed in 12% vessels in the proliferative phase, increasing to 32% and 39% in the early and mid secretory phases, respectively but was seen in only 11% of αSMA^+ vessels in the late secretory phase (Figure 3.6C). In the controls, while smoothelin was expressed in 36% of the αSMA^+ vessels in proliferative endometrium, this decreased to 17% in the early secretory phase and decreased further to only 5% αSMA^+ vessels in the late secretory phase. In contrast, in HMB smoothelin remained stable in proliferative (18%), early secretory (18%), mid secretory (23%) phases, before increasing to be expressed in 47% αSMA^+ vessels in the late secretory phase (Figure 3.6D). There was an apparent inverse relationship between staining for smoothelin and calponin in the αSMA^+ vessels in the late secretory phase in both control and HMB groups (Figure 3.6C-D). While smoothelin was increased in αSMA^+ vessels (P=0.03), calponin was decreased (P=0.008) in HMB compared with controls. The most marked difference between control and HMB groups in calponin and smoothelin staining was in the late secretory phase.

Double-labelling immunohistochemistry (N=5 samples per subject group) was undertaken to investigate smoothelin and calponin stain in conjunction with the endothelial cell markers CD31 or UEA-1, respectively, in the stratum functionalis of late secretory phase endometrium (Figure 3.6E-I). The proportion of vessels identified using EC markers positive for calponin and smoothelin was expressed as a percentage of the total number of vessels in the stratum

Figure 3.6: Vascular smooth muscle cell (VSMC) differentiation markers in control and HMB endometrium across the menstrual cycle. **(A-D)** Bar charts represent mean percentage of vessels immunopositive for different VSMC markers in each phase of the menstrual cycle for control and HMB. **(E)** Bar chart represents mean (±SEM) percentage of smoothelin+ or calponin+ vessels respectively in the LSP for both control and HMB. Double immunolabeling of endometrial blood vessels in **(F, H)** control and **(G, I)** HMB. **(F, G)** Calponin and **(H, I)** smoothelin are silver/grey and endothelial cells, **(F, G)** UEA-1 and **(H, I)** CD31 in brown. Black arrows indicate calponin$^+$/UEA-1$^+$ and smoothelin$^+$/CD31$^+$ vessels. Images were captured at 200x original magnification.

functionalis. This demonstrated that the proportion of vessels expressing calponin was significantly decreased (*P*=0.001) in HMB compared with controls (Figure 3.6E). No significant differences were identified using the endothelial markers that expressed smoothelin.

3.4.2 Pattern of stromal, glandular and vascular proliferation during the menstrual cycle

The pattern of stromal, glandular and vascular proliferation in the menstrual cycle in control endometrium has been compared spatio-temporally (Figure 3.7A-C).

Stromal proliferation: Stromal proliferation was highest in the PP luminal region, reducing in ESP (*P*=0.008), before increasing again in LSP. The stratum functionalis followed the same pattern of stromal proliferation as was seen in the luminal region. Stromal proliferation was reduced in the stratum basalis; LSP showed the maximum proliferation, which was higher than in ESP (*P*=0.001). Finally, superficial myometrium showed negligible stromal proliferation which was significantly lower than in the luminal region (MSP *P*=0.005, LSP *P*=0.007) (Figure 3.7A).

Glandular proliferation: Glandular proliferation was also highest in the PP in the luminal region, gradually decreasing in ESP, MSP to be lowest in LSP (*P*=0.006 compared with PP). The stratum functionalis showed the same pattern; glandular proliferation was highest in PP and lowest in LSP (*P*=0.006 compared with PP). Although no difference was seen in glandular proliferation between the luminal region and the stratum functionalis, the stratum basalis showed lower glandular proliferation in PP compared with the luminal region (*P*=0.02) (Figure 3.7B).

Vascular proliferation: Proliferation in the muscular vessels ($MyHC^+$) differed across the menstrual cycle as well as between the layers. Vascular proliferation in the luminal region was the highest in PP compared with ESP (*P*=0.008). In the stratum functionalis vascular proliferation reduced from PP to ESP, but gradually increased in LSP. Vascular proliferation was negligible in the myometrium; compared with the luminal region (*P*=0.002 in PP) and the stratum functionalis (ESP *P*=0.004, MSP *P*=0.002, LSP *P*=0.004) (Figure 3.7C).

Figure 3.7: Graphical representation (mean ± SEM) of pattern of immunohistochemical staining for proliferation marker Ki67. Ki67 staining between phases (N=3, each phase) of the control menstrual cycle (proliferative, early secretory, mid secretory, late secretory) and in the different tissue layers (luminal, stratum functionalis, stratum basalis, myometrium) for **(A)** stroma, **(B)** glands **(C)** and vessels. Bars labelled with the same letter are significantly different from each other. (a-e) represent differences between phases within each layer, (A-G) represent differences between layers, within each phase. $^{a,\ e}P$=0.008, ^{b}P=0.001, $^{c,\ d}P$=0.006; ^{A}P=0.005, ^{B}P=0.007, ^{C}P=0.015, $^{D,\ F}P$=0.002, $^{E,\ G}P$=0.004. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant difference.

3.4.3 Spatio-temporal pattern of vascular staining for different EC markers in the menstrual cycle is altered in HMB

The different EC markers displayed different patterns of staining in the endometrium and myometrium (Figure 3.8A-P).

3.4.3.1 Pattern of vascular staining for each EC marker between the phases of the menstrual cycle in the uterine tissue layers

UEA-1: In the luminal region, UEA-1 reactivity was higher in LSP compared to MSP (P=0.006; Figure 3.9A). UEA-1 reactivity in the stratum functionalis was lower in MSP compared with ESP (P=0.002). In contrast, in stratum basalis and superficial myometrium there were no menstrual cycle differences for UEA-1 (Figure 3.9A).

F8RA: In all regions F8RA staining was unchanged from PP to MSP, but was reduced in LSP compared with ESP (luminal region P=0.006, stratum functionalis P=0.003, stratum basalis P=0.003, and myometrium P=0.002; Figure 3.9B).

CD34: CD34 staining within endometrial layers and superficial myometrium was stable across the different phases of the menstrual cycle (Figure 3.9C).

CD31: CD31 staining remained similar from PP to MSP before increasing in LSP compared with PP (Figure 3.9D) in the luminal region (P=0.007) and stratum functionalis (P=0.002). In contrast, CD31 staining in the myometrium remained similar from PP to MSP before decreasing in LSP compared with PP (P=0.003). In stratum basalis, CD31 staining did not vary with menstrual cycle phase (Figure 3.9D).

Figure 3.8: Staining for endothelial cell (EC) markers in the uterus. Layers of the uterus (myometrium, stratum basalis, stratum functionalis, luminal) showing vessels stained positive for (**A-D**) UEA-I, (**E-H**) F8RA, (**I-L**) CD34 and (**M-P**) CD31 during the mid secretory phase of the control menstrual cycle. Images were captured at 200x original magnification.

Figure 3.9: Graphical representation (mean ± SEM) of Quickscore assessment of immunohistochemical staining for endothelial cell (EC) marker stainingbetween phases (N=5, each phase) of the normal menstrual cycle in the tissue layers (luminal, stratum functionalis, stratum basalis, myometrium) for each EC marker (**A-D**) and between the different EC markers (UEA-1, F8RA, CD34, CD31) in the tissue layers for each menstrual cycle phase (**E-H**). Bars labelled with the same letter are significantly different from each other. (a-t) represent differences between phases or factors, within each layer, (A-J) represent differences between layers, within each phase. $^{a,\,c}P$=0.006, $^{b,\,f,\,h,\,t}P$=0.002, $^{d,\,e,\,l,\,j,\,s}P$=0.003, ^{g}P=0.007, $^{k,\,l,\,n,\,r}P$=0.001, $^{m,\,q}P$<0.001, ^{o}P=0.005, ^{p}P=0.008; $^{A,\,I}P$<0.001, $^{B,\,C,\,H}P$=0.001, $^{D,\,E,\,G,\,J}P$=0.002, ^{F}P=0.004. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant difference.

3.4.3.2 Pattern of vascular EC marker staining between the uterine tissue layers in each phase of the menstrual cycle

The pattern of staining for different EC markers showed a similar trend of a steady increase from the luminal region to the superficial myometrium during the menstrual cycle (Figure 3.8A-P).

Proliferative phase: UEA-1 reactivity and F8RA staining was lower in the luminal region compared with myometrium (UEA-1 $P<0.001$, F8RA $P=0.002$; Figure 3.9B). The pattern of CD34 staining differed (Figure 3.9C) with staining being lower in the luminal area compared with stratum functionalis ($P=0.002$). CD31 staining showed a similar trend with lower staining in the luminal area compared with myometrium ($P<0.001$; Figure 3.9D).

Early secretory phase: There was increased reactivity for UEA-1 ($P=0.001$; Figure 3.9A), F8RA ($P=0.002$; Figure 3.9B) and CD31 ($P=0.002$; Figure 3.9D) from the luminal region to myometrium. No difference between the tissue layers was observed for CD34 staining (Figure 3.9C).

Mid-secretory phase: Staining or reactivity of the different EC markers in the MSP showed no significant changes between the tissue layers (Figure 3.9A-D).

Late secretory phase: There was increased reactivity for UEA-1 ($P=0.001$; Figure 3.9A), F8RA ($P=0.004$; Figure 3.9B) and CD34 ($P=0.001$; Figure 3.9C) from the luminal region to myometrium. In contrast, CD31 staining remained unchanged between the endometrial layers and superficial myometrium (Figure 3.9D).

3.4.3.3 Comparative pattern of the different EC marker staining in the phases of the menstrual cycle in each uterine tissue layer

Luminal region: CD34 showed highest staining in PP compared with CD31 ($P=0.003$; Figure 3.9E) and in ESP compared with UEA-1 reactivity ($P=0.001$; Figure 3.9F). CD31 showed highest staining in MSP compared with UEA-1 reactivity ($P=0.008$; Figure 3.9G) and in LSP compared with F8RA ($P<0.001$; Figure 3.9H). F8RA staining was absent in the luminal layer in the LSP.

Stratum functionalis: CD34 showed highest staining compared with F8RA in PP ($P=0.001$; Figure 3.9E) and ESP ($P=0.005$; Figure 3.9F). CD31 staining was the

higher than F8RA in LSP (*P*=0.001; Figure 3.9H). Staining for F8RA in LSP was scarce.

Stratum basalis: Staining for CD31 was higher in PP than F8RA (*P*=0.001; Figure 3.9E). The reactivity of the four EC markers was similar in ESP (Figure 3.9F), MSP (Figure 3.9G) and LSP (Figure 3.9H) apart from F8RA, which showed lower staining compared with CD34 in LSP (*P*=0.003; Figure 3.9H).

Myometrium: Myometrium showed variation in the reactivity of the EC markers with CD31 being higher than F8RA in PP (*P*<0.001; Figure 3.9E) and UEA-1 being higher than F8RA in LSP (*P*=0.002; Figure 3.9H).

3.4.3.4 Differences between the different vascular EC marker staining correlates to the proliferative state of the endometrial vessels

An immunohistochemical labelling study (N=3 samples in each phase) was undertaken to investigate whether differences seen in the pattern of reactivity between the four different EC markers correlated to the proliferative state ($Ki67^+$ staining, Figure 3.10A-D) of these vessels. In the luminal region, the percentage of UEA-I^+ and $F8RA^+$ vessels proliferating ($Ki67^+$) was similar within PP/ESP and MSP/LSP. Strikingly there was a reduction in the percentage of UEA-1^+ (*P*=0.009) and $F8RA^+$ (*P*=0.04, Figure 3.10E) proliferating vessels ($Ki67^+$) in the MSP/LSP compared with PP/ESP. In the luminal region and stratum functionalis, UEA-1^+ vessels constituted the highest proportion (70%) of proliferating vessels in PP reducing to 51% in LSP. This was followed by CD31 (46% in PP, reducing to 25% in LSP) and CD34 (40% in PP, reducing to 22% in LSP). Strikingly, F8RA showed the lowest proportion (34%) of proliferating vessels in PP further lowering to 14% in LSP. F8RA was significantly lower in both luminal region and stratum functionalis in PP, ESP and LSP (*P*=0.05 for all), compared with UEA-1 (Figure 3.10F-G).

3.4.3.5 Pattern of vascular EC staining during the menstrual cycle is altered in HMB

The mean Quickscore and standard error in the control and HMB groups are shown in Table 3.1. The pattern of reactivity of the different EC markers varied considerably in HMB compared with controls. Amongst the EC markers, CD34 was most differently expressed in HMB, when compared with control samples.

Figure 3.10: Staining for vascular Ki67 in the control menstrual cycle. (**A-D**) $Ki67^{+}$ vessels indicated by the dotted circles in proliferative, early secretory, mid secretory and late secretory phase. Images were captured at 200x original magnification. Graphical representation (mean ± SEM) of the percentage assessment of immunohistochemical staining for proliferation (Ki67, N=3, each phase) in vessels expressing UEA-1, F8RA, CD34 and CD31 in the control menstrual cycle in (**E, F**) luminal region and (**G**) stratum functionalis. (**E**) Mann-Whitney U test determined statistical significance between PP/ESP and MSP/LSP. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant difference between factors. (A-G) represent differences between factors, within each phase. $^{A,B,C,E,F,G}P$=0.05, ^{D}P=0.046.

Interestingly, the majority of differences were detected in the LSP.

Luminal region: Compared with controls, in HMB ECs showed reduced UEA-1 reactivity in the PP (P=0.008) and LSP (P=0.008). Similarly, reduced CD31 staining was observed in HMB in both MSP (P=0.008) and LSP (P=0.008). In contrast, compared with controls, staining for F8RA (P=0.008) and CD34 (P=0.008) in the luminal area was increased in HMB in LSP, although staining for F8RA was still low (Table 3.1).

Stratum functionalis: Staining or reactivity of the four EC markers varied between controls and women with HMB, especially in MSP and LSP (Table 3.1). In particular in HMB ECs were less reactive to UEA-1 in PP (P=0.008) and LSP (P=0.008) and also expressed lower CD31 in both MSP (P=0.008) and LSP (P=0.008). In contrast, staining for CD34 was increased in MSP (P=0.008) in HMB (Table 3.1).

Stratum basalis: Staining or reactivity of EC markers in stratum basalis also differed in HMB but the changes were less extensive and were only seen for CD34 and CD31. While CD34 staining was increased in ESP (P=0.008) and LSP (P=0.008), CD31 staining was reduced in LSP (P=0.008) (Table 3.1).

Myometrium: UEA-1 reactivity in myometrium did not differ between control and HMB samples. F8RA staining was decreased in ESP (P=0.008) in HMB but CD34 staining was increased in all menstrual cycle phases in HMB (PP P=0.008, ESP P=0.008, MSP P=0.008, LSP P=0.008). CD31 staining in LSP (P=0.008) was also increased in HMB compared with controls (Table 3.1).

Table 3.1: Mean immunohistochemical Quickscore (standard error) for EC markers in endometrial vessels in control and HMB

	Luminal (N=5)		**Stratum Functionalis** (N=5)		**Stratum Basalis** (N=5)		**Myometrium** (N=5)	
	Control	**HMB**	**Control**	**HMB**	**Control**	**HMB**	**Control**	**HMB**
UEA-1								
PP	**2.60[a] (0.24)**	**1.00[a] (0.00)**	**5.00[b] (0.00)**	**2.60[b] (0.40)**	6.00 (0.32)	5.00 (0.55)	9.40 (0.40)	9.20 (0.37)
ESP	2.60 (0.60)	1.80 (0.49)	5.40 (0.40)	3.60 (0.81)	7.00 (0.63)	5.80 (0.49)	9.00 (0.63)	8.60 (0.81)
MSP	1.20 (0.20)	2.40 (0.40)	3.20 (0.49)	4.20 (0.58)	4.60 (1.29)	5.80 (0.73)	6.40 (1.66)	8.80 (0.58)
LSP	**3.20[c] (0.20)**	**1.00[c] (0.00)**	**4.60[d] (0.24)**	**2.20[d] (0.20)**	6.60 (0.40)	4.20 (0.37)	9.80 (0.73)	6.00 (0.55)
F8RA								
PP	3.20 (0.20)	4.00 (0.55)	3.80 (0.37)	4.40 (0.40)	5.40 (0.24)	6.20 (0.37)	6.60 (0.24)	7.20 (0.20)
ESP	4.00 (0.63)	3.00 (0.00)	5.00 (0.55)	2.60 (0.24)	6.60 (0.40)	4.80 (0.20)	**8.40[e] (0.40)**	**4.80[e] (0.37)**
MSP	2.00 (0.84)	2.60 (0.24)	3.20 (0.80)	3.80 (0.49)	4.20 (1.07)	4.40 (0.24)	4.80 (1.24)	6.00 (0.32)
LSP	**0.00[f] (0.00)**	**1.40[f] (0.24)**	0.60 (0.24)	1.80 (0.20)	3.20 (0.20)	2.00 (0.45)	3.80 (0.49)	2.60 (0.68)
CD34								
PP	5.80 (0.37)	6.00 (0.63)	8.60 (0.40)	7.40 (0.24)	7.20 (0.20)	8.20 (0.49)	**8.00[g] (0.00)**	**10.00[g] (0.45)**
ESP	8.60 (0.40)	7.60 (0.24)	7.80 (0.20)	8.60 (0.24)	**7.80[h] (0.20)**	**9.00[h] (0.00)**	**7.80[i] (0.20)**	**10.60[i] (0.24)**
MSP	5.60 (1.08)	8.00 (0.45)	**6.00[j] (0.63)**	**8.80[j] (0.20)**	6.60 (1.66)	9.40 (0.24)	**6.40[k] (1.60)**	**10.60[k] (0.24)**
LSP	**5.80[l] (0.20)**	**8.00[l] (0.32)**	6.80 (0.20)	8.60 (0.40)	**7.40[m] (0.24)**	**9.40[m] (0.24)**	**8.40[n] (0.24)**	**10.60[n] (0.24)**
CD31								
PP	2.20 (0.37)	2.80 (0.20)	4.60 (0.40)	5.00 (0.00)	9.00 (0.00)	7.80 (0.80)	11.00 (0.00)	9.80 (0.80)
ESP	6.20 (0.49)	6.80 (0.20)	7.00 (0.00)	6.80 (0.20)	8.60 (0.40)	8.60 (0.24)	10.20 (0.37)	9.60 (0.24)
MSP	**7.40[o](0.24)**	**2.80[o] (0.20)**	**7.00[p] (0.00)**	**5.00[p] (0.00)**	6.00 (1.52)	7.60 (0.68)	5.60 (1.40)	9.00 (0.55)
LSP	**7.40[q] (0.40)**	**5.00[q] (0.00)**	**7.40[r] (0.40)**	**5.00[r] (0.00)**	**7.20[s] (0.20)**	**4.20[s] (0.49)**	**5.60[t] (0.40)**	**8.80[t] (0.20)**

Values represent mean (±SEM); [a-t]*P*=0.008, data in bold with same superscript are significantly different from each other, while those without are non-significant. HMB-Heavy menstrual bleeding; PP-Proliferative phase; ESP-Early secretory phase; MSP-Mid secretory phase; LSP-Late secretory phase

3.4.4 Spatio-temporal pattern of vascular extracellular matrix (ECM) staining in normal menstrual cycle is altered in HMB

The pattern of staining for the ECM components varied both during the different menstrual cycle phases and between myometrium and the different endometrial layers (Figure 3.11A-L).

3.4.4.1 Pattern of vascular ECM staining in the phases of the control menstrual cycle in each uterine tissue layer

Stratum functionalis: Osteopontin staining was highest in PP, decreasing in MSP (P=0.003; Figure 3.12A). Staining for laminin, fibronectin and collagen IV in stratum functionalis remained similar throughout the menstrual cycle (Figure 3.12B-D).

Stratum basalis: The stratum basalis showed both lower staining for some ECM components and variation compared with stratum functionalis. Osteopontin staining remained unchanged during the menstrual cycle phases (Figure 3.12A). Laminin was scarcely expressed in stratum basalis at all menstrual cycle phases (Figure 3.12B). Fibronectin staining was also low and was stable apart from a decrease in LSP compared with MSP (P=0.005; Figure 3.12C). While collagen IV was absent in PP stratum basalis, it increased in ESP and remained stable thereafter in MSP and LSP (Figure 3.12D).

Myometrium: Myometrial ECM staining did not differ across the menstrual cycle with stable staining of osteopontin, laminin, fibronectin and collagen IV (Figure 3.12A-D).

3.4.4.2 Pattern of vascular ECM staining between the uterine tissue layers in each phase of the normal menstrual cycle

Vascular staining of the different ECM components differed between stratum functionalis, stratum basalis and myometrium (Figure 3.11A-L).

Proliferative phase: Vascular osteopontin staining was highest in stratum functionalis compared with myometrium (P=0.002; Figure 3.12A). Laminin staining was low in PP and did not differ between the three layers (Figure 3.12B). Fibronectin staining did not differ between the three layers

Figure 3.11: Staining for extracellular matrix components in the uterus. Uterine layers (myometrium, stratum basalis, stratum functionalis), showing vessels immunopositive for (**A-C**) osteopontin, (**D-F**) laminin, (**G-I**) fibronectin and (**J-L**) collagen IV during the mid secretory phase of the control menstrual cycle. Arrows indicate vascular extracellular matrix components. Images were captured at 200x original magnification.

Figure 3.12: Immunohistochemical staining for pattern of staining for vascular extracellular matrix (ECM) markers in the control menstrual cycle. Graphical representation (mean ± SEM) of Quickscore assessment of ECM markers between phases (N=5, each phase) of the control menstrual cycle (proliferative, early secretory, mid secretory, late secretory) in the tissue layers (stratum functionalis, stratum basalis, myometrium) for each ECM marker (osteopontin, laminin, fibronectin, collagen IV) **(A-D)**. (*a*, *b*) represent differences between phases or factors, within each layer, (A-H) represent differences between layers, within each phase. ^{a}P=0.003, ^{b}P=0.005; ^{A}P=0.002, ^{B}P=0.02, ^{C}P=0.005, ^{D}P=0.003, ^{E}P=0.004, ^{F}P=0.001, ^{G}P=0.01, ^{H}P=0.01. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant difference.

(Figure 3.12C). Collagen IV was absent from the stratum basalis in PP, with higher staining in myometrium (*P*=0.01; Figure 3.12D).

Early secretory phase: Osteopontin staining did not differ between the stratum functionalis, stratum basalis and myometrium (Figure 3.12A). Vascular laminin staining was low throughout but was higher in stratum functionalis compared with stratum basalis (*P*=0.005; Figure 3.12B). Fibronectin staining was also higher in stratum functionalis compared with stratum basalis (*P*=0.003; Figure 3.12C). Similarly collagen IV showed highest staining in stratum functionalis with lowest staining in myometrium (*P*=0.01; Figure 3.12D).

Mid secretory phase: Osteopontin staining was higher in myometrium than stratum functionalis (*P*=0.02; Figure 3.12A). Laminin staining was low in MSP and did not differ between the layers (Figure 3.12B). Unlike laminin, fibronectin was highly present in the stratum functionalis in MSP but was decreased in myometrium (*P*=0.004; Figure 3.12C). In contrast, collagen IV staining in MSP was low in both stratum functionalis and myometrium (Figure 3.12D).

Late secretory phase: There was no variation in staining between the three layers for osteopontin, laminin and collagen IV (Figure 3.12A,B,D). However, fibronectin staining was higher in stratum functionalis compared with stratum basalis (*P*=0.002; Figure 3.12C).

3.4.4.3 Pattern of vascular ECM staining during the menstrual cycle is altered in HMB

The Quickscore (mean and standard error) for each ECM component for the control and HMB groups are shown in Table 3.2. Differences in ECM component staining between HMB and controls were most common in the stratum basalis followed by the stratum functionalis and all variations were observed in the secretory phases. Amongst the ECM components, collagen IV showed most differences in HMB, followed by osteopontin and fibronectin (Table 3.2).

The pattern of vascular laminin staining did not differ between controls and women with HMB throughout the menstrual cycle. Compared with controls, vascular fibronectin staining was reduced in MSP stratum basalis (*P*=0.006) in HMB (Table 3.2).

Compared with controls, in HMB osteopontin staining was increased in the LSP (P=0.008) in the stratum functionalis but decreased in the ESP in stratum basalis (P=0.008; Table 3.2). The pattern of collagen IV staining differed most between HMB and controls and was decreased compared with controls in the stratum functionalis in ESP (P=0.008) and in the stratum basalis in ESP (P=0.007) and MSP (P=0.002; Table 3.2). In the myometrium, however, collagen IV staining remained unaltered compared with controls.

Table 3.2: Mean immunohistochemical Quickscore (standard error) for ECM components in endometrial vessels in control and HMB

	Stratum Functionalis (N=5)		Stratum Basalis (N=5)		Myometrium (N=5)	
	Control	**HMB**	**Control**	**HMB**	**Control**	**HMB**
Osteopontin						
PP	11.00 (0.00)	9.20 (0.66)	7.80 (0.49)	7.20 (0.37)	6.80 (0.20)	6.80 (0.58)
ESP	9.60 (0.60)	8.00 (0.95)	**9.40[a] (0.24)**	**6.00[a] (0.84)**	8.80 (0.58)	7.20 (0.49)
MSP	6.40 (0.24)	7.20 (0.97)	7.40 (0.40)	6.40 (1.40)	7.80 (0.20)	5.80 (1.07)
LSP	**7.20[b] (0.49)**	**11.20[b] (0.37)**	6.40 (0.75)	7.40 (0.24)	7.00 (0.84)	7.40 (0.51)
Laminin						
PP	1.40 (0.68)	3.60 (0.81)	0.20 (0.20)	0.40 (0.24)	1.40 (0.40)	1.20 (0.20)
ESP	2.00 (0.45)	1.60 (0.68)	0.00 (0.00)	0.20 (0.20)	0.40 (0.24)	1.20 (0.37)
MSP	1.40 (0.40)	5.40 (1.50)	0.60 (0.40)	0.40 (0.24)	1.00 (0.32)	0.40 (0.24)
LSP	3.20 (1.39)	3.40 (1.47)	0.40 (0.40)	1.60 (0.40)	1.20 (0.20)	1.20 (0.20)
Fibronectin						
PP	6.60 (1.29)	4.40 (1.47)	1.40 (0.68)	1.20 (0.49)	3.60 (1.21)	2.00 (0.95)
ESP	8.00 (0.32)	5.40 (1.03)	1.20 (0.20)	1.60 (0.87)	2.20 (0.49)	1.00 (0.55)
MSP	8.20 (0.80)	4.80 (1.32)	**3.20[c] (0.20)**	**1.20[c] (0.49)**	2.20 (0.73)	0.60 (0.60)
LSP	6.20 (0.37)	7.20 (1.32)	0.40 (0.40)	3.40 (1.03)	2.00 (0.00)	3.00 (1.00)
Collagen IV						
PP	2.40 (0.60)	1.40 (0.68)	0.00 (0.00)	0.00 (0.00)	3.00 (0.63)	3.80 (0.20)
ESP	**7.00[d] (0.71)**	**3.40[d] (0.40)**	**4.60[e] (0.60)**	**1.20[e] (0.20)**	3.60 (0.40)	3.60 (0.60)
MSP	1.80 (0.37)	4.80 (1.50)	**4.60[f] (0.24)**	**1.60[f] (0.98)**	2.00 (0.77)	3.20 (1.39)
LSP	6.20 (1.16)	3.20 (0.73)	3.60 (0.60)	2.80 (0.58)	4.00 (0.32)	6.20 (0.58)

[a,b,d]P=0.008, [c]P=0.006, [e]P=0.007, [f]P=0.002; data in bold with same superscript are significantly different from each other, while those without are non-significant. PP-Proliferative Phase; ESP-Early Secretory Phase; MSP-Mid Secretory Phase; LSP-Late Secretory Phase

3.4.5 Menstrual cycle distribution of leukocytes is altered in HMB

3.4.5.1 Distribution of leukocytes during the menstrual cycle is altered in HMB

Endometrial samples (N=5 each menstrual cycle phase) were immunostained for CD3 (T cells and uNK cells), CD8 (cytotoxic T cells), CD14 (macrophages), CD56 (uNK cells), CD83 (dendritic cells) and FOXP3 (regulatory T cells, Tregs) (Figure 3.13A-X). During the normal menstrual cycle $CD3^+$ cells accounted for 5.7% of total stromal cells in the PP, which decreased in the ESP (P=0.01) before increasing in the LSP (P=0.01), compared with PP (Figure 3.14). Additionally, compared with ESP, the percentage of $CD3^+$ cells was increased in both MSP (P=0.001) and LSP (P<0.001). However, no significant variation was observed in the percentage of $CD8^+$ cytotoxic T cells and $CD14^+$ macrophages throughout the control menstrual cycle, and $CD83^+$ dendritic cells and $FOXP3^+$ Tregs remained scarce throughout (Figure 3.14). $CD56^+$ uNK cells were least common in PP (2.13 ± 0.31) and ESP (2.09 ± 0.24), but their percentage increased in MSP (6.34 ± 0.57, P=0.002) and peaked in LSP (16.95 ± 1.10, P<0.001) compared with PP. Again, compared with ESP, the proportion of $CD56^+$ uNK cells was increased in both MSP (P=0.002) and LSP (P<0.001; Figure 3.14).

During the menstrual cycle of women with HMB $CD8^+$ T cells, macrophages, dendritic cells and regulatory T cells, showed no difference as a proportion of the endometrial stromal cells compared with controls (Figure 3.14B-C, Figure 3.14E-F). The $CD3^+$ cell population was reduced in the MSP (P=0.01), but increased in the LSP (P=0.01) in HMB (Figure 3.14A). The $CD56^+$ uNK cell population showed the most alteration in HMB (Figure 3.14D). While the percentage of $CD56^+$ cells was increased in both PP (P<0.01) and ESP (P<0.02) in HMB compared with controls, in the LSP the proportion of uNK cells was reduced in HMB (P<0.01).

3.4.5.2 Altered uNK cell staining in HMB is due to an alteration in the proportion of CD56+CD3+ cell population

A double immunohistochemical labelling study (N=3 samples per group for each of MSP and LSP) was undertaken to investigate whether the proportion of $CD56^+$ positive cells, which express CD3 is altered in HMB (Figure 3.15A-D). The percentage of $CD56^+$ cells within the total $CD3^+$ cell population was reduced in the stratum functionalis in the LSP in women with HMB, compared with controls (P=0.05). Moreover, the proportion of $CD3^+CD56^+$ double positive cells within the total $CD56^+$ cell population reduced in the stratum functionalis in the LSP in women with HMB, compared with controls (P=0.05) (Figure 3.15E-F).

Figure 3.13: Immunohistochemical labeling for leukocytes in the stratum functionalis of endometrium in HMB. Arrows indicate $CD3^+$ cells (**A-D**), $CD8^+$ T cells (**E-H**), $CD14^+$ macrophages (**I-L**), $CD56^+$ uNK cells (**M-P**), $CD83^+$ dendritic cells (**Q-T**), and $FOXP3^+$ regulatory T cells (**U-X**), during the proliferative, early secretory, mid secretory and late secretory phases of the menstrual cycle. Images were captured at 200x original magnification.

Figure 3.14: Endometrial leukocyte population as a proportion of the total stromal cells at each phase of the menstrual cycle in controls and in HMB. Proportion of $CD3^+$ cells (**A**), $CD8^+$ T cells (**B**), $CD14^+$ macrophages (**C**), $CD56^+$ uNK cells (**D**), $CD83^+$ dendritic cells (**E**) and $FOXP3^+$ regulatory T cells (**F**) in stratum functionalis of the endometrium across the phases of the menstrual cycle in controls and in HMB (N=5 in each phase). The line graphs show mean ± standard error of mean (SEM) of the percentage population of leukocytes as a proportion of total stromal cells at each phase of the menstrual cycle. Non-parametric Mann-Whitney U test, with Bonferroni correction for multiple comparisons was used to determine significance.

Figure 3.15: Double immunolabeling for $CD3^+$ cells and $CD56^+$ uNK cells in the mid secretory and late secretory phases in stratum functionalis of control (**A, B**) and HMB samples (**C, D**). $CD3^+$ cells are red and $CD56^+$ cells are silver. The black arrows indicate $CD3^+CD56^+$ cells. Images were captured at 400x original magnification. Bar chart with a standard error mean (SEM) represents the mean percentage of $CD3^+CD56^+$ cells as a proportion of all $CD3^+$ cells (**E**) and that of $CD3^+CD56^+$ cells as a proportion of all $CD56^+$ cells (**F**) in control and HMB samples. Mann-Whitney U test was used to determine statistical significance.

3.5 DISCUSSION

3.5.1 Vascular smooth muscle content and differentiation during the menstrual cycle

The current study showed that the muscle content of vessels in the stratum functionalis measured as a proportion of the vascular cross-sectional area increased from the proliferative to the late secretory phase (Figure 3.3). However, this pattern did not alter between control and HMB groups (Figure 3.4). Moreover, vascular calponin staining in relation to both the total number of vessels and to αSMA^{+} vessels was significantly decreased in women with HMB, suggesting an alteration in vascular smooth muscle cell differentiation status (Figure 3.6). Vascular smooth muscle cells form an integral part of the structure and normal functioning of the endometrial spiral arteries and arterioles and therefore may play an important role in abnormal bleeding disorders such as HMB. Understanding the healthy functioning of the uterus, especially the endometrial vasculature may thus provide significant clues to advance the development of effective, and less invasive treatments, to improve the quality of life for thousands of women suffering from HMB.

Previous studies have suggested that the structure and/or function of endometrial spiral arteries and arterioles may play a role in HMB, but little is known about the underlying mechanisms. Abberton *et al*. (1999b) and Hurskainen *et al*. (1999) reported that menorrhagic women had increased volume and rate of blood flow through the endometrial vessels. This increased blood flow rate may be explained by either increased vasodilation or poor vasoconstriction. If vasodilation is caused by relaxation of the vascular smooth muscle cells of the vessel wall, it is possible that differences may lie in the vascular muscle content and thickness in HMB endometrium.

The current study examined whether the thickness of VSMCs differed between controls and women with HMB during the different menstrual cycle phases. Previous studies have shown no difference in the endometrial microvascular density in women with HMB compared with controls (Abberton *et al*., 1999b). This study has extended these findings to demonstrate that, while the percentage of muscularised blood vessels increased across the menstrual

cycle, there was no difference between controls and women with HMB. In addition, in αSMA$^+$ vessels, the proportion of vascular cross-sectional area accounted for by VSMCs did not differ between controls and women with HMB (Figure 3.3-3.4). This result is partially consistent with that of Abberton *et al*. (1999a) who reported no difference in the muscle thickness of spiral arterioles between control and menorrhagic tissue during the menstrual cycle, although there was a decrease in muscle thickness in straight arterioles in the late secretory phase in HMB. These differences may be explained by the fact that our study included both straight and spiral arteries/arterioles, while Abberton *et al*. (1999a) analysed these separately. Furthermore, assessment of vessel muscle content by Abberton *et al*. (1999a) was based on the number of VSMC layers surrounding the vessel rather than as a ratio of the total vessel cross sectional area, as reported in the current study. Overall, these data suggest that the number and muscle content of endometrial blood vessels is not substantially altered in HMB and that any underlying vascular abnormalities are more subtle.

VSMCs play a major role in contraction and control of blood flow by monitoring vascular tonicity and diameter and consequently in maintenance of blood pressure. They express a range of proteins as differentiation markers. αSMA is one of the earliest markers of VSMC differentiation, while calponin and h-Caldesmon are expressed later (Glukhova *et al*., 1986; Glukhova *et al*., 1990; Frid *et al*., 1992; Owens, 1995). Myosin heavy chain 1 (MyHC1) is expressed prior to MyHC2 (Kocher *et al*., 1991; Aikawa *et al*., 1993; Miano *et al*., 1994; Owens, 1995; Owens *et al*., 2004), whereas smoothelin is expressed in 10-11 week placental tissue (van der Loop *et al*., 1997). Combining these a chronological pattern of staining for VSMC differentiation markers could be deduced as αSMA, h-Caldesmon and calponin, MyHC1, smoothelin and finally MyHC2. However, it is not known whether the order of this sequential differentiation is similar in endometrial vessels to other vessels; nor is it known whether a change in the differentiation status can potentially lead to altered vascular function.

Desmin is the major intermediate filament in smooth muscle and is thought to contribute to maintaining mechanical strength and contractility (reviewed by

Paulin and Li *et al*. 2004). However, desmin was not expressed by endometrial blood vessels at any stage of the menstrual cycle, perhaps suggesting an alternative mechanism of maintaining mechanical integrity and contractile function in these vessels. MyHC (both SM1 and SM2 were detected by the antibody used) was the most abundantly detected VSMC marker throughout the menstrual cycle in both subject groups, while the staining patterns of smoothelin and calponin were the most highly variable, both with menstrual cycle phase and between control and HMB groups. This difference suggests a change of differentiation status of VSMCs in HMB that may reflect altered function, particularly in the late secretory phase of the menstrual cycle just prior to menstruation. In contrast to our findings, Abberton *et al*. (1999a) reported decreased MyHC staining in spiral arterioles in the early secretory phase in HMB (Abberton *et al*., 1999a). However, as stated, our study included spiral and straight arterioles and recorded MyHC staining only in vessels that expressed αSMA, while Abberton *et al*. (1999a) measured MyHC staining independent of αSMA using a quick scoring method: integrated optical density x proportional area. While both methods have merits, by analysing staining for VSMC differentiation markers in relation to all αSMA^+ vessels, we hoped to also detect differences in the chronological pattern of differentiation in the VSMCs between control and HMB samples.

There was an inverse relationship between smoothelin and calponin staining in the late secretory phase; the proportion of αSMA^+/$smoothelin^+$ vessels increased in the late secretory phase in HMB compared with controls, whereas that of αSMA^+/$calponin^+$ vessels decreased (Figure 3.3). Double immunohistochemical labelling also showed a decrease in the proportion of calponin expressing vessels in HMB, compared with the total number of vessels detected using endothelial markers. The fact that some of these differentiation markers manifest a more restricted distribution than others in endometrial vessels raises an important question; do the differences in distribution indicate their stage of differentiation or do they reflect functional differences? Further future functional studies examining these possibilities are therefore warranted, which could in turn indicate potential therapeutic targets.

Smoothelin is a major contributor to the contractile properties of VSMCs and has two isoforms; smoothelin A, expressed in viscera, and smoothelin B, which is specifically present in VSMCs (Rensen *et al.*, 2002; van Eys *et al.*, 2007). Rensen *et al.* (2008) reported that smoothelin B deficient mice had significantly reduced arterial contractility and increased basal mean arterial pressure (MAP), which may be attributed to a simultaneous reduction in vasodilation. The rate of muscle contraction is determined by acto-myosin cross-bridge cycling and the rate of muscle shortening (shortening velocity); the co-operation between these acto-myosin cross-bridges in turn depends on proteins such as h-Caldesmon and calponin (Butler and Siegman, 1998; Fitzsimons *et al.*, 2001; Morgan and Gangopadhyay, 2001). Interestingly, smoothelin B consists of a 'calponin homology domain', which is capable of binding to αSMA in VSMCs (van Eys *et al.*, 2007). In support of this, Schildmeyer *et al.* (2000) showed that αSMA null mice had abnormal vasoconstriction as well as impaired vasodilation. Therefore, deficiency or aberrant expression of smoothelin B may result in impaired vasodilation (Li and Hui, 2009). Increased smoothelin may facilitate contractility and vasodilation leading to increased blood flow. In this study we have shown such an increase in smoothelin in the late secretory phase, perhaps indicating increased vasocontraction and vasodilation as an underlying mechanism for HMB.

Calponin h1 (or basic calponin) is a smooth muscle specific protein, which binds to actin and plays a significant role in regulation of the contractile apparatus in VSMCs (Walsh *et al.*, 1993; Lu *et al.*, 1995; Stafford *et al.*, 1995). In aortic smooth muscle, shortening velocity is a direct measure of rate of vessel contraction and consequently blood flow; increased shortening velocity increases the rate of vessel contraction, thereby increasing the rate of blood flow per unit time. Takahashi *et al.* (2000) reported that calponin plays a role in regulating cross-bridge cycling and shortening velocity and consolidated previous findings that calponin slowed shortening velocity in both tonic and phasic smooth muscle (Jaworowski *et al.*, 1995; L-Mezgueldi and Marston, 1996; Malmqvist *et al.*, 1997; Takahashi *et al.*, 2000). The same group also reported that decreased calponin leads to an increase in cross-bridge cycle, i.e. increased shortening velocity and therefore increased rate of contraction and

blood flow (Fujishige *et al.*, 2002). In the present study there was a significant decrease in calponin in the late secretory phase of the menstrual cycle in HMB (Figure 3.3). It is possible that reduced calponin leads to increased shortening velocity leading to an increased rate of contraction and blood flow in HMB. This hypothesis is also in line with our findings on smoothelin; increased smoothelin expression may facilitate contractility and vasodilation leading to increased blood flow. It should be noted, however, that in contrast with the pump vessels investigated in the studies described above, the endometrial vessels are thought to be resistance vessels and the mechanism of blood flow in these vessels may therefore differ. An alternative explanation for the present results could be that the changes in the pattern of expression of the differentiation markers may have caused a functional change in the endometrial vasculature, such that these vessels operate in a pumping manner similar to those in the cardiovascular system.

Vascular smooth muscle cell differentiation results from the co-ordinated transcriptional activation of an array of contractile genes including calponin, MyHC, αSMA. Most of these genes carry the transcription binding code, CArG box (Minty and Kedes, 1986; Sun *et al.*, 2006). This binds to the serum response factor (SRF) transcription factor with differing affinity, thus regulating its activation (Miano *et al.*, 2007). However SRF expression is not specific to VSMCs; cell specific activation is achieved through the expression of a SMC specific co-factor, myocardin (MYCOD) (Wang *et al.*, 2001; Chen *et al.*, 2002). In VSMCs, the CArg-SRF-MYCOD acts as the primary transcriptional switch, which maintains a physiologically normal functioning SMC phenotype (Long *et al.*, 2008). The fact that these contractile genes share a co-ordinated regulation may lead to the assumption that their expression pattern (presence or absence) may also be co-ordinated. However, our results indicate a decrease of calponin without any significant alteration in expression of the other contractile proteins between the control and HMB groups. One explanation for this could be differences in the CArG positioning between calponin and the other contractile protein encoding genes (Miano *et al.*, 2000). Moreover, calponin has another layer of complexity such that the positioning of CArG elements must also follow a strict positional relationship with other genomic elements (Long *et al.*, 2011).

Therefore the specific dysregulation of calponin expression shown in this study indicates the possibility of a novel calponin regulatory pathway.

3.5.2 Stromal, glandular and vascular proliferation during the normal menstrual cycle

The current study showed that proliferation, as marked by Ki67 expression, in the stroma, glandular epithelium or vessels, was highest in general in the luminal region, closely followed by the stratum functionalis. Strikingly, proliferation was lower in the stratum basalis and scarce or negligible in the superficial myometrium (Figure 3.7). Although several studies have previously compared stromal and glandular proliferation across the phases of the menstrual cycle in the context of endometriosis (Jones *et al.*, 1995), cyclins/cyclin-dependant kinases (Shiozawa *et al.*, 1996), endometrial granulated lymphocytes (Jones *et al.*, 1998), steroid receptors and apoptosis (Dahmoun *et al.*, 1999; Mertens *et al.*, 2002), endometrial polyps (Maia *et al.*, 2004) and out of phase endometrium from infertile and recurrent abortion patients (Meresman *et al.*, 2010), comparison of stromal, glandular and vascular proliferation has never been done between the layers of the endometrium and superficial myometrium. In the current study glandular proliferation across the menstrual cycle was in agreement with previous findings (Shiozawa *et al.*, 1996; Jones *et al.*, 1998; Dahmoun *et al.*, 1999; Maia *et al.*, 2004). The present study showed that stromal proliferation was highest in PP, reducing significantly in ESP, before rising back in the LSP (Figure 3.7). This is in line with the observations of Shiozawa *et al.* (1996), but it differs from those observed by Jones *et al.* (1995, 1998) and Dahmoun *et al.* (1999): in contrast with our findings these studies reported no difference between PP and ESP and a significant decrease in LSP, followed by an increase in the premenstrual phase. These differences may to some extent be explained by differences in the methodologies used. Firstly, in these studies samples were grouped as proliferative (days 5-13), early secretory (days 14-22) and late secretory (day 23 onwards), whereas we had four groups separated histologically; proliferative, early secretory, mid secretory and late secretory. Secondly, the previous studies had counted numbers of cells positively stained for Ki67 as a percentage of the total stromal cell population, whereas the current study used

a Quickscore method taking into consideration both the percentage of cells stained and their staining intensity. Finally the previous studies had examined either superficial endometrium or endometrium as a whole, whereas the present study assessed endometrium as three separate regions; the luminal region, stratum functionalis and stratum basalis. It is of interest to highlight, that the current study revealed significant differences between the luminal region and stratum functionalis compared with stratum basalis.

The increase in stromal, glandular and vascular proliferation from superficial myometrium towards the surface or luminal region of the endometrium shown in the current study, highlights the differences in developmental stage of the stroma, glands and vessels between the tissue layers. Moreover it indicates that there is minimal growth in the myometrium followed by stratum basalis and majority of the stromal, glandular and vascular remodeling takes place in the stratum functionalis and the luminal region. Importantly, taken together it suggests that at the end of menstruation, when a new layer of stratum functionalis regenerates, the stroma, glands and vessels grow outwards and that the less mature and more proliferative ends of the vessels, glands or layers of stroma remain nearest to the surface.

3.5.3 Vascular EC staining during the menstrual cycle

The current study showed that vascular endothelial cell markers were differentially expressed across the menstrual cycle and between the different endometrial layers and myometrium in controls. Moreover, distinct differences were observed in HMB for UEA-1, F8RA, CD34 and CD31 (Figure 3.9).

ECs in existing blood vessels migrate and form tubules marking the first step in blood vessel development and extension (Girling and Rogers, 2005). ECs communicate with VSMCs under the action of angiogenic growth factors and receptors, which in turn increases their interaction with the ECM and as a result stabilises the structure of the blood vessel (Kayisli *et al.*, 2006; Demir *et al.*, 2010). Importantly, during the menstrual cycle, depending on tissue requirements, angiogenesis is spatio-temporally regulated (Gargett and Rogers, 2001). Therefore, if angiogenesis is tightly regulated during the menstrual cycle,

it is possible that there exists a distinct pattern of EC marker staining and that any alteration in this pattern may underlie HMB.

Previous studies in our laboratory had observed differential reactivity/expression with UEA-1, F8RA, CD34 and CD31 with variation between different layers of the endometrium. In the present study we have shown distinct patterns for individual EC markers across the menstrual cycle. CD31 and CD34 were expressed at the highest levels (Figure 3.9). CD31 has previously been reported to be expressed by both large and small endometrial vessels (Rees *et al.*, 1993; Zhang *et al.*, 2002). In contrast, CD34 primarily detected small and medium vessels and capillaries, while UEA-1 was reactive with all types of vessels (Zhang *et al.*, 2002). F8RA was reported to be expressed only by larger vessels (Rees *et al.*, 1993) with weak staining across the menstrual cycle (Zhang *et al.*, 2002). Rees *et al.* (1993) documented no variation in staining for CD31, CD34 or F8RA either across the menstrual cycle or between endometrium and myometrium. In contrast, in agreement with Zhang *et al.* (2002) we observed menstrual cycle differences in EC staining for each marker investigated (Figure 3.9). Differences between our study and that of Zhang *et al.* (2002), are likely to reflect their division of the proliferative phase into early, mid and late, while we assessed proliferative phase endometrium as a whole. Moreover, we used a modified Quickscore system to analyse the immunohistochemical staining, while their analysis was based on a four-point scale (absent, weak, moderate and strong). In the present study, UEA-1 reactivity was present throughout the menstrual cycle and the level of CD34 staining remained almost unaltered during the menstrual cycle. F8RA was weakly expressed in the endometrium and decreased during the secretory phase, especially in the luminal region and stratum functionalis in LSP (Figure 3.9). Although this contrasts with Rees *et al.* (1993), who found no difference between the phases, it agrees with earlier findings by both Zhu and Zhao (1988) and Zhang *et al.* (2002). CD31 staining increased from PP to LSP in the luminal region and the stratum functionalis, again in agreement with Zhang *et al.* (2002) but contrasting with Tawia *et al.*, (1993), who did not detect any alteration in CD31 staining across the menstrual cycle. Tawia *et al.* (1993) quantified staining intensity in the entire endometrium, whereas the Quickscore

method used in the present study assessed both staining intensity and the proportion of positively stained vessels separately in the luminal, functional and basal regions of endometrium. To summarise, staining for each EC marker differed in a spatio-temporal manner; therefore care must be exercised in selecting EC markers for studies of endometrial vessels, especially in the superficial functional parts of the endometrium.

UEA-1 reactivity differed from F8RA, CD34 and CD31 staining within the same phase of the menstrual cycle specially in the luminal region and stratum functionalis (Figure 3.9). To investigate whether these differences reflected altered vessel development, Ki67 was used to assess the proportion of proliferating vessels separately in the stratum functionalis and luminal region for each of the four EC markers. UEA-1 was the best marker of ECs in proliferating vessels, whereas F8RA was expressed by the lowest proportion of proliferating vessels with decreasing stain from ESP to LSP. The proportion of UEA-1$^+$ and F8RA$^+$ proliferating vessels reduced from the proliferative/early secretory phase to the mid/late secretory phase, suggesting that developing vessels within the endometrial stratum functionalis may not be easily detectable with traditional EC markers (Figure 3.10). Combined together, the differences revealed a potential vascular developmental stage dependant staining for EC markers in endometrial vessels: UEA-1 (most proliferating, least mature vessels) followed by CD31/CD34 (less proliferative, medium sized vessels) and finally F8RA (least proliferating, most mature, large vessels).

There were also differences in UEA-1 reactivity and staining for F8RA, CD34 and CD31 between the layers of the endometrium and superficial myometrium. Although CD34 staining was relatively consistent throughout, expression/reactivity of other EC markers was highest in myometrium, decreasing towards the surface, with least staining in the luminal region (Figure 3.9). This is in contrast with Rees *et al*. (1993), who reported no difference in staining for CD31, CD34 and F8RA between endometrium and myometrium. Differences in the present study were mainly in the luminal region, which was not studied separately in the previous study, with no differences between the stratum functionalis/stratum basalis compared with the myometrium. The spatial difference in staining for EC markers in endometrium at different cycle phases

and within different endometrial layers may reflect the vascular developmental stage. Since endometrial blood vessels have been implicated in HMB, could an alteration of this staining pattern also underlie HMB?

While differences were observed in all four markers, vascular staining for CD34, a transmembrane glycoprotein, was increased in HMB in both endometrium and myometrium. Interestingly, a previous study using double staining for CD34 and proliferating cell nuclear antigen (PCNA) reported increased endometrial EC proliferation in HMB (Kooy *et al.*, 1996), perhaps highlighting dysregulation of $CD34^+$ endometrial vessels in the context of vascular development in HMB. In contrast, UEA-1^+ and $CD31^+$ ECs were generally reduced in HMB. CD31 has been implicated in the binding of leukocytes to ECs as well as in maintaining cell-cell contact (Tabibzadeh and Poubouridis, 1990). Since both UEA-1 and CD31 may stain small and large vessels, an alteration in $CD31^+$ EC staining may indicate dysregulation from an early stage of angiogenesis, possibly giving rise to leaky vessels from weaker lumen formation. F8RA was reduced in ESP myometrium but increased in LSP in the luminal region in HMB. F8RA enables platelet adhesion to injured vessel walls (Sixma and de Groot, 1991); altered staining may be indicative of adverse endothelial changes in these superficial endometrial vessels in HMB. Combining the results on alteration in staining of the different vascular EC markers and the differential staining for EC markers according to vascular proliferation it can be speculated that there is dysregulation of endometrial vascular endothelium in HMB. This may alter EC-VSMC signalling, which in turn may affect recruitment and/or differentiation of VSMCs ultimately resulting in dysfunctional vascular tone and consequently blood flow.

3.5.4 Vascular ECM staining during the menstrual cycle

Angiogenesis is regulated both spatially and temporally in the cycling endometrium and vascular ECM must also alter to sustain these vascular changes, although little is known about the mechanisms underlying ECM regulation in the endometrium. In agreement with Kelly *et al.* (1995), in the present study vascular laminin staining was low throughout the menstrual cycle, with few spatial or temporal differences. Fibronectin staining was also relatively

stable across the phases, with highest staining in the stratum functionalis (Figure 3.12). Our results for fibronectin and laminin in the stratum functionalis are similar to those of Bilalis *et al*. (1996), the only difference being their complete absence of both at LH+10. Since we examined ESP, MSP and LSP endometrium staged using histologic criteria (Noyes *et al*., 1975) rather than endometrium precisely timed from the LH surge, this difference can be explained. Osteopontin was expressed at high levels in the stratum functionalis with little variation in the secretory phase. In contrast, collagen IV staining varied in both stratum functionalis and stratum basalis, with increased levels in the secretory phase (Figure 3.12).

While these results are similar to those of Bilalis *et al*. (1996), they differ from Kelly *et al*. (1995), who concluded that the number of collagen IV^+ vessels remained similar across the menstrual cycle. Kelly *et al*. (1995) examined the number of vessels/mm^2 in whole endometrium, whereas we assessed the proportion and staining intensity of vessels in the entire tissue section and separately assessed stratum functionalis and basalis. Another more recent study reported increased collagen IV staining in endometrial vessels from the proliferative to the secretory phase in whole endometrium (Oefner *et al*., 2015), while we demonstrated increased collagen IV staining in the ESP and LSP in stratum functionalis. Furthermore if our data from stratum functionalis and basalis are combined we also demonstrate a trend for increased collagen IV staining from PP to MSP, although there are many other methodological differences between these studies, including antibody specificity, endometrial staging and image quantification. The ECM is known to interact with cellular receptors determining cell-shape, migration, proliferation and even apoptosis (Timpl, 1996). Regulation of normal angiogenesis for the maintenance of normal function may result in distinctly separate ECM signature in the stratum functionalis, stratum basalis and myometrium.

A major function of vascular ECM is vascular stability and if ECM component staining is tightly regulated in endometrial vasculature any variation in this ECM signature could lead to altered vessel stability, which could impact on abnormal bleeding in HMB. Laminin and fibronectin staining in stratum functionalis and myometrium were comparable in controls and HMB. Both laminin and

fibronectin staining in the stratum basalis was higher in LSP in HMB, although fibronectin was decreased in MSP in HMB, compared with controls; reflecting altered vascular structural properties in HMB. Fibronectin is known to promote MMP-2 staining resulting in ECM breakdown during menses (Hoffmann *et al.*, 2006). However, when fibronectin is targeted by MMP-3 and broken into proteolytic fragments, it has been shown to induce apoptosis (Fukai *et al.*, 1995). Therefore, increased fibronectin staining in LSP stratum basalis in HMB may reflect increased local ECM breakdown and/or enhanced cellular apoptosis. However, the amount of fibronectin remained unaltered in the stratum functionalis in HMB. It can be speculated that compared with endometrium from control women, in HMB, vessels arising from the stratum basalis at the end of menses have altered ECM composition. Finally, this cyclic variation of laminin and fibronectin staining is also an indication that their production is under the direct or indirect regulation of ovarian steroid hormones, oestrogen and progesterone.

Collagen IV staining was generally decreased in both stratum functionalis and stratum basalis in HMB. Collagen IV networks are a major architectural feature of the vascular ECM and maintain mechanical stability. Therefore, a decrease in collagen IV would suggest a weaker vascular structure in HMB, while the increased myometrial collagen IV staining may represent a compensatory mechanism to stabilise the vascular bed.

Previous studies have shown that human endometrial glandular and luminal osteopontin staining is elevated in MSP (Apparao *et al.*, 2001). In this study vascular osteopontin staining was higher in ESP than in LSP in controls but this pattern was reversed in HMB, where osteopontin staining was reduced in ESP stratum basalis and increased in LSP stratum functionalis. Osteopontin is induced in VSMCs in cardiovascular diseases and increased osteopontin has been implicated in renal diseases and linked with the activity of several oncogenes (Waller *et al.*, 2010). Activated lymphocytes and macrophages synthesize osteopontin, accounting for its elevated staining in traumatised tissue (Sodek *et al.*, 2000). Thus, increased osteopontin staining in the LSP could reflect increased breakdown of endometrial vascular ECM. A study using mice aortic VSMCs has shown that osteopontin down-regulates calponin,

through an extracellular signalling pathway (Gao *et al.*, 2012). It can be proposed that osteopontin may down-regulate calponin staining in LSP endometrium in HMB. The temporal dysregulation may indicate a mechanistic basis for osteopontin as a regulator of the endometrial vascular structure-function relationship, which may underlie HMB.

3.5.5 Leukocyte distribution during the menstrual cycle

Menstruation is an immunological process (Finn, 1986; Salamonsen and Woolley, 1999; Jones *et al.*, 2004; Berbic and Fraser, 2013) and immune cells constitute a significant component of the non-pregnant endometrial stromal and epithelial layers (Morris *et al.*, 1985; Bulmer and Earl, 1987; Bulmer *et al.*, 1991; Hunt, 1994; Dallenbach-Hellweg, 2012). $CD56^+$ uNK cells, $CD14^+$ macrophages and $CD3^+$ T cells form the most prominent leukocyte subsets in the endometrium (Morris *et al.*, 1985; Kamat and Isaacson, 1987; Bulmer *et al.*, 1991; Hunt, 1994; Lachapelle *et al.*, 1996). Leukocyte populations have been implicated in remodelling and growth of blood vessels within the endometrium, and the endometrial spiral arterioles in turn have been implicated in the pathogenesis of HMB in relation to their premature breakdown, failure to repair, vascular smooth muscle cell differentiation, vessel number and morphology (Abberton *et al.*, 1999a; Abberton *et al.*, 1999b; Hurskainen *et al.*, 1999; Rogers and Abberton, 2003; Kawai-Kowase and Owens, 2007; Biswas Shivhare *et al.*, 2014). The aim of the present study was to compare endometrial leukocyte populations during the menstrual cycle in the stratum functionalis of women with HMB, compared with controls. The results demonstrated altered leukocyte numbers in the endometrium of women with HMB at different phases of the menstrual cycle.

Regulatory T cells ($FOXP3^+$), which play a crucial role in controlling and suppressing immune responses, especially to sustain successful implantation (Berbic *et al.*, 2010), were rare at all menstrual cycle phases and no dynamic alteration was observed in HMB (Figure 3.14). Endometrial macrophages may play a role in a wide array of activities important for sustaining fertility; tissue degradation during menses, phagocytic activity to clear endometrial debris, tissue repair/regeneration, and reconstruction of tissue structure (Aplin *et al.*,

2008; Thiruchelvam *et al.*, 2013). As a proportion of all endometrial cells, macrophage numbers have been previously reported to increase from 1-2% in the proliferative phase to 3-5% in the secretory phase and 6-15% during the menstrual phase (Salamonsen and Woolley, 1999; Salamonsen *et al.*, 2002; Thiruchelvam *et al.*, 2013). We did not observe any significant change in macrophage numbers during the menstrual cycle (Figure 3.14). There are several possible explanations for the difference in these results. The previous data corresponded to $CD68^+$ macrophages, while we assessed $CD14^+$ macrophage numbers; CD68 is a cytoplasmic marker while CD14 is a cell surface marker, hence their pattern of staining may not be directly comparable. Moreover, the $CD68^+$ macrophage numbers were assessed as a proportion of all endometrial cells, while we expressed $CD14^+$ macrophages as a proportion of endometrial stromal cells (Salamonsen and Woolley, 1999). Mature $CD83^+$ dendritic cells have also been proposed to play a role in remodelling fertile cycling endometrium (Bengtsson *et al.*, 2004). Our results are in line with previous findings showing that mature dendritic cells are most abundant in the late secretory phase (Rieger *et al.*, 2004), but there were no significant differences in either macrophages or dendritic cells between control and HMB endometrium (Figure 3.14). Taken together these results support our proposal that HMB is associated with spatio-temporal dysregulation of blood flow due to abnormal vasoconstriction rather than due to failed tissue repair.

The number of endometrial $CD3^+$ cells was reduced in the mid secretory phase and increased in the late secretory phase in HMB compared with controls (Figure 3.14). However, $CD3^+$ antibodies for paraffin embedded sections detect the epsilon (ε) chain of human CD3, which may also be expressed by a proportion of $CD56^+$ uNK cells (King *et al.*, 1998b). Therefore we performed double immunohistochemical labelling to differentiate between $CD3^+$ T cells and $CD56^+$ uNK cells expressing cytoplasmic CD3ε (Figure 3.15). Double immunohistochemical labelling was required to differentiate between $CD3^+$ T cells and $CD56^+$ uNK cells expressing cytoplasmic CD3ε. Compared with controls, the proportion of $CD3^+$ cells among the total number of $CD56^+$ cells was increased and the proportion of single $CD56^+$ cells amongst the total $CD3^+$ cells was decreased in the late secretory phase in HMB (Figure 3.15). The

increase in $CD3^+$ cells in the late secretory phase in HMB did not therefore represent an increase in $CD3^+$ T cells but was accounted for by altered expression of CD3ε by the $CD56^+$ cells.

$CD56^+$ uNK cells are the most prevalent leukocyte population in the stratum functionalis during the mid and late secretory phases, although they are present in small numbers in proliferative endometrium (Hamperl and Hellweg, 1958; Bulmer *et al.*, 1991). In the present study, while uNK cell numbers were increased in the proliferative and early secretory phases in HMB their numbers did not increase to the same extent in the late secretory phase, resulting in a lower proportion of uNK cells compared with controls. Additionally, CD3/CD56 double-labelling immunohistochemistry confirmed that the uNK cell population in the late secretory phase in HMB showed increased staining of CD3 compared with controls. This may reflect altered uNK cell differentiation in HMB. The mechanisms that underlie the dramatic increase in uNK cell numbers in the mid and late secretory phases of the menstrual cycle are not fully understood and may involve both influx from peripheral blood and local differentiation and proliferation (Koopman *et al.*, 2003; Eidukaite *et al.*, 2004; Zhang *et al.*, 2005; Lynch *et al.*, 2007). Uterine NK cells have been implicated in a wide range of functions; cytokine and angiogenic growth factor secretion, immune function, regulation of trophoblast invasion, early stages of spiral artery remodelling and regulation of endometrial bleeding (Lash *et al.*, 2006; Smith *et al.*, 2009; Lash *et al.*, 2010; Lash and Bulmer, 2011; Wilkens *et al.*, 2013). Importantly, uNK cells secrete angiogenic growth factors such as Ang1, Ang2, VEGF-C, PlGF and TGF-β1, which play important roles in vascular development (Li *et al.*, 2001; Lash *et al.*, 2006).

Therefore, differences in leukocyte populations and an altered state of uNK cell differentiation detected in the present study may reflect altered leukocyte recruitment and/or *in situ* differentiation and proliferation in women with HMB. The functional consequences of altered endometrial leukocyte numbers in women with HMB may impact on endometrial vascular development and/or the way in which the endometrium prepares for menstruation. We have previously reported a positive association between uNK cell density and vascular maturation in women with RM and recurrent implantation failure (Quenby *et al.*,

2009). In the current study, uNK cells were significantly reduced in the late secretory phase of the menstrual cycle in HMB, linking to our previous results demonstrating reduced vascular development in HMB (Biswas Shivhare *et al.*, 2014). This study investigated leukocytes in the stratum functionalis of the endometrium, on the basis that it is at this site that endometrial leukocytes vary during the menstrual cycle. However, future studies investigating their expression in the stratum basalis and myometrium with a comparison between control women and those with HMB may be beneficial. A further interest will be to examine whether uNK cell function such as cytokine production is changed between mid and late secretory phases in the non-pregnant endometrium.

3.6 CONCLUSION

In conclusion, endometrial vascular muscle content was unchanged during the menstrual cycle in HMB compared with controls. However, there were characteristic differences in late secretory phase endometrium in the differentiation stage of VSMCs between HMB and control endometrium, with smoothelin being increased and calponin staining decreased in muscularised vessels in HMB and calponin staining also being reduced in relation to total vessel number. This altered VSMC differentiation may lead to altered function, which could contribute to abnormal bleeding. These results also indicate calponin as a potential therapeutic target, but detailed functional studies are required before this can be deduced. The pattern of staining for endometrial vascular EC and ECM was also altered during the different phases of the control menstrual cycle and in HMB. While laminin and fibronectin did not alter to a great extent, osteopontin was generally increased and collagen IV staining was decreased in the uterine vessels in HMB. The study suggests that vascular ECM is continuously remodelled at the different phases of the menstrual cycle. Altered vascular EC and ECM component staining may indicate dysregulated development and structural instability of endometrial vessels and consequently altered function, which could contribute to abnormal bleeding in HMB. The results also highlight a potentially dysregulated osteopontin-calponin pathway inviting further functional studies. Moreover temporal differences were observed in the leukocyte populations in HMB endometrium, in particular uNK cell numbers were significantly reduced in the late secretory phase, perhaps implicating reduced/altered vascular maturation at the onset of menstruation underlying HMB.

Collectively, results from these immunohistochemical studies of the uterine vasculature revealed key alterations in blood vessel development, crucially drawing attention to the significance of VSMC differentiation, the spatio-temporal pattern of ECs and ECM staining and the uNK cell population and differentiation status in the pathogenesis of HMB. In light of the fact that blood vessel maturation and development is potentially dysregulated in HMB and that development of these vessels is principally regulated by a network of key AGFs

and their receptors such as PDGF-BB, PDGFRα, PDGFRβ, TGFβ1, TGFβRI and TGFβRII, it is of great interest and significance to consequently investigate the pattern of staining for these AGFs and their receptors in the context of HMB and potentially their role in VSMC differentiation and vascular ECM staining in the endometrium.

CHAPTER 4

ANGIOGENIC GROWTH FACTORS AND BLOOD VESSEL DEVELOPMENT IN WOMEN WITH HEAVY MENSTRUAL BLEEDING

4 ANGIOGENIC GROWTH FACTORS AND BLOOD VESSEL DEVELOPMENT IN WOMEN WITH HEAVY MENSTRUAL BLEEDING

4.1 INTRODUCTION

The endometrium, a dynamic tissue with regenerative ability, undergoes proliferation, differentiation and breakdown every month during the menstrual cycle. The growing endometrium therefore relies heavily on a blood supply from newly developing blood vessels or angiogenesis. The endometrium is one of the two tissues in women where angiogenesis takes place on a physiological basis (Smith, 1998). Abnormal development and flow in these endometrial blood vessels has been implicated in various reproductive disorders such as RM, recurrent implantation failure, unexplained infertility and HMB (Goswamy *et al.*, 1988; Steer *et al.*, 1994; Habara *et al.*, 2002; Quenby *et al.*, 2009). Additionally, these studies, discussed in Chapter 3, have highlighted the significance of these vessels in HMB especially with respect to VSMC differentiation (Biswas Shivhare *et al.*, 2014), EC and ECM component expression.

The carefully timed cyclic angiogenesis and remodelling, which forms the backbone of monthly endometrial growth and regression results from a complex network of interactions between hormones and pro or anti-angiogenic growth factors. Pro-angiogenic factors activate or promote formation of new blood vessels, while anti-angiogenic factors inhibit blood vessel formation (Folkman and Klagsbrun, 1987). Although oestrogen and progesterone primarily regulate endometrial angiogenesis (Iruela-Arispe *et al.*, 1999; Hague *et al.*, 2002), their action is indirectly mediated by several growth factors expressed by the diverse cell population in the endometrium. Sprouting, pruning and/or intussusception during angiogenesis involves proliferation and migration of ECs (Risau, 1997), driven by a complex interaction of well-defined families of angiogenic growth factors, which include the VEGF family, consisting of VEGF-A (Charnock-Jones *et al.*, 1993; Donnez *et al.*, 1998), VEGF-B, and VEGF-C (Mints *et al.*, 2002; Moller *et al.*, 2002), VEGF-D, VEGF-E, placental growth factor (PlGF) and the receptors VEGF-R1, VEGF-R2 and VEGF-R3 (Smith, 2001; Jain, 2003; Hoeben *et al.*, 2004; Tammela *et al.*, 2005; Lash *et al.*, 2012); the fibroblast growth

factor (FGF) family (Presta, 1988; Ferriani *et al.*, 1993; Sangha *et al.*, 1997; Presta *et al.*, 2005); TGFβ1 and its receptors, TGFβRI and TGFβRII, which have been shown to play a role in vascular stabilisation and VSMC differentiation (Dickson *et al.*, 1995; Pepper, 1997; Massague *et al.*, 2000; Distler *et al.*, 2003). As these new vessels form, a population of leading ECs provide directionality and a population of lagging ECs form the lumen of the vessel (Kamei *et al.*, 2006). Subsequently, the newly formed vessels undergo maturation; capillaries recruit pericytes, whereas larger vessels recruit VSMCs, which requires the PDGF family, including PDGF-A, PDGF-B, PDGF-C and PDGF-D and their receptors, PDGF-Rα and PDGF-Rβ (Lindahl *et al.*, 1997; Betsholtz, 2004). The PDGF signalling pathway plays a critical role in recruitment of mesenchymal cells, which differentiate into VSMCs in response to TGFβ signalling (ten Dijke and Arthur, 2007). Finally, an extended network of vessels is formed which continue to mature and remodel under the influence of other cytokines and growth factors, blood flow and tissue specific requirements. Amongst other growth factors, which play a role in vascular stabilisation, maturation and remodelling are the angiopoietins, consisting of Ang1, and its receptor Tie-2 and Ang2, which inhibits Ang1 binding promoting vascular breakdown (Maisonpierre *et al.*, 1997; Witzenbichler *et al.*, 1998; Zhang *et al.*, 2001). The TGFβ, PDGF, VEGF and angiopoietin signalling pathways and their specific roles in vascular development have been discussed in detail in section 1.2.4.

VEGFs, TGFβs and PDGFs are expressed in the endometrium and their expression has been studied previously (Akhurst *et al.*, 1990; Charnock-Jones *et al.*, 1993; Shifren *et al.*, 1996; Donnez *et al.*, 1998; Sharkey *et al.*, 2000; von Wolff *et al.*, 2000; Aase *et al.*, 2001; Moller *et al.*, 2002; Piestrzeniewicz-Ulanska *et al.*, 2002; Hirchenhain *et al.*, 2003; Rogers and Abberton, 2003; Malik *et al.*, 2006; Lee *et al.*, 2008; Girling and Rogers, 2009; Omwandho *et al.*, 2010; Lash *et al.*, 2012; Li *et al.*, 2013). Although these AGFs are key to angiogenesis and are expressed during the human menstrual cycle, with cyclic variation suggesting phase specific role in angiogenesis, the role of these AGFs and their expression has not been investigated in HMB.

4.2 HYPOTHESES AND AIMS

4.2.1 Hypotheses

Taking into consideration the critical roles of both TGFβ1 and its receptors TGFβRI and TGFβRII, and PDGF-BB and its receptors PDGFRα and PDGFRβ in vascular development, VSMC differentiation and ECM dependant activation or spatial diffusion and bioavailability, we hypothesised that:

1. The spatio-temporal pattern of AGFs in endometrial stroma, glands, ECs and VSMCs during the normal menstrual cycle is altered in women with HMB.

2. Abnormal endometrial VSMC differentiation and vascular ECM component staining is associated with altered AGF expression.

4.2.2 Aims

To test the above hypotheses we aimed to:

1. Compare the pattern of staining for the AGFs and their receptors in stroma, glands, ECs and VSMCs in the endometrium and superficial myometrium, across the menstrual cycle in controls and women with HMB.

2. Compare the staining for VSMC differentiation markers and vascular ECM components in proliferative endometrium and superficial myometrium in controls with those treated with AGFs or their respective neutralising antibodies.

4.3 EXPERIMENTAL DESIGN

The experimental design adopted to achieve the specific aims set out in section 4.2.2, is illustrated in Figure 4.1 and the specific materials and methods used have been described below.

4.3.1 Human tissue

Biopsies of endometrium with superficial myometrium were obtained with informed consent from women undergoing hysterectomy as described in section 2.1.1.2. Endometrial explant cultures with AGFs and their respective neutralising antibodies were carried out as described in section 2.2.6.8.

4.3.2 Immunohistochemistry and antibodies used

Immunohistochemistry was performed as described in section 2.2.3.1. Details of source, pretreatment, dilution and incubation times for all primary antibodies used in immunohistochemistry are provided in Table 2.2.

4.3.3 Quantitative image analysis

4.3.3.1 Staining for AGFs in the endometrium and superficial myometrium

Entire, full thickness immunostained tissue sections (N=5) in each of PP, ESP, MSP and LSP from both controls and women with HMB were analysed semi-quantitatively for the different AGFs in stroma, glands, ECs and VSMCs, using a modified 'Quickscore' method (section 3.3.3.2) by an observer who was blinded to the origin of the sample. Vessels identified by their surrounding layer(s) of smooth muscle cells and with a visible lumen were included in the analysis. The superficial myometrium, stratum basalis (within one x200 field adjacent to the myometrium) and stratum functionalis were scored separately for each cell type.

4.3.3.2 VSMC differentiation marker and ECM component staining in endometrial and superficial myometrial explant cultures

Staining for VSMC differentiation markers, EC marker (CD31) and ECM components in vessels in the entire tissue section of the explant cultures of proliferative phase samples from controls (N=3 myometrium, N=2 endometrium) was assessed by an observer, who was blinded to the origin of the samples.

Figure 4.1: Experimental design to investigate angiogenic growth factors and blood vessel development in women with HMB.

Vessels identified by their surrounding layer(s) of smooth muscle cells and with a visible lumen were included in the analysis. Expression of VSMC differentiation markers and vascular ECM components were assessed using open-source ImageJ software package in combination with the 'cell counter' plugin as the percentage of vessels positively stained for each marker compared with the total number of vessels in the entire tissue identified by $CD31^+$ staining.

4.3.4 Statistical analysis

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc.). Data are presented as means ± standard error of mean (SEM) and differences are considered statistically significant at $P \leq 0.05$. Specific tests used are given in the results.

4.4 RESULTS

4.4.1 Spatio-temporal pattern of vascular AGF staining in the control menstrual cycle and in HMB

4.4.1.1 PDGF-BB and its receptors in the menstrual cycle

PDGF-BB: PDGF-BB staining in stromal cells was minimal throughout the menstrual cycle within stratum functionalis, stratum basalis and myometrium (Figure 4.2a). Stromal PDGF-BB staining was also low in the myometrium, and there were no differences between the menstrual cycle phases (Figure 4.3A). PDGF-BB staining in the glandular epithelial cells was minimal in both stratum functionalis and stratum basalis and staining did not change during the menstrual cycle (Figure 4.2a, Figure 4.3B).

Vascular ECs showed moderate to high staining for PDGF-BB in both endometrium and myometrium (Figure 4.2a). Staining was unchanged during the menstrual cycle; although PDGF-BB staining in myometrium was higher than in stratum functionalis in PP (P=0.01), and LSP (P=0.009) (Figure 4.3C).

VSMCs showed low PDGF-BB expression, which remained unchanged during the menstrual cycle in both endometrium and myometrium (Figure 4.2a). However, PDGF-BB staining in myometrium was higher than in stratum functionalis in PP (P=0.004), ESP (P=0.003) and LSP (P=0.004) (Figure 4.3D).

PDGFRα: PDGFRα staining was moderate to high throughout the menstrual cycle (Figure 4.2b). Stromal PDGFRα staining was moderate in endometrium and myometrium and did not change during the menstrual cycle in myometrium and stratum basalis. In stratum functionalis PDGFRα staining was higher in LSP than in ESP (P=0.007) and was lower than myometrium in MSP (P=0.007) (Figure 4.4A).

In the glandular epithelial cells, PDGFRα staining was moderate to high throughout the menstrual cycle in both stratum functionalis and stratum basalis (Figure 4.2b). PDGFRα staining was higher in the PP than MSP in both stratum functionalis (P=0.006) and stratum basalis (P=0.007) (Figure 4.4B).

Figure 4.2a: Staining for PDGF-BB in the endometrium and myometrium during the menstrual cycle. Stromal cells (SC), glandular epithelial cells (GE), VSMCs and ECs were stained positive for PDGF-BB (**A1**-**A4**, **B1**-**B4**). Images were captured at 200x original magnification.

Figure 4.2b: Staining for PDGFRα in the endometrium and myometrium during the menstrual cycle. Stromal cells (SC), glandular epithelial cells (GE), VSMCs and ECs were stained positive for PDGFRα (**A1**-**A4**, **B1**-**B4**). Images were captured at 200x original magnification.

Figure 4.2c: Staining for PDGFRβ in the endometrium and myometrium during the menstrual cycle. Stromal cells (SC), glandular epithelial cells (GE), VSMCs and ECs were stained positive for PDGFRβ (**A1**-**A4**, **B1**-**B4**). Images were captured at 200x original magnification

Figure 4.3: Graphical representation of immunohistochemical staining for PDGF-BB during the menstrual cycle. (**A-D**) Quickscore assessment (mean ± SEM) of PDGF-BB (N=5, each phase, each group) in controls and in HMB for stromal cells (**A**), glandular epithelial cells (**B**), ECs (**C**) and VSMCs (**D**). Bars labelled with the same letter are significantly different from each other. (a-e) represent differences between layers, within each phase; ^{a}P=0.01, ^{b}P=0.009, $^{c,e}P$=0.004, ^{d}P=0.003. (A-F) represent differences between control and HMB; $^{A\text{-}F}P$=0.008. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant differences between phases or layers in control. Mann-Whitney U test, with Bonferroni correction determined significant differences between control and HMB.

PDGFRα staining in vascular ECs was moderate to high and remained unaltered throughout the menstrual cycle in both endometrium and myometrium (Figure 4.2b), although staining was higher in the myometrium compared with stratum functionalis in both PP (P=0.003) and ESP (P=0.01) (Figure 4.4C).

VSMCs showed low to moderate PDGFRα staining during the menstrual cycle in both endometrium and myometrium (Figure 4.2b). Staining was highest in LSP, compared with PP in both stratum functionalis (P=0.006) and myometrium (P=0.007). Moreover, PDGFRα staining in myometrium was higher than stratum functionalis in PP (P=0.014) (Figure 4.4D).

PDGFRβ: Stromal PDGFRβ staining was moderate throughout the menstrual cycle in both endometrium and myometrium (Figure 4.2c). In stratum functionalis, PDGFRβ staining was highest in stratum basalis and myometrium in LSP compared with PP (P=0.005), although staining remained unchanged during the menstrual cycle. PDGFRβ staining was higher in stratum basalis compared with stratum functionalis in both PP (P=0.002) and MSP (P=0.011) and compared with myometrium in ESP (P=0.003) (Figure 4.5A).

In the glandular epithelial cells, PDGFRβ was moderately expressed throughout the menstrual cycle in both stratum functionalis and stratum basalis (Figure 4.2c). No changes were observed during menstrual cycle, although staining was higher in stratum functionalis compared with stratum basalis in PP (P=0.016) (Figure 4.5B).

PDGFRβ staining was moderate to high in vascular ECs and low to minimal in VSMCs in both endometrium and myometrium during the menstrual cycle (Figure 4.2c). No differences were observed between menstrual cycle phases or tissue layers in either ECs (Figure 4.5C) or VSMCs (Figure 4.5D).

Figure 4.4: Graphical representation of immunohistochemical staining for PDGFRα during the menstrual cycle. (**A-D**) Quickscore assessment (mean ± SEM) of PDGFRα (N=5, each phase, each group) in controls and in HMB for stromal cells (**A**), glandular epithelial cells (**B**), ECs (**C**) and VSMCs (**D**). '*P*' values shown in graph represent significant differences between phases within each layer. Bars labelled with the same letter are significantly different from each other. (a-d) represent differences between layers, within each phase; ^{a}P=0.007, ^{b}P=0.003, ^{c}P=0.01, ^{d}P=0.014. (A-Q) represent differences between control and HMB; $^{A\text{-}Q}$P=0.008. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant differences between phases or layers in control. Mann-Whitney U test, with Bonferroni correction determined significant differences between control and HMB.

Figure 4.5: Graphical representation of immunohistochemical staining for PDGFRβ during the menstrual cycle. (**A-D**) Quickscore assessment (mean ± SEM) of PDGFRβ staining (N=5, each phase, each group) in controls and in HMB for stromal cells (**A**), glandular epithelial cells (**B**), ECs (**C**) and VSMCs (**D**). '*P*' value shown in graph represents significant difference between phases within each layer. Bars labelled with the same letter are significantly different from each other. (a-d) represent differences between layers, within each phase; ^{a}P=0.002, ^{b}P=0.011, ^{c}P=0.003, ^{d}P=0.016. (A-C) represent differences between control and HMB; $^{A\text{-}C}P$=0.008. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant differences between phases or layers in control. Mann-Whitney U test, with Bonferroni correction determined significant differences between control and HMB.

4.4.1.2 TGFβ1 and its receptors in the menstrual cycle

TGFβ1: TGFβ1 staining in the stromal cells was low throughout the normal menstrual cycle in both endometrium and myometrium (Figure 4.6a) and there were no differences between either the phases or the tissue layers (Figure 4.7A).

In the glandular epithelial cells, TGFβ1 staining was low throughout the menstrual cycle (Figure 4.6a) and there were no differences between the menstrual cycle phases in both stratum functionalis and stratum basalis. However, in ESP, TGFβ1 staining was higher in stratum functionalis compared with stratum basalis (P=0.015) (Figure 4.7B).

TGFβ1 staining was low in the vascular ECs and VSMCs in both endometrium and myometrium during the menstrual cycle (Figure 4.6a) and there were no differences between phases or tissue layers in ECs (Figure 4.7C). However, in ESP, TGFβ1 staining in VSMCs was lower in stratum functionalis compared with myometrium (P=0.010) (Figure 4.7D).

TGFβRI: TGFβRI staining in the stromal cells was moderate throughout the menstrual cycle in endometrium and myometrium (Figure 4.6b). While no differences in staining were observed during the menstrual cycle, TGFβRI staining was higher in stratum functionalis, compared with stratum basalis in PP (P=0.012) and compared with myometrium in LSP (P=0.006) (Figure 4.8A).

TGFβRI was moderately expressed in the glandular epithelial cells throughout the menstrual cycle in stratum functionalis and stratum basalis (Figure 4.6b). While no differences in staining were observed between the phases of the menstrual cycle, TGFβRI staining was higher in stratum functionalis compared with stratum basalis in LSP (P=0.016) (Figure 4.8B).

TGFβRI was moderately expressed in the vascular ECs and did not alter during the menstrual cycle in both endometrium and myometrium (Figure 4.6b). However, TGFβRI staining was higher in stratum functionalis compared with stratum basalis in PP (P=0.009) and compared with myometrium in LSP (P=0.010) (Figure 4.8C).

Figure 4.6a: Staining for TGFβ1 in the endometrium and myometrium during the menstrual cycle. Stromal cells (SC), glandular epithelial cells (GE), VSMCs and ECs were stained positive for TGFβ1 (**A1**-**A4**, **B1**-**B4**). Images were captured at 200x original magnification.

Figure 4.6b: Staining for TGFβRI in the endometrium and myometrium during the menstrual cycle. Stromal cells (SC), glandular epithelial cells (GE), VSMCs and ECs were stained positive for TGFβRI (**A1**-**A4**, **B1**-**B4**). Images were captured at 200x original magnification.

Figure 4.6c: Staining for TGFβRII in the endometrium and myometrium during the menstrual cycle. Stromal cells (SC), glandular epithelial cells (GE), VSMCs and ECs were stained positive for TGFβRII (**A1**-**A4**, **B1**-**B4**). Images were captured at 200x original magnification.

Figure 4.7: Graphical representation of immunohistochemical staining for TGFβ1 during the menstrual cycle. (**A-D**) Quickscore assessment (mean ± SEM) of TGFβ1 staining (N=5, each phase, each group) in controls and in HMB for stromal cells (**A**), glandular epithelial cells (**B**), ECs (**C**) and VSMCs (**D**). Bars labelled with the same letter are significantly different from each other. (a, b) represent differences between layers, within each phase; ^{a}P=0.015, ^{b}P=0.010. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant differences between phases or layers in control. Mann-Whitney U test, with Bonferroni correction determined significant differences between control and HMB.

VSMCs showed low to moderate staining of TGFβRI during the menstrual cycle in both endometrium and myometrium (Figure 4.6b). TGFβRI staining in stratum basalis was higher than in stratum functionalis in both PP (P=0.006) and ESP (P=0.009) (Figure 4.8D).

TGFβRII: Stromal TGFβRII staining was low to minimal throughout the menstrual cycle in endometrium and myometrium (Figure 4.6c) with no differences between phases or tissue layers (Figure 4.9A).

TGFβRII staining in glandular epithelial cells, was low and did not change during the menstrual cycle in both stratum functionalis and stratum basalis. No alterations were observed between the tissue layers (Figure 4.6c, Figure 4.9B).

TGFβRII was moderately expressed in vascular ECs, with no differences between tissue layers during the menstrual cycle (Figure 4.6c). While in stratum functionalis, staining was highest in ESP compared with LSP (P=0.004), no such changes were observed in either stratum basalis or myometrium (Figure 4.9C).

TGFβRII staining in the VSMCs was moderate to low throughout the menstrual cycle in endometrium and myometrium (Figure 4.6c) and no differences were observed between phases or tissue layers (Figure 4.9D).

Figure 4.8: Graphical representation of immunohistochemical staining for TGFβRI during the menstrual cycle. (**A-D**) Quickscore assessment (mean ± SEM) of TGFβRI staining (N=5, each phase, each group) in controls and in HMB for stromal cells (**A**), glandular epithelial cells (**B**), ECs (**C**) and VSMCs (**D**). Bars labelled with the same letter are significantly different from each other. (a-g) represent differences between layers, within each phase; ^{a}P=0.012, $^{b,\,f}P$=0.006, ^{c}P=0.016, $^{d,\,g}P$=0.009, ^{e}P=0.010. (A-F) represent differences between control and HMB; $^{A\text{-}F}P$=0.008. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant differences between phases or layers in control. Mann-Whitney U test, with Bonferroni correction determined significant differences between control and HMB.

Figure 4.9: Graphical representation of immunohistochemical staining for TGFβRII during the menstrual cycle. (**A-D**) Quickscore assessment (mean ± SEM) of TGFβRII staining (N=5, each phase, each group) in controls and in HMB for stromal cells (**A**), glandular epithelial cells (**B**), ECs (**C**) and VSMCs (**D**). '*P*' value shown in graph represents significant difference between phases within each layer. Bars labelled with the same letter are significantly different from each other. (A-C) represent differences between control and HMB; $^{A\text{-}C}P$=0.008. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction determined significant differences between phases or layers in control. Mann-Whitney U test, with Bonferroni correction determined significant differences between control and HMB.

4.4.1.3 Staining for PDGF-BB and its receptors is altered in HMB

PDGF-BB: Stromal PDGF-BB staining in stratum basalis was lower in ESP in HMB, compared with controls (*P*=0.008) (Figure 4.3A). Glandular epithelial staining for PDGF-BB did not differ between HMB and controls (Figure 4.3B). PDGF-BB staining in the vascular ECs was reduced in HMB compared with controls in stratum functionalis in MSP (*P*=0.008), in stratum basalis in MSP (*P*=0.008) and LSP (*P*=0.008) and in myometrium in PP (*P*=0.008) and LSP (*P*=0.008) (Figure 4.3C). staining of PDGFB-BB in VSMCs was unaltered in HMB compared with controls (Figure 4.3D).

PDGFRα: Stromal PDGFRα staining in stratum basalis was lower in LSP in HMB, compared with controls (*P*=0.008). staining in myometrium was lower in both ESP (*P*=0.008) and LSP (*P*=0.008) in HMB (Figure 4.4A). Glandular epithelial staining for PDGFRα in PP and ESP was reduced in HMB compared with controls, in both stratum functionalis and stratum basalis (*P*=0.008 for all) (Figure 4.4B). In HMB compared with controls, PDGFRα staining in ECs was reduced in stratum functionalis in MSP, in stratum basalis in PP, ESP, MSP and LSP and in myometrium in PP, ESP and LSP (*P*=0.008 for all) (Figure 4.4C). While PDGFRα staining in VSMCs in stratum functionalis was unaltered in HMB compared with controls, it was reduced in both stratum basalis and myometrium in LSP (*P*=0.008 for all) (Figure 4.4D).

PDGFRβ: While stromal PDGFRβ staining was increased in HMB in PP stratum functionalis (*P*=0.008), it was decreased in PP stratum basalis (*P*=0.008) compared with controls. No differences were observed in myometrium (Figure 4.5A). Glandular epithelial staining of PDGFRβ did not differ in HMB compared with controls (Figure 4.5B). staining of PDGFRβ in ECs was reduced in HMB compared with controls in ESP stratum basalis (*P*=0.008), but no changes were observed in stratum functionalis or myometrium (Figure 4.5C). PDGFRβ staining in VSMCs did not differ between HMB and controls (Figure 4.5D).

4.4.1.4 Staining for TGFβ1and its receptors is altered in HMB

TGFβ1: TGFβ1 staining in stromal cells, glandular epithelial cells, ECs and VSMCs was unaltered in HMB compared with controls (Figure 4.7A-D).

TGFβRI: TGFβRI staining in stroma was unaltered in HMB, compared with controls in stratum functionalis and myometrium. However, in stratum basalis, stromal TGFβRI staining in PP (P=0.008) was increased in HMB (Figure 4.8A). Glandular epithelial staining for TGFβRI was lower in HMB compared with controls in LSP stratum functionalis (P=0.008) but no alteration was observed in stratum basalis (Figure 4.8B). TGFβRI staining in ECs in MSP stratum functionalis was reduced in HMB compared with controls (P=0.008) but no alteration was observed in stratum basalis or myometrium (Figure 4.8C). TGFβRI staining in VSMCs was unaltered in HMB in stratum functionalis and myometrium but was reduced in stratum basalis in PP, ESP and MSP (P=0.008 for all) (Figure 4.8D).

TGFβRII: TGFβRII staining in stromal cells was reduced in HMB compared with controls in PP and ESP stratum functionalis (P=0.008 for all) and in PP myometrium (P=0.008) (Figure 4.9A). No alterations in TGFβRII staining were observed in the glandular epithelial cells, ECs or VSMCs in HMB compared with controls (Figure 4.9B-D).

4.4.2 Effect of AGFs on endometrial VSMC differentiation

Preliminary observations from the endometrial and myometrial explants cultured for 72 hr with 10ng/ml of PDGF-BB, anti-PDGFBB, TGFβ1 or anti-TGFβ1 showed patterns of alteration in the various VSMC differentiation markers, but none were statistically significant due to the small sample numbers in these preliminary studies (Figure 4.10, Figure 4.11A-B).

h-Caldesmon: Around 30% $CD31^+$ endometrial vessels also expressed h-Caldesmon in the untreated control group (Figure 4.10, Figure 4.11A). This was reduced when treated with either PDGF-BB (~10%) or anti-PDGFBB (~20%) (Figure 4.11A). The percentage of $CD31^+$ endometrial vessels expressing h-

Caldesmon was also reduced when treated with either TGFβ1 (~20%) or anti-TGFβ1 (~12%) compared with the untreated group (Figure 4.11A).

In the myometrial explants, around 95% $CD31^+$ vessels also expressed h-Caldesmon in the untreated control group (Figure 4.10, Figure 4.11B) and this was unaltered after treatment with PDGF-BB (100%), anti-PDGFBB (~90%), TGFβ1 (~90%) or anti-TGFβ1 (~90%) (Figure 4.11B).

Smoothelin: Around 10% $CD31^+$ endometrial vessels also expressed smoothelin in the untreated control group (Figure 4.10, Figure 4.11A); this was reduced after treatment with PDGF-BB (<5%), anti-PDGFBB (<5%) TGFβ1 (<5%) or anti-TGFβ1 (<5%), but none were statistically significant due to the small sample numbers in these preliminary studies (Figure 4.11A).

In the myometrial explants, around 40% $CD31^+$ vessels also expressed smoothelin in the untreated control group (Figure 4.10, Figure 4.11B) and this did not change after treatment with PDGF-BB (~30%), anti-PDGFBB (~25%), TGFβ1 (~20%) or anti-TGFβ1 (~30%) (Figure 4.11B).

Calponin: Around 15% $CD31^+$ endometrial vessels also expressed calponin (Figure 4.10, Figure 4.11A) and this was reduced after treatment with PDGF-BB (<5%), anti-PDGFBB (<5%), TGFβ1 (<5%) or anti-TGFβ1 (<5%) (Figure 4.11A).

Around 60% $CD31^+$ vessels in superficial myometrium also expressed calponin in the untreated control group (Figure 4.10, Figure 4.11B), which was unaltered after treatment with PDGF-BB (~60%), but was increased after treatment with anti-PDGFBB (~80%), TGFβ1 (~80%) or anti-TGFβ1 (~80%) (Figure 4.11B).

4.4.3 Effect of AGFs on endometrial vascular ECM component expression

Preliminary observations from endometrial and myometrial explants cultured for 72 hr with 10ng/ml of PDGF-BB, anti-PDGFBB, TGFβ1 or anti-TGFβ1 showed no alteration in vascular ECM component staining (Figure 4.11C-D, Figure 4.12).

Collagen IV: In the endometrial and myometrial explants, 100% $CD31^+$ vessels expressed collagen IV in the untreated control group (Figure 4.12, Figure

4.11C-D) and this remained unaltered in both endometrium and myometrium after treatment with PDGF-BB (100%), anti-PDGFBB (100%), TGFβ1 (100%) or anti-TGFβ1 (100%) (Figure 4.11C-D).

Osteopontin: Around 100% $CD31^+$ vessels in the endometrium and superficial myometrium expressed osteopontin in the untreated control group (Figure 4.12, Figure 4.11C-D) and this did not alter after treatment with PDGF-BB (100%), anti-PDGFBB (~90% in endometrium, 100% in myometrium), TGFβ1 (100%) or anti-TGFβ1 (100%) (Figure 4.11C-D).

Figure 4.10: Staining for VSMC differentiation markers h-Caldesmon, smoothelin and calponin in endometrial and superficial myometrial explants. Arrows indicate vessels in untreated control endometrium and myometrium (**A1**-**A6**) or after treatment with 10ng/ml of PDGF-BB (**B1**-**B6**), anti-PDGFBB (**C1**-**C6**), TGFβ1 (**D1**-**D6**) and anti-TGFβ1 (**E1**-**E6**). Images were captured at 200x original magnification.

Figure 4.11: Stacked cluster chart showing % CD31 positive vessels stained for VSMC differentiation markers and ECM components in endometrial and superficial myometrial explants. h-Caldesmon, smoothelin, calponin (**A**,**B**) and collagen IV and osteopontin (**C**,**D**) in the endometrium (**A**,**C**; N=2) and myometrium (**B**,**D**; N=3) of untreated control or treated for 72 hr with 10ng/ml of each of PDGF-BB, anti-PDGFBB, TGFβ1 and anti-TGFβ1. Pairwise Kruskal-Wallis one way ANOVA, with Bonferroni correction was used to determine significant differences between control and treated groups for myometrium; no significant differences were observed.

Figure 4.12: Staining for ECM components collagen IV and osteopontin in endometrial and superficial myometrial explants. Arrows indicate vessels in untreated control endometrium and myometrium (**A1**-**A4**) or treated for 72 hr with 10ng/ml of each of PDGF-BB (**B1**-**B4**), anti-PDGFBB (**C1**-**C4**), TGFβ1 (**D1**-**D4**) and anti-TGFβ1 (**E1**-**E4**). Images were captured at 200x original magnification.

4.5 DISCUSSION

4.5.1 Spatio-temporal pattern of vascular AGF staining in normal menstrual cycle and in HMB

Angiogenesis takes place in the endometrium throughout the different phases of the menstrual cycle, in accordance with the requirements of this dynamic tissue (Girling and Rogers, 2005; Girling and Rogers, 2009). While the process of blood vessel growth and development in endometrium remains under the master control of steroid hormones, angiogenic growth factors such as PDGF-BB, TGFβ1 and their corresponding receptors may directly regulate EC proliferation and/or apoptosis, interaction with and integrity of ECM components, as well as recruitment and maturation of VSMCs, which ultimately results in vascular development and maturation (Gold *et al.*, 1994; Dickson *et al.*, 1995; Lindahl *et al.*, 1997; Pepper, 1997; Massague *et al.*, 2000; Tabibzadeh, 2002; Distler *et al.*, 2003; Betsholtz, 2004). Importantly, previous studies as well as those outlined in Chapter 3 have indicated dysregulation of endometrial vascular development and maturation in bleeding disorders such as HMB (Kooy *et al.*, 1996; Abberton *et al.*, 1999a; Biswas Shivhare *et al.*, 2014). Although some studies have previously examined the pattern of staining for endometrial PDGF-BB, TGFβ1 and their respective receptors in the normal menstrual cycle (Chegini *et al.*, 1992; Gold *et al.*, 1994; von Wolff *et al.*, 2000; Omwandho *et al.*, 2010; Lash *et al.*, 2012), to our knowledge this has not been reported for HMB.

The current study demonstrated that in controls, PDGF-BB was minimally expressed in the stroma, glandular epithelial cells and VSMCs, but was moderately expressed in the ECs (Figure 4.3), while both its receptors PDGFRα and PDGFRβ were moderately expressed in stroma, glandular epithelial cells and ECs but minimally in VSMCs (Figures 4.4-4.5). Although not statistically significant, stromal staining for both receptors appeared to increase as the menstrual cycle progressed from PP to LSP and PDGFRα staining in VSMCs also increased from PP to LSP (Figure 4.4). This phase specific pattern may be explained by the fact that PDGF-BB is known to be associated with recruitment and maturation of VSMCs; hence as the menstrual cycle progresses and the

newly formed vessels require VSMC recruitment, ECs start to express specific AGFs and receptors to aid vascular maturation. In contrast, PDGF-BB staining in glandular epithelium was unchanged across the menstrual cycle and both PDGFRα and PDGFRβ stainings were decreased in the glandular epithelial cells from PP to LSP (Figures 4.3-4.5). This is in agreement with Chegini *et al*. (1992), who reported that PDGFRβ staining was highest in PP and decreased considerably in the secretory phase. Moreover, the results on staining for PDGF-BB, PDGFRα and PDGFRβ across the menstrual cycle are mainly in line with those reported by Lash *et al*. (2012); the only difference between the two studies, though not significant, was that the current study observed very low PDGF-BB staining in stroma and glands, while Lash *et al*. (2012) had reported no expression.

In controls, there was low TGFβ1 staining and moderate TGFβRI and TGFβRII staining in the endometrial stroma, glandular epithelium and ECs. While TGFβ1 and TGFβRII staining was minimal in VSMCs, TGFβRI was moderately expressed. Although not statistically significant, TGFβ1 staining in stroma increased and that in the glandular epithelium decreased from PP to LSP (Figure 4.7-4.9). In contrast, Chegini *et al*. (1994) reported highest TGFβ1 in the endometrial tissues from late proliferative and early secretory to late secretory phases, while the current study showed highest in the MSP/LSP with levels remaining similar to LSP in PP, although the observed differences could be due to variation in antibody specificity and/or endometrial staging. On the other hand, the current results for TGFβ1, TGFβRI and TGFβRII staining in stratum functionalis and stratum basalis collectively are similar to those demonstrated by Lash *et al*. (2012) with some variations in the absolute scores. The phase specific pattern of staining for TGFβ1 and its receptors is indicative of their steroid hormone dependant regulation, and potentially roles in angiogenesis during the menstrual cycle.

In addition, there was altered AGF staining in HMB. In general compared with controls, in HMB, staining for PDGF-BB, PDGFRα, PDGFRβ, TGFβ1, TGFβRI and TGFβRII were all decreased across the menstrual cycle in both endometrium and myometrium, especially in the stromal cells and ECs, with glandular epithelium and VSMCs showing less pronounced changes (Figures

4.3-4.5, Figures 4.7-4.9). While PDGF-BB and its receptors are involved mainly in VSMC recruitment, TGFβ1 and its receptors regulate both VSMC recruitment and differentiation (Massague, 1998; Lindahl *et al.*, 1999; Lebrin *et al.*, 2005). Therefore, a reduction in both of these AGF families would point towards alteration, not in the activation phase, but in the resolution phase constituting vessel stability and VSMC recruitment thereby leading to vascular maturation. This in turn may suggest that the alteration/reduction in AGF production by these different endometrial cell populations may contribute towards poor maturation and consequently dysregulated vascular development in HMB.

While ECs from Tgfbr2 (or ALK5) deficient mice were found to be defective in fibronectin production and migration in culture (Larsson *et al.*, 2001), data from the ECM study described in Chapter 3 (section 3.4.4.3) have shown reduced fibronectin in endometrium of women with HMB. This is a further indication of the potential critical effects of altered levels of AGFs with respect to changes in vascular ECM composition, ultimately resulting in altered vascular development, which may underlie HMB. Surprisingly, stromal staining for PDGFRβ in PP stratum functionalis and that of TGFβRI in PP stratum basalis, were increased in HMB compared with controls, indicating an alteration not only in the levels of but also in the timing of AGF staining for in the endometrial stromal cells in HMB. The current study suggests that reduced AGF expression may contribute towards decreased vascular maturation in HMB. Moreover our previous results have shown a decrease in the uNK cell population during the LSP (section 3.4.5) in HMB. Collectively our data suggests that in HMB endometrial vascular maturation is reduced alongside the uNK cell population as well as AGF expression. Previous studies in the laboratory showed increased endometrial vascular maturation in RM alongside both an increased endometrial stromal uNK cell population (Quenby *et al.*, 2009) and altered AGF staining (Lash *et al.*, 2012). In particular staining of vascular TGFβ1, which is the key player in vessel maturation, was reported to be increased in RM, while the current study showed that there was an overall pattern of a decrease in TGFβ1 staining in HMB compared with controls. Although differences exist between the two studies in terms of endometrial staging and grouping methodology, the contrasting results highlight that while decreased vascular development and maturation with

respect to VSMC differentiation may alter vascular function leading to excessive blood loss in the form of HMB, increased vascular maturation may also result in increased blood flow resulting in increased oxygen at the early implantation site proving detrimental to the developing embryo and placenta. Both studies showed a decrease in staining for vascular PDGF-BB, PDGFRα, TGFβRI and TGFβRII in HMB and RM, compared with controls during mid/late secretory phases. This may suggest that the structure and function alterations seen in endometrial blood vessels in HMB or RM may share common as well as distinct pathways, especially in downstream signalling for differential expression of AGFs such as TGFβ1. Therefore further studies are required, especially investigating the growth factors, which are differentially expressed between HMB and RM and also on the interaction between the different AGFs in the endometrium, which may provide crucial clues to elucidating a structure function relationship for endometrial blood vessels.

4.5.2 Effect of AGFs on endometrial VSMC differentiation and ECM staining

The results in section 4.4.1 and previous studies have indicated an alteration in endometrial AGF staining in HMB and reproductive disorders such as RM (von Wolff *et al.*, 2000; Lash *et al.*, 2012). Endometrial and myometrial explants were used as an *in vitro* model to study the effects of altered levels of AGFs in endometrial vascular development. The effects of PDGF-BB and TGFβ1 on VSMC differentiation and ECM component staining in explants of proliferative endometrium and superficial myometrium were investigated to assess whether the developing vessels of proliferative endometrium undergo altered development and maturation on elimination of or exposure to altered levels of PDGF-BB and TGFβ1. Preliminary data from the current study showed a reduction in h-Caldesmon, smoothelin and calponin staining both on elimination of or exposure to different levels of PDGF-BB and TGFβ1 (Figure 4.11). Interestingly, the myometrial explant cultures did not reveal such differences for h-Caldesmon and smoothelin, although calponin was increased after treatment with PDGF-BB, TGFβ1 and anti-TGFβ1 (Figure 4.11). These results, although preliminary, may suggest two possibilities; VSMC differentiation with respect to h-Caldesmon, smoothelin and calponin staining is regulated by PDGF-BB and/or TGFβ1 and that endometrial vessels are more receptive to alterations in

PDGF-BB and TGFβ1 levels than those in superficial myometrium. Furthermore, vascular development in endometrium is perhaps differently regulated than that in myometrium casting doubt on the applicability of myometrial explants and myometrial vessels as appropriate models for the study of vascular development in endometrial bleeding disorders such as HMB. Additionally the current study found no differences in staining for ECM components on elimination of or exposure to different levels of PDGF-BB and TGFβ1 (Figure 4.11). These preliminary results may suggest either a PDGF-BB, TGFβ1 independent regulation of endometrial/myometrial ECM component expression, or that the levels (10ng/ml) of PDGF-BB and TGFβ1 utilised in the current study were not adequate to bring about any alteration. Moreover, although treated with increased levels of AGF (physiological concentration range between 0.1-4ng/ml), the absolute amount absorbed by the tissue and consequently the respective cellular components is uncertain. In summary, the results from the explant culture study, although preliminary, have indicated the possibility of utilising endometrial explant culture as a method to investigate the effects of altered levels of AGFs on vascular development in the endometrium. However in future these experiments must be repeated with larger sample size prior to drawing any specific conclusions.

4.6 CONCLUSION

In conclusion, PDGF-BB, TGFβ1 and their corresponding receptors are differentially stained spatio-temporally in the uterus during the phases of the menstrual cycle, highlighting their dependence on steroid hormones and their critical role in normal uterine vascular development. Moreover, this distinct pattern of AGF staining was altered (principally reduced) in HMB, suggesting that reduced levels of AGF may contribute towards reduced or altered vascular maturation and stability underlying HMB.

Preliminary results from the explant culture study showed that altered levels or inhibition of AGF action may affect VSMC differentiation, especially in endometrium; although this may not be the case for ECM components. Importantly, the results suggested that since the endometrial vessels are sensitive to alteration in the levels of AGF, they may be more suited to the investigation of vascular development in HMB, compared with those in myometrium. These preliminary studies also highlighted the potential of the explant culture system for future studies investigating roles of AGFs on endometrial vascular development.

Collectively the results on the role of AGFs in endometrial vascular development are in agreement with the results described in Chapter 3, which show altered VSMC differentiation, EC, ECM component staining and importantly an altered endometrial stromal uNK cell population, all of which point towards a dysregulated endometrial vascular development, rather than early vascular breakdown underlying HMB.

The results on the role of AGFs in endometrial vascular development, especially vascular maturation and VSMC differentiation, draw attention to the need for further study of their effects on EC-VSMC association leading to VSMC recruitment and maturation. Although such functional studies are crucial, they are not achievable *in vivo* and due to limited technology currently available, have not been investigated *in vitro*. This highlights the importance of development and validation of *in vitro* assays for angiogenesis, specifically suited for visualisation and investigation of the dynamic EC-VSMC association and how this may be affected by alterations in the levels of AGFs.

CHAPTER 5

IN VITRO EC-VSMC ASSOCIATION ASSAY

5 *IN VITRO* EC-VSMC ASSOCIATION ASSAY

5.1 INTRODUCTION

The human endometrium is one of the very few tissues in the adult human, where physiological angiogenesis takes place. During the menstrual cycle, following shedding, the endometrium is renewed every 28 days, with angiogenesis being central to this process. The complex mechanism of blood vessel development includes angiogenesis which involves degradation of ECM, EC proliferation, migration and tubule formation, followed by arteriogenesis involving recruitment of pericytes or VSMCs, further vascular maturation and importantly formation of complex branching networks. Since angiogenesis is integral to the normal physiology of the endometrium it is indisputable that it may be implicated in endometrial pathologies. The significance of angiogenesis in the endometrium and consequently its effects on the menstrual cycle or implications in its pathological states have been further discussed in sections 1.2, 1.4.1 and its role in heavy menstrual bleeding have been investigated through immunohistochemical studies in Chapter 3 and Chapter 4.

Importantly, our studies have implicated dysregulated angiogenesis with a possible role of AGFs underlying HMB. Although crucial to the normal functioning of the endometrium and consequently for reproduction, understanding of the process of endometrial blood vessel development has remained limited, due to constraints in availability of animal models or technological advancements. Therefore, although current literature outlines the interplay of mechanisms which co-ordinate angiogenesis, it also highlights the need for further research to understand the molecular regulators of vascular patterning and guidance and how any such guidance cues come together to develop a new vessel. However, this requires a detailed understanding of the complex molecular mechanisms underlying the process, which in turn is dependant on *in vitro* or *in vivo* assays aiding testing of hypotheses and screening of target molecules. The significance of angiogenesis in tumour growth and consequently the need for identification of specific anti-angiogenic molecules for novel cancer therapy along with the growing need for non-

invasive technology fuelled advancements in microscopy and bioimaging techniques in the context of studying angiogenesis.

Angiogenesis is a dynamic process and therefore although important, static end point assays often offer limited information, such as indication of sustained effect or recovery, evidencing a need for development of assays, which may permit dynamic imaging of angiogenesis with high spatio-temporal resolution. Importantly, *in vitro* dynamic imaging of angiogenesis has the potential to monitor and predict responses to angiogenic factors in real time, thus opening doors to identification of candidate molecules and critically determining their functions in angiogenesis. Two-dimensional assays are unable to study EC activation, the process of invasion, sprouting or branching from parent tubules. This has lead to the development of three-dimensional assays utilising collagen or fibrin matrices *in vitro* (Martin and Murray, 2009), which have been shown to help retain EC characteristics, thereby responding differently to different stimuli, compared with two-dimensional assays. Additionally, these 3D assays were further utilised to study spread of vessels formed by ECs and EC invasion in the presence of angiogenic stimuli (Martin and Murray, 2009). Although utilising these assays helped to investigate EC proliferation, migration, tubule formation and proteolysis, to our knowledge no assay has ever been used to study the combined effects of EC mediated tubule formation followed by VSMC recruitment and how this process may be affected by different AGFs.

Angiogenesis can be targeted at different levels, such as EC migration, tubule formation, VSMC recruitment etc. Therefore an *in vitro* assay must also be sensitive, developed specifically depending on the context of the scientific query. The *in vitro* assay developed in this study aims to investigate the following scientific questions; firstly, how do ECs communicate with VSMCs to recruit them onto the already formed EC tubules, secondly can this EC-VSMC interaction be affected by AGFs and finally, if so could this depend on altered AGF secretion, mRNA expression by ECs and VSMCs or VSMC differentiation? To be able to study these questions the assay must allow initiation of EC mediated tubule formation, be suited for addition of VSMCs, and importantly allow continuous imaging over a prolonged period of time allowing dynamic visualisation of the EC-VSMC interaction and the effects of AGFs.

5.2 AIMS

Understanding the functional role of angiogenesis in the pathogenesis of HMB first requires an insight into the basic process of angiogenesis, which is initiated through EC proliferation, migration and tubule formation followed by VSMC recruitment and differentiation. Investigation of these steps involved in angiogenesis requires an appropriate *in vitro* assay, which is dynamic and thus able to quantitatively investigate VSMC recruitment following EC mediate tubule formation with high resolution. Therefore, we aimed to:

1. Develop and optimise an *in vitro* assay to study EC-VSMC association.

2. Optimise the *in vitro* assay to study effects of VEGF-C treatments on EC-VSMC association.

3. Analyse AGF gene expression or secretion profiles of ECs and VSMCs cultured on matrigel compared with plastic, as well as differentiation of VSMCs cultured on matrigel when treated with VEGF-C.

5.3 EXPERIMENTAL DESIGN

The experimental design is illustrated in Figure 5.1 and specific materials and methods used are described below.

5.3.1 Human aorta cell line

Human ECs (section 2.2.6.5) and VSMCs (section 2.2.6.6) were cultured through sequential passaging as detailed in section 2.2.6.

5.3.2 Enzymatic disaggregation of human saphenous veins

Human SVs were enzymatically disaggregated in order to isolate ECs and VSMCs. The process of enzymatic disaggregation along with preparation of cell culture treatments and supernatants has been described in section 2.2.6.7, Figure 2.6.

5.3.3 Assay description

The initiation of tubule formation is based on differentiation of ECs on a basement membrane matrix such as matrigel (Kubota *et al.*, 1988). ECs differentiate and form tubule-like 'honeycomb' structures on matrigel but are unable to do the same on glass. Therefore we utilised matrigel in our assay, making it suitable for EC-mediated honeycomb formation. This required assay optimisation on two levels: thickness of the matrigel to achieve uniform coating and the seeding density of ECs used. The next step was the addition of VSMCs. Again, this step required optimisation to determine the seeding density of VSMCs used without disrupting the already formed tubules. Finally, dynamic imaging of the assay set-up required optimisation of the imaging parameters such as use of the correct objective, time between each frame captured and importantly keeping an environment suitable for the cell-culture while imaging is being carried out. The principal steps involved in the *in vitro* assay are outlined in Figure 5.2 and the seeding density for ECs to form honeycomb structures on matrigel is shown in Figure 5.3.

Figure 5.1: Experimental design to develop and optimise an *in vitro* assay aiding investigation of EC-VSMC association and how this process is affected by angiogenic growth factors.

Figure 5.2: Principal steps involved in the *in vitro* tubule formation and EC-VSMC association assay.

Figure 5.3: Seeding density of saphenous vein derived endothelial cells on glass (**A1-A4**) and matrigel (**B1-B4**). Images were captured at 100x magnification. Arrows indicate tubule or honeycomb structure formation. Note that $6x10^3$/well (**B4**) was chosen as optimal. (**B5**) Higher than optimal number of ECs clustered to disrupt efficient tubule formation.

5.3.4 Assay optimisation

5.3.4.1 Matrigel thickness

Matrigel is a basement membrane matrix extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumour rich in ECM proteins such as laminin, collagen IV and entactin. The assay used growth factor reduced matrigel that contained small amounts of growth factors (Table 5.1), which allowed formation of honeycomb structures. The layer of coating played an important role, since uneven layer formation due to the hydrophobicity of the chamber slide disrupted the EC-honeycomb arrangement, while large amounts affected imaging, by increasing the working distance of an objective. Therefore a relatively thin (5-7µl/ 0.8cm^2) even layer was deemed suitable for this assay.

Table 5.1: Amounts of growth factors in Growth Factor Reduced BD Matrigel

GROWTH FACTOR	CONCENTRATION
Epidermal growth factor (EGF)	< 0.5ng/ml
Insulin growth factor (IGF-1)	5ng/ml
Nerve growth factor (NGF)	< 0.2ng/ml
PDGF	< 0.5pg/ml
TGFβ	1.7ng/ml
VEGF	< 1.5ng/ml

5.3.4.2 Seeding density of cells

The appropriate seeding density of ECs was critical for honeycomb formation as well as for quantitative computer aided image analysis, the sensitivity of which depended on accuracy in automatic cell counting. Therefore, while less than adequate EC seeding density resulted in little tubule formation (Figure 5.3), high numbers jeopardised quantitative image analysis. Similarly, seeding density of VSMCs had an effect on disruption of EC-honeycomb structures and image analysis. While low numbers of VSMC were not sufficient for studying EC-VSMC interactions, high numbers resulted in collapsing EC-honeycomb structures as well as inaccurate image analysis. Therefore, both ECs and VSMCs were seeded at 6x 10^3/well.

5.3.5 Vital dyes and confocal time-lapse microscopy

The ECs and VSMCs were incubated at 37℃, 5% CO_2 for 30 min in cell tracker CFSE (5mM stock diluted 1:5000 in EC basal medium; Invitrogen ♯C34554) and CMRA (10mM stock diluted 1:2000 in VSMC basal medium; Invitrogen ♯C34551), respectively. The stained cells were washed in PBS (pH 7.4) and left to incubate at 37℃, 5% CO_2 for ≥ 30 min, before trypsinisation and centrifugation (200g for 5 min with brake at 3). The cell pellets were then re-suspended in the appropriate cell culture media and plated in 8-well chamber slides. Prior to cell plating, 4 wells of the 8-well chamber slide were coated with matrigel, while the remaining 4 were left uncoated for the control group on glass. In the first instance ECs were plated and after 4.5 hr incubation at 37℃, VSMCs in cell media or treatment media were added. Following a further 1 hr incubation, time-lapse live imaging was performed on a Nikon Eclipse-i laser-scanning confocal microscope, equipped with 37℃ incubator and 5% CO_2 supply, using 20x oil objective, 10x eyepiece and excitation wavelengths of 492nm (CFSE) and 548nm (CMRA). Live imaging was carried out for up to 10 hr and images were captured every 15 min.

5.3.6 Quantitative image analysis

5.3.6.1 EC-VSMC interaction on glass and matrigel

The digital time-lapse images were analysed using the NIS Elements Ar software package (Nikon Instruments Inc.). In order to study EC-VSMC interaction on glass and matrigel, we aimed to analyse: (a) alterations in the number and velocity with which an EC or VSMC moved on glass or matrigel during the course of the 10 hr assay and (b) the number of other ECs or VSMCs which one EC come in contact with in the 10 hr assay.

The number of ECs or VSMCs in a frame was analysed through automatic surface rendering aided by manual threshold adjustment (Figure 5.4A-C, Table 5.2) so that only ECs or VSMCs were included for analysis, respectively, using Imaris 7.3 software (Bitplane AG, Switzerland). The automatic surface rendering also allowed for the automatic analysis of average velocity for ECs or VSMCs on glass and matrigel over the 10 hr course of the assay. To study EC-VSMC

interactions, 10 ECs or VSMCs were randomly chosen from each video and tracked to investigate the number of the same cell type (EC-EC, VSMC-VSMC) and the other cell type (EC-VSMC) each interacted with during the course of the assay. Images were analysed between 2-10 hr, to eliminate any false alterations due to stage displacement in the microscope. The same procedure was followed for N=3 time-lapse movies.

Table 5.2: Surface rendering and manual thresholding parameters for IMARIS software analysis of EC:VSMC time-lapse recordings

PARAMETER	VALUE (EC)	VALUE (VSMC)
Diameter of largest sphere (Background elimination)	20.00µm	20.00µm
Manual threshold (Signal detection)	24.18	24.18
Automatic threshold B (Signal detection)	3619.03	3515.48
Tracking algorithm (EC, VSMC tracking, velocity)	Autoregressive motion	Autoregressive motion
Tracking maximum distance (Maximum distance a cell may travel between frames)	100.00µm	200.00µm
Tracking maximum gap size (Maximum frames a cell may not be tracked)	2 steps	4 steps

Figure 5.4: Quantitative image analysis utilised for the *in vitro* assay. (**A-C**) show surface rendering using IMARIS software package and (**D**) shows types of honeycombs (yellow arrows, dotted lines; **D1** shows complete honeycomb, **D2** shows incomplete honeycomb and **D3** shows perimeter of a complete honeycomb.

5.3.6.2 Assessment of EC-VSMC interaction on matrigel

In order to study EC-VSMC interaction on matrigel, the following parameters were included: (a) total number of honeycomb (or tubule) structures with ≥3 arms, (b) the number of complete honeycombs (with an enclosed area), (c) the perimeter of the complete honeycombs and finally (d) the percentage of VSMC coverage of the complete honeycombs determined as described below.

The digital time-lapse movies were analysed using the open-source ImageJ software package in combination with the 'cell counter' plugin to manually count the total number of honeycombs, complete honeycombs and measure the perimeter of three randomly chosen honeycombs (Figure 5.4) every 2 hr from the start of the assay (t=15 min) up to 10 hr. The percentage of VSMC coverage was analysed by measuring the total length of one complete honeycomb covered with VSMCs with respect to the perimeter of the honeycomb. The length of VSMC coverage inside (*y*) and outside (*z*) the honeycomb was added and a percentage VSMC coverage was obtained out of twice the honeycomb perimeter (*x*), as shown below.

$$\%VSMC\ coverage = \frac{y+z}{2x} \times 100$$

5.3.7 Assay application

Applications of the *in vitro* assay include assessment of the effects of treatment with AGFs and neutralising antibodies on EC-VSMC interaction and percentage VSMC coverage (VSMC recruitment) on the honeycomb structures.

Functional understanding of the effects of AGF treatment can be achieved through analysis of AGF gene expression by ECs and VSMCs, and AGF secretion by ECs cultured on plastic compared with matrigel. This can potentially help to identify key genes that may play a role in VSMC recruitment by ECs. VSMC differentiation is central to EC-VSMC interaction and VSMC recruitment. Hence the effect of AGF treatment on VSMC differentiation can be assessed by investigating the protein expression for VSMC differentiation markers (smoothelin and calponin; section 3.4.1.2) or h-Caldesmon gene expression.

5.3.7.1 Treatment with AGFs and neutralising antibodies

In the *in vitro* assay, SV derived ECs were first plated into the 8-well chamber slides in EC culture medium. The chamber was incubated for 4.5 hr at 37℃ and 5%CO_2, which allowed ECs to attach to the matrigel layer. Excess EC culture medium was carefully discarded using a pipette and VSMCs diluted in VSMC culture medium containing VEGF-C or anti-VEGFC neutralising antibodies (Appendix B) were added to each well as shown in Figure 5.2. Following this the chamber was imaged as described in section 5.3.5 and analysed as described in section 5.4.2.

5.3.7.2 AGF gene expression and secretion

SV derived ECs and VSMCs (only qRT-PCR) were separately cultured on 6-well plates with or without matrigel coating. Cells were used to extract RNA for qRT-PCR, while supernatants were collected for analysing AGF secretion using ELISA/angiogenesis array. SV derived VSMCs were also cultured in 8-well chamber slides coated with matrigel in control VSMC culture medium or treated with 1ng/ml and 10ng/ml VEGF-C or anti-VEGFC for 10 hr to investigate the effect of VEGF-C on VSMC differentiation, in terms of calponin and smoothelin expression.

5.3.7.3 qRT-PCR

Total RNA was extracted from the ECs and VSMCs that had been grown on plastic and matrigel, using the TRIZOL protocol as described in section 2.2.7. Real-time RT–PCR was performed using TaqMan Universal PCR MasterMix as described in section 2.2.7.4. A list of TaqMan probe/primer sets utilized has been listed in Table 2.5. All probe/primer sets were previously validated for use with a laboratory standard total RNA extracted from early pregnancy decidua. The $2^{-\Delta\Delta CT}$-method was used to calculate relative fold changes in gene expression as described in section 2.2.7.6.

5.3.7.4 ELISA and Quantibody Human Angiogenesis Array

Sandwich ELISA and Quantibody Human Angiogenesis Array were used to study the effects of matrigel on the profile of secreted AGFs by ECs. Sandwich

ELISA was performed as described in section 2.2.9.1 and Quantibody Human Angiogenesis Array was performed as described in section 2.2.10.

5.3.8 Statistical analysis

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc.). Data are presented as means ± standard error of mean (SEM) and differences are considered statistically significant at $P \leq 0.05$. Specific tests used are given in the results.

5.4 RESULTS

5.4.1 EC-VSMC association on glass and matrigel

5.4.1.1 ECs and VSMCs differ in number and velocity when cultured on glass and matrigel

When cultured on glass the number of disconnected (cell not in contact with any other cells) SV ECs and VSMCs and their velocity remained unchanged during the 10 hr *in vitro* assay. However, when these cells were cultured on matrigel both ECs and VSMCs decreased in number and velocity (Figure 5.5). The number of disconnected ECs was halved (P=0.018) and their velocity was also reduced (P=0.018). The number and velocity of VSMCs were also reduced (P=0.020, P=0.043 respectively; Figure 5.5).

5.4.1.2 ECs and VSMCs interact more with own cell type than the other

ECs and VSMCs derived from aorta or SVs were cultured *in vitro* for 10 hr. ECs derived from aorta and SV interacted with other ECs and VSMCs in a similar manner (Figure 5.6A, Figure 5.6B). ECs were observed to interact significantly more with other ECs than with VSMCs on both glass and matrigel for both the aorta cell line (glass P=0.001, matrigel P=0.02; Figure 5.6A) and ECs from SVs (glass P=0.01, matrigel P=0.03; Figure 5.6B). VSMCs derived from aorta and SV interacted differently with other VSMCs and ECs (Figure 5.6A, Figure 5.6B). Aorta VSMCs were observed to interact significantly more with other VSMCs than with ECs on both glass and matrigel (glass P<0.001, matrigel P=0.001; Figure 5.6A), but no such differences were observed for SV VSMCs on both glass and matrigel (Figure 5.6B). Importantly, ECs formed honeycomb structures to which VSMCs were recruited only on matrigel and not on glass (Figure 5.6C).

5.4.1.3 ECs and VSMCs from human aorta and saphenous vein differentially express AGF genes when cultured on glass and matrigel

Angiopoietin family and receptor: Ang1, Ang2 and Tie2 mRNA remained unaltered when cultured on matrigel compared with plastic in ECs from aorta or SV (Figure 5.7A).

Figure 5.5: Graphical representation (mean ± SEM) of fold change in number and velocity of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). Saphenous vein derived (**A**) ECs and (**B**) VSMCs were cultured on glass and matrigel (N=3 each group) for up to 10 hr. Mann-Whitney U test determined significant difference between glass and matrigel while Pairwise Kruskal-Wallis one way ANOVA determined significant difference between different time points.

Figure 5.6: Graphical representation (mean ± SEM) of percentage of endothelial cells (ECs), which interacted with other ECs or with vascular smooth muscle cells (VSMCs). Cellular interaction during the course of a 10 hr assay (N=3 each group) using cells from human (**A**) aorta and (**B**) saphenous vein. Mann-Whitney U test determined significant difference in EC:EC, EC:VSMC, VSMC:EC and VSMC:VSMC interaction between glass and matrigel as well as between aorta and saphenous vein. (**C**) ECs (green) form honeycomb structures indicated by arrows, to which VSMCS (red) are recruited, only when they are cultured on matrigel (**C6-C10**), but not on glass (**C1-C5**).

Figure 5.7: Graphical representation (mean ± SEM) of fold change in mRNA expression of angiogenic growth factors. **(A-D)** endothelial cells (ECs) and **(E-H)** vascular smooth muscle cells (VSMCs) from human aorta or saphenous vein cultured on matrigel compared with plastic (N=3 each group). Mann-Whitney U test determined significant difference in fold change mRNA expression on matrigel compared with plastic (indicated by a-k; [a]P=0.015, [b,f,k]P=0.05, [c,j]P=0.016, [d]P=0.03, [e]P=0.022, [g]P=0.006, [h,i]P=0.004, [b]P=0.05).

In aorta VSMCs Ang1, Ang2 and Tie2 mRNA expression remained unaltered when cells were cultured on matrigel compared with plastic (Figure 5.7E). In SV derived VSMCs, Ang1 and Ang2 mRNA expression remained unaltered, while Tie2 was increased over two-fold (P=0.006) when cultured on matrigel compared with plastic (Figure 5.7E).

VEGF family and receptors: VEGF-A, VEGF-C, VEGFR1 and VEGFR2 mRNA expression in ECs from aorta or SVs remained unaltered when cultured on matrigel compared with plastic (Figure 5.7B). However, VEGFR3 mRNA expression was increased over six-fold in aorta ECs (P=0.015) and over three-fold in SV derived ECs (P=0.05; Figure 5.7B).

VEGF-C, VEGFR1 and VEGFR2 mRNA expression in VSMCs from aorta or SVs remained unaltered when cultured on matrigel compared with plastic (Figure 5.7F). VEGF-A increased over two-fold in SV derived VSMCs (P=0.004) but remained unaltered for aortic VSMCs, when grown on matrigel compared with plastic.

PDGF-BB and receptors: PDGF-BB mRNA expression in ECs from aorta or SVs remained unaltered when cultured on matrigel compared with plastic (Figure 5.7C). PDGFRα expression increased over ten-fold in SV derived ECs (P=0.016), but was unaltered in aorta ECs when cultured on matrigel compared with plastic (Figure 5.7C). PDGFRβ expression increased in ECs from both aorta (P=0.030) and SVs (P=0.022; Figure 5.7C).

PDGF-BB and PDGFRα mRNA expression in VSMCs from aorta or SVs remained unaltered when cultured on matrigel compared with plastic (Figure 5.7G). PDGFRβ was increased in SV derived VSMCs (P=0.004), but remained unaltered in aorta VSMCs, when cultured on matrigel compared with plastic (Figure 5.7G).

TGFβ1 and receptors: TGFβRI and TGFβRII mRNA in ECs from aorta or SVs remained unaltered when cultured on matrigel compared with plastic (Figure 5.7D). However, TGFβ1 expression was increased over four-fold in ECs derived from SVs (P=0.05) but remained unaltered in aorta ECs, when grown on matrigel compared with plastic.

TGFβRI mRNA in VSMCs from aorta or SVs remained unaltered when cultured on matrigel compared with plastic (Figure 5.7H). VSMCs from SV showed an increase in mRNA of TGFβ1 (*P*=0.016) and TGFβRII (*P*=0.05) when cultured on matrigel compared with plastic (Figure 5.7H).

5.4.1.4 No alteration in AGF secretion by saphenous vein derived ECs when cultured on matrigel, compared with plastic

In SV derived ECs, fold change in secretion of Angiogenin, Ang1, Ang2, VEGF-A, VEGF-C, PIGF, PDGF-BB, TGFβ1, bFGF, HGF, leptin, EGF and HB-EGF remained unaltered between groups cultured on matrigel compared with those cultured on plastic (Figure 5.8A-D).

5.4.2 Effect of VEGF-C on EC-VSMC association and VSMC differentiation *in vitro*

5.4.2.1 Effect of VEGF-C on EC-VSMC association

Number and velocity of ECs and VSMCs: The number of SV derived ECs decreased at 10 hr compared with that at 2 hr in all groups (control *P*=0.018, 10ng/ml VEGF-C *P*=0.033, 10ng/ml anti-VEGF-C *P*=0.018; Figure 5.9A). The EC velocity remained unchanged in samples treated with 1ng/ml VEGF-C, 10ng/ml VEGF-C or 10ng/ml anti-VEGFC, but was decreased in the untreated controls (*P*=0.018; Figure 5.9B).

The number of SV derived VSMCs decreased at 10 hr compared with that at 2 hr in the controls (*P*=0.02) and after treatment with 10ng/ml anti-VEGFC (*P*=0.035; Figure 5.9C). In addition, the number of VSMCs in the anti-VEGFC treated group was decreased at 10 hr compared with 4 hr (*P*=0.02) and at 8 hr compared with 2 hr (*P*=0.044) and 4 hr (*P*=0.028; Figure 5.9C). VSMC velocity decreased in the controls at 8 hr compared with 2 hr (*P*=0.043) and in the 10ng/ml VEGF-C treated group at 10 hr compared with 2 hr (*P*=0.025; Figure 5.9D).

Total number of honeycombs: The total number of honeycombs (≥3 arms) was reduced at 10 hr compared with 15 min in the untreated control group (*P*=0.035) and after treatment with 10ng/ml VEGF-C (*P*=0.024; Figure 5.10A-B).

Figure 5.8: Graphical representation (mean ± SEM) of fold change in angiogenic growth factor secretion in SV derived endothelial cells (ECs). (**A-D**) Saphenous vein derived ECs cultured on matrigel (N=3 each group). Mann-Whitney U test determined significant difference between fold changes in AGF secretion on matrigel compared with plastic. No significant differences were observed.

Figure 5.9: Graphical representation (mean ± SEM) of fold change in number and velocity of VEGF-C treated endothelial cells (EC) and vascular smooth muscle cells (VSMC) on matrigel. Fold change in saphenous vein derived EC (**A**) number and (**B**) velocity and VSMC (**C**) number and (**D**) velocity (N=3 each group) at different time points relative to 2 hr between untreated control or VEGF-C treatments, on matrigel. Pairwise Kruskal-Wallis one way ANOVA determined significant difference between different time points and between untreated control or VEGF-C treatments. Bars with same letters are significantly different from each other; $^{A,C}P$=0.018, ^{B}P=0.033, $^{D,F}P$=0.020, ^{E}P=0.035, ^{G}P=0.044, ^{H}P=0.028, ^{I}P=0.043, ^{J}P=0.025.

Figure 5.10: Graphical representation (mean ± SEM) of fold change in VEGF-C treated (EC) honeycombs (HC) and vascular smooth muscle cell (VSMC) coverage. Fold change at different time points relative to 15 min in number of (**A, B**) total HC with ≥3arms, (**A, C**) complete honeycomb, (**A, D**) perimeter of complete honeycomb and (**E**) percentage of VSMC coverage of complete honeycomb by saphenous vein derived ECs and VSMCs (N=3 each group). Pairwise Kruskal-Wallis one way ANOVA determined significant difference between different time points and between untreated control or VEGF-C treatments. Bars with same letters are significantly different from each other. 'P' values represent differences between different time points; ^{A}P=0.035, ^{B}P=0.024, ^{C}P=0.050, ^{D}P=0.033, $^{E,F}P$=0.047.

Number of complete honeycombs: The number of complete honeycombs was reduced at 10 hr compared with 15 min in the untreated control group (P=0.05) but was unaltered in the groups treated with 1ng/ml VEGF-C, 10ng/ml VEGF-C and anti-VEGFC (Figure 5.10C).

Honeycomb perimeter: The perimeter of complete honeycombs increased at 8 hr compared with 15 min in the control group (P=0.033). The perimeter of complete honeycombs was greater after 8 hr (P=0.05) and 10 hr (P=0.05) treatment with 10ng/ml VEGF-C compared with 15 min (Figure 5.10D).

VSMC coverage: The percentage VSMC coverage of honeycombs appeared to increase over time, although this was not statistically significant in control or VEGF-C treated groups (Figure 5.10E).

5.4.2.2 Effect of VEGF-C on VSMC differentiation

Calponin and smoothelin expression: SV derived VSMCs were cultured in 8-well chamber slides coated with matrigel in control VSMC culture medium, 1ng/ml VEGF-C, 10ng/ml VEGF-C and anti-VEGFC for up to 10 hr. The number of calponin$^+$ or smoothelin$^+$ VSMCs as determined by IHC, remained unaltered when treated with different concentrations of VEGF-C or its neutralising antibody (Figure 5.11A).

h-Caldesmon mRNA expression: SV derived VSMCs were cultured overnight in 6-well dishes coated with matrigel in control VSMC culture medium, 1ng/ml VEGF-C, 10ng/ml VEGF-C and anti-VEGFC. h-Caldesmon mRNA (fold change compared with untreated controls) as determined by qRT-PCR, remained unaltered in VSMCs treated with different concentrations of VEGF-C or its neutralising antibody (Figure 5.11B).

Figure 5.11: Graphical representation (mean ± SEM) of fold change in calponin^{+} or smoothelin^{+} in saphenous vein derived vascular smooth muscle cells (VSMCs). Fold change compared with untreated control (**A**) in percentage calponin^{+} or smoothelin^{+} VSMCs and (**B**) in H-Cal mRNA in saphenous vein derived VSMCs cultured on matrigel (N=3 each group). Pairwise Kruskal-Wallis one way ANOVA. No significant differences were observed.

5.5 DISCUSSION

5.5.1 EC-VSMC association assay

Angiogenesis is central to the normal menstrual cycle and dysregulated angiogenesis has been implicated in HMB. In order to understand the mechanisms underlying dysregulated angiogenesis in HMB, it is first necessary to study the basic steps in angiogenesis; EC proliferation, migration, tubule formation, VSMC recruitment and vascular maturation. ECs and VSMCs are present in vascular beds of different tissues and although they have similarities with one another such as the cobblestone morphology typical of ECs, tissue-specific requirements drive them towards distinct heterogeneous cell populations, differing at the biochemical, molecular and consequently functional levels. This highlights the importance of the source of cells used in an *in vitro* assay, and that the best fit may be from vessels that are perhaps functionally the closest match to those in the specific scientific query being investigated.

ECs mostly remain quiescent in adults until activated by AGFs such as VEGFs, following which they undergo proliferation, migration and tubule formation, marking the initiation of angiogenesis. ECs have been harvested from large vessels such as the aorta, umbilical and saphenous veins through mechanical removal or enzymatic disaggregation such as collagenase digestion (Jaffe *et al.*, 1973). However, since the endothelium of larger vessels may differ from tissue microvasculature, techniques have also been established for isolation of ECs from endometrial vasculature for utilisation in *in vitro* models (Schatz *et al.*, 2000). Although brain or adipose tissues are suited for microvascular EC isolation (Wagner and Matthews, 1975; Dorovini-Zis *et al.*, 1991) due to their high vascular density as well as ease of disaggregation, the endometrial microvasculature still remains challenging. This is due to the lower density of vessels, vascular or branching size and rigidity, the presence of a mixed cell population and importantly endometrial tissue availability.

The other key player in vascular development is the VSMC, whose recruitment onto the endothelial tubule leads to vascular maturation and allows blood circulation. VSMCs have previously been isolated from umbilical veins or arteries (Campbell and Campbell, 1993) and their phenotypic modulation *in vitro*

has also been discussed in detail elsewhere (Campbell and Campbell, 2012). The two commonly used techniques used to isolate VSMCs from arteries or veins are enzymatic disaggregation of the vessel or tissue explantation and subsequent cell isolation. While isolation using the explantation method may provide VSMC colonies that may be kept for longer periods, it is not suitable for endometrial tissue as explained earlier.

A number of *in vitro* assays may be utilised to study angiogenesis; however the choice of the most appropriate cell type or assay is ultimately based on the combination of a number of factors including tissue, cell, and reagent availability, cost of equipment, time taken for an experimental setup and importantly the skills required and reproducibility. Taking into consideration the above-mentioned factors, the first choice for this study was to utilise the widely studied human aorta EC and VSMC cell lines. However, these primary cells were often observed to de-differentiate in culture, with loss of characteristics. SV derived ECs and VSMCs were the next choice, since SVs are a good source of ECs and VSMCs and were relatively easy and sustainable to source. Moreover, SV ECs have been reported to retain their characteristics for up to 15 passages (Scoumanne *et al.*, 2002) and we had also observed that ECs and VSMCs isolated from SVs retained their characteristics for longer as determined by cell characterisation (section 2.2.6.3); hence SV derived ECs and VSMCs were chosen for this assay.

Finally, while the results from Chapter 3 and Chapter 4 highlighted the importance of vascular maturation, VSMC differentiation and AGF expression in the endometrium, to our knowledge, no existing *in vitro* assay was applicable to further investigate these processes. Hence this study aimed to developed an angiogenesis assay, based on EC differentiation on matrigel to form 'honeycomb' tubular structures, to specifically target vascular maturation through EC mediated VSMC recruitment and investigate the effects of AGFs on EC-VSMC association and VSMC differentiation. Moreover a comparison of ECs and VSMCs, when cultured on plastic and matrigel gave some insight into the roles PDGF-BB and TGFβ1 governing the process of vascular maturation.

Number and velocity of ECs and VSMCs: The current study demonstrated that the number and velocity of ECs and VSMCs reduced over time when cultured

on matrigel (Figure 5.5). This can be explained by the fact that ECs come in contact with other ECs and organise themselves into honeycomb structures, which increase in number forming bigger networks with time, leaving fewer numbers and space between isolated ECs, resulting in slower movement in space. Furthermore, when VSMCs are added to the wells with ECs, with time, they are drawn towards the EC honeycomb structures, where they form attachments and start to cover the honeycomb structures. Therefore, as more and more VSMCs attach to the honeycombs, fewer numbers of isolated VSMCs remain present and in addition, with reduced free space (due to EC mediated honeycomb structure formation), their average velocity is reduced.

EC-VSMC interaction: The results showed that ECs and VSMCs are more likely to interact with their own cell type than with the other when cultured on both glass and matrigel (Figure 5.6). Firstly, this may suggest that ECs and VSMCs release mediators in the culture medium, which can be detected by their own cell type, therefore reaching and drawing neighbouring cells as well as cells that are further away. While ECs are also involved in VSMC recruitment into the honeycombs, they may secrete cell specific mediators and therefore demonstrate distinct relationships with other ECs or VSMCs. Since this interaction remains unaltered when cultured on glass or matrigel, it may indicate that this EC mediated cell specific communication *in vitro* may not be dependent on the interaction with extracellular matrix interactions; however EC mediated honeycomb structure formation and VSMC recruitment is only observed on matrigel. Therefore it may be that although ECs and VSMCs are interacting with one another independent of the matrix matrigel, their ability to form stable attachments forming honeycombs or VSMC coverage is affected by the presence of matrigel; further study is required, which may specifically address the role of ECM in EC-VSMC association.

AGF gene expression: Matrigel was observed to alter ECs and VSMCs in terms of their number, velocity and interaction between each other; ECs formed honeycomb structures and allowed VSMC coverage only when cultured on matrigel. This is perhaps explained by the differences in the pattern of AGF mRNA in ECs and VSMCs when cultured with or without matrigel (Figure 5.7). PDGFRβ is particularly expressed on VSMCs or pericytes and communicates

through PDGF-BB released from ECs; mice lacking PDGF-BB and PDGFRβ were shown to be VSMC deficient and died at birth (Lindahl *et al.*, 1997). This perhaps suggests that when cultured on matrigel, VSMCs have an increased PDGFRβ, which enables them to be more sensitive towards PDGF-BB expression on ECs, thereby leading to VSMC coverage on EC honeycombs.

TGFβ1 and its receptor TGFβRII play important roles in regulating cell proliferation, adhesion, differentiation and migration in the activation phase of angiogenesis while inhibiting EC migration and proliferation, imparting stability at the resolution phase (Goumans *et al.*, 2009). TGFβ1 was increased in both ECs and VSMCs when cultured on matrigel, which perhaps reiterates the fact that it not only plays a role in mediating EC migration, proliferation and honeycomb structure formation, but also later in helping maintain the structure after addition of VSMCs. The Ang1-Tie2 signalling pathway is critical for vascular development; in particular Tie2 has been proposed to be important for EC formation and mice lacking Tie2 die embryonically, due to poor vascular organisation and perivascular cell coverage (Dumont *et al.*, 1994; Sato *et al.*, 1995). This may explain why when cultured on matrigel once ECs have formed the honeycomb structures, VSMCs expressing higher Tie-2 are drawn towards them resulting in VSMC coverage.

VEGF-A crucially regulates angiogenesis; homozygous and heterozygous VEGF-A knockout mice die embryonically due to immature blood vessel formation (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996). Therefore an increase in VEGF-A expression in VSMCs cultured on matrigel may indicate altered vascular maturation *in vitro*. VEGFR3 is expressed in ECs at the tips of angiogenic sprouts (Tammela *et al.*, 2008), which may partially explain its increase in *in vitro* culture on matrigel, which is an active site for EC migration and honeycomb/tubule formation. Interestingly, VEGFR3 signalling is initiated through VEGF-C or VEGF-D binding, although VEGF-D has primarily been implicated in lymphangiogenesis. Therefore an increase in VEGFR3 by both ECs and VSMCs may also suggest VEGF-C as a potential mediator for tubule formation, VSMC recruitment and differentiation, and EC-VSMC association. Additionally, both the PDGF-BB receptors and TGFβ1 were also increased,

which together with VEGF-C may contribute towards EC-VSMC association, highlighting the significance of further investigation into their effects *in vitro*.

AGF secretion: Although SV derived ECs, when cultured on matrigel, showed a significant increase in VEGFR3, PDGFRα and PDGFRβ and TGFβ1 gene expression, matrigel had no effect on AGF secretion by these ECs. This may suggest that while matrigel may affect AGF expression in ECs perhaps indirectly making them sensitive to paracrine AGF signalling, it is unable to directly regulate AGF secretion by ECs thereby not directly affecting EC-EC or EC-VSMC communication. This may also be the reason why no alteration in EC-EC, EC-VSMC interaction was observed when ECs were cultured with or without matrigel. However, these are preliminary results and further experiments must be performed before any definite conclusion may be drawn.

5.5.2 Effect of VEGF-C on EC-VSMC association and VSMC differentiation

Angiogenic growth factors such as VEGF along with TGFβ and PDGF are some of the most important factors in vascular development, homeostasis, stabilisation and maturation. Amongst the VEGF family, VEGF-A remains the most widely studied member and key to angiogenesis. However, VEGF-C has been suggested to regulate VEGF-A expression, thereby promoting angiogenesis in cancer (Gacche and Meshram, 2014). VEGF-C binds to VEGFR3, but also to VEGF2 with low affinity (Hoeben *et al.*, 2004; Olsson *et al.*, 2006). Binding of VEGF-C to sVEGFR2 prevents VEGFR3 binding resulting in inhibition of EC proliferation (Koch *et al.*, 2011). Although VEGFR3 is mostly restricted to lymphatic ECs in adults, ECs present in tissues or sites of active angiogenesis, especially the 'tip cells' of angiogenic sprouts express VEGFR3 (Koch *et al.*, 2011), and presumably initiate signalling via binding of VEGF-C. Importantly, Tie2 expressing macrophages localise at vascular branching points and induce connection between EC tubule structures by VEGF-C secretion and consequently activation of the VEGFR3 signalling pathway in the tip cells (Fagiani and Christofori, 2013). Interestingly, an *in vivo* study using a combination of antibodies against VEGFR3 and VEGF-C was shown to be efficient in inhibiting VEGFR3 signalling and vascular network formation (Tvorogov *et al.*, 2010; P. Saharinen *et al.*, 2011). Again, unpublished data from

our laboratory has suggested a role for VEGF-C in tubule formation. The *in vitro* angiogenesis assay described above aided investigation of the effects of VEGF-C on EC-VSMC association and VSMC differentiation, governing angiogenesis.

VEGF-C in EC-VSMC association: Results from the *in vitro* assay showed that VEGF-C and anti-VEGFC treatments did not affect the number of disconnected ECs or VSMCs compared with untreated controls (Figure 5.9). However, the reduction in EC velocity over time in the control group was not observed in any of the VEGF-C treated groups. Additionally, the different VEGF-C treatments did not affect the number of total and complete honeycombs or the size of their perimeter (Figure 5.10). Although this may not suggest any specific role for VEGF-C, the results from this study are preliminary and require further optimisation in terms of the concentration of VEGF-C treatments and also a larger sample size to counteract variability.

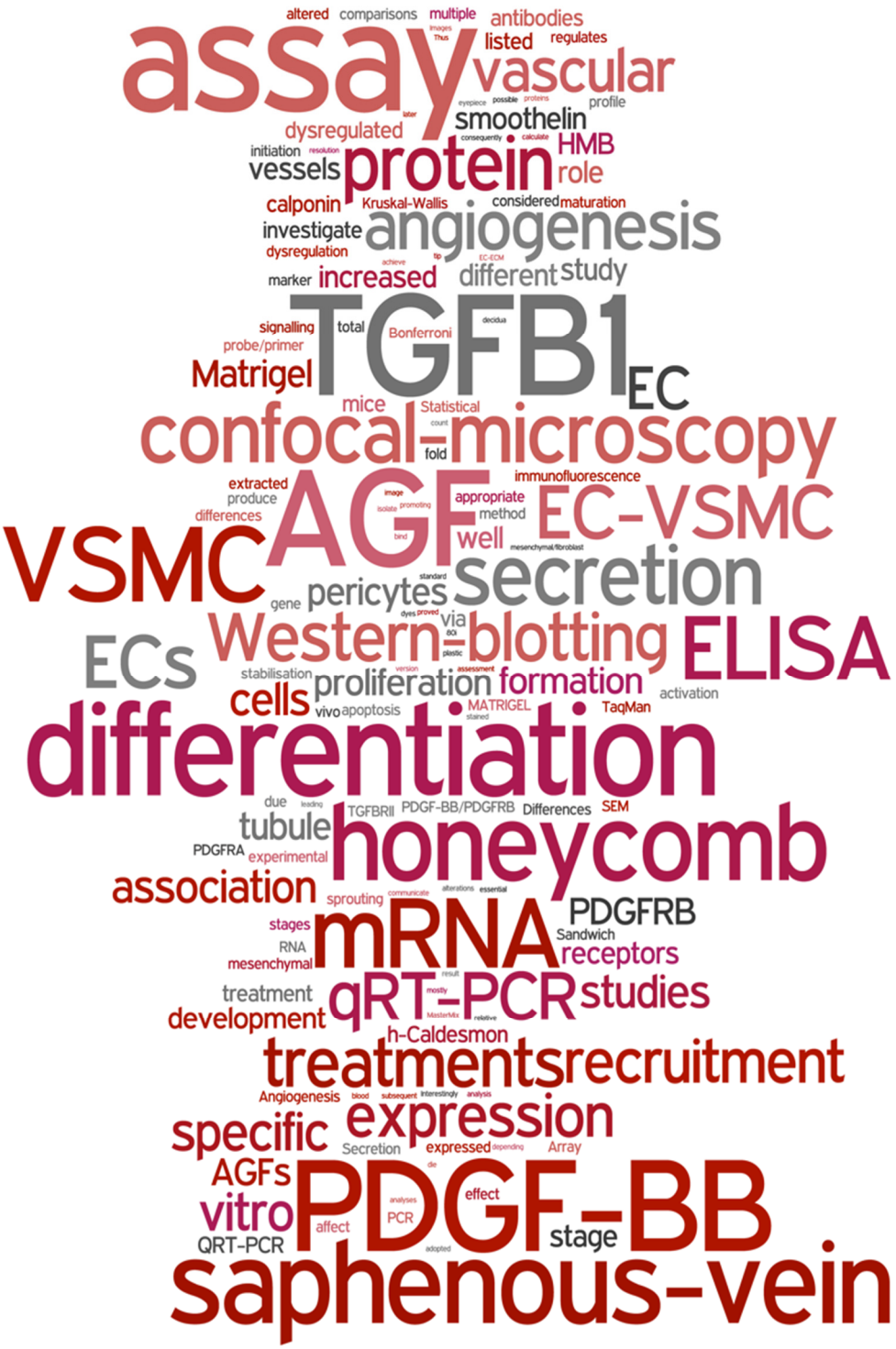
VEGF-C in VSMC differentiation: In order to investigate whether VEGF-C might affect EC-VSMC association by regulating VSMC differentiation, VSMCs were exposed to different VEGF-C treatments for the same length of time as during the *in vitro* assay. In particular the VSMC differentiation markers calponin and smoothelin were implicated in HMB perhaps underlying dysregulated vascular maturation. Therefore it was of interest to study whether VEGF-C affected the expression of these factors in VSMCs *in vitro*. However, the number of VSMCs expressing calponin and smoothelin remained unaltered in all treatment groups compared with untreated controls (Figure 5.11). Although 1ng/ml or 10ng/ml are the standard treatment concentrations utilised to study effects of molecules on physiological processes, this may need further optimisation, perhaps through a dose response assay. These data may not suggest a role for VEGF-C in VSMC differentiation *in vitro*, but the small sample size in this study may have affected the results due to variation; therefore inviting further research with a larger sample size.

5.6 CONCLUSION

The current study demonstrated that although both aorta and SV derived ECs and VSMCs were suitable for tubule formation and the study of EC-VSMC association, distinct differences existed in their AGF gene expression and their response to VEGF-C treatments. Therefore care should be taken to compare or draw conclusions from studies using cells from different sources in relation to the process *in vivo*. While ECs and VSMCs from different tissue vasculature may be phenotypically different at the molecular and consequently functional level, no gold standard assay or cell type currently exists. Moreover unless an *in vitro* angiogenesis assay utilises ECs and VSMCs from the specific vessels on which the scientific query is based on the results may not be directly translatable to the *in vivo* situation. However, as discussed earlier the nature of the endometrial vasculature makes it challenging to study and while methodology exists for isolation of endometrial EC no such methodology is currently available for endometrial VSMCs. Therefore, in the absence of a gold standard model and cells derived from the endometrial vasculature, it is only possible to develop and optimise an assay with cells from larger vessels such as the saphenous vein, keeping in mind factors such as cell characterisation, sustainability and reproducibility. Results from the application of the assay, has provided us with preliminary results, which would need to be tested with a larger sample size and if possible in future compared with cells from the endometrial vasculature before any definitive conclusion or direction can be drawn. Nonetheless, the assay was successfully optimised along with the development of a semi-automated image analysis protocol and shows promise for application to further study effects of other AGFs such as PDGF-BB and TGFβ1 on EC-VSMC association, VSMC recruitment and differentiation. Importantly, further research into the development of *in vitro* angiogenesis assays and utilising the quantitative data from these assays will help us to understand the basic angiogenic process and open doors to finding candidate molecules with potential clinical applications.

CHAPTER 6

EFFECTS OF ANGIOGENIC GROWTH FACTORS ON EC-VSMC ASSOCIATION AND VSMC DIFFERENTIATION *IN VITRO*



6 EFFECTS OF ANGIOGENIC GROWTH FACTORS ON EC-VSMC ASSOCIATION AND VSMC DIFFERENTIATION *IN VITRO*

6.1 INTRODUCTION

Angiogenic growth factors such as PDGF-BB and TGFβ1 play significant roles in regulation of blood vessel development including initiation of EC proliferation, migration, tubule formation, recruitment and differentiation of pericytes or VSMCs and vascular stabilisation. PDGF-BB binds to both PDGFRα and PDGFRβ; PDGF-BB and PDGFRβ are the most widely studied members of the PDGF family. PDGFRα is expressed in mesenchymal cell progenitors in lung, skin and intestine and is less well studied, but has been implicated in mesenchymal or fibroblast derived pathologies (Andrae *et al.*, 2008). PDGF-BB and PDGFRβ are mostly expressed in developing vessels, where quiescent ECs, especially the tip cells, express both PDGF-BB and PDGFRβ and communicate with perivascular cells, which express PDGFRβ (Gerhardt and Betsholtz, 2003). Moreover, *in vitro* studies have shown that stimulating ECs with PDGF-BB induces sprouting, proliferation and tubule formation (Battegay *et al.*, 1994), while studies in mice have shown that disruption of the PDGF-BB/PDGFRβ pathway results in weakened vascular structures and dilated vessels, due to irregular proliferation of ECs and loss of pericytes (Andrae *et al.*, 2008). Consistent with this, increased PDGFRβ expression correlated with VEGF-A and VEGFR2 overexpression that resulted in increased sprouting, along with increased pericyte recruitment and vascular development (Magnusson *et al.*, 2007). Collectively these studies highlight a specific role for PDGF-BB in promoting VSMC recruitment and vascular maturation though the PDGF-BB/PDGFRβ pathway.

PDGF-BB plays a role specifically in VSMC recruitment and vascular maturation, while TGFβ1 regulates VSMC recruitment as well as differentiation (Lebrin *et al.*, 2005). TGFβ1 may bind to and mediate signalling via TGFβRI or TGFβRII receptors. TGFβ1 has been shown to stimulate or inhibit angiogenesis *in vivo* and *in vitro*, depending on the experimental design and the specific stage of angiogenesis being studied (Goumans *et al.*, 2009). Furthermore, TGFβ1 has been shown to induce, as well as protect against, EC apoptosis;

while EC proliferation is an essential step in angiogenesis, especially at the activation stage, apoptosis of ECs via TGFβ1 is also crucial, since its inhibition leads to abnormal vessel formation (Ferrari *et al.*, 2009). Additionally, both TGFβ1 and VEGF have been implicated in increased EC differentiation, EC-ECM interaction thereby regulating vascular stabilisation, development and remodelling (Davis and Senger, 2008; Ferrari *et al.*, 2009; Margadant and Sonnenberg, 2010). During the resolution phase of angiogenesis, mesenchymal cells undergo differentiation to become pericytes or VSMCs, via EC induced TGFβ1 signalling. Consequently, pericytes or VSMCs are recruited onto the EC tubules resulting in muscularised and mature vessels (ten Dijke and Arthur, 2007; Shi and Chen, 2014). The critical role of TGFβ1 in VSMC recruitment is further indicated by studies in mice, which showed that the absence of TGFβ receptors TGFβRII or ALK5 was embryonically lethal due to vascular defects (Jiao *et al.*, 2006). Thus it is evident that TGFβ1 is critical both in the initiation, activation as well as later stages of angiogenesis.

The stage specific reliance of angiogenesis on AGFs highlights that dysregulation at any of these stages may give rise to vascular diseases (ten Dijke and Arthur, 2007; Demoulin and Essaghir, 2014; Heldin, 2014). Vascular dysregulation, including increased EC proliferation is implicated in HMB (Kooy *et al.*, 1996; Abberton *et al.*, 1999a; Abberton *et al.*, 1999b; Hurskainen *et al.*, 1999). In addition, we have shown dysregulated VSMC differentiation in terms of altered smoothelin and basic calponin staining (Chapter 3) (Biswas Shivhare *et al.*, 2014) and altered (primarily reduced) staining of PDGF-BB, TGFβ1 and their respective receptors in the endometrium of women with HMB (Chapter 4). AGFs such as PDGF-BB, TGFβ1, EGF and FGF promote VSMC differentiation; VSMC differentiation is regulated by the transcriptional activation of genes including calponin (CNN), smoothelin (SMTN), MyHC and αSMA through the transcription factor SRF and SMC specific co-factor myocardin (Wang *et al.*, 2001; Chen *et al.*, 2002). A secreted glycoprotein, cellular repressor of E1A-stimulated genes (CREG), has been shown to play important roles in regulating proliferation, migration and differentiation of VSMCs (Han *et al.*, 2009; Han *et al.*, 2010; Shi and Chen, 2014). In light of the importance of VSMC differentiation in dysregulated angiogenesis underlying HMB and the critical

roles of AGFs in angiogenesis, it is of interest to investigate the role of these AGFs on VSMC recruitment leading to EC-VSMC association and on subsequent VSMC differentiation. The absence of an appropriate *in vivo* model or *in vitro* assay to study dysregulated angiogenesis underlying HMB necessitated the development of the *in vitro* angiogenesis assay shown in Chapter 5. Using this assay we are now able to study the effects of PDGF-BB and TGFβ1 on EC-VSMC association, VSMC recruitment and differentiation.

6.2 HYPOTHESES AND AIMS

6.2.1 Hypotheses

PDGF-BB and TGFβ1 critically regulate angiogenesis; the expression of these AGFs and their receptors has been shown to be altered in HMB alongside dysregulated angiogenesis and VSMC differentiation. Therefore we hypothesised that:

1. PDGF-BB and TGFβ1 treatment may affect EC-VSMC association.

2. PDGF-BB and TGFβ1 treatment may result in alteration of AGF secretion and gene expression by ECs and VSMCs.

3. PDGF-BB and TGFβ1 treatment may affect VSMC differentiation.

6.2.2 Aims

To test the above hypotheses we aimed to:

1. Assess the effect of PDGF-BB and TGFβ1 treatment on EC-VSMC association *in vitro*.

2. Assess the effect of PDGF-BB and TGFβ1 treatment *in vitro* on AGF mRNA expression and protein secretion by both ECs and VSMC.

3. Assess the effect of PDGF-BB and TGFβ1 treatment on *in vitro* VSMC differentiation.

6.3 EXPERIMENTAL DESIGN

The experimental design adopted to achieve the specific aims set out in section 6.2.2, has been illustrated in Figure 6.1 and the specific materials and methods used are described below.

6.3.1 Enzymatic disaggregation of human saphenous veins

Human SVs were enzymatically disaggregated in order to isolate ECs and VSMCs used in the *in vitro* assay. The process of enzymatic disaggregation along with preparation of cell culture treatments and supernatants has been described in section 2.2.6.7, Figure 2.6.

6.3.2 EC-VSMC association and tubule formation assay

In vitro EC-VSMC association and tubule formation assay was performed as described in sections 5.3.3 and 5.3.4 and Figure 5.2.

6.3.2.1 Treatment with AGFs and neutralising antibodies

The method for AGF treatments (PDGF-BB, TGFβ1, neutralising antibodies) has been described in section 5.3.7.1 and the constituents of the treatment media have been described in Appendix B.

6.3.2.2 Vital dyes and confocal time-lapse microscopy

The vital dyes and microscopy parameters used for the *in vitro* assay have been described in section 5.3.5.

6.3.2.3 Quantitative image analysis

Quantitative image analysis for EC-VSMC association and tubule formation was been carried out as described in section 5.3.6.

6.3.3 qRT-PCR

qRT-PCR was used to investigate the effect of AGFs on mRNA of a number of genes in ECs and VSMCs. Total RNA was extracted from the ECs and VSMCs, treated overnight with PDGF-BB or TGFβ1, using the TRIZOL protocol as described in section 2.2.7. Real-time RT–PCR was performed using TaqMan Universal PCR MasterMix as described in section 2.2.7.4. A list of TaqMan probe/primer sets utilized has been listed in Table 2.5. All probe/primer

Figure 6.1: Experimental design to study the effect of PDGF-BB and TGFβ1 on EC-VSMC association and VSMC differentiation *in vitro*.

sets were previously validated for use with a laboratory standard total RNA extracted from early pregnancy decidua. The $2^{-\Delta\Delta CT}$-method was used to calculate relative fold changes in gene expression as described in section 2.2.7.6.

6.3.4 ELISA and Quantibody Human Angiogenesis Array

Sandwich ELISA and Quantibody Human Angiogenesis Array were used to study the effect of AGF treatment on the profile of secreted AGFs by ECs and VSMCs. Sandwich ELISA was performed as described in section 2.2.9.1 and Quantibody Human Angiogenesis Array was performed as described in section 2.2.10.

6.3.5 Western blotting

Western Blotting was used to investigate the effects of AGF treatments on VSMC differentiation marker (calponin, h-Caldesmon and smoothelin). Western blotting was performed as described in section 2.2.8 and the antibodies used have been listed in Table 2.6.

6.3.6 Immunofluorescence, Immunocytochemistry

Western blotting proved unsuitable to detect calponin, h-Caldesmon and smoothelin taking into consideration the amount of starting material available. Therefore, immunofluorescence was used next to study the effect of AGF treatments on VSMC differentiation markers as described in section 2.2.5. However ECs and VSMCs were highly auto-fluorescent; therefore use of immunofluorescence could have masked the quantification of any possible alterations in the intensity of staining for the VSMC differentiation markers. Therefore, immunocytochemistry was performed using an indirect avidin-biotin technique to study the effect of AGF treatment on VSMC differentiation (calponin, h-Caldesmon and smoothelin) as described in section 2.2.3.1 and the antibodies used have been listed in Table 2.2.

6.3.6.1 Quantitative image analysis

Immunostained slides were independently blinded prior to assessment. Images were quantified as fold change compared with the control group, of percentage

of positively stained cells (by count) for calponin or smoothelin in comparison with the total number of cells in five randomly selected fields of views within each well using the ImageJ software package.

6.3.7 Statistical analysis

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc.). Data are presented as means ± standard error of mean (SEM) and differences are considered statistically significant at $P \leq 0.05$. Specific tests used are given in the results.

6.4 RESULTS

6.4.1 Role of PDGF-BB and TGFβ1 on EC-VSMC association *in vitro*

6.4.1.1 Effect of PDGF-BB on EC-VSMC association

Number and velocity of ECs, VSMCs: The number of ECs remained unaltered throughout the 10 hr assay in all PDGF-BB treated and untreated groups (Figure 6.2A). EC velocity was unchanged throughout in the control and groups treated with 10ng/ml PDGF-BB or 10ng/ml anti-PDGFBB, but was decreased in the group treated with 1ng/ml PDGF-BB at 10hr, compared with 2 hr (P=0.035) and 4 hr (P=0.022; Figure 6.2B).

The number of VSMCs remained unaltered throughout the 10 hr assay in all PDGF-BB treated and untreated groups (Figure 6.2C). VSMC velocity was unchanged throughout for the groups treated with 1ng/ml and 10ng/ml PDGF-BB, but decreased at 10 hr compared with 2 hr in both the controls (P=0.009) and after treatment with 10ng/ml anti-PDGFBB (P=0.006; Figure 6.2D).

Total number of honeycombs: The total number of honeycombs (≥3 arms) was reduced at 10 hr compared with 15 min in the control group (P=0.012) and in the groups treated with 1ng/ml PDGF-BB (P=0.021) or 10ng/ml anti-PDGFBB (P=0.046; Figure 6.3A). However, the total number of honeycombs remained unaltered after treatment with 10ng/ml PDGF-BB.

Number of complete honeycombs: The number of complete honeycombs reduced from 2 hr to 10 hr but was not statistically significant in either control or PDGF-BB treated groups (Figure 6.3B).

Honeycomb perimeter: The perimeter of complete honeycombs in the group treated with 10ng/ml anti-PDGFBB increased after 6 hr (P=0.016) and 10 hr (P=0.017; Figure 6.3C) compared with 15min. However it remained unaltered for the other groups.

VSMC coverage: The percentage of VSMC coverage of honeycombs appeared to increase over time in all groups, although this was not statistically significant in either control or PDGF-BB treated groups (Figure 6.3D).

Figure 6.2: Graphical representation (mean ± SEM) of fold change in number and velocity of PDGF-BB treated endothelial cells (EC) and vascular smooth muscle cells (VSMC) on matrigel. Fold change in saphenous vein derived EC (**A**) number and (**B**) velocity and VSMC (**C**) number and (**D**) velocity (N=3 each group) at different time points relative to 2 hr between untreated control or PDGF-BB treatments, on matrigel. Pairwise Kruskal-Wallis one way ANOVA determined significant difference between different time points and between untreated control or PDGF-BB treatments. Bars with same letters are significantly different from each other; ^{A}P=0.035, ^{B}P=0.022, ^{C}P=0.009, ^{D}P=0.006.

Figure 6.3: Graphical representation (mean ± SEM) of fold change in PDGF-BB treated (EC) honeycombs (HC) and vascular smooth muscle cell (VSMC) coverage. Fold change at different time points relative to 15 min in number of (**A**) total HC with ≥3arms, (**B**) complete HC, (**C**) perimeter of complete HC and (**D**) percentage of VSMC coverage of complete HC by saphenous vein derived ECs and VSMCs (N=3 each group). Pairwise Kruskal-Wallis one way ANOVA determined significant difference between different time points and between untreated control or PDGF-BB treatments. Bars with same letters are significantly different from each other. 'a' represents difference between treatments, 'A-E' represent difference between time points; ^{a}P=0.012, ^{A}P=0.012, ^{B}P=0.021, ^{C}P=0.046, ^{D}P=0.016, ^{E}P=0.017.

6.4.1.2 Effect of TGFβ1on EC-VSMC association

Number and velocity of ECs, VSMCs: The number of ECs decreased at 10 hr compared with 2 hr in the control group (*P*=0.013) and after treatment with 10ng/ml anti-TGFβ1 (*P*=0.013; Figure 6.4A). EC velocity remained unaltered throughout the 10 hr assay for control and treatment groups (Figure 6.4B).

The number of VSMCs decreased at 10 hr compared with 2 hr in the group treated with 10ng/ml anti-TGFβ1 (*P*=0.006), but remained unchanged for the other groups (Figure 6.4C). VSMC velocity remained unaltered throughout the 10 hr assay for control, 10ng/ml TGFβ1 and anti-TGFβ1 groups, but decreased for the group treated with 1ng/ml TGFβ1 at 10 hr compared with 2 hr (*P*=0.005; Figure 6.4D).

Total number of honeycombs: The total number of honeycombs (≥3 arms) was reduced at 10 hr compared with 15 min in the control group (*P*=0.005) and after treatment with 1ng/ml TGFβ1 (*P*=0.001), 10ng/ml TGFβ1 (*P*=0.006) and 10ng/ml anti-TGFβ1 (*P*=0.003; Figure 6.5A).

Number of complete honeycombs: The number of complete honeycombs was reduced at 10 hr compared with 15 min in the control group (*P*=0.040) and after treatment with 1ng/ml TGFβ1 (*P*=0.022), 10ng/ml TGFβ1 (*P*=0.028) and 10ng/ml anti-TGFβ1 (*P*=0.010; Figure 6.5B).

Honeycomb perimeter: The perimeter of complete honeycombs increased in the control group (8 hr *P*=0.003; 10 hr *P*=0.004) and after treatment with 10ng/ml anti-TGFβ1 (8 hr *P*=0.007; 10 hr *P*=0.002), compared with 15 min (Figure 6.5C). The perimeter of complete honeycombs remained unaltered in the other groups.

VSMC coverage: The percentage VSMC coverage of honeycombs appeared to increase over time, although this was not statistically significant in control or TGFβ1 treated groups (Figure 6.5D).

Figure 6.4: Graphical representation (mean ± SEM) of fold change in number and velocity of TGFβ1 treated endothelial cells (EC) and vascular smooth muscle cells (VSMC) on matrigel. Fold change in saphenous vein derived EC (**A**) number and (**B**) velocity and VSMC (**C**) number and (**D**) velocity (N=3 each group) at different time points relative to 2 hr between untreated control or PDGF-BB treatments, on matrigel. Pairwise Kruskal-Wallis one way ANOVA determined significant difference between different time points and between untreated control or TGFβ1 treatments. Bars with same letters are significantly different from each other; $^{A,B}P$=0.013, ^{C}P=0.006, ^{D}P=0.005.

Figure 6.5: Graphical representation (mean ± SEM) of fold change in TGFβ1 treated (EC) honeycombs (HC) and vascular smooth muscle cell (VSMC) coverage. Fold change at different time points relative to 15 min in number of (**A**) total HC with ≥3arms, (**B**) complete HC, (**C**) perimeter of complete HC and (**D**) percentage of VSMC coverage of complete HC by saphenous vein derived ECs and VSMCs (N=3 each group). Pairwise Kruskal-Wallis one way ANOVA determined significant difference between different time points and between untreated control or TGFβ1 treatments. Bars with same letters are significantly different from each other. 'A-L' represent difference between time points; ^{A}P=0.005, ^{B}P=0.001, ^{C}P=0.006, $^{D,I}P$=0.003, ^{E}P=0.040, ^{F}P=0.022, ^{G}P=0.028, ^{H}P=0.010, ^{J}P=0.004, ^{K}P=0.007, ^{L}P=0.002.

6.4.2 Role of PDGF-BB and TGFβ1 on EC-VSMC gene expression and secretion profile *in vitro*

6.4.2.1 Effect of PDGF-BB on EC AGF gene expression and secretion

AGF mRNA (Ang1, Ang2, Tie2, VEGF-A, VEGF-C, VEGFR1-3, PDGF-BB, PDGFRα, PDGFRβ, TGFβ1, TGFβRI and TGFβRII) was unaltered in ECs treated with different concentrations of PDGF-BB or its neutralising antibody compared with controls (Figure 6.6A-D).

AGF secretion (Angiogenin, Ang1, Ang2, VEGF-A, VEGF-C, PIGF, PDGF-BB, TGFβ1, bFGF, HGF, leptin, EGF and HB-EGF) was also unaltered in ECs treated with different concentrations of PDGF-BB or its neutralising antibody compared with controls (Figure 6.6E-H).

6.4.2.2 Effect of TGFβ1 on EC AGF gene expression and secretion

AGF mRNA was unaltered in ECs treated with different concentrations of TGFβ1or its neutralising antibody (Figure 6.7A-D), except for PDGF-BB, which was increased over two-fold when treated with 10ng/ml TGFβ1, compared with controls (P=0.03; Figure 6.7C).

AGF secretion was unchanged in ECs treated with different concentrations of TGFβ1 or its neutralising antibody (Figure 6.7E-H).

6.4.2.3 Effect of PDGF-BB on VSMC AGF gene expression and secretion

AGF mRNA (Figure 6.8A-D) and secretion (Figure 6.8E-H) did not alter in VSMCs treated with different concentrations of PDGF-BB or its neutralising antibody.

Figure 6.6: Graphical representation (mean ± SEM) of fold change in (**A-D**) mRNA and (**E-H**) secretion of angiogenic growth factors by PDGF-BB treated saphenous vein derived endothelial cells (N=3 each group) cultured on matrigel. Pairwise Kruskal-Wallis one way ANOVA determined significant difference in fold change between control or PDGF-BB treatments. No significant differences were observed.

Figure 6.7: Graphical representation (mean ± SEM) of fold change in (**A-D**) mRNA and (**E-H**) secretion of angiogenic growth factors by TGFβ1 treated saphenous vein derived endothelial cells (N=3 each group) cultured on matrigel. Pairwise Kruskal-Wallis one way ANOVA determined significant difference in fold change between control or TGFβ1 treatments. 'a' represents difference between control and 10ng/ml TGFβ1 treatment; [a]*P*=0.03.

Figure 6.8: Graphical representation (mean ± SEM) of fold change in (**A-D**) mRNA and (**E-H**) secretion of angiogenic growth factors by PDGF-BB treated saphenous vein derived vascular smooth muscle cells (N=3 each group) cultured on matrigel. Pairwise Kruskal-Wallis one way ANOVA determined significant difference in fold change between control or PDGF-BB treatments. No significant differences were observed.

6.4.2.4 Effect of TGFβ1 on VSMC AGF gene expression and secretion

AGF mRNA was unaltered in VSMCs treated with different concentrations of TGFβ1 or its neutralising antibody compared with controls (Figure 6.9A-D).

AGF secretion did not change in VSMCs treated with different concentrations of TGFβ1 or its neutralising antibody (Figure 6.9E-H), except for TGFβ1, which was increased over two-fold when treated with 10ng/ml TGFβ1 compared with controls (*P*=0.019; Figure 6.9G).

6.4.3 Role of PDGF-BB and TGFβ1 on VSMC differentiation *in vitro*

Using Western blot analysis calponin and h-Caldesmon was undetectable in VSMCs, even though positive control samples gave appropriately sized bands (Figure 6.10A2; Figure 6.10B2). Non-specific bands were detected for smoothelin in pregnant myometrial tissue extract (10-40µg; Figure 6.10C1), with specific bands only detected when 100µg total protein was loaded (Figure 6.10B2). However 100µg of total protein was unavailable from isolated VSMCs; western blotting analysis was therefore unsuitable for investigating the effects of PDGF-BB or TGFβ1 on VSMC differentiation.

The number of calponin$^+$ or smoothelin$^+$ VSMCs, as determined by immunocytochemistry, remained unaltered after treatment with different concentrations of PDGF-BB or TGFβ1 or their neutralising antibody (Figure 6.11A-B).

mRNA of different genes involved in VSMC differentiation; myocardin (Myocd), CREG1, CREG2, SRF, CNN1, CNN2 and SMTN remained unaltered in VSMCs treated with different concentrations of PDGF-BB or TGFβ1, except for a slight increase in SMTN after treatment with 10ng/ml PDGF-BB (*P*=0.023; Figure 6.11C-D).

Figure 6.9: Graphical representation (mean ± SEM) of fold change in (**A-D**) mRNA and (**E-H**) secretion of angiogenic growth factors by TGFβ1 treated saphenous vein derived vascular smooth muscle cells (N=3 each group) cultured on matrigel. Pairwise Kruskal-Wallis one way ANOVA determined significant difference in fold change between control or TGFβ1 treatments. 'a' represents difference between control and 10ng/ml TGFβ1 treatment; ^{a}P=0.019.

Figure 6.10: Western blot analysis for calponin, smoothelin and h-Caldesmon. (**A1, B1**) respectively shows calponin (~29kDa) and h-Caldesmon (~110kDa) in non-pregnant (NP1) and pregnant (P1-2) myometrial protein extract (10µg). (**C1**) shows non-specific bands for smoothelin (~110kDa) in pregnant myometrial protein extract (10-40µg). (**A2**) shows undetectable calponin in saphenous vein derived vascular smooth muscle cells (VSMCs) (V1-6; 14µg). (**B2**) shows undetectable h-Caldesmon in aortic VSMCs (10-40µg). (**C2**) shows smoothelin specific bands around 110kDa only in P1-2 (100µg protein). (**A3, B3, C3**) shows napthol blue black staining of PVDF membranes corresponding to **A2, B2** and **C2** respectively.

Figure 6.11: Graphical representation (mean ± SEM) of fold change in protein and mRNA of differentiation markers in saphenous vein derived vascular smooth muscle cells (VSMCs) treated with PDGFBB or TGFβ1. Fold change, compared with control in (**A-B**) percentage of calponin^{+} or smoothelin^{+} saphenous vein VSMCs and (**C-D**) mRNA of different genes involved in VSMC differentiation; myocardin (Myocd), cellular repressor of E1A-stimulated genes (CREG1, CREG2), SRF, calponin (basic-CNN1, CNN2), and smoothelin (SMTN) in VSMCs cultured on matrigel (N=3 each group). Pairwise Kruskal-Wallis one way ANOVA determined significant difference between control or (**A-C**) PDGFBB, and (**B-D**) TGFβ1 treatments. 'a' represents difference between control and 10ng/ml PDGFBB treatment; ^{a}P=0.023.

6.5 DISCUSSION

Angiogenesis plays a critical role in the normal menstrual cycle, the dysregulation of which is implicated in HMB with altered AGF expression and VSMC differentiation (Chapters 3, 4). The importance of studying angiogenesis led to the development and optimisation of an *in vitro* angiogenesis assay capable of studying dynamic EC-VSMC associations and the possible effects of AGFs on the process of VSMC differentiation and recruitment of VSMCs to EC honeycomb structures (Chapter 5). Therefore this chapter utilised the *in vitro* assay to study the potential roles of PDGF-BB and TGFβ1 on EC-VSMC association and further investigate if alterations observed in EC-VSMC association are due to altered AGF secretion and/or mRNA expression by ECs, VSMCs and VSMC differentiation.

6.5.1 Effect of PDGF-BB and TGFβ1on EC-VSMC association

PDGF-BB in EC-VSMC association: Results from the *in vitro* assay did not show any effect of PDGF-BB treatment on the number of disconnected ECs and VSMCs over time (Figure 6.2). No reduction in numbers of disconnected ECs over time i.e. a higher number of isolated ECs in culture may suggest reduced honeycomb formation; thereby also reducing VSMC attachment onto the honeycombs and consequently leading to a greater number of disconnected VSMCs over time. This may be caused primarily by two factors: first PDGF-BB treatment and secondly cell seeding density. If unaltered numbers of ECs or VSMCs is an effect of PDGF-BB, then the numbers must differ from the control group. However there was no alteration in the number of ECs or VSMCs over time in the PDGF-BB control group; therefore the unaltered number of ECs or VSMCs is not due to an effect of PDGF-BB. On the other hand if there were greater number of cells (both ECs and VSMCs) at the start of the experiment, even after honeycomb formation and VSMC recruitment, there would be a higher number of isolated cells left in the medium, which would remain relatively unaltered over time, as suggested by the results; although this is unlikely since the seeding density was the same for all *in vitro* studies. PDGF-BB (1ng/ml) was shown to reduce EC velocity while anti-PDGFBB (10ng/ml) reduced VSMC velocity at 10hr; this may also be due to higher numbers of cells and

consequently a lower amount of space within the culture, however this reduction in velocity did not differ significantly from the control group (Figure 6.2).

The assay was unable to detect any effect of PDGF-BB on the number of total honeycombs, complete honeycombs, perimeter of honeycombs and VSMC coverage, compared with those in the control group (Figure 6.3). Interestingly, the number of total honeycombs and complete honeycombs in the group treated with 10ng/ml PDGF-BB remained stably higher at 10 hr compared with the control and other treatment groups, albeit not statistically significant (Figure 6.3). Since growth factor treatment medium was added after the formation of EC honeycombs (4.5 hr after seeding of ECs), this might suggest that PDGF-BB may help to sustain honeycomb structures i.e. EC-EC attachment *in vitro*. Coincidentally, stimulation of ECs with PDGF-BB has previously been shown to induce sprouting, proliferation and tube formation *in vitro* (Battegay *et al.*, 1994).

TGFβ1 in EC-VSMC association: The *in vitro* assay was unable to detect any effect of TGFβ1 on the number and velocity of ECs. Anti-TGFβ1 slightly reduced the number of VSMCs and 1ng/ml TGFβ1 reduced VSMC velocity at 10 hr in the assay, although neither of these were different from those seen in the control group (Figure 6.4). Additionally, reduction in the number of total honeycombs, complete honeycombs and an increase in honeycomb perimeter was observed at 10 hr, although these changes were not statistically different to those observed in the respective control groups (Figure 6.5). TGFβ1 also had no effect on VSMC coverage *in vitro*. The reduction in the number of honeycombs may suggest a disruptive effect of TGFβ1 treatment on EC-EC attachment. However a reduction also seen in the anti-TGFβ1 group, may suggest otherwise. Interestingly an increase in honeycomb perimeter in the anti-TGFβ1 group, although not statistically significant, possibly suggested that as the perimeter of the honeycombs becomes large, a relatively low number of them are sustained over time i.e. the larger the honeycombs the smaller their number in culture and vice versa. Interestingly, depending on the experimental setup, TGFβ1 has been shown to stimulate or inhibit angiogenesis *in vivo* and *in vitro* (Goumans *et al.*, 2009). Results from this study are preliminary and may not direct any definitive conclusion; future studies with a larger sample size may

help to understand possible roles of TGFβ1 on EC-VSMC association. Meanwhile, these preliminary results from the *in vitro* study raise the question of further optimisation of the assay to determine its suitability for investigating EC-VSMC association and VSMC recruitment and the effects of AGFs on this process.

One of the factors which may have affected results of the *in vitro* assay is the AGF treatment concentration. Although the study included the concentrations of 1ng/ml and 10ng/ml, it is uncertain whether these levels were sufficient to induce any effect on EC-VSMC association. Moreover, studies in the past have utilised AGFs at concentrations of up to 100ng/ml and neutralising antibodies up to 50ng/ml to study their effects on cell migration, proliferation etc., albeit the type of cells or tissue of origin of ECs/VSMCs varied. Perhaps absolute quantification of the amount of endogenously expressed or secreted AGFs by these cells in culture for specific lengths of time could help to determine the range of AGF treatment concentrations to use, especially for the neutralising antibodies. Therefore this invites further optimisation of the existing assay including determination of a dose-response relationship between the concentration and length of time for the AGF treatment and its effect on EC-VSMC association and VSMC recruitment *in vitro* as assessed through the percentage of VSMC coverage.

Another factor affecting the results could be inter and intra experiment variability; this would include differences in the number of honeycombs formed and the number of each type of cells at the start of an experiment; although to avoid this bias the current study looked at relative change, in future an increase in sample size should benefit interpretation of results. Finally in order to increase sensitivity of the assay, perhaps an automated image analysis protocol may be established such that tubule length (honeycomb arm) rather than honeycomb perimeter is determined at each time point throughout the assay along with the length of co-localisation between ECs and VSMCs (on the tubule) depicting VSMC recruitment. This would not only reduce user variability but also increase the accuracy of determination of the percentage of VSMC coverage. Collectively, the present assay would not only require optimisation

with respect to determining a dose-response relationship for each AGF, but would also benefit from a modified fully automated analysis protocol.

6.5.2 Effect of PDGF-BB and TGFβ1 on AGF gene expression and secretion by ECs and VSMCs

Secretion of AGFs and mRNA expression in ECs was minimally affected by TGFβ1, while not affected by PDGF-BB. TGFβ1 treatment (10ng/ml) increased EC PDGF-BB mRNA expression by two-fold (Figures 6.6-6.7). This suggests that TGFβ1 modulates the transcription of PDGF-BB although whether this is also translated and/or activated leading to further downstream effects requires further investigation. If TGFβ1 mediated alteration in the transcription of PDGF-BB in ECs in fact leads to further downstream effects, any alteration in EC-VSMC association shown in the *in vitro* study using TGFβ1 could possibly be a combined result of TGFβ1 and PDGF-BB on ECs.

Secretion of AGFs and mRNA expression in VSMCs was minimally affected by TGFβ1, while not affected by PDGF-BB. TGFβ1 treatment (10ng/ml) led to a two-fold increase in TGFβ1 secretion by VSMCs (Figure 6.8-6.9). This TGFβ1 mediated TGFβ1 secretion by VSMCs may affect VSMC differentiation and recruitment by ECs *in vitro*. Thus any alteration in EC-VSMC association shown in the *in vitro* study using TGFβ1 may be due to its effect on ECs as well as VSMCs.

6.5.3 Effect of PDGF-BB and TGFβ1 on VSMC differentiation

PDGF-BB treatment (10ng/ml) led to an increase in smoothelin mRNA expression in VSMCs *in vitro*, but this may not have been translated since no alteration in smoothelin expression was observed. Additionally, PDGF-BB did not alter calponin expression (Figure 6.11). This is striking since PDGF is known to promote maturation of blood vessels through VSMC recruitment and/or differentiation. Moreover, TGFβ1 treatment had no effect on either mRNA or expression of calponin, smoothelin or other genes regulating VSMC differentiation (Figure 6.11). This may indicate that alterations seen in the *in vitro* study with respect to EC-VSMC association and consequent VSMC

recruitment may not be due to altered VSMC differentiation. Although not statistically significant, TGFβ1 treatment appeared to increase while PDGF-BB appeared to downregulate mRNA expression for myocardin, which is the SMC specific transcriptional co-factor for genes regulating SMC differentiation; thereby possibly still directing a role for these AGFs in VSMC differentiation *in vitro* (Figure 6.11). As described previously, one factor that could have affected both the protein and gene expression results is the concentration of AGF treatments. Moreover, it should be noted that performance of IHC on cells grown on matrigel might have resulted in non-specific staining, yielding false positively stained cells, which might have masked any alteration in smoothelin or calponin expression by PDGF-BB treatment. One possible solution could be to recover the AGF treated cells from matrigel using the BD cell recovery solution (♯354253, BD Biosciences) followed by immunostaining. Since this solution allows for non-enzymatic cell separation, cells should maintain any biochemical alteration such as differentiation marker expression, which can then be analysed through IHC. It may also be beneficial to culture cells on glass bottom culture dishes, instead of the small 8-well chamber slides, which would allow a larger number of cells for culture and consequently the option to investigate protein expression for several different differentiation markers. Additionally supernatants can directly be collected from these cultures for further analysis of the AGF secretion profile.

6.6 CONCLUSION

The current study suggested that PDGF-BB and TGFβ1 might affect EC-VSMC association *in vitro* through altering EC, VSMC number or velocity. However the study did not detect any effect of PDGF-BB and TGFβ1 treatments on maintaining EC mediated honeycomb formation; although TGFβ1 might alter honeycomb formation in terms of their number and perimeter. The study did not demonstrate any effect of PDGF-BB on secretion and mRNA expression of AGFs by ECs and VMSCs while it showed that TGFβ1 specifically induced PDGF-BB mRNA expression in ECs and TGFβ1 secretion by VSMCs. The results revealed minimal effects of PDGF-BB and TGFβ1 on VSMC differentiation *in vitro*, although they did hint at alterations in myocardin and smoothelin gene expression. The *in vitro* studies discussed here and in Chapter 5 did not allow any definite conclusions to be drawn in terms of the effects of VEGF-C, PDGF-BB, and TGFβ1 on EC-VSMC association, VSMC differentiation and/or AGF secretion and mRNA expression. Therefore further optimisation of methodology and increased sample size is required prior to assay validation. It should perhaps also be noted that although the complex process of angiogenesis is regulated by each of these factors, their effect on angiogenesis is a result of the combination of their specific roles, which are not necessarily independent of each other. A possible synergistic role of VEGF-C, PDGF-BB and TGFβ1 could perhaps also be taken into account in future experiments, possibly incorporating multiple combinations of AGF treatments such as PDGFBB-TGFβ1 etc. Nonetheless development and validation of *in vitro* models to study vascular maturation remains vital towards unravelling underlying complexities of angiogenesis the dysregulation of which may result in diseases such as heavy menstrual bleeding.

CHAPTER 7

GENERAL DISCUSSION AND FUTURE STUDIES

Life can only be understood backwards;
but it must be lived forwards.

Søren Kierkegaard

7 GENERAL DISCUSSION AND FUTURE STUDIES

7.1 DISCUSSION

Heavy menstrual bleeding affects 30% of women of reproductive age disturbing their quality of life alongside posing a heavy financial burden on the NHS; yet not much is known or understood about its underlying mechanisms. A few studies in the past had implicated a role of uterine blood vasculature in the pathogenesis of HMB (Kooy *et al.*, 1996; Abberton *et al.*, 1999a; Abberton *et al.*, 1999b; Hurskainen *et al.*, 1999). Considering the importance of blood vessel development in menstrual bleeding it is unsurprising that dysregulated angiogenesis may underlie menstrual bleeding disorders such as HMB. On the basis of the key findings the current study set out to investigate two principal queries:

1. To what extent is the uterine blood vessel development impaired in women with HMB?

2. How is this vascular impairment affected by angiogenic growth factors?

7.1.1 Impaired blood vessel development in women with heavy menstrual bleeding

Blood vessels are primarily made up of ECs, VSMCs and the ECM; the development of which heavily relies on the tissue specific composition of the vascular bed i.e. the endometrial or myometrial stroma. Since the menstrual cycle involves active tissue breakdown and repair and is considered to be an inflammatory process, an important regulator of endometrial angiogenesis is its stromal leukocyte population. Additionally while VSMCs and ECs have been implicated in HMB (Kooy *et al.*, 1996; Abberton *et al.*, 1999a; Abberton *et al.*, 1999b), uNK cells have been associated with reproductive disorders; particularly blood vessel development in the endometrium of women with RM (Quenby *et al.*, 2005; Lash *et al.*, 2011a) and have been shown to regulate endometrial bleeding by maintaining arterial stability (Wilkens *et al.*, 2013).

The current study showed for the first time that ECs in the uterine blood vasculature of women with HMB show altered pattern of staining (Table 3.1), which were associated with their proliferative state (Figure 3.10). This may be

suggestive of an altered vascular endothelium, which in turn may affect EC-VSMC association and subsequently VSMC differentiation; this may result in immature, weaker or leaky vessels or altered vascular tone affecting the rate of blood flow underlying HMB. In line with this theory, the study demonstrated for the first time an altered state of VSMC differentiation, especially in terms of smoothelin and calponin expression (Figure 3.6). Interestingly decreased calponin had been shown to result in an increase in cross-bridge cycle, i.e. increased shortening velocity and therefore an increased rate of contraction and blood flow (Fujishige *et al.*, 2002). Therefore the reduction in vascular calponin staining shown in the current study may also suggest an increased rate of contraction and blood flow in HMB, while an increase in smoothelin staining may facilitate contractility and vasodilation, again, leading to increased blood flow in HMB. However, endometrial vessels are thought to be resistance vessels and the mechanism of blood flow in these vessels may differ from those in the cardiovascular system; although changes shown in the current study may well indicate an underlying functional change in the endometrial vessels causing them to operate similar to those in the cardiovascular system.

A key finding of the present study is that the reduction in calponin in the late secretory phase of the menstrual cycle was also shown to associate with increased osteopontin expression (Figure 3.12). Osteopontin, an ECM component is induced in VSMCs in cardiovascular diseases and is elevated in traumatised tissue (Sodek *et al.*, 2000; Waller *et al.*, 2010). But importantly it has been shown to downregulate calponin expression (Gao *et al.*, 2012); thus suggesting osteopontin as a regulator of endometrial vascular structure-function relationship, possibly underlying HMB. Additionally the increased osteopontin could also be indicative of increased vascular breakdown in HMB, which along with decreased collagen IV staining may again suggest weaker vasculature in HMB. Along with alterations observed in the structure of the endometrial blood vessels, the current study also revealed an altered endometrial uNK cell population during the menstrual cycle and critically the alteration in the late secretory phase in HMB showed altered staining of CD3 by the uNK cells (Figures 3.14-3.15). Since uNK cells are implicated in a wide range of functions; cytokine and growth factor secretion (Ang1, Ang2, VEGF-C, PIGF and TGF-β1),

immune function, regulation of trophoblast invasion and also in the initial stages of spiral artery remodelling, the results from our study may suggest altered AGFs in the endometrial stroma further affecting endometrial angiogenesis. Importantly uNK cells are positively correlated with vascular maturation. Therefore a decrease in uNK cell number in the late secretory phase of HMB may indicate reduced vascular maturation at the onset of menstruation underlying HMB.

7.1.2 Role of AGFs in dysregulated angiogenesis underlying heavy menstrual bleeding

7.1.2.1 Altered AGF staining in uterine tissue layers in women with heavy menstrual bleeding

Blood vessel development is a complex multistep process under the strict regulation of AGFs. Additionally our previous studies suggested that altered AGF staining in the uterine tissue layers may underlie the dysregulated angiogenesis observed in HMB. Investigation of AGFs in fact showed distinct alterations in AGF staining with respect to the stroma, glands and the blood vessels. In HMB, staining for PDGF-BB (Figure 4.3), PDGFRα (Figure 4.4), PDGFRβ (Figure 4.5), TGFβ1 (Figure 4.7), TGFβRI (Figure 4.8) and TGFβRII (Figure 4.9) were all decreased across the menstrual cycle in both endometrium and myometrium, especially in the stromal cells and ECs followed by the glandular epithelium and VSMCs. Critically the reduction in both the PDGF and TGFβ family of AGFs suggested that the dysregulation in angiogenesis in HMB may not be during the activation phase of angiogenesis; instead it is perhaps during the resolution phase thereby affecting vessel stability, VSMC differentiation and consequently vascular maturation. Moreover, altered AGF expression also affects ECM production by ECs in culture, which may add to the significance of these findings and also support the results from our studies on altered vascular ECM composition in HMB. Rather than an early breakdown, collectively the results from the IHC studies in HMB distinctly highlight that altered AGF staining associated with dysregulated vascular maturation in terms of EC-VSMC interaction and subsequent VSMC differentiation underlies HMB.

7.1.2.2 *In vitro* model to study EC-VSMC association

An alteration in AGFs and a role of altered vascular maturation indicated by our previous studies fuelled the need for investigation of the roles of AGFs in angiogenesis, especially on EC-VSMC association, VSMC recruitment and VSMC differentiation. Although existing angiogenesis assays have studied EC migration, proliferation and mediated tubule formation, to our knowledge, no assay has ever been optimised to look at the dynamic EC-VSMC interaction and VSMC recruitment following EC tubule formation. Therefore for the first time in this present study we have established an *in vitro* assay together with a semi-automated quantitative image analysis protocol, for studying EC-VSMC interaction, with possible applications including the study of effects of AGFs on angiogenesis (Figure 5.2).

7.1.2.3 Effect of AGFs on EC-VSMC association and VSMC differentiation *in vitro*

The final aim of the current study was utilisation of the *in vitro* angiogenesis assay to study the effects of AGFs on EC-VSMC association, VSMC recruitment and critically VSMC differentiation. The primary purpose of this assay is justified by the previous studies, which revealed an altered state of VSMC differentiation in association with altered AGF staining suggesting dysregulated angiogenesis underlying HMB. The results from the angiogenesis assay were inconclusive, in part due to small sample size, in terms of the specific effects of VEGF-C (Figures 5.9-5.10), PDGF-BB (Figures 6.2-6.3) and TGFβ1 (Figures 6.4-6.5) on EC-VSMC association and VSMC recruitment; although they may hint at a disruptive role of TGFβ1 on EC mediated honeycomb formation. In order to further validate any findings from the *in vitro* assay, the study investigated the effects of these AGFs on mRNA and secretion of AGFs by ECs and VSMCs; these revealed that TGFβ1 increased PDGF-BB mRNA in ECs and TGFβ1 secretion by VSMCs while PDGF-BB had no effect on either mRNA or secretion of AGFs. This suggests that TGFβ1 may alter AGF gene expression and secretion *in vitro*, which may in turn affect EC-VSMC interaction. However, these preliminary results from the *in vitro* study must be repeated with further functional studies prior to drawing definitive conclusions.

7.2 LIMITATIONS AND FUTURE STUDIES

7.2.1 Immunohistochemical study of the uterine vasculature

Immunohistochemical studies utilising endometrial biopsies from women undergoing hysterectomy provided us with important insights into the extent of endometrial vascular impairment along with implications of the role of endometrial leukocyte populations and AGF expression in women with HMB. In this context it must be noted that the current study included both straight and spiral arterioles within the endometrium, a further distinction of which, as performed by Abberton *et al*. (1999a), could possibly reveal any distinctions underlying HMB.

The endometrial biopsies utilised in the present study showed inter-patient variability; it would therefore be ideal if material from the same patient could have been used throughout the different phases of the menstrual cycle possibly through endometrial pipelle biopsies. However since pipelle biopsies may often only retrieve superficial endometrium, this would limit the study to investigating the endometrium as a whole rather than examining specific differences in the stratum basalis and stratum functionalis as indicated by the present study. Moreover acquiring endometrial samples from the same patient would be more difficult with respect to obtaining consents as well as proving time consuming.

This study demonstrated altered VSMC differentiation in the endometrial vessels in HMB; in future these results could be validated, utilising laser capture microdissection. Following microdissection mRNA or protein may be extracted from these cells to further investigate any alteration in the levels of different genes or proteins regulating VSMC differentiation respectively. Moreover the importance of microRNAs (miRs) in functional regulation of EC as well as VSMC may suggest further studies investigating their alterations would be worthwhile, perhaps also utilising laser capture microdissection in isolating endometrial vessels. Since vascular maturation and VSMC differentiation was the central aim of this thesis, the current study investigated alterations in staining for PDGF-BB and TGFβ1 and their receptors in HMB. However both VEGF-A and VEGF-C have also been suggested to regulate angiogenesis. Therefore in future studying the pattern of staining for these AGFs along with

their respective receptors may also provide further insights into the roles of AGFs in vascular impairment in HMB.

7.2.2 EC-VSMC association and VSMC differentiation *in vitro*

The *in vitro* assay developed in this study, utilised cells isolated from SVs from patients undergoing cardiac bypass surgery. The choice of these cells depended on factors such as sample availability, ease of isolation and cost, but most importantly the observation that SV derived ECs and VSMCs retained their characteristics longer than the commonly used human aorta cell lines. However whether these cells are the best representatives for the endometrial vascular ECs and VSMCs could perhaps be investigated further. At present there are three major sources of ECs and VSMCS; human aorta, SVs and umbilical cord. In future a direct comparison could be made between ECs and VSMCs from these with respect to expression of characteristic proteins further validated through western blotting or mRNA expression and finally a secretion profile using angiogenesis arrays or ELISAs. Moreover since both ECs and VSMCs from aorta cell lines de-differentiated much earlier than those from SVs, again, the above profiles could perhaps be repeated between passages, which would give a more detailed analysis of the de-differentiation timeline for cells from each of these sources. Not considering time or cost, if possible this could be taken a step further; the above protein and gene expression profile of ECs and VSMCs could also be studied after treatments with different AGFs which may in turn give insight into differences in sensitivity to AGF signalling amongst cells from the different sources. While these studies may prove time consuming they may help to draw some conclusions on the suitability of each of these cell types for specific angiogenesis assays, where due to practical limitations cells cannot be isolated from the tissue specific vessels.

Results from the *in vitro* assay provided some insights into the effects of matrigel on ECs and VSMCs; while the study investigated the AGF secretion profiles of ECs cultured on matrigel compared with plastic, due to sample unavailability this was not done for VSMCs. Although matrigel had a more observable effect on ECs marked by distinct honeycomb structure formation, future studies could investigate its role on VSMCs. Additionally the results from

the study of EC-VSMC interactions and VSMC recruitment and the role of AGFs *in vitro* must be repeated with larger sample size. Again, the current study only examined the effects of PDGF-BB and TGFβ1 on mRNA and secretion of AGFs by ECs and VSMCs; this could perhaps also be repeated utilising VEGF-C to further validate the effects of VEGF-C on EC-VSMC association and VSMC recruitment and differentiation. Moreover apart from the AGFs studied *in vitro*, the assay can be used in future to study the effects of other key growth factors such as VEGF-A. Finally, possible synergistic roles of AGFs on angiogenesis may suggest further studies utilising combinations of AGF treatments in addition to treating with individual AGFs. Importantly the *in vitro* assay must be further optimised in terms of the concentration and length of time for the AGF treatments along with a possible fully automated image analysis protocol prior to any future applications.

7.3 SUMMARY

The primary aim of this exploratory study was to investigate vascular impairment in HMB and investigate the role of AGFs in dysregulated angiogenesis. This has been achieved and the study represents a significant move forward for understanding the mechanisms underlying this condition. The study has identified VSMC differentiation with respect to dysregulated calponin-osteopontin pathway as key to dysregulated angiogenesis in HMB, in association with altered EC-VSMC association, AGF distribution and leukocyte populations (Figure 7.1). Furthermore an *in vitro* angiogenesis assay has been developed to investigate dynamic effects of AGFs on EC-VSMC association, key to VSMC recruitment and differentiation; ultimately directing vascular maturation, which in turn has been implicated in HMB. Findings from this study along with development of the *in vitro* assay has provided key directions towards future investigations which may ultimately lead to treatment advances, promising a better quality of life for women with HMB.

Figure 7.1: Key outcomes from the investigation of uterine blood vessel development in heavy menstrual bleeding.

Appendix A1: Patient information sheet and consent form

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Newcastle University

Patient information sheet

Tissue samples for research into pregnancy complications to be stored in the Newcastle Uteroplacental Tissue Bank.

Introduction.

Most women have a straightforward pregnancy and deliver a healthy baby around their expected date. Unfortunately some women miscarry. Others go into labour and deliver early (preterm birth) or develop problems with the placenta (afterbirth) resulting in high blood pressure or small babies. These later problems can have a major impact on the health of the mother and/or her baby.

Our research involves studying cells from the uterus (womb), cervix and placenta (afterbirth) in order to better understand how these cells work, what controls or regulates them and why sometimes these control mechanisms go wrong. Researchers at Newcastle University are coordinating a local collection of tissue samples from the uterus and placenta for use in research projects so we can provide better care to women in the future. We would like to ask your permission to donate tissue samples to the collection. It is important for you to understand why the research is being done and what it will involve before you decide whether to take part. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear.

The research work will not directly benefit you, but may help patients with pregnancy problems in the future. You do not have to give permission for your samples to be stored. If you decline it will not affect your medical care in any way.

Any research we do requires the approval of a Research Ethics Committee. We occasionally send samples to other UK research teams but only if their projects have been approved by a Research Ethics Committee. No personal details which could be used to identify you will be available to any researcher.

What will happen to me if I take part?

We are asking you to take part because you are having a **hysterectomy**. If you do decide to take part you will be asked to sign a consent form. A copy of your consent and this information sheet will be given to you to keep.

In order to undertake this study we need 3 or 4 small samples, each about the size of a pea, from the muscle layer and neck of the uterus (cervix). These are collected after the uterus (womb) has been removed and will not interfere with the detailed pathological examination.

In order to make the best use of these important samples, more than one laboratory study may be undertaken on each sample. Samples may be stored prior to study and will represent a gift to the research team.

What will happen to my specimens?

Samples will be stored in the Newcastle Uteroplacental Tissue Bank. Samples may be stored for many years before they are used. The samples may be used to extract DNA (the genetic information inside a cell) which would also be stored. Samples are stored with a code number, and the only link between the sample code and your identity details is stored in a secure file.

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Newcastle University

Researchers will have some information about your health but will have no personal details which could identify you.

Similarly, any results from each research project will not be traceable back to you. All information on your samples will be kept at the Newcastle Uteroplacental Tissue Bank in accordance with national guidelines (The Data Protection Act and Caldicott guidelines)

What processes are in place to approve use of the donated specimen?

Before the start of any research work, written approval is required from the Tissue Bank Manager ProfessorRobson. He will be advised by Tissue Bank Governance Committee, chaired by Professor Davison. The Governance Committee will assess requests to use samples from the Newcastle Uteroplacental Tissue Bank. Voting members of the committee will include an independent non-consultant clinical assessor, an independent scientist and a clinical obstetrician from outside the research team. The governance committee will be responsible for ensuring that projects are scientifically sound, worthwhile and adequately funded.

What will happen to the results of the research study?

We anticipate the results will be published in medical journals. You will not be identified in any report or publication.

What are the benefits and risks of taking part?

You will not directly benefit from taking part in the study but the knowledge gained will help patients in the future. As the uterus (womb) has already been removed there are no risks involved in taking the samples. The research samples are very small compared to the rest of the uterus which will examined as part of your routine care. If evidence of disease with immediate clinical relevance were found in the research samples it is extremely unlikely that the same problem would not have been seen in the larger clinical sample but we would always make sure your clinical team were aware so they could advise the most appropriate management.

Do I have to take part?

No. Your participation is entirely voluntary. Whether or not you decide to allow your specimens to be stored for research purposes will not affect you being given the best possible treatment. If at any time in the future you change your mind and do not wish the specimens to be used for research, then as soon as you have told your doctor, any samples remaining will be disposed of, usually by incineration, but we will use the information collected up to the time of your withdrawal.

What if there is a problem?

If you have a concern about any aspect of this study, you should ask to speak to the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure (or Private Institution). Details can be obtained from the hospital.

Further information

If you have any questions about the study please contact: Dr. M. Smith or Professor S. Robson (Tel: 0191 2824132)

Thank you for reading this information sheet

PIS Non Pregnant v4 Oct 2012

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Newcastle University

CONSENT FOR GIFTING OF CLINICAL SAMPLES

Title of Project: Tissue samples for research into pregnancy complications to be stored in the Newcastle Uteroplacental Tissue Bank

Name of Researcher: Professor S. Robson, Newcastle Uteroplacental Tissue Bank

I agree to donate the following samples for research (initial appropriate boxes)

Myometrial biopsies ☐ Cervix ☐ Vaginal swab ☐

I understand that relevant sections of my medical notes and data collected during the study, may be looked at by researchers, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records **YES / NO**

If **YES,** please initial each of the following statements:

- I have read and understood the relevant patient information sheet/s. (Please take a copy home with you to keep) ☐
- I have had an opportunity to discuss the Tissue Bank and ask any questions ☐
- I understand that I am free to withdraw consent for use of my tissue at any time without having to give a reason and without affecting my medical care ☐
- I agree to donate small samples specifically for research purposes and for these to be stored for future use in approved research projects ☐
- I understand that I will not be told the results of any tests carried out on my sample. ☐
- I give permission for my medical information to be stored ☐
- I agree for my anonymised medical information to be used analysis of the study results ☐

Name + MRN of Patient	Date	Signature
Name of Person taking consent	Date	Signature

1 form for patient; 1 form to be kept with hospital notes; 1 form for Biobank

Consent NP v4 Oct 2012

Appendix A2: Sample information form

Biobank #

Uterine Cell Signalling Group Tissue Collection

Date of sample collection…… Hospital Number……

Gestation (wk + day)…… Age……

Gravidity …… Parity…… Height ……Weight ……BMI ……

Ethnicity …… Term BMI……

Birthweight ……M / F Smoker: True ☐ False ☐

Birth Centile ……

Consented:

Placenta ☐
Amnion/chorion ☐
Placental bed ☐
Myometrium Upper ☐ Lower ☐
Cervix ☐

Samples Collected

Placenta ☐
Amnion/chorion ☐
Placental bed ☐
Myometrium Upper ☐ Lower ☐
Cervix ☐

(PTO for other outcomes)

Delivery:

Vaginal delivery ☐ Elective CS ☐ Indication……
Emergency CS ☐ Dilatation……cm) Indication……
Hysterectomy ☐ LMP …… Indication……
TOP ☐
ERPC ☐

Drugs:

This patient does not take **any** prescribed medications ☐

Prostaglandin	True	☐	False	☐
Oxytocin	True	☐	False	☐
Steroids (within 48h)	True	☐	False	☐
Tocolytic (within 48h)	True	☐	False	☐
Progesterone (inc Mirena)	True	☐	False	☐
GnRH analogue	True	☐	False	☐

Other medications:

Other medical conditions:

Preclampsia ☐ Diabetes ☐ Prolonged ROM ☐ Infection ☐

Consent:

I confirm that this patient has given informed written consent for this sample

True ☐ False ☐

Signed……Print name……

Thank you for filling in this form. Please ring RVI Ext 24132 for sample collection

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Appendix A3: Patient medications in the control group

Drug	Function
Fluoxetine	Fluoxetine (also known by the tradenames Prozac, Sarafem, and Fontex, among others) is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class, used to treat premenstrual dysphoric disorder.
Salbutamol	Salbutamol (INN) or albuterol (USAN) is a short-acting β2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and chronic obstructive pulmonary disease.
Tramadol	is a centrally acting synthetic opioid analgesic used to treat moderate to moderately severe pain.
Amitriptyline	Amitriptyline (Tryptomer, Elavil, Tryptizol, Laroxyl, Saroten, Sarotex, Lentizol, Endep) is a tricyclic antidepressant (TCA). It is the most widely used TCA and may be efficacious for the treatment of depression.These types of tricyclics have been used historically for the treatment of migraines, tension headaches, anxiety, psychosis, aggression and violent behavior.
Paracetamol	Paracetamol is classified as a mild analgesic. It is commonly used for the relief of headaches and other minor aches and pains and is a major ingredient in numerous cold and flu remedies.
Fentanyl	Fentanyl is a potent, synthetic opioid analgesic with a rapid onset and short duration of action.
Oramorph	Oramorph oral solution contains the active ingredient morphine sulphate, which is a type of medicine called an opioid painkiller.
Mesalazine	Mesalazine (INN, BAN), also known as mesalamine (USAN) or 5-aminosalicylic acid (5-ASA), is an anti-inflammatory drug used to treat inflammatory bowel disease, such as ulcerative colitis and mild-to-moderate Crohn's disease.[2] Mesalazine is a bowel-specific aminosalicylate drug that acts locally in the gut and has its predominant actions there, thereby having few systemic side effects.
Lansoprazole	Lansoprazole reduces the amount of acid produced in the stomach. The common side effects are digestive system discomfort (feeling sick, stomach ache, diarrhoea) and headache. These effects are mild and do not last long
Dihydrocodeine	Dihydrocodeine is a semi-synthetic opioid analgesic prescribed for pain, severe dyspnea, or as an antitussive, either alone or compounded with aspirin or paracetamol, as in co-dydramol.
Ferrous sulphate	This medicine is used to treat and prevent iron deficiency anaemia. It belongs to a group of medicines called iron supplements.
Omeprazole	Omeprazole is a proton pump inhibitor used in the treatment of dyspepsia, peptic ulcer disease (PUD), gastroesophageal reflux disease (GORD/GERD), laryngopharyngeal reflux (LPR) and Zollinger–Ellison syndrome.
Levothyroxine	It is also used to treat thyroid hormone deficiency, and occasionally to prevent the recurrence of thyroid cancer.
Citalopram	Citalopram is an antidepressant drug of the selective serotonin reuptake inhibitor (SSRI) class. It has U.S. Food and Drug Administration (FDA) approval to treat major depression.
Mefenamic acid	Mefenamic acid is a non-steroidal anti-inflammatory drug used to treat pain, including menstrual pain. It is typically prescribed for oral administration.

Appendix A4: Patient medications in the HMB group

Drug	Function
Nurofen	Nurofen is the brand name of a range of pain-relief medication. There are 11 variants of Nurofen, all of which contain the analgesic ibuprofen as the active ingredient
Co-codamol	Co-codamol (BAN) is a compound analgesic consisting of a combination of codeine phosphate and paracetamol (acetaminophen). Co-codamol tablets are used for the relief of mild to moderate pain
Thyroxine	Thyroxine is the main hormone produced by the thyroid gland. It plays an important role in regulating many of the body's metabolic functions.
Penicilin	Penicillin is a group of antibiotics derived from Penicillium fungi. Penicillin antibiotics are the first drugs that were effective against syphilis, and infections caused by staphylococci and streptococci.
Mefanemic acid	Mefenamic acid is a non-steroidal anti-inflammatory drug used to treat pain, including menstrual pain. It is typically prescribed for oral administration.
Salbutamol	Salbutamol (INN) or albuterol (USAN) is a short-acting β2-adrenergic receptor agonist used for the relief of bronchospasm in conditions such as asthma and chronic obstructive pulmonary disease.
Paracetamol	Paracetamol is classified as a mild analgesic. It is commonly used for the relief of headaches and other minor aches and pains and is a major ingredient in numerous cold and flu remedies.
Fluiticasone	Fluticasone is used to treat symptoms of allergic rhinitis and perennial nonallergic rhinitis. These symptoms include sneezing and stuffy, runny, or itchy nose. It works by preventing and decreasing inflammation (swelling that can cause other symptoms) in the nose.
Citalopram	Citalopram is an antidepressant drug of the selective serotonin reuptake inhibitor (SSRI) class. It has U.S. Food and Drug Administration (FDA) approval to treat major depression.
Omeprazole	Omeprazole is a proton pump inhibitor used in the treatment of dyspepsia, peptic ulcer disease (PUD), gastroesophageal reflux disease (GORD/GERD), laryngopharyngeal reflux (LPR) and Zollinger–Ellison syndrome.
Fluoxetine	Fluoxetine (also known by the tradenames Prozac, Sarafem, and Fontex, among others) is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class, used to treat premenstrual dysphoric disorder.
Ferrous fumarate	This medicine is used to treat and prevent iron deficiency anaemia. It belongs to a group of medicines called iron supplements.
Fludrocortisone	Fludrocortisone, a corticosteroid, is used to help control the amount of sodium and fluids in your body. It is used to treat Addison's disease and syndromes where excessive amounts of sodium are lost in the urine. It works by decreasing the amount of sodium that is lost (excreted) in your urine.
Bendroflumethiazide	Bendroflumethiazide belongs to a group of medicines called thiazide diuretics. A diuretic is a medicine, which increases the amount of urine that you pass out from your kidneys. They are also used to clear excess fluid from your body in conditions where your body retains more than it needs, called oedema.
Ramipril	Ramipril is an angiotensin-converting enzyme (ACE) inhibitor, used to treat high blood pressure and congestive heart failure.
Propranolol	Propranolol (INN) is a sympatholytic non-selective beta-blocker. Sympatholytics are used to treat anxiety and panic.
Levothyroxine	It is also used to treat thyroid hormone deficiency, and occasionally to prevent the recurrence of thyroid cancer.

Appendix B: Reagents and solutions

Most chemicals and reagents, used in the study were obtained from Sigma-Aldrich Co., Dorset, UK, unless stated otherwise.

Mayer's Haematoxylin:

Mayer's Haematoxylin solution was prepared by adding the ingredients listed in Table B1 to 2L distilled water.

Table B1: Preparation of Mayer's Haematoxylin

REAGENT (SOURCE)	QUANTITY
Haematoxylin (#261103G, BDH, Poole UK)	2.0g
Potassium alum (#237086)	100.0g
Sodium iodite (#S4007)	0.4g
The above chemicals were dissolved in 2L distilled water at room temperature overnight and then the following were added.	
Chloral Hydrate (#C8383)	100.0g
Citric acid (#C7129)	2.0g
The above solution was brought to the boil, left overnight and filtered prior to use.	

Scott's tap water substitute:

Scott's tap water substitute solution was prepared, by adding the reagents listed in Table B2 to 5L distilled water at room temperature.

Table B2: Preparation of Scott's tap water substitute

REAGENT (SOURCE)	QUANTITY
Sodium bicarbonate (#S5761)	17.5g
Magnesium sulphate (#M7506)	100.0g
The above chemicals were dissolved in 2L distilled water at room temperature.	

DPX Mounting medium:

DPX synthetic mounting medium (♯SEA-0304-00A) was obtained from CellPath Ltd., Newtown, UK).

Eosin:

Eosin (♯HT110316) was obtained from Raymond A Lamb, London, now under Fisher Scientific, Loughborough, UK.

Ethanol:

Ethanol (99%) (♯E/0650DF/17), was obtained from Fisher Scientific. 95% and 70% alcohol solutions were prepared by diluting the above with distilled water appropriately.

3-Aminopropyltriethoxysilane (APES) coated glass slides:

Glass microscope slides (♯MAF-0102-03A, CellPath, Ltd.) were washed in diluted detergent and rinsed several times with water. The slides were then immersed in distilled water and washed in 99% methanol (♯M/4000/17, Fisher Scientific). Following this they were air dried prior to coating with APES solution. The slides were then immersed sequentially into acetone only, acetone and APES solution (196ml acetone ♯154598, 4ml APES solution ♯A3648), acetone only and distilled water. Finally slides were air dried overnight at room temperature and stored at 4℃ until required.

Citrate buffer (pH6.0):

Citrate buffer solution was prepared by adding the ingredients listed in Table B3 to 10L distilled water at room temperature.

Table B3: Recipe for preparation of citrate buffer solution

INGREDIENT (SOURCE)	QUANTITY
Citric acid (♯C7129)	21.0g
2M Sodium hydroxide (♯S8045)	130.0ml
The above chemicals were dissolved in 10L distilled water overnight at room temperature. The solution was adjusted to pH6.0 before use.	

p-Xylene:

p-Xylene (♯134449) was obtained from Sigma-Aldrich Co.

3,3'- Diaminobenzidine tetrachloride (DAB) solution:

One DAB tablet (♯D5905) was added to 0.2ml 1% aqueous H_2O_2 (♯31642) in 10ml TBS pH7.6 and left to dissolve. This was filtered using 5-13µm qualitative filter paper (♯516-0815, VWR International, Leicestershire, UK) prior to applying to the slides.

Ethylenediaminetetraacetic acid (EDTA) buffer (pH8.0):

Ethylenediaminetetraacetic acid (EDTA) buffer solution was prepared by dissolving 2.26g EDTA (♯10093, VWR International) in 5L distilled water overnight at room temperature. The solution was adjusted to pH8.0 before use.

Phosphate buffered saline (PBS):

Phosphate buffered saline (PBS) solution was prepared by dissolving five PBS tablets (♯P4417) in 1L distilled water at room temperature. Depending on the type of application the solution was adjusted to pH7.2, pH7.5, pH7.4 or pH7.6 and if required sterile filtered through a 0.2µm syringe filter (♯190-2520, Nalgene Fisher Scientific), autoclaved and stored at 4°C.

Calcium chloride stock solution:

Calcium chloride ($CaCl_2$) stock solution (4%) was prepared, by dissolving 4g calcium chloride (♯C3306) in 100ml distilled water at room temperature. 5ml aliquots were stored at -20°C, until required.

Trypsin stock solution:

Trypsin stock solution (4%) was prepared by dissolving 4g trypsin (♯215240, BD Biosciences, Oxford, UK) in 100ml distilled water at room temperature. 5ml aliquots were stored at -20°C, until required.

Tris buffered saline (TBS):

Tris buffered saline (TBS; 0.1M Tris, 0.5M saline) solution (pH7.6) was prepared by adding the reagents listed in Table B4 to 10L distilled water at room temperature.

Table B4: Preparation of tris buffered saline solution

REAGENT (SOURCE)	QUANTITY
Sodium chloride (♯S3014)	80.0g
Tris (♯252859)	6.05g
1N HCl (♯H1758)	38.0ml
The above chemicals were dissolved in 10L distilled water overnight at room temperature. The solution was adjusted to pH7.6 before use.	

Trypsin-$CaCl_2$ working solution:

One aliquot (5ml) each of the trypsin and $CaCl_2$ stock solutions was diluted in 200ml distilled water and the working solution was adjusted to pH7.8.

Hydrogen peroxide:

Hydrogen peroxide (H_2O_2) (♯31642) was obtained from Sigma-Aldrich Co., Dorset, UK.

Vectastain Elite mouse IgG ABC kit:

The Vectastain Elite mouse IgG ABC kit (♯PK-6102) was used as per manufacturer's instructions (Table B5)

Table B5: User instructions for Vectastain Elite mouse IgG ABC kit

REAGENT	QUANTITY
Blocking serum	Vectastain normal horse serum (15µl) diluted in 1ml TBS
Secondary antibody	Vectastain normal horse serum (15µl) and Vectastain Biotinylated anti-mouse IgG antibody (5ul) diluted in 1ml TBS
Tertiary antibody	Vectastain reagent A (20µl) and Vectastain reagent B (20µl) diluted in 1ml TBS

Vectastain Elite rabbit IgG ABC kit:

The Vectastain Elite rabbit IgG ABC kit (♯PK-6101) was used as per manufacturer's instructions (Table B6)

Table B6: User instructions for Vectastain Elite rabbit IgG ABC kit

REAGENT	QUANTITY
Blocking serum	Vectastain normal goat serum (15µl) diluted in 1ml TBS
Secondary antibody	Vectastain normal goat serum (15µl) and Vectastain Biotinylated anti-rabbit IgG antibody (5ul) diluted in 1ml TBS
Tertiary antibody	Vectastain reagent A (20µl) and Vectastain reagent B (20µl) diluted in 1ml TBS

Vector NovaRED peroxidase (HRP) substrate kit:

Vector NovaRED peroxidase (HRP) substrate kit (♯SK-4800) was used as per manufacturer's instructions (Table B7)

Table B7: User instructions for Vector NovaRED peroxidase substrate kit

REAGENT	QUANTITY
Reagent 1	2 drops, mixed well
Reagent 2	1 drops, mixed well
Reagent 3	1 drops, mixed well
Hydrogen peroxide	1 drops, mixed well
The above reagents were added to 5ml distilled water, and the final solution was applied to the slides.	

Vector SG peroxidase (HRP) substrate kit:

Vector SG peroxidase (HRP) substrate kit (♯SK-4700) was used as per manufacturer's instructions (Table B8)

Table B8: User instructions for Vector SG peroxidase substrate kit

REAGENT	QUANTITY
SG chromogen	3 drops, mixed well
Hydrogen peroxide	3 drops, mixed well
The above reagents were added to 5ml PBS (pH7.5), and the final solution was applied to the slides.	

Ultramount aqueous permanent mounting medium:

Ultramount aqueous permanent mounting medium (♯S1964) was obtained from Dako, Cambridgeshire, UK.

Endothelial cell culture medium:

Endothelial cell culture medium was prepared by adding reagents from the Endothelial Cell Growth Kit-BBE (♯PCS-100-040, LGC Standards Ltd, Teddington, UK) listed in Table B9 to 475ml Vascular Cell Basal Medium (♯PCS-100-030, LGC Standards Ltd).

Table B9: Preparation of endothelial cell culture medium

REAGENT	QUANTITY	FINAL CONCENTRATION
Bovine brain extract (BBE)	1.0ml	0.2%
rh EGF	0.5ml	5ng/ml
L-glutamine	25.0ml	10mM
Heparin sulphate	0.5ml	0.75Units/ml
Hydrocortisone hemisuccinate	0.5ml	1µg/ml
Fetal bovine serum (FBS)	10.0ml	2%
Ascorbic acid	0.5ml	50µg/ml
The above reagents were added to 475ml Vascular Cell Basal Medium and stored at 4℃ for use.		

Vascular smooth muscle cell culture medium:

Vascular smooth muscle cell culture medium was prepared by adding the reagents listed in Table B10 to 450ml Ham's F-12K (Kaighn's) Medium (♯30-2004, LGC Standards Ltd).

Table B10: Preparation of vascular smooth muscle cell culture medium

REAGENT (SOURCE)	QUANTITY	FINAL CONCENTRATION
Fetal bovine serum (FBS) (♯F7524)	50.0ml	10%
Endothelial cell growth supplement (♯E0760)	15.0mg	30µg/ml
Ascorbic acid (♯A4403)	25.0mg	50µg/ml
HEPES (♯H0887)	5.0ml	1%
TES (♯T5691)	1.15g	2.3mg/ml
Insulin-transferrin-sodium selenite (ITS; ♯I1884)	5.0ml	1%
Amphotericin (♯A2942)	5.0ml	1%
Penicillin Streptomycin (♯P0781)	5.0ml	1%
The above reagents were added to 450ml Ham's F-12K (Kaighn's) medium and stored at 4℃ for use.		

RPMI 1640 incomplete medium:

RPMI 1640 incomplete medium was prepared by adding the reagents listed in Table B11 to 200ml RPMI 1640 medium (♯R0883).

Table B11: Preparation of RPMI incomplete medium

REAGENT (SOURCE)	QUANTITY	FINAL CONCENTRATION
L-glutamine (♯G7513)	2.0ml	0.4mM/ml
Penicillin Streptomycin (♯P0781)	2.0ml	1%
The above reagents were added to 200ml RPMI 1640 medium and stored at 4℃ for use.		

RPMI 1640 Complete medium:

RPMI 1640 complete medium was prepared by adding 10% vol/vol heat inactivated fetal bovine serum (♯F7524) to the RPMI Incomplete medium described above.

DMEM-F12 Complete medium:

Dulbecco's modified eagle (DMEM) complete medium was prepared by adding the reagents listed in Table B12 to 200ml DMEM medium (♯D6421).

Table B12: Preparation of DMEM complete medium

REAGENT (SOURCE)	QUANTITY	FINAL CONCENTRATION
Ham's F-12K (Kaighn's) Medium (♯30-2004, LGC Standards Ltd).	200.0ml	1:1, ratio by volume
Fetal bovine serum, heat inactivated (♯F7524)	20.0ml	10%
L-glutamine (♯G7513)	2.0ml	0.4mM/ml
Penicillin Streptomycin (♯P0781)	2.0ml	1%
The above reagents were added to 200ml DMEM and stored at 4℃ for use.		

Cell freezing medium:

Cell freezing medium was prepared by adding 20% vol/vol DMSO (♯D2650) to FBS solution (♯F7524). 1ml aliquots were stored at -20℃, until required.

Citric acid solution:

Citric acid solution was prepared by dissolving 21mg (10mM) or 42mg (20mM) citric acid (♯C7129) in 10ml distilled water. The solution was adjusted to pH3.0, filtered through a sterile 0.2µm syringe filter (♯190-2520, Nalgene Fisher Scientific) and stored at 4℃.

TGFβ1 tissue or cell treatment medium:

TGFβ1 (♯100-21, Peprotech EC Ltd., London, UK) and anti TGFβ1 (♯ab92486, Abcam, Cambridgeshire, UK) were individually diluted with DMEM-F12 complete medium (for tissue) or endothelial cell culture medium or vascular cell culture medium to prepare 1ng/ml, 10ng/ml final concentration of treatment media. Since TGFβ1 stock solution (10µg/ml) was diluted in acid and PBS, to prepare culture medium for the control group, 10mM citric acid was first diluted to 2mM in sterile PBS (pH7.4), which was further diluted to a final concentration of 2µM with DMEM-F12 complete medium (for tissue) or endothelial cell culture medium or vascular cell culture medium for the respective control groups.

PDGF-BB tissue or cell treatment medium:

PDGF-BB (♯130-094-630, Miltenyi Biotec Ltd., Surrey, UK) and anti PDGF-BB (♯ab9704, Abcam) was individually diluted with DMEM-F12 complete medium

(for tissue) or endothelial cell culture medium or vascular cell culture medium to prepare 1ng/ml, 10ng/ml final concentration of treatment media. Since PDGF-BB stock solution (10µg/ml) and was diluted in acid, to prepare culture medium for the control group, 20mM citric acid was diluted to a final concentration of 20µM with DMEM-F12 complete medium (for tissue) or endothelial cell culture medium or vascular cell culture medium for the respective control groups.

Endothelial cell digestion medium:

Endothelial cell digestion medium was prepared by adding Type I collagenase (♯C2674), 0.1% volume, to 10ml sterile PBS (pH7.4). 500µl aliquots were stored at -20℃.

Vascular smooth muscle cell digestion medium:

Vascular smooth muscle cell digestion medium was prepared by adding (i) 0.1% vol/vol of type I collagenase, (ii) 0.02% vol/vol of elastase (♯E7885) and (iii) 0.02% vol/vol of DNase (♯D5025) to 10ml sterile PBS (pH7.4). 500µl aliquots were stored at -20℃.

BD Matrigel Basement Membrane Matrix:

Matrigel Basement Membrane Matrix (♯354234) for the *in vitro* cell association and tubule formation assay was obtained from BD Biosciences, Bedford, UK. 100µl aliquots were prepared and stored at -20℃, until required.

VEGF-C cell treatment medium:

VEGF-C (♯2179-VC, R&D Systems Ltd., Abingdon, UK) and anti VEGF-C (♯sc-9047, Santa Cruz Biotechnology, Heidelberg, Germany) were individually diluted with endothelial cell culture medium or vascular smooth muscle cell culture medium as required to prepare 1ng/ml and 10ng/ml final concentrations of cell treatment media. Endothelial cell culture medium or vascular smooth muscle cell culture medium was used as culture medium for the control group respectively.

Amresco 10% gel:

Amresco 10% gel (♯M256) was obtained from VWR International, Leicestershire, UK.

Protein extraction buffer (3X):

Protein extraction buffer (3X) was prepared by adding the reagents listed in Table B13 to 100ml distilled water.

Table B13: Preparation of protein extraction buffer (3X)

REAGENT (SOURCE)	QUANTITY	FINAL CONCENTRATION
Tris-HCl (♯73253)	2.27g	62.5mM
SDS (♯L3771)	6.0g	2%
Sucrose (♯84097)	30.0g	10%
The above reagents were added to 100ml distilled water to make up 3X protein extraction buffer. The solution was adjusted to pH6.8 before use.		

Protein extraction buffer (1X):

Protein extraction buffer (1X) was prepared by diluting protein extraction buffer (3X) in distilled water and adding 1% vol/vol protease inhibitor (♯P8340) and 0.5% vol/vol phosphatase inhibitor (♯P5726).

Protein loading buffer:

Protein loading buffer was prepared by combining the reagents listed in Table B14.

Table B14: Preparation of protein loading buffer

REAGENT (SOURCE)	QUANTITY	FINAL CONCENTRATION
Tris (♯252859)	2.0ml	0.5M
SDS (♯L3771)	4ml	10%
Bromophenol blue (♯B0126)	2.0ml	1%
99% Glycerol (♯G5516)	2.0ml	20%
The above reagents were combined and the final volume was brought up to 10ml by addition of distilled water, if required.		

Protein loading buffer (reducing):

Protein loading buffer (reducing) was prepared by adding 5% vol/vol mercaptoethanol (♯161-0710, BioRad, Hertfordshire, UK) to the protein loading buffer described above.

Running buffer:

Running buffer (20X) (♯M256) was obtained from VWR International. Running buffer (1X) was prepared by diluting running buffer (20X) in distilled water.

Transfer buffer:

Transfer buffer was prepared by adding the reagents listed in Table B15 to 2L distilled water.

Table B15: Preparation of transfer buffer

REAGENT (SOURCE)	QUANTITY	FINAL CONCENTRATION
Tris (♯252859)	11.62g	5.81g/L
Glycine (♯G8898)	5.86g	2.93g/L
SDS (♯L3771)	0.75g	0.38g/L
99% Methanol (♯M/4000/17, Fisher Scientific)	400.0ml	200ml/L
The above reagents were added to 2L distilled water and the final solution was stored at 4℃ till use.		

Tris buffered saline with Tween20 (TBST):

Tris buffered saline with Tween20 (TBST) was prepared by adding 0.05% vol/vol Tween20 to TBS pH7.6.

Blocking buffer (5%):

Blocking buffer (5%) was prepared by dissolving 5g non-fat dry powdered milk in 20ml TBST.

Wash buffer (0.05% PBS with Tween20):

Wash buffer was prepared by adding 0.05% vol/vol Tween20 (500µl) to 1L of PBS (pH7.6).

Reagent diluent:

Reagent diluent was prepared by adding 10g BSA (1% final concentration) (♯441555J, VWR International) to 1L PBS (pH7.6).

Substrate solution:

Substrate solution was prepared by combining 1:1 ratio vol/vol colour Reagent A (H_2O_2, ♯895000; R&D Systems Ltd., Abingdon, UK) and Reagent B (Tetramethylbenzidine, ♯895001; R&D Systems Ltd.).

Stop solution (2N Sulphuric acid):

Stop solution (2N Sulphuric acid) ♯339741, was obtained from Sigma-Aldrich.

TGFβ1 blocking solution (5% PBST):

TGFβ1 blocking solution was prepared by adding 5% vol/vol Tween 20 to PBS (pH7.4) and sterile filtered using a 0.2µm syringe filter (♯190-2520, Nalgene Fisher Scientific).

TGFβ1 Reagent diluent:

TGFβ1 reagent diluent consisted of 5% charcoal stripped FBS (♯F7524) sterile filtered using a 0.2µm syringe filter (♯190-2520, Nalgene Fisher Scientific).

TGFβ1 acid activation solution (1N HCl):

TGFβ1 acid activation solution was prepared by diluting 8.33ml 12N (36%) concentrated HCl (♯H1758), in 91.67ml deionised water.

TGFβ1 acid neutralisation solution (1.2N NaOH/ 0.5M HEPES):

TGFβ1 acid neutralisation solution was prepared by adding (i) 12ml 10N NaOH (♯S8045), (ii) 11.9g HEPES (♯H0887), to 75ml deionised water and bringing the final volume up to 100ml.

Quantibody® Human Angiogenesis Array 1:

The Quantibody® Human Angiogenesis Array 1 (♯QAH-ANG-1) was obtained from RayBiotech, Norcross, U.S.A). Components of this array detect Angiopoietin-2 (Ang2), Epidermal growth factor (EGF), basic Fibroblast growth factor (bFGF), Heparin binding Fibroblast growth factor (HB-FGF), Hepatocyte

growth factor (HGF), Leptin, Platelet-derived growth factor (PDGF-BB), Placental growth factor (PlGF) and Vascular endothelial growth factor (VEGF) (Table B16).

Table B16: Components of Quantibody Human Angiogenesis Array 1

ITEM	DESCRIPTION
1	Quantibody array glass chip
2	Sample diluent
3	20X Wash Buffer I
4	20X Wash Buffer II
5	Lyophilized cytokine standard mix
5	Detection antibody cocktail
6	Cy3 equivalent dye-conjugated streptavidin

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