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The Human Endometrium

Studies on Angiogenesis and Endometriosis

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Abstract

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Angiogenesis is thought to play a pivotal role in the cycling endometrium. Coordinated by oestrogen and progesterone, endometrial blood vessel development is primarily mediated by vascular endothelial growth factor-A (VEGF-A), which promotes endothelial cell (EC) proliferation and protects ECs from induced apoptosis. Studying changes at transcript level in human endometrial endothelial cells (HEECs) in response to mitogenic and inhibitory stimuli is one way towards understanding the regulation of physiological endometrial angiogenesis.

Endometriosis, the presence of endometrial-like tissue outside the uterine cavity, is a common gynaecological disorder in women of reproductive age, often causing pelvic pain and reduced fertility. Chronic inflammation in the peritoneal environment and defective endometrial protein expression are some of the contributors to the complex pathophysiology of endometriosis. The aim of this work was to study the changes in the transcriptome induced by VEGF-A and partial serum deprivation in primary HEECs, and to investigate biochemical factors associated with subfertility and chronic pelvic pain in endometriosis patients.

Exposing primary HEECs to VEGF-A, and serum withdrawal was found to regulate transcripts associated with survival, migration, apoptosis and progression through the cell cycle, when assessed using microarray technology and bioinformatic tools. A subset of 88 transcripts was reciprocally regulated under the two experimental conditions; thus probably important in HEEC biology.

Higher endometrial epithelial staining scores of oestrogen receptor-α and reduced staining of progesterone receptors were seen in subfertile endometriosis patients. Lower levels of the receptivity biomarker leukaemia inhibitory factor (LIF) and its receptor, as well as signs of dysregulated αB-crystallin expression and increased peritoneal fluid concentrations of interleukin (IL)-1α and IL-6 were associated with reduced pregnancy rates.

Endometriosis patients with chronic pelvic pain had higher levels of vasoactive intestinal peptide (VIP) in eutopic endometria and in endometriotic lesions compared with patients without chronic pain. The presence of chronic pelvic pain was also associated with increased concentrations of VIP and IL-6 in peritoneal fluid.

The present results may constitute a basis for further investigation of regulatory pathways in endometrial angiogenesis as well as for studies of endometrial receptivity and pain in women with endometriosis.

Keywords: endometrium, angiogenesis, endothelial cell, endometriosis, receptivity, implantation, oestrogen receptor, progesterone receptor, crystallin, interleukin, vasoactive intestinal peptide, peritoneal fluid

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"Ever tried. Ever failed. No matter.
Try again. Fail again. Fail better."
Samuel Beckett

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Moberg C, Catalano RD, Charnock-Jones DS[†], Olovsson M[†]. (2010) VEGF-A and Serum Withdrawal Induced Changes in the Transcript Profile in Human Endometrial Endothelial Cells. *Reprod Sci*, 17(6):590-611

II Moberg C[†], Bourlev V[†], Ilyasova N, Olovsson M. (2014) Levels of oestrogen receptor, progesterone receptor and αB-crystallin in eutopic endometrium in relation to pregnancy in women with endometriosis. *Hum Fertil (Camb)*, 18(1):30-7

III Moberg C[†], Bourlev V[†], Ilyasova N, Olovsson M. (2015) Endometrial expression of LIF and its receptor and peritoneal fluid levels of IL-1α and IL-6 in women with endometriosis are associated with the probability of pregnancy. *Arch Gynecol Obstet*, 292(2):429-37

IV Bourlev V[†], Moberg C[†], Ilyasova N, Davey E, Kunovac Kallak T, Olovsson M. Vasoactive intestinal peptide is upregulated in women with endometriosis and chronic pelvic pain. *Manuscript*

[†]These authors contributed equally to the work.

Cover illustration:
Photomicrograph of human mid-secretory phase endometrium. Glandular epithelium stained with antibody against αB-crystallin.

Contents

Abbreviations

αBCr	AlphaB-Crystallin
ASRM	American Society for Reproductive Medicine
CPP	Chronic pelvic pain
E2	17β-Oestradiol
EC	Endothelial cell
ER	Oestrogen receptor
gp130	Glycoprotein 130
GnRH	Gonadotrophin-releasing hormone
HEEC	Human endometrial endothelial cell
HUVEC	Human umbilical vein endothelial cell
IL	Interleukin
IVF	In vitro fertilisation
LH	Luteinising hormone
LIF	Leukaemia inhibitory factor
LIFR	Leukaemia inhibitory factor receptor
PR	Progesterone receptor
RCOGP	Research Center for Obstetrics, Gynecology and Perinatology
SHR	Steroid hormone receptor
uNK	Uterine natural killer cell
VEGF	Vascular endothelial growth factor
VIP	Vasoactive intestinal peptide
VPAC1	VIP and PACAP receptor 1
WOI	Window of implantation

Introduction

The human endometrium, the mucosal lining of the uterine cavity, is histologically stratified into two functionally different layers: the permanent basal layer (*stratum basalis*) adjacent to the myometrium and the superficial functional layer (*stratum functionalis*). The basal layer contains the vasculature supplying the functional layer with blood and lymphatic vessels, the acini of the endometrial glands and a stroma. The functional layer is lined by luminal epithelium and contains the superficial parts of the endometrial glands, stroma and vessels. Both layers contain endometrial immune cells such as macrophages and uterine natural killer (uNK) cells.

Under the influence of ovarian steroids, mainly 17β-oestradiol (E2) and progesterone, the endometrium grows and becomes receptive to the implantation of a blastocyst. If no implantation occurs, the superficial layer of the endometrium is shed, which constitutes the onset of the menstrual cycle. During this cycle, the endometrium undergoes a large number of morphological and physiological changes, e.g. cell growth and differentiation, formation of new blood vessels (angiogenesis) and expression of proteins necessary for receptivity. Detailed knowledge of the regulatory mechanisms of this process is necessary for understanding and treating endometrial disorders such as bleeding disturbances, infertility and endometriosis.

General aspects of angiogenesis

Angiogenesis is defined as the formation of new capillary blood vessels from pre-existing microvessels (Folkman 1985, Tammela et al. 2008). One mechanism, denoted sprouting angiogenesis, involves generation of subpopulations of endothelial cells (ECs); leading 'tip cells', which respond to paracrine growth factor guidance, and trailing 'stalk cells' which form the vascular branches (Koch and Claesson-Welsh 2012). New blood vessels are also the result of intussusception, i.e. pre-existing vessels internally dividing into two smaller vessels by penetration of smooth muscle cells through the endothelium, and elongation of existing vessels without the formation of new vascular junctions, e. g. in the endometrium (Girling and Rogers 2005, Carmeliet and Jain 2011). More recently, a form of angiogenesis in both adults and in mammalian embryos involving circulating bone marrow-derived endothelial progenitor cells – a process known as vasculogenesis –

has been described (Carmeliet and Jain 2011, Koch and Claesson-Welsh 2012) (Figure 1).

In adult humans, physiological angiogenesis only occurs in the ovaries, uterus and placenta during the reproductive years (Carmeliet 2005). Examples of pathological conditions involving angiogenesis, besides wound healing and repair, are carcinogenesis and inflammatory disorders such as rheumatoid arthritis and endometriosis (Groothuis et al. 2005, Carmeliet and Jain 2011, Konisti et al. 2012).

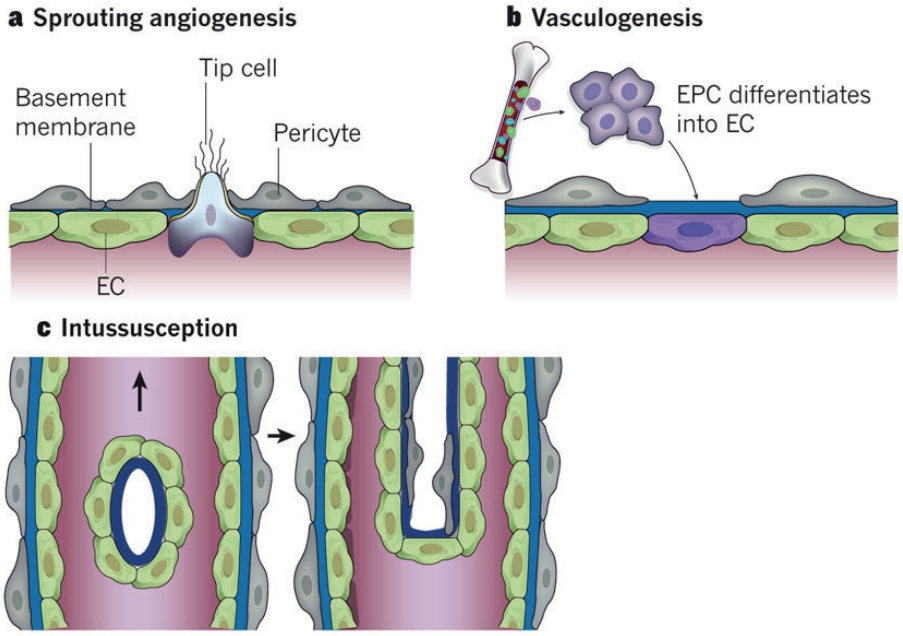

Figure 1. Modes of vessel formation. New blood vessels can be formed by sprouting angiogenesis (**a**), recruitment of bone-marrow-derived and/or vascular-wall-resident endothelial progenitor cells (EPC) that differentiate into endothelial cells (EC) (**b**), or by internal division of vessels, known as intussusception (**c**). Adapted from Carmeliet and Jain 2011. Reproduced with permission from Nature Publishing Group.

The VEGF superfamily of polypeptides is the most important stimulator of angiogenesis, activating vascular and lymphatic ECs through receptor tyrosine kinases (VEGFRs) (Smith et al. 2015). The VEGF family consists of six members: VEGF-A, -B, -C, -D, the Orf virus-derived VEGF-E and PlGF (placental growth factor) with VEGF-A, hereafter referred to as VEGF, being a key regulator in physiological blood vessel growth (Ferrara et al. 2003, Shibuya 2009). VEGF has mitogenic, pro-survival and permeability-enhancing effects in ECs that are of importance in newly formed vessels and inflammation (Senger et al. 1983, Ferrara 2009). *In vitro*, it has the ability to prevent apoptosis in ECs induced by serum starvation (Gerber et al. 1998). VEGF binds to and activates VEGFR-1 and -2, where activation of VEGFR-

2 on vascular ECs is the primary stimulator of EC proliferation (VEGFR-1 having only about one-tenth of the tyrosine kinase activity of VEGFR-2) (Shibuya 2009). In addition to its angiogenic activity, VEGF has several other properties such as haematopoietic effects and promotion of monocyte chemotaxis.

Although VEGF is the most important pro-angiogenic growth factor, there is a broad range of other signalling molecules essential for the formation and stability of the vascular network, e. g. fibroblast growth factors (FGFs), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-beta) and the angiopoietins (Gerwins et al. 2000, Papetti and Herman 2002, Eklund and Saharinen 2013, Fernandez-Alonso et al. 2015).

Endothelial cell-proliferation, survival and apoptosis-regulation

VEGF is an EC mitogen and its stimulatory effects on EC proliferation are mainly mediated through binding to VEGFR-2. This promotes receptor dimerisation and induces activation of Ras-dependent and -independent pathways (Zachary 2001). Binding of VEGF to VEGFR-2 also induces the phosphatidylinositol 3'-kinase (PI3K)/AKT intracellular signal transduction pathway affecting EC migration, survival, permeability and apoptosis, essential for forming and maintaining a vascular tube (Gerber et al. 1998, Zachary 2001, Koch and Claesson-Welsh 2012). The serine-threonine protein kinase isoform AKT1 is the predominantly expressed isoform in ECs and induces the pro-survival protein Bcl-2 and phosphorylates the Bcl-2-associated agonist of cell death (BAD) (Rusinol et al. 2004). When phosphorylated, BAD binds to cytoplasmic 14-3-3 proteins and its pro-apoptotic function is temporarily inhibited (Zha et al. 1996). Bcl-2 itself inhibits apoptosis by antagonising its pro-apoptotic family members BAX and BAK (Dlugosz et al. 2006).

The complex regulation of the PI3K/AKT signalling pathway also involves activation by the cytokine macrophage migration inhibitory factor (MIF) and insulin-like growth factor 1 (IGF-1) (Hellstrom et al. 2001, Amin et al. 2003, Lue et al. 2007). Binding of IGFs to IGF receptors is modulated by IGF binding proteins (IGFBPs). Negative regulatory action on angiogenesis through inhibition of VEGF- and IGF-1-induced AKT phosphorylation, independent of the type 1 IGF receptor, has been shown in HUVECs in the presence of IGF binding protein 3 (IGFBP3) (Franklin et al. 2003).

Endometrial angiogenesis

The human endometrium receives its blood supply from the arcuate arteries, branching off radially at intervals and the terminal branches divide into straight and spiral arterioles. The straight arterioles supply the basal layer of the endometrium from which regrowth of the functional layer (the superficial two-thirds of the endometrium) takes place during the interval between menstruations. The spiral arterioles penetrate the entire functional layer, supplying the endometrial glands and stroma, and have the ability to change functionally in response to ovarian steroids. In other words, the endometrial vascular bed, in contrast to that in most other tissues, grows and regresses during the different phases of the menstrual cycle and thus provides a dynamic model of physiological angiogenesis (Kayisli et al. 2004). During the oestrogen-driven proliferative phase, vessel elongation is the major angiogenic mechanism in the endometrium, whereas intussusception and possibly sprouting are the two dominating forms of angiogenesis in the secretory phase, with progesterone as the predominant ovarian steroid hormone. The angiogenic potential of the endometrium seems to vary over the menstrual cycle with the highest increase in the early- to mid-proliferative phase and throughout the secretory phase (Maas et al. 2001).

In response to rapidly decreasing progesterone levels following corpus luteum demise in the late-secretory phase, the functional layer of the endometrium undergoes tissue breakdown and is shed. Immediately, vascular repair commences and bleeding is usually stopped at day five of the cycle [reviewed in (Maybin and Critchley 2015)]. The regulation of this process is not fully known but it apparently does not depend on oestrogen support. VEGF-encoding mRNA levels are greatly increased in menstrual endometrium, possibly in part induced by endometrial hypoxia due to spiral artery vasoconstriction, although absolute evidence of endometrial hypoxia at menses does not exist (Charnock-Jones et al. 1993, Sharkey et al. 2000, Maybin and Critchley 2015). However, because of methodological limitations and great variation in endothelial cell proliferation rates in endometrial samples taken from women at the same cycle phase, absolute knowledge of endometrial vascular formation throughout the menstrual cycle is still lacking (Girling and Rogers 2005).

That endometrial angiogenesis is locally regulated is supported by the results of a study showing proliferation of human umbilical vein ECs (HUVECs) following treatment with the supernatant from cultured endometrium (Print et al. 2004). One of the soluble factors in this supernatant was VEGF. Interestingly, production of VEGF did not correlate to the menstrual phase in which the endometrium was collected, further supporting the theory that endometrial VEGF expression is not predominantly steroid hormone-regulated. Indeed, local endometrial VEGF production may partly occur in neutrophils and macrophages (Girling and Rogers 2005).

Human endometrial endothelial cells (HEECs) express oestrogen receptor beta (ERβ) mRNA and protein (Critchley et al. 2001, Lecce et al. 2001) and progesterone receptor (PR) mRNA (Iruela-Arispe et al. 1999, Krikun et al. 2005). Oestradiol has been shown to enhance the proliferative response of cultured HEECs to VEGF and FGF-2 and induce the expression of mRNAs associated with intermediate signalling and enzymatic angiogenic pathways, although there is no evidence of a direct angiogenesis-promoting effect on HEECs (Iruela-Arispe et al. 1999, Girling and Rogers 2005, Krikun et al. 2005). Both VEGF and FGF-2 proteins and their receptors have been identified in human endometrium (Moller et al. 2001). Furthermore, E2 significantly increases VEGF mRNA in a dose-dependent manner in human endometrial stromal cells, supporting the paracrine-signalling model of endometrial angiogenesis (Bausero et al. 1998).

There is controversy over the role of progesterone in endometrial vascular formation. In response to the oestrogen peak during the proliferative phase, the expression of PRs is increased. The subsequent rise in circulating progesterone levels during the secretory phase downregulates ERs, suggesting an anti-proliferative effect on endothelial cells. However, during the secretory phase, angiogenesis is not arrested and occurs through the elongation and coiling of the spiral arteries. Despite the lack of conclusive evidence of the existence of PRs in HEECs (Krikun et al. 2005), progesterone has been reported to both inhibit and stimulate HEEC proliferation *in vitro* (Vazquez et al. 1999, Kayisli et al. 2004).

Pathophysiology of endometriosis

Pathogenesis

The processes leading to establishment of endometriosis are still awaiting a definitive mechanistic explanation. Evidence indicates combined contributions of menstrual effluent, pelvic peritoneum and peritoneal cavity fluid in the implantation and maintenance of endometrial fragments in the peritoneal surfaces of the female pelvis (Young et al. 2013). According to 'Sampson's hypothesis', viable endometrial fragments are transported by way of retrograde menstruation through the Fallopian tubes into the pelvic cavity, where the cells implant in the peritoneum and on the ovaries (Sampson 1927). Much later, this hypothesis was augmented with evidence of evasion from immune clearance and decreased apoptosis in the implanted endometrial cells (Burney and Giudice 2012). The implantation theory was subsequently expanded to include scar endometriosis (i.e. after Caesarean sections and laparoscopy), lymphatic spread to locations outside the abdominal cavity and experimentally induced endometriosis in rodents and primates after xenotransplants of endometrial or endometriotic cells (Koninckx et al. 1999).

Another mechanism, still supported as regards ovarian endometriosis, is coelomic metaplasia of the mesothelial cells covering the ovarian surface. Metaplastically transformed epithelium has been demonstrated to invaginate into the ovarian cortex and hence be in continuum with the endometrial-like tissue on the inside of ovarian endometriomas (Donnez et al. 1996).

A more recent theory is the migration to ectopic sites, and subsequent differentiation into endometriotic tissue, of bone-marrow-derived stem cells and endometrial progenitor cells from the *basalis* layer of the endometrium (Hufnagel et al. 2015). These latter cells are believed to play a part in the endometrial self-renewal process. The stem cell theory might explain some of the extra-abdominal locations, including the deep infiltrating endometriosis (DIE) phenotype. This form of endometriosis has also been proposed to be derived from Müllerian remnants, at least in the pouch of Douglas and the uterosacral ligaments (Vercellini et al. 2014).

Steroid hormone receptors in eutopic and ectopic endometrium

Both oestrogen and progesterone receptor isoforms (ERα/β and PR-A/-B) are expressed in the functional layer of the human endometrium. Together they mediate the steroid hormone effects on endometrial proliferation and differentiation during the menstrual cycle. The different endometrial cell compartments display cyclical variations in their nuclear steroid hormone receptor (SHR) expression in response to the alterations in circulating levels of oestrogen and progesterone throughout the cycle.

The ERs and PRs belong to the superfamily of intracellular hormone ligand-activated transcription factors. Upon ligand binding, the ligand-receptor complexes form homo- or heterodimers and enter the nucleus, where they bind to specific DNA sequences called oestrogen-/progesterone-response elements (Gruber et al. 2002, Patel et al. 2015). Simultaneous binding to nuclear receptor co-activators or repressors modulates the SHR-initiated transcription of genes downstream of the steroid hormone-responsive promoters. Oestradiol and progesterone thus regulate the expression of a large number of genes during the menstrual cycle (Kao et al. 2002).

Oestrogen receptors

Oestradiol is a key hormone as regards proliferation of the endometrium, as well as endometriosis lesions. Immunohistochemical (IHC) staining, using monoclonal antibodies, and *in situ* hybridization techniques have shown ERα to be the more abundant isoform of oestrogen receptor in the endometrium and it undergoes the most evident cyclical changes in its expression. The highest ERα mRNA and protein levels are seen in both glandular epithelial and stromal cells during the late-proliferative phase (menstrual cycle day 10–14), subsequently declining in the secretory phase in response to increasing progesterone levels (Lessey et al. 1988, Sasano et al. 1999). ERα is also expressed in human uterine vessels, mainly in the smooth muscle cells of the vascular walls (Lecce et al. 2001). There is substantial evidence that E2-induced endometrial proliferation and PR expression is mediated exclusively through ERα (Brosens et al. 2004, Patel et al. 2015).

A more enigmatic role in normal endometrium seems to be played by ERβ. It displays a similar temporal expression pattern to ERα, with the exception of late-secretory-phase stroma, where ERβ is the predominant ER subtype (Critchley et al. 2001). Expression of ERβ transcripts is induced by ligand-activated ERα and PR and has been demonstrated in all endometrial cell types (Brandenberger et al. 1999, Brosens et al. 2004). Although mRNA levels are lower, controversy exists over the ERβ protein levels compared with those of ERα. A restricting action of ERβ on the mitogenic effect of E2 on human endometrium has been suggested (Hapangama et al. 2015).

As previously stated, ERβ is the main, or only, ER isoform expressed in endometrial endothelial cells and it is also more abundant in the vascular walls during the mid-secretory menstrual phase, suggesting a regulatory role in endometrial angiogenesis. At the time of implantation, uNK cells expressing ERβ invade the stroma. However, it is not clear whether this cyclical increase in uNK cells is hormonally regulated (Critchley and Saunders 2009).

Eutopic endometrium in women with endometriosis seems to differ from normal endometrium in SHR expression and function in several respects. Because of the often-observed subfertility in these women, a number of studies have been focused on the period when the endometrium is the most re-

ceptive. The endometrium becomes receptive during the mid-secretory menstrual phase 6–9 days after the luteinising hormone (LH) peak, the so-called window of implantation (WOI), which occurs during cycle days ~19–23 (Paiva et al. 2009) (Figure 2).

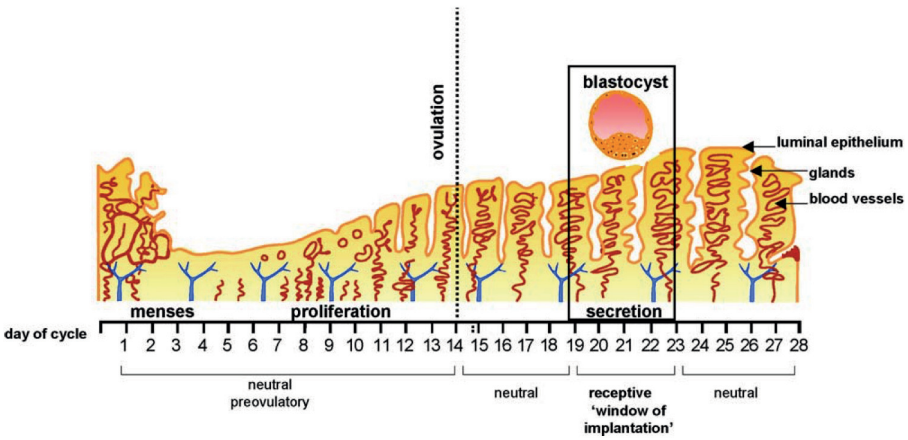

Figure 2. Endometrial remodelling during the different phases of the menstrual cycle showing periods of receptivity and non-receptivity ('neutral'). Adapted from Paiva et al. 2009. Reproduced with permission from Elsevier Ltd.

Lessey and colleagues found higher levels of ERα immunostaining in luminal and glandular epithelial cells in mid-secretory endometrial biopsy samples obtained from infertile women with endometriosis compared with normal fertile controls (Lessey et al. 2006). This implies a reduced response to progesterone in these endometria. Biopsy samples were selected on the basis of a lack of αvβ3 integrin expression, believed to further contribute to infertility. However, this aberrant ERα expression was not seen in a similar semi-quantitative study in which IHC evaluation of endometrial and endometriotic tissue biopsy samples was employed (Hudelist et al. 2005).

Endometriotic tissue differs from normal endometrium in several respects. Stromal cells in endometriosis lesions have the capacity to produce E2 through endogenous steroidogenesis (including action of the enzyme aromatase) effectively making it independent of ovarian E2 production (Bulun et al. 2012). In primary endometriotic stromal cells and endometriosis implants, ERβ is the predominant form of ER, probably as a result of hypomethylation of the ERβ promoter. This has led investigators to examine ERα/ERβ ratios in normal *vs.* eutopic endometrium, but the results are conflicting. Increased ERβ immunostaining in eutopic endometria from mostly nulliparous women has been reported although not reproduced in subsequent mRNA-based studies, with the exception of a decreased ERα/ERβ splice variant 1 ratio in the proliferative phase (Hudelist et al. 2005, Juhasz-Boss et

al. 2011). The greatest decrease in the ERα–ERβ ratio has been observed in ovarian endometriomas (Huhtinen et al. 2012).

Progesterone receptors

Progesterone exerts specific effects, through PRs, on the human endometrium during the secretory menstrual phase. The pre-ovulatory proliferation of the functional layer is inhibited and it is morphologically and functionally transformed into a secretory and oedematous tissue receptive to the implantation of a blastocyst. One mechanism behind the inhibition of oestrogen-driven endometrial growth is induction of 17β-hydroxysteroid dehydrogenase type 2 in epithelial cells. This enzyme converts E2 to the less oestrogenic oestrone (Bulun et al. 2006). Several putative biomarkers of endometrial receptivity have been identified as being progesterone-regulated; glycodelin, IGF-binding protein 1, integrins and complement proteins (Young and Lessey 2010). Some of these proteins are upregulated as a result of a direct progesterone effect on the epithelium and some, at least partly, are mediated by stromal cells sending paracrine signals involving retinoids to the glandular epithelium (Bulun et al. 2015).

A single gene, which is translated into the truncated PR-A and full-length PR-B, encodes the human PR isoforms, and progesterone effects are generally considered to be the result of the combined activity of the two receptor subtypes (Patel et al. 2015). Owing to the common amino-acid sequence of the two isoforms, many earlier studies have involved the use of antibodies raised against total PR or the longer isoform PR-B. Only recently has an antibody purportedly specific to PR-A become commercially available, inferring challenges when comparing results from different studies.

In the functional layer of the human endometrium, early studies of PR distribution showed prominent epithelial immunostaining in the mid- to late-proliferative phase, rapidly declining shortly post-ovulation. Stromal cells, in contrast, maintained positive PR staining throughout the secretory phase (Press et al. 1988). In a later study, involving the use of a semi-quantitative dual fluorescence technique, Mote and co-workers found both PR isoforms to be increasingly expressed during glandular proliferation, peaking in the late-proliferative phase (Mote et al. 1999). A similar expression pattern was seen in the stroma, albeit with a predominance for PR-A. PR staining in secretory-phase endometrium displayed the expected progesterone-induced reduction but with an unexpected discordance regarding the temporal decline of the two subtypes; PR-B levels were increased slightly in mid-secretory epithelium, correlating with the second oestrogen peak. Dual fluorescence staining revealed PR-A to be responsible for the previously observed prevailing PR immunostaining in secretory-phase stroma. A recent proteomics analysis has also confirmed the decrease in PR-B abundance in glandular epithelium during the secretory phase (Hood et al. 2015). Both PR isoforms are responsible for mediating the pro-gestational and anti-inflammatory ac-

tions of progesterone on the human endometrium and murine data suggests a hyperplasia-inducing effect of PR-B, which is repressed by PR-A (Patel et al. 2015).

There is no consensus regarding how PR function differs between normal and eutopic endometria. Gene expression profiling by microarray of endometria from women with endometriosis has shown dysregulation of several progesterone target genes during the window of implantation providing evidence of impaired progesterone action *in vivo* (Kao et al. 2003). A reduced PR-B/PR-A ratio and decreased expression of hydrogen peroxide-inducible clone-5 (Hic-5), a PR co-activator, may alter PR signalling and further contribute to the progesterone resistance often observed in endometriosis (Aghajanova et al. 2009b).

The abundance of ERβ may be at least partly responsible for the suppression of PR expression in endometriosis. Several reports exist of abnormal progesterone signalling in both eutopic endometrium and ectopic lesions: PR-responsive genes, including transcripts important for endometrial receptivity, are dysregulated in the secretory-phase endometrium; levels of PR-A are higher in eutopic endometrium of endometriosis patients than in women without disease regardless of menstrual phase; total PR expression is higher in eutopic endometrium than in peritoneal lesions; there is attenuated PR-induced capacity to metabolise E2 to oestrone, thereby increasing oestrogen-mediated proliferation and angiogenesis; and failure of progesterone to downregulate eutopic endometrial matrix metalloproteinases (MMPs) during the secretory phase (Osteen et al. 2005, Bulun et al. 2010, Bulun et al. 2012, Bedaiwy et al. 2015, Greene et al. 2016).

Pelvic inflammation

Pelvic inflammation plays a major role in the pathophysiology of endometriosis. Among the numerous cytokines and other pro-inflammatory factors elevated in peritoneal fluid (PF) of women with endometriosis are tumour necrosis factor (TNF)-α, IL-1α, IL-1β, IL-6, IL-8, IL-33, COX-2, prostaglandins and MMPs (Kondera-Anasz et al. 2005, Akoum et al. 2008, Santulli et al. 2012, Arosh et al. 2015, McKinnon et al. 2015). Concomitantly with inflammation and establishment of endometriotic lesions is neuroangiogenesis, the *de novo* formation of neuronal and vascular networks (Morotti et al. 2014). Both eutopic and ectopic endometrial tissue express nerve growth factor (NGF), as well as several other neurogenic factors. Peritoneal fluid from endometriosis patients stimulates neurite outgrowth from root ganglia *in vitro* and this process is significantly reduced by NGF inhibitors (Barcena de Arellano et al. 2011). Based on the first reports of increased nerve fibre density in eutopic endometria from women with endometriosis, immunostaining for neuronal markers in endometrial biopsy samples was proposed as a minimally invasive diagnostic tool for the disease (Bokor et al.

2009). In more recent work, investigators have not been able to reproduce the initial results of this diagnostic method. Thus, at present, although most experts agree that nerve fibres in endometriotic lesions, particularly DIE, are a cause of perimenstrual and chronic pain, controversy exists over whether the nerve fibres in eutopic endometrium are related specifically to endometriosis or to uterine pain symptoms in general (Morotti et al. 2014, Tarjanne et al. 2015).

Regardless of the origin of an endometriotic lesion, angiogenesis is a prerequisite for the establishment of endometriosis and is closely linked to the inflammatory state of the disease. Endometriotic and immune cells secrete angiogenic factors such as VEGF, transforming growth factor (TGF)-β, IL-6 and $PGF_{2\alpha}$, which can be found in PF (Bourlev et al. 2010, Asante and Taylor 2011, Rakhila et al. 2016). Also, as in eutopic endometrium, vessel formation is under oestrogenic control, since E2 induces ectopic stromal cell production of VEGF (Zhang et al. 2016).

The enhanced angiogenesis in endometriosis is illustrated by the results of a study conducted by the Reproductive Molecular Research Group in Cambridge, UK (Hull et al. 2003). They found a larger proportion of tissue occupied by blood vessels in peritoneal endometriotic lesions than in the functional layer of eutopic endometria from the same women. The ectopic vessels were predominantly of an immature phenotype and lacked pericytes. According to the authors, these newly formed vessels are therefore responsive to both VEGF-stimulated proliferation, and apoptosis following experimentally induced VEGF withdrawal.

Other research groups have also used IHC-based analysis of microvessel density as a means to study angiogenesis in relation to endometriosis. Applying computerised image analysis of tissues stained with anti-CD31 antibody, a somewhat increased number of microvessels in peritoneal and DIE lesions compared with eutopic endometria from women with endometriosis has been seen (Jondet et al. 2006). In eutopic and superficial ectopic biopsy samples from endometriosis patients treated with progestins, vessels were significantly fewer than in untreated patients, but the vascular area was increased. This finding indicates that progestins induce a reduction in number and dilation of small vessels in these tissues. Since this effect and signs of pseudodecidualisation were much less evident in deep lesions, the authors concluded that DIE is less responsive to progestin than eutopic endometrium and peritoneal endometriosis. Furthermore, the number of microvessels per mm^2 in endometrial samples from healthy women in a preceding work by Jondet and colleagues, employing the same techniques (Jondet et al. 2005), was lower than in the women in the latter study, in line with the hypothesis that angiogenesis increases in eutopic endometrium of endometriosis patients (Healy et al. 1998).

Endometriosis and fertility

If the pathogenesis of endometriosis is enigmatic, it is no less true when one tries to evaluate the possible detrimental effects of endometriosis on fertility. Aberrant expression or epigenetic regulation of several candidate markers for endometrial receptivity have been reported although there is no consensus regarding their relative importance. Among the most investigated biomarkers are integrin αvβ3, homeobox A10 (HOXA10) and leukaemia inhibitory factor (LIF) (Garcia-Velasco et al. 2015). Integrin αvβ3 and its ligand osteopontin are expressed on the cell surface of the luminal epithelium, suggesting a role in initial endometrium-embryo signalling and adhesion at implantation. Homeobox proteins are pleiotropic transcription factors that regulate downstream target genes important for endometrial receptivity and decidualisation (Zhu et al. 2013, Xu et al. 2014). These genes include those encoding integrin αvβ3 and LIF, which was demonstrated by knock-down of *Hox* genes in a mouse model, resulting in reduced implantation rates and decreased *Lif* and *Itgb3* expression (Xu et al. 2014). However, translation of mouse model data to humans must be done with great care. Nevertheless, HOXA10, HOXA11 and LIF are upregulated during the mid-secretory menstrual phase in normal endometrium and altered expression in women with endometriosis has been associated with impaired fertility (Dimitriadis et al. 2006, Du and Taylor 2015). Moreover, lack of integrin expression, possibly due to a direct effect exerted by PF factors, has been reported in infertile endometriosis patients (Lessey 2002). A number of these factors are listed above.

Levels of IL-1β are increased and its soluble receptors sIL1R2 and inhibitory sIL1RAcP are decreased in PF from endometriosis patients (Michaud et al. 2011). The reciprocal regulation of IL-1β and sIL1R2 was most pronounced in the secretory phase and in infertile patients with pelvic pain (Akoum et al. 2008). Reduced expression of sIL1RAcP in eutopic endometrium has also been demonstrated, suggesting a defect in the capability of endometrial cells to counter-regulate IL-1-mediated inflammation (Guay et al. 2011).

Levels of IL-1α have been found to be increased in PF from infertile women with endometriosis and concentrations were found to correlate with the severity of the disease (Kondera-Anasz et al. 2005). No detectable levels were seen in healthy controls. Although no hypothesis was presented as to whether IL-1α directly contributed to the women's infertility, the authors postulated that IL-1α upregulates the expression of other cytokines, such as TNF-α and IL-6. Compared with the relative abundance of research entries on IL-1β and endometriosis, the significance of IL-1α is still a somewhat unknown territory.

Interference with oocyte quality and decreased sperm function has been attributed to IL-10, TNF-α and IL-6 (Gupta et al. 2008). Of particular inter-

est in endometriosis-related subfertility is the proposed mechanism of a combined interaction of IL-6 and the soluble IL-6 receptor, also present in PF, binding free gp130 in sperm and reducing motility (Yoshida et al. 2004). Pro-inflammatory cytokines are also considered to be major contributors to the lower abdominal pain often experienced by endometriosis patients. Levels of IL-6 in PF correlated positively with dysmenorrhoea and pelvic pain as well as recurrence of symptoms after surgery in a cohort of women undergoing laparoscopy for ovarian endometriomas (Velasco et al. 2010). Reduction of pain symptoms and decreased IL-6 mRNA in PR-A- or PR–B-expressing endometriotic epithelial cell lines after treatment with the synthetic progestin dienogest provide evidence of PR-mediated production of IL-6 in endometriosis (Grandi et al. 2016).

In this thesis, the roles of ERs, PRs, LIF and interleukins 1α and 6 in some aspects of endometriosis are discussed in more detail. In addition, the small heat-shock protein αB-crystallin, hitherto unrelated to endometriosis, and the inflammation-associated neuropeptide vasoactive intestinal peptide are subjects of observational study.

AlphaB-crystallin in reproduction

The α-crystallins A and B belong to a family of small heat-shock proteins first identified in the mammalian ocular lens. Whereas αA-crystallin expression is mainly confined to the eye, αB-crystallin/HspB5 (αBCr) distribution is widespread in the body (Narberhaus 2002, Haslbeck et al. 2016). Crystallins act as molecular chaperones, forming hetero- and homo-oligomers that prevent the intracellular aggregation of unfolded or misfolded proteins during translation and translocation (Parcellier et al. 2005).

Upregulation of αBCr is associated with several neurodegenerative disorders where deposition of aggregated proteins occurs, such as Alzheimer's, Parkinson's and Alexander diseases (Parcellier et al. 2005, Reddy and Reddy 2015). Increased expression and/or activation through phosphorylation of αBCr in response to a wide variety of cellular stress events, such as heat shock, oxidative stress and radiation, as well as in certain tumours, has also been described (Zuo et al. 2014, Reddy and Reddy 2015). Under these conditions, αBCr prevents apoptosis, especially through inhibition of the proteolytic maturation of caspase-3 and by binding to the Bcl-2-associated proteins BAX and Bcl-X(S) (Mao et al. 2004, Kamradt et al. 2005).

Evidence of the immunomodulating properties of αBCr can be gained from a study where injection of αBCr in a mouse inflammation model reduced the production of the pro-inflammatory cytokines interleukin-1α and TNF-α (Masilamoni et al. 2005). Furthermore, αBCr has pro-survival effects on endothelial cells and regulates tube morphogenesis in tumour vessels (Dimberg et al. 2008).

The role of αBCr in the female reproductive tract is enigmatic and has been the subject of only a few studies. It was demonstrated in secretory-phase endometrial epithelium by Gruidl and co-workers while searching for differently expressed mRNAs possibly associated with endometrial receptivity (Gruidl et al. 1997). They found a progressive increase in αBCr mRNA and protein expression from around post-ovulatory-day (POD) 5, peaking at POD 14. A similar temporal pattern for mRNA abundance was also seen in baboon endometria. Using polyclonal antibodies, αBCr immunostaining was first seen in the luminal epithelium in the early-secretory phase, gradually increasing in intensity and demonstrated also in glands in mid- and late-secretory-phase endometrial specimens. No mRNA or protein was detected in proliferative-phase samples, prompting the authors to propose a role for αBCr in endometrial receptivity.

Microarray analyses of transcript changes in human endometrium at the WOI compared with POD 2–3 have demonstrated an up to fourfold upregulation of the *CRYAB* gene, encoding the αBCr protein (Riesewijk et al. 2003, Mirkin et al. 2005). However, other investigators, using similar techniques and materials, have not found differential regulation of this gene, making the

interpretation of the findings difficult (Carson et al. 2002, Borthwick et al. 2003).

The Human Protein Atlas [www.proteinatlas.org (Uhlen et al. 2015)] shows medium- to high-level antibody staining for αBCr localized to the cytosolic compartment in endometrial glands from women of fertile age. Uneven staining distribution is seen where some cells show high immunoreactivity, whereas other cells are unstained within the same gland. This is consistent with previous findings as well as with results presented in this thesis (Gruidl et al. 1997, Moberg et al. 2015).

In vitro decidualisation of mouse uterine stromal cells and exposure to progesterone are associated with elevated *Cryab* mRNA levels although the hormonal regulation of αBCr in mouse uterus *in vivo* is still unclear (Tian et al. 2013, Zuo et al. 2014).

Leukaemia inhibitory factor and endometrial receptivity

The interleukin (IL)-6 family protein leukaemia inhibitory factor (LIF) and its receptor heterodimer complex, consisting of the low-affinity LIFRβ and shared cytokine co-receptor glycoprotein 130 (gp130) chains, are expressed in many tissues in mammals and they have a broad range of activities (Kimber 2005, Nicola and Babon 2015). LIF signalling is transduced foremost through JAK1/STAT3 but also the PI3K/Akt and MAPK pathways, stimulating proliferation and differentiation in both embryonic stem cells and developing cells in growing or adult individuals, as well as following injury. Depending on the pathway, it can have pro-survival effects in some cells and induce apoptosis in others. LIF signalling is negatively regulated by suppressors of cytokine signalling (SOCS) proteins, targeting the JAK/STAT cascade. Despite being such a pleiotropic cytokine, LIF knock-out mice show relatively few developmental defects and many LIF functions may be redundant or executed by other inducers of the same signalling pathways (Nicola and Babon 2015).

When LIF and its receptor (here referred to as LIFR) were identified in the epithelium of human secretory-phase endometria from fertile women, it was suggested that LIF plays an important role in embryo implantation (Charnock-Jones et al. 1994, Cullinan et al. 1996, Sherwin et al. 2002). Previous work had identified LIF as an absolute requirement for implantation in mice, since the uterus in LIF -/- females is not receptive to blastocysts (Stewart et al. 1992). This inspired further investigation of different aspects of fertility/infertility in humans related to LIF and its receptor in the endometrium. LIF and LIFR immunostaining in the endometrial epithelium increases after ovulation, reaching their maximum levels on days LH +6 to LH +9, coinciding with fully developed pinopodes on luminal epithelial cells, indicating receptive endometrium (Aghajanova et al. 2003). It is therefore

assumed that progesterone is a major regulator of endometrial LIF production. This assumption is supported by evidence of reduced epithelial LIF expression during the WOI in women treated with the progesterone receptor modulator mifepristone immediately after ovulation (Danielsson et al. 1997). However, local modulation of LIF production and secretion by, among other factors, heparin-bound epidermal growth factor (HB-EGF), prokineticin 1, human chorionic gonadotrophin (hCG), insulin-like growth factor (IGF)-1 and -2 and pro-inflammatory cytokines points to a complex systemic and paracrine regulatory network (Paiva et al. 2009).

Although the absolute significance of LIF and LIFR/gp130 in endometrial receptivity is unclear and their expression may not be vital for embryo implantation in humans, a considerable amount of work has been invested in studying their roles in infertility. Most studies, however, are descriptive rather than presenting causality, sometimes with conflicting results. Lower concentrations of LIF in uterine flushings, obtained in the mid-secretory menstrual phase, from women with unexplained infertility *vs*. normal fertile women have been measured. Cultured endometrial epithelial cells from fertile women also produced the highest amounts of LIF when sampled in the early-secretory phase (Laird et al. 1997). Moreover, in women with unexplained infertility, decreased epithelial LIF, LIFR and gp130 expression has been detected in mid-secretory endometrial biopsy samples and a simultaneous loss of LIF and αvβ3 integrin was more specific to poor reproductive outcome than loss of αvβ3 integrin alone (Aghajanova et al. 2009a, Franasiak et al. 2014).

An indication of the negative effects on LIF production by inflammatory processes comes from the observation that hydrosalpinx reduces endometrial LIF expression during the WOI in infertile women. Accordingly, endometrial LIF levels are restored after salpingectomy (Seli et al. 2005). On the other hand, a study by Makker and co-workers revealed no differences in LIF immunostaining when examining mid-secretory endometrial biopsy samples from a heterogeneous cohort of infertile women with mild endometriosis, tubal and immunological factors, as well as normal fertile controls (Makker et al. 2009).

Besides their presence in endometrial epithelium, LIF, LIFR and gp130 are expressed in human preimplantation embryos and in Fallopian tube epithelium (Charnock-Jones et al. 1994, Sharkey et al. 1995, Wanggren et al. 2007). Autocrine and paracrine functions of LIF/LIFR in embryo attachment and implantation have been suggested, as well as modulation of the immune tolerance to the implanting blastocyst (Wanggren et al. 2007, Aghajanova 2010). Further support for the role of LIF in the earliest stages of pregnancy comes from studies where a polyethylene glycated LIF antagonist has been shown to inhibit implantation in mice and in an *in vitro* 3D model of human receptive endometrium (White et al. 2007, Lalitkumar et al. 2013). In addition, LIF and LIFR appear to be important for successful decidualisation of

human and murine endometrial stromal cells, trophoblast adhesion and invasion of the decidua (Paiva et al. 2009).

LIF in endometriosis

As described above, accumulating data suggest that in women with endometriosis, the eutopic endometrium aberrantly expresses biomolecules important for receptivity contributing to sub- or infertility. There is a lack of conclusive evidence supporting the hypothesis that defective LIF expression is a cause of implantation failure in cases of unexplained infertility, and even more so as regards endometriosis. Only a few studies exist and the participating patients are generally presented as 'primary infertile'.

In 2006, two independent reports on infertility related to eutopic endometrial dysregulation of IL-11 and LIF during the WOI were published. In a small series of endometrial biopsy samples from infertile women with rASRM (1997) stage I–IV endometriosis, Dimitriadis and colleagues found reduced epithelial LIF immunostaining compared with that in a control group of healthy fertile women undergoing laparoscopic tubal ligation (Dimitriadis et al. 2006). The second study concerned secreted LIF in uterine flushings and *LIF* mRNA expression (RT-PCR) in the endometrium (Mikolajczyk et al. 2006). There was a tendency towards lower secreted LIF levels in infertile women with rASRM stage I–II endometriosis than in a control group of women without visible endometriosis at laparoscopy, but the results did not reach statistical significance. Furthermore, the number of women without detectable LIF in uterine flushings was higher among the endometriosis patients. The authors found no difference in *LIF* mRNA transcript levels among the groups. Both of these studies included very few women with endometriosis (n=7 and n=14 respectively), raising questions about their relevance as regards general conclusions about the role of LIF in endometriosis-related subfertility and also possibly preventing the small observed differences seen in secreted LIF levels from reaching significance.

Ruling out all causes of infertility other than endometriosis must be regarded as near impossible, as is isolating a defective endometrium as the main cause in women with the disease. A methodological weakness in this area of research is that both women with primary infertility and those who have given birth previously may be affected by several fertility-impairing factors besides endometriosis. Excluding women with primary infertility from studies may reduce the risk of mixing endometriosis-related subfertility with other causes such as uterine anomalies and infertility of genetic or immunological origin.

Vasoactive intestinal peptide and its receptor VPAC1

Vasoactive intestinal peptide (VIP) is a key player in regulation of inflammation and in the parasympathetic nervous system. Originally identified as a vasodilator peptide in the upper intestine (Said and Mutt 1970), VIP is now recognised as a ubiquitous neuropeptide, widely distributed throughout the body. It is involved in a variety of biological activities in the nervous, endocrine and immune systems; vasodilation, smooth muscle relaxation, secretion, hormone release, inhibition of macrophage and lymphocyte migration and production of pro-inflammatory cytokines upon activation, just to name a few (Delgado et al. 2004, Gomariz et al. 2006). VIP is a 28-amino acid peptide, belonging to the same family of gastrointestinal and nervous system peptides as glucagon and growth hormone-releasing factor. It is produced in parasympathetic and sensory nerve fibres, intrinsic enteric VIPergic neurons, lymphocytes, granulocytes and mast cells (Delgado et al. 2004).

The biological effects of VIP are mediated through two class B G-protein-coupled receptors, VPAC1 and VPAC2. Like VIP, VPAC receptors are widely distributed and are found in both the CNS and in most peripheral tissues. In the immune system, VPAC1 is constitutively expressed in monocytes, macrophages and T lymphocytes, whereas VPAC2 expression is induced as part of an immune response (Langer 2012). Both VPAC receptor subtypes are expressed in normal endometrium and myometrium (Bajo et al. 2000). Endometrial carcinomas predominantly express VPAC1 in glands and VPAC2 in the stroma, whereas leiomyomas preferentially express VPAC2 (Bajo et al. 2000, Reubi et al. 2000).

There are very few reports concerning VIP and VPAC function in the uterus. Cultured human endometrial stromal cells (HESCs) constitutively express VIP and VPAC1. Progesterone stimulates endogenous VIP production in HESCs but does not seem to modulate VPAC1 expression (Grasso et al. 2014). Cultivation of HESCs in the presence of VIP induced decidual morphology, production of decidualisation markers and the chemokine RANTES, involved in T cell recruitment. Hence, the authors of the study concluded that VIP, modulated by progesterone, induces a microinflammatory environment in the stroma and contributes to the decidualisation process (Grasso et al. 2014). It has also been proposed that VIP plays a role in the endometrial immune tolerogenic response, required for blastocyst implantation (Gallino et al. 2016).

Evidence of a role of VIP in peripheral pain transmission in endometriosis has emerged since its discovery a decade ago as one of the neurotransmitters in the sensory and autonomic nerve fibres in the endometrium, endometriotic lesions and adjacent tissue (Tokushige et al. 2006, Kelm Junior et al. 2008, Wang et al. 2009a). The majority of these nerve fibres are unmyelinated C fibres and a small proportion consists of myelinated Aδ fibres (Miller and Fraser 2015). Sensory C fibres are nociceptors and contribute to the dull,

throbbing, and in some cases, over time, chronic pelvic pain often experienced by endometriosis sufferers.

That VIP is directly involved in pain generation was demonstrated by intra-articular injection of VIP in rat knee joints, causing pain-associated behaviour. The VIP-generated pain response was inhibited by co-administration of the VPAC antagonist VIP_{6-28}, as was the pain behaviour associated with induced osteoarthritis in the same study (McDougall et al. 2006). In humans, reduced VPAC1 expression on circulating monocytes correlated with increased IL-6 expression and joint symptoms in patients with early arthritis, further confirming the key role of the VIP/VPAC system in chronic inflammatory diseases (Seoane et al. 2016).

Even though the pathogenesis of endometriosis in many ways differs from the mechanisms behind autoimmune diseases, one of its main features is the chronic inflammatory state in the pelvic environment. It is therefore not unlikely that VIP and VIP receptors are involved in an anti-inflammatory response to activation of the local immune system. The known increased density of VIPergic nerve fibres and probable invasion of VIP-secreting mono-/lymphocytes in endometriosis are likely to be reflected in the local presence of VIP. This reasoning led us to investigate whether or not VIP in eutopic and ectopic endometrium, as well as in PF of women with endometriosis is related to the degree of inflammation, expressed as PF levels of IL-6, and the occurrence (or not) of chronic pelvic pain (CPP).

Aims

The general aim of this thesis was to investigate aspects of physiological endometrial angiogenesis and proposed effects of endometriosis on female fertility and chronic pelvic pain, by achieving the following specific research objectives:

I. To assess the effect of exposure to VEGF-A and partial serum withdrawal on the transcript profile of cultured primary human endometrial endothelial cells.

II. To investigate the eutopic endometrial levels of ER, PR and alphaB-crystallin in relation to the chance of spontaneous pregnancy in women with minimal to moderate endometriosis.

III. To investigate whether eutopic endometrial expression of LIF, LIFR, gp130 and peritoneal fluid levels of IL-1α and IL-6 are related to the probability of spontaneous pregnancy in women with minimal to mild endometriosis.

IV. To test the hypothesis that chronic pelvic pain in women with minimal to moderate endometriosis is related to inflammation and increased expression of vasoactive intestinal peptide (VIP) in eutopic endometrium, endometriotic lesions and in peritoneal fluid.

Materials & Methods

Study population

Paper I

Twelve women of fertile age undergoing hysterectomy for benign indications were included in this study. They were generally healthy, non-smokers and had a history of proven fertility. All women had provided Pap smears (benign) and none had been using hormonal or intrauterine contraceptives for at least three months prior to surgery.

Paper II

The women in this study were included while being investigated for secondary infertility at the Research Center for Obstetrics, Gynecology and Perinatology, Moscow, Russian Federation. In all, 38 women, 27–35 years of age, with regular menstrual cycles and laparoscopically verified endometriosis agreed to participate. Assessment of the extent of endometriosis according to rASRM criteria was performed and 25 women were found to have stage I–II and 13 women stage III disease. For further clinical data, see Paper II (Table 1).

A control group of age-matched healthy women (n=18) was recruited among patients undergoing laparoscopic sterilisation. They showed no signs of endometriosis and had not been using any hormonal contraceptive or intrauterine device for at least six months.

A venous blood sample and an endometrial biopsy sample were obtained on the day of surgery, performed in the secretory phase of the menstrual cycle, from all women in both the study and the control groups.

Women in the study group underwent injections with goserelin acetate every 28–30 days for three months according to the local RCOGP protocol, beginning at the first menstruation post-surgery. Spontaneous pregnancies within 12 months after completed endometriosis treatment were recorded.

Paper III

For this study, 65 women were included according to the same criteria as in Paper II except that the extent of endometriosis was limited to rASRM stage I–II. An endometrial biopsy sample and a peritoneal fluid (PF) sample were

obtained during surgery, timed to the secretory phase of the menstrual cycle. After two months of goserelin acetate therapy and a wash-out period, 24 women (36.4%) agreed to undergo a second endometrial biopsy (Figure 3).

The cumulative on-going pregnancy rate in all 65 participating women (occurring in spontaneous ovulatory cycles) within the following six months was registered, after which the majority of the women not having conceived wished to proceed with assisted reproduction procedures.

A group of healthy women (*n*=23), age-matched to the study patients, undergoing laparoscopic tubal ligation served as controls and agreed to provide an endometrial biopsy sample and a PF sample. All participants in the study and control groups reported regular menstrual cycles and no use of hormonal contraceptives for at least three months prior to inclusion.

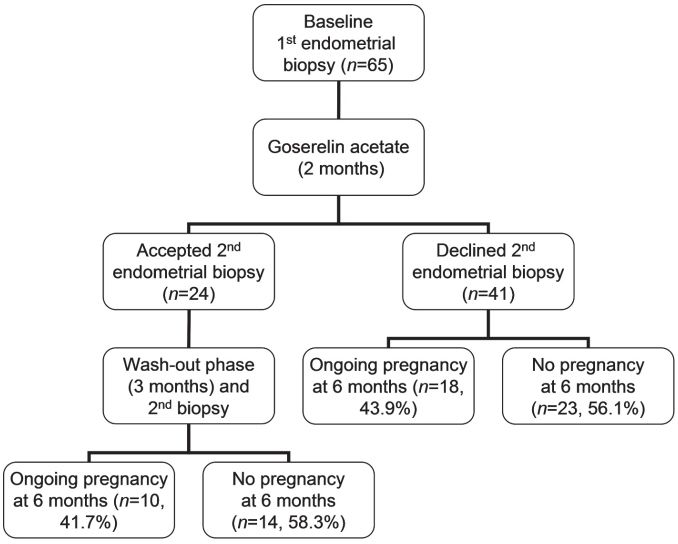

Figure 3. Flowchart showing women's progress through Study III.

Paper IV

Eighty-five women diagnosed with rASRM stage I–III endometriosis at laparoscopy while being investigated for pain and/or subfertility were enrolled in the study. They had regular menses and no recent hormone use or endometrial pathology. Endometrial biopsy and peritoneal fluid samples were obtained as in Paper III, but no separation of different menstrual phases, based on histological dating of the biopsy samples, was performed. In addition, ectopic peritoneal lesions were excised and included in the analyses.

The patients were asked to grade the intensity of their chronic pelvic pain, defined as more or less daily pain for six months or more, on a continuous 0–10 ('no pain' – 'worst imaginable pain') visual analogue scale (VAS). Thirty-four women had no chronic pain (referred to as E/CP-) and 53 report-

ed chronic pelvic pain with a mean value of 4.8 on the pain VAS (E/CP+). More demographic data are to be found in Paper IV (Table 1).

A control group of 53 healthy women, age-matched to the study patients and undergoing laparoscopic tubal ligation, was used. Women who had been using hormonal contraception in the three-month period before laparoscopy were excluded. Endometrial biopsy specimens and PF samples were obtained on the day of surgery, as in the study group of endometriosis patients.

Methods

Isolation, characterisation and culture of cells

Endometrial tissue samples from twelve hysterectomy specimens were collected under sterile conditions, dated by a pathologist (six from the proliferative phase and six from the secretory phase) and assessed as being normal. Endometrial endothelial cells were isolated using a slightly modified version of a previously published protocol (Bredhult et al. 2007). Briefly, the tissue samples were cut into small pieces and enzymatically digested and filtered before the addition of CD31-coated Dynabeads to the cell suspension. Bead-coated HEECs were collected and cultured in EGM-2MV + Bullet Kit culture medium, supplemented with 5% FBS, to confluence. A small proportion of early passage cells were used for EC immunocytochemical characterisation with a panel of antibodies against known EC antigens (Table 1).

All 12 cell cultures were divided into two subsets (treatment and matched controls) where one subset was exposed to VEGF (or vehicle control) and the other subset was subject to partial serum starvation by reducing the FBS content to 2% (or no change) for 24 h. This setup allowed for paired statistical analysis where each treated cell culture had its own control.

Four replicates of each subset were pooled and total RNA was extracted using TRIzol Reagent according to manufacturer's instructions. No distinction was made between cells isolated from proliferative- or secretory-phase endometrium.

Table 1. *Primary antibodies used for immunocytochemistry of isolated primary human endometrial endothelial cells.*

Target protein	**Catalogue number**	**Dilution**
von Willebrand factor	M0616	1:25
CD31	GA610	1:40
CD34	GA632	1:10
CD54	F7143	1:25
CD105	M3527	1:10

Antibodies purchased from Dako, Agilent Technologies Sweden AB

Microarray analysis

After quantification and assessing RNA integrity, double-stranded complementary DNA (cDNA) was made using the SMART synthesis protocol. For amplification of cDNA, an AmpliTaq DNA Polymerase kit (with Buffer II) was used (15 cycles). Fluorescence labelling of the amplified cDNA product was then performed by means of a BioPrime DNA Labeling system. After removal of unincorporated fluorescent dCTPs, the cDNA was hybridised to microarray slides for 16–18 hours at 50° C.

The slides were prepared in-house at the microarray core facility of the Department of Pathology, University of Cambridge, UK, using the Oligator Human Genome RefSet oligonucleotide library. The RefSet library contains 22,740 70-mer oligonucleotide probes including negative controls, targeting 20,726 genes in the human genome, including 1,310 splice variants.

Scanning of the slides was carried out on a GenePix 4100A Microarray scanner and images were captured using GenePix Pro 4.1 software. The dual-channel images were then imported to BlueFuse for Microarrays 3.1 open platform software for removal of noise and uncertain probe signals before conversion to numerical data.

Immunohistochemistry [Papers II and III]

Immunostaining was performed on sections of formalin-fixed, paraffin-embedded endometrial biopsy samples. Incubation with blocking serum was followed by incubation with primary antibodies (Table 2) overnight at 4° C. Irrelevant IgG was used as a negative control. Secondary antibodies were biotinylated horse anti-mouse, goat anti-rabbit and horse anti-goat antibodies, purchased from Vector Laboratories Inc., USA.

Sections were incubated with secondary antibody for 60 min at RT and rinsed before avidin/biotin labelling using VECTASTAIN Elite ABC kits. Detection of specific staining was performed by using the chromogen 3,3-diaminobenzidine (DAB) and sections were counterstained with Mayer's haematoxylin.

A Nikon Labophot-2 microscope was used for examination of stained sections and images were captured with Image-Pro Plus acquisition software. Assessment of staining and distribution was carried out using the semi-quantitative histological score (HSCORE) system, calculated according to the following equation: $[\Sigma Pi\ (i+1)]/100$, where i = the intensity of staining (i.e. 0 [weak staining] to 3 [most intense staining]) and Pi = the percentage of stained cells for each intensity, ranging from 0–100% (McCarty et al. 1986).

Table 2. *Primary antibodies used in immunohistochemistry and Western blot.*

Target protein	Catalogue number	Supplier	Method and dilution
Estrogen receptor α	sc-786	Santa Cruz Biotechnology	IHC 1:200
Estrogen receptor β	51–7700	Invitrogen	IHC 1:200
Progesterone receptor A+B	M3569	Dako	IHC 1:50
Progesterone receptor B	sc-811	Santa Cruz Biotechnology	IHC 1:100
Crystallin αB	ADI-SPA-222	Enzo Life Sciences	IHC 1:75
Leukaemia inhibitory factor	MAB250	R&D Systems	IHC 1:25
Leukaemia inhibitory factor receptor α	AF-249-NA	R&D Systems	IHC 1:250
Glycoprotein 130	AF-228-NA	R&D Systems	IHC 1:25
Suppressor of cytokine signalling 1	sc-7006	Santa Cruz Biotechnology	IHC 1:75
CD31	GA610	Dako	IHC 0.3µg/ml
Vasoactive intestinal peptide	AB982	EMD Millipore	IHC 1:100 WB 1:500

Immunohistochemistry [Paper IV]

Preparation of sections from formalin-embedded biopsy samples and incubation with antibodies was carried out according to the protocol described in Papers II and III. Primary antibodies used for studying immunolocalisation and staining intensity are listed in Table 2. The sections were then rinsed and incubated with avidin/biotin-labelled horse anti-mouse secondary antibody using VECTASTAIN Elite ABC kits followed by detection of staining with DAB. For negative controls the primary antibody was omitted.

Microvessel density (MVD) calculation was performed with the help of a microscope grid slide for the examination of five randomly chosen fields, each measuring 0.109 mm^2. The stained vessels were counted, extrapolated and expressed as the number of vessel intersections per mm^2.

Immunohistochemical detection of VIP expression in endometrial and endometriosis tissue was performed using the EnVision G|2 System/AP, Rabbit/Mouse (Permanent Red) according to the manufacturer's instructions. Digital image capture and analysis was carried out as previously described (Papers II and III). A VIP density score was expressed as mean staining intensity (on a scale of 0–3) × % of area per mm^2.

Serum analysis

Serum levels of oestradiol and progesterone were measured using the IMMULITE 1000 Immunoassay system.

Analysis of peritoneal fluid

Undiluted PF samples were spun in a refrigerated centrifuge before being stored at -70 °C until analysed. Levels of PF IL-1α, IL-6 and VIP were determined using Quantikine ELISA and Phoenix Pharmaceuticals EIA kits. Colour intensities were measured using a SpectraMax 250 spectrophotometric microplate reader.

RNA extraction and quantitative real-time PCR

Total RNA was extracted with RNeasy mini kits and RNA concentrations were measured with a NanoDrop spectrophotometer. RNA integrity was assessed with the Agilent 2100 Bioanalyzer system. Only samples with an RNA integrity number of 5 or more were included.

Complementary DNA (cDNA) synthesis was carried out by using SuperScript VILO Master Mix. Real-time qPCR was performed using TaqMan Fast Universal PCR Master Mix and TaqMan Gene Expression Assays on a StepOnePlus™ Real-Time PCR System according to the manufacturers' protocols using primers for vasoactive intestinal peptide (VIP; Hs00175021-m1) and human β-actin (ACTB; 4326315E) as endogenous control. Mean primer efficiency and normalized gene expression were calculated using LinRegPCR and comparative C_T methods, modified from those originally described by Kallak and colleagues (Kallak et al. 2015).

Western blot

Proteins were isolated in RIPA buffer from endometrial and endometriosis tissue from a subset of endometriosis patients and healthy control women and used for western blots. Denatured protein samples were separated using a NuPAGE® 4–12% gel system and transferred to PVDF membranes. The membranes were blocked in LI-COR Odyssey blocking buffer, followed by overnight incubation at 4 °C with primary antibody (Table 2). After washing, the membranes were incubated with fluorescently labelled secondary antibodies and protein bands were visualized using an Odyssey Infrared imager.

Statistics

In Paper I, numerical data output from BlueFuse for Microarrays 3.1 software were normalised using the Linear Models for Microarray data (LIMMA) software package in the 'R' statistical computing environment (http://bioinf.wehi.edu.au/limma). Rank products and Cyber-T algorithms for paired two-conditions data were used for identifying differentially expressed cDNA transcripts and both are developed for microarrays with transcripts expressed under two experimental conditions Selected transcripts in

the study had to pass both tests. 'Rank products', a non-parametric test for replicated microarray experiments, does not assume that the data are normally distributed and has proven to be reliable in determining the significance level of each gene even in noisy data (Breitling et al. 2004). Cyber-T (http://cybert.ics.uci.edu/) implements a Bayesian *t* test to compute a regularised variance of the measurements associated with each probe under the two conditions (Kayala and Baldi 2012). It also calculates the posterior probability of differential expression (PPDE), estimating the rates of false positives and false negatives on the array based on a threshold below which *P* values are considered significant and representative of (fold-) change.

The output files of the statistical analyses were imported into Microsoft Excel as gene lists with fold changes and *P* values, and gene ontology (GO) analysis was performed by uploading the gene lists to FatiGO, a web-based integrative platform for the analysis (for example) of transcriptomics including functional profiling (http://babelomics.bioinfo.cipf.es/). Over- and underrepresented GO categories in the gene lists compared with the whole arrays were identified using GOstat (http://gostat.wehi.edu.au). For the creation of functional gene networks, the gene lists were uploaded to Ingenuity® Pathway Analysis (IPA®) v5.0 software (http://www.qiagen.com/ingenuity), searching the Ingenuity Knowledge Base for known gene relationships in the literature.

Statistical analysis in Papers II–IV was performed using IBM SPSS Statistics 10 and 20 software. The χ^2 test was used for comparing demographic data and clinical characteristics between groups of endometriosis patients. The Mann–Whitney *U* test was used for non-parametric comparison of mean values from the immunostaining data and from the western blot, qPCR, serum and peritoneal fluid analyses. When comparing more than two groups, the Kruskal–Wallis test was used. If group means were significantly different in the Kruskal–Wallis test, pairwise comparisons were carried out by use of the Mann–Whitney *U* test. *P* values of <0.05 were considered significant.

Ethics approval

The Regional Ethics Review Board in Uppsala (Uppsala University secretariat) approved the study protocol of Paper I. All women were informed about the aim of the study and gave their informed consent.

Approval for the study protocols of Papers II–IV was obtained from the Ethics Committee at the RCOGP, Moscow, and informed consent was received from all participants. All investigations were carried out according to the Declaration of Helsinki.

Summary of results

Paper I

The growth rate varied between EC isolates but all cell cultures were confluent within five to seven days. There were no signs of Dynabeads interfering with cell growth or adhesion and the beads were lost after two passages. Characterisation of ECs showed that cells from all isolates expressed one or more of the examined antigens (Paper I, Table 1).

In total, 593 transcripts with significant fold changes were identified in the VEGF-treated cell cultures. Of these, 546 were found to be upregulated and 47 were downregulated. A search for endothelium-related probes revealed transcripts encoding proteins known to be expressed in vascular ECs, e.g. fms-related tyrosine kinase 1/VEGFR1 [*FLT1*], angiopoietin 2 [*ANGPT2*] and endothelial differentiation-related factor 1 [*EDF1*].

Uploading the gene list to FatiGO resulted in grouping of the VEGF-regulated transcripts according to gene ontology categories. Only the GO category 'Iron ion binding' was found to be significantly overrepresented in the GOstat analysis. No underrepresented GO categories were found in the VEGF data set.

Analysis of the data from the HEEC cultures exposed to 24-h partial serum deprivation yielded a total of 440 differentially regulated transcripts meeting the statistical criteria. In contrast to the VEGF gene list, upregulated transcripts were fewer than the downregulated ones (177 vs. 263). Transcripts encoding proteins known or believed to promote cell proliferation were generally downregulated. Examples of these are macrophage migration inhibitory factor [*MIF*], marker of proliferation Ki-67 [*MKI67*] and baculoviral IAP repeat-containing 5 [*BIRC5*]. Further, transcripts associated with gene products described as inhibiting cell growth (e.g. insulin-like growth factor binding protein 3 [*IGFBP3*] and tumour protein p53-inducible nuclear protein 1 [*TP53INP1*]) were, with a few exceptions, upregulated.

The 440 transcripts were associated with GO categories, as with the VEGF-regulated set. GOstat analysis revealed two overrepresented GO terms, 'Regulation of progression through cell cycle' and 'Microtubule-based process'. The only significantly underrepresented GO category in the serum withdrawal data set was 'Cell communication'.

Uploading the VEGF-regulated data set to IPA® produced several gene networks. The two most significant networks in the hierarchy were merged for more convenient presentation (Figure 4). The merged networks comprised 45 nodes encoding proteins involved in cell death and survival, notably serine-threonine protein kinase AKT1 [*AKT1*], BCL2-antagonist of cell death [*BAD*], BCL2-associated X protein [*BAX*], insulin-like growth factor binding protein 3 [*IGFBP3*], macrophage migration inhibitory factor [*MIF*], 14-3-3β protein [*YWHAB*] and tumour necrosis factor [*TNF*]. Together with the pro- and anti-apoptosis-related transcripts upregulated in the VEGF data set, but not incorporated in the top gene networks (e.g. insulin-like growth factor 1 [*IGF1*], the mitochondrial enzyme hexokinase 1 [*HK1*], B-cell CLL/lymphoma 6 [*BCL6*], programmed cell death 5 [*PDCD5*] and DNA polymerase delta 4 [*POLD4*]), the data indicates a complex interplay of cell-fate regulation in growth factor-stimulated HEECs *in vitro*.

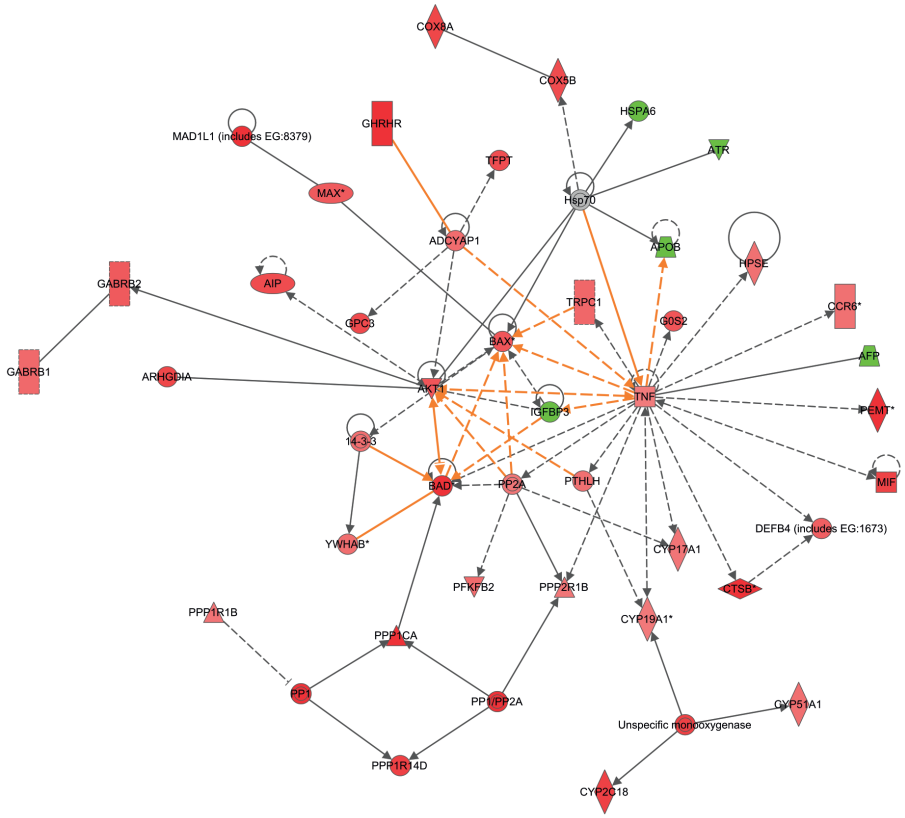

Figure 4. Ingenuity® pathway analysis network showing regulatory relationships among differentially regulated transcripts in human endometrial endothelial cells after 24 h exposure to VEGF-A. Upregulated transcripts are marked in red and downregulated in green. The colour intensity reflects the level of regulation.

Ingenuity® pathway analysis performed with the set of 440 transcripts from the partially serum-deprived HEECs generated networks representing genes mainly known to regulate cell cycle progression (e.g. cell division cycle 20 [*CDC20*], cyclin-dependent kinases 1 and 2 [*CDK1*, *CDK2*], the CDK2-dephosphorylating cyclin-dependent kinase inhibitor 3 [*CDKN3*] and cyclin B1 [*CCNB1*]) (Figure 5), and genes associated with cell death and survival (Paper I, Figure 3).

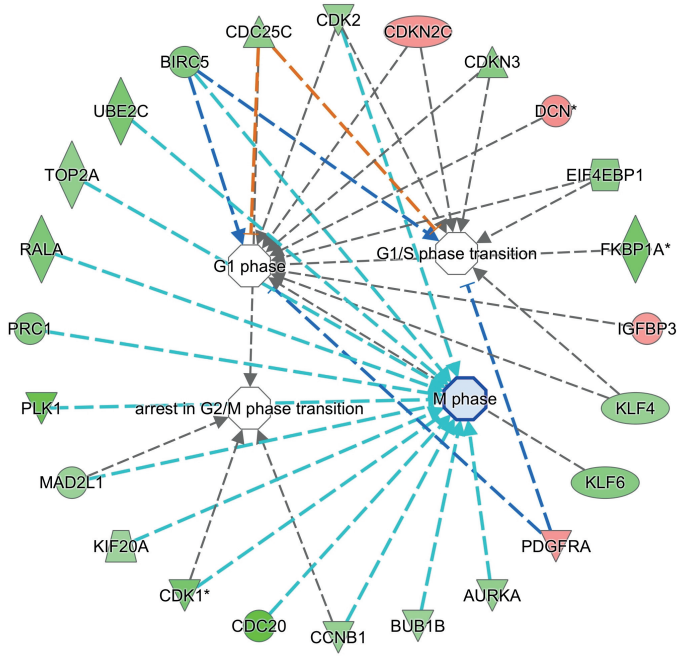

Figure 5. Ingenuity® pathway analysis network diagram illustrating annotated interactions between genes categorised by IPA® functions 'Progression through cell cycle' and 'Cell cycle arrest' in human endometrial endothelial cells affected by 24 h partial serum deprivation. Note that a majority of the transcripts are downregulated. Nodes designated as in Figure 4.

Comparison of the gene lists derived from the VEGF and serum withdrawal experiments revealed 89 overlapping transcripts, of which 88 were reciprocally regulated (i.e. upregulated by one of the treatments and downregulated by the other). Several of the previously presented mRNAs encoding proteins related to cell survival and apoptosis as well as cell cycle progression were among the 88 transcripts. Some of these, such as the transcripts of the *HK1* and *YWHAB* genes have not previously been well characterised in ECs.

Paper II

Of the 38 women included in the study as a result of laparoscopically verified endometriosis, 17 (44.7%) became pregnant during the 12-month follow-up period. Among those without a recorded pregnancy, the number of women with rASRM stage III endometriosis was somewhat elevated but not significant.

The most elevated ERα HSCORE values (in both luminal (Table 3) and in glandular epithelium) were seen in women with endometriosis and no pregnancy, being about twice as high as those in the controls. Endometriosis patients who became pregnant during the study also displayed slightly stronger ERα immunostaining than the healthy controls.

Positive ERβ immunoreactivity was seen in all examined endometria but differences between the two groups of endometriosis patients and/or controls were not significant.

The total PR HSCORE values in luminal (Table 3) and glandular epithelium were higher in women with endometriosis who succeeded in becoming pregnant during the study, versus women with no pregnancy, and similar to those in the controls. Specific staining for PR-B was stronger in both epithelial compartments in fertile endometriosis patients compared with those without a recorded pregnancy and non-significantly higher than in the controls.

Examination of steroid hormone receptor staining in the stroma revealed no significant differences between study groups with the exception of marginally stronger staining for PR-B in biopsy samples from the endometriosis patients with a subsequent pregnancy compared with the group of women without proven fertility.

Table 3. *Semi-quantitative immunostaining (HSCORE ±SD) of oestrogen and progesterone receptors in luminal endometrial epithelium.*

Receptor	**Endometriosis (np)**	**Endometriosis (p)**	**Controls**
ERα	2.18 (±0.81)*	1.49 (±0.17)**	1.12 (±0.25)
ERβ	1.51 (±0.12)	1.62 (±0.34)	1.33 (±0.09)
PR A+B	1.20 (±0.19)*	1.97 (±0.84)	1.80 (±0.23)
PR-B	1.42 (±0.25)*	2.30 (±0.43)	1.85 (±0.21)

'np' and 'p'=endometriosis patients without and with a recorded pregnancy during the study, *P<0.05 *vs*. endometriosis (p); **P<0.05 *vs*. control endometrium

AlphaB-crystallin immunostaining was detected only in the cytosolic intracellular compartment in the epithelium, with no staining seen the in stroma, endothelial cells or blood cells (Figure 6). Moreover, staining among cells differed in intensity within the same gland, consistent with previous findings (Gruidl et al. 1997).

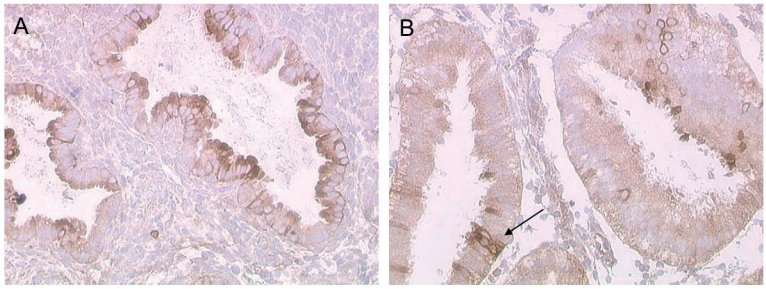

Figure 6. Representative immunostainings for αB-crystallin in mid-secretory menstrual phase endometria from patients in the (A) 'medium' and (B) 'low' HSCORE subgroups. Arrow denotes glandular epithelial cells with positive staining.

No significant overall differences in αBCr staining levels were seen between endometriosis patients and women without the disease. When αBCr HSCOREs were stratified into 'high', 'medium' and 'low', the majority of women who became pregnant during the observation period were found in the 'medium' range (n=14, 82.3%). Importantly, of the 18 control women with proven fertility, 16 (88.8%) also displayed αBCr HSCOREs in the 'medium' range.

No difference was detected in serum oestradiol levels between the two groups of women with endometriosis or controls. However, mean serum concentrations of progesterone in women with endometriosis were lower in the confirmed-pregnancy group compared with patients who did not conceive during the study (22 ± 14 (SD) *vs*. 58 ± 12 nmol/l; P<0.05), but neither group of patients differed significantly from the controls (39 ± 17 nmol/l) in this respect.

Paper III

The percentage of recorded pregnancies among the 41 women in the study who underwent one endometrial biopsy (*n*=18, 43.9%) was no different from that in the 24 women who agreed to have a second biopsy sample taken (*n*=10, 41.7%) (Figure 3). Nor did comparison of their respective clinical characteristics reveal any significant differences.

Immunostaining of luminal and glandular endometrial epithelium showed positive reactions for LIF, LIFR, gp130 and SOCS1 in all examined tissue samples. HSCOREs were calculated for LIF, LIFR and gp130 and values from the glandular compartment generally supported the findings in the luminal epithelium.

Immunoreactivity of LIF was distinct in the luminal epithelia obtained from endometriosis patients who became pregnant during the six-month follow-up period (E/P group) and from controls. Women with endometriosis and no recorded pregnancy (E/NP group) had significantly lower HSCOREs in the biopsy samples taken both preoperatively and after goserelin acetate treatment. The staining pattern for the LIF receptor was similar to that seen with LIF and luminal epithelium HSCOREs were elevated in E/P patients compared with E/NP patients. Control women exhibited the most intense LIF and LIFR immunostaining overall (Figure 7).

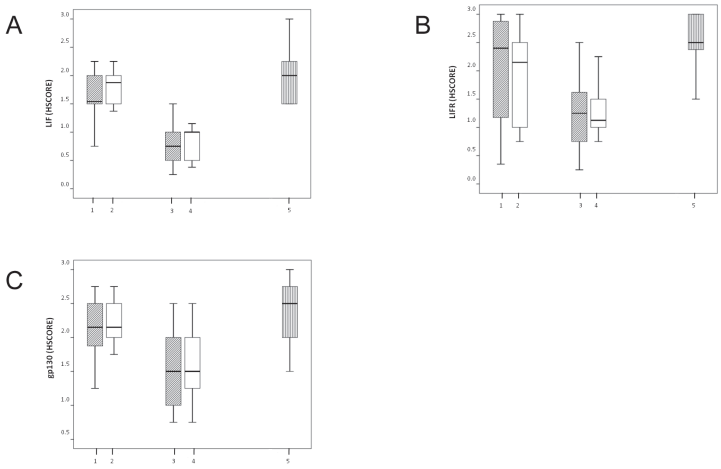

Figure 7. Semi-quantitative immunostaining (HSCOREs) for (A) leukaemia inhibitory factor (LIF); (B) LIF receptor (LIFR); and (C) glycoprotein 130 (gp130) in human mid-secretory luminal endometrial epithelium. 1) preoperative biopsy samples; 2) biopsy samples obtained 3 months after goserelin acetate treatment from endometriosis patients who became pregnant during the study; 3) and 4) denote biopsy samples obtained as in 1–2 from endometriosis patients with no recorded pregnancy during the study; 5) biopsy samples from healthy control women.

Immunohistochemical expression of gp130 in luminal epithelia was slightly stronger in the 28 preoperative endometrial biopsy samples and in the 10 second biopsy samples from E/P patients compared with E/NP patients. Endometrial specimens from control women showed a tendency towards more pronounced gp130 staining but it was not significantly greater than that in E/P patients. Immunolocalisation of SOCS1 to the luminal epithelium largely confirmed previous findings (Aghajanova et al. 2009a).

Interleukin-1α levels in PF from E/NP patients were higher than in E/P patients (72.0 *vs*. 49.7 pg/ml) and more than three times increased compared with controls (19.4 pg/ml). Peritoneal fluid levels of IL-6 in the E/NP patients were significantly elevated, around twice as high as in E/P patients and in the controls (88.6 *vs*. 47.2 pg/ml, and controls, 41.1 pg/ml).

Paper IV

No significant differences in demographic data were found between the two groups of endometriosis patients with (E/CP+) and without (E/CP-) chronic pelvic pain except that E/CP+ patients reported a higher prevalence of dysmenorrhea and combined dysmenorrhea and dyspareunia. The mean intensity of their self-assessed chronic pain was 4.8 on the 0–10 visual analogue scale (VAS).

Immunostaining with anti-CD31 antibody revealed that endometriosis patients had higher MVD in eutopic endometrium than controls (Figure 8). Patients in the E/CP+ group displayed the highest MVD in endometriotic lesions. Furthermore, staining for VIP showed a similar pattern with the highest densities in both eutopic and ectopic endometrium in E/CP+ cases. In fact, VIP density in endometriotic tissue was 4.5-fold increased in E/CP+ compared with E/CP- patients. The lowest MVD and VIP density values overall were seen in healthy women.

Western blotting revealed one band of approx. 20 kDa corresponding to VIP in eutopic and ectopic endometrial tissue lysates from a subset of the study women. Fluorescent quantification analysis, normalized to β-actin, revealed that VIP protein expression was elevated in endometriosis patients compared with controls. The difference in VIP levels between the two groups of endometriosis patients was most pronounced in ectopic endometria where the VIP/β-actin ratio was more than threefold higher in E/CP+ than in E/CP- patients.

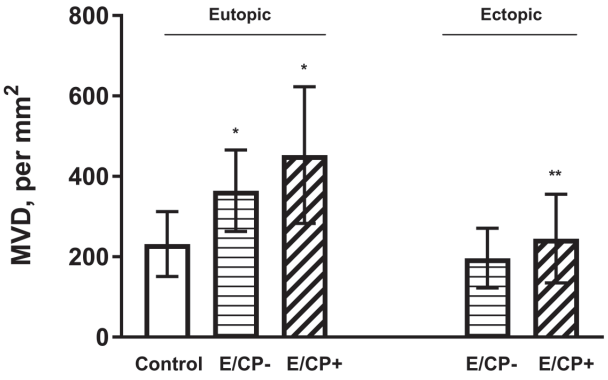

Figure 8. Microvessel density, expressed as the number of microvessel intersections per mm^2, in eutopic and ectopic endometrium, as determined by immunohistochemistry using an anti-CD31 antibody. **P* <0.05 *vs.* control endometrium; ***P* <0.05 *vs.* E/CP- cases.

RT-qPCR analysis of endometrial samples from a subset of the women in the study did not reveal a difference in VIP mRNA levels between eutopic endometrial samples from E/CP- patients and from control women. An increase in mRNA transcripts was detected in E/CP+ patients compared with the other two study groups, whereas relative VIP mRNA expression in ectopic lesion specimens from E/CP+ cases was clearly elevated.

The mean concentration of VIP was 1.1 pg/ml in PF from controls. Levels of VIP were significantly elevated in E/CP- patients (9.3 pg/ml) compared with controls and the highest concentrations of VIP were detected in the E/CP+ group (26.4 pg/ml) (Figure 9).

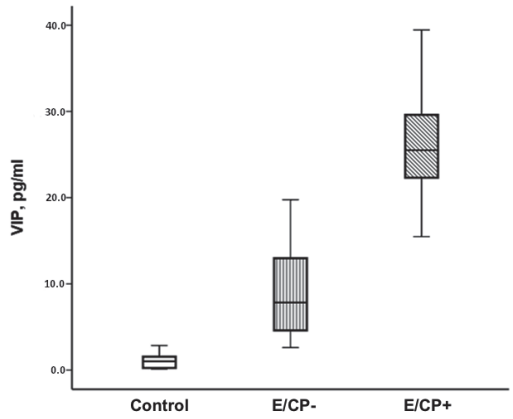

Figure 9. Levels of vasoactive intestinal peptide in peritoneal fluid of healthy control women and of endometriosis patients without (E/CP-) and with (E/CP+) chronic pelvic pain. *P* <0.05 between groups.

Peritoneal fluid concentrations of IL-6 were examined in a subset of study patients and compared with levels in the same control group as in Study III. A significant difference in PF levels of IL-6 was observed when comparing healthy controls (*n*=23) with E/CP- patients (41.1 pg/ml vs. 57.2 pg/ml; *n*=37). A marked increase in PF concentrations of IL-6 was seen in PF from E/CP+ patients (79.4 pg/ml; *n*=28) compared with those in E/CP- group.

Discussion

Methodological considerations

Paper I

In this study, only 12 women were included, which by most experimental standards is a small number. This was due to a number of reasons; the wish to include an equal number of patients from the proliferative and secretory menstrual phases, which meant exclusion of women with irregular bleeding patterns, that some HEEC isolates did not grow sufficiently and also real-world limitations such as the high cost and labour needed for establishing primary cell cultures and performing microarrays manually in a laboratory abroad. On the other hand, the included women were all non-smokers and had not used any hormonal therapies for three months, which otherwise would have affected the results.

Primary HEECs are more likely to resemble the *in vivo* phenotype than immortalised cells. However, there are some disadvantages that one must bear in mind when working with primary cell cultures. Contamination with other cell types, such as fibroblasts, must be avoided in the isolation process and the slow growth rate of HEECs can make establishing cultures of sufficient size time-consuming. Both of these difficulties were encountered during the work and resulted in disposal of several HEEC isolates. The protocol used for isolation of HEECs in Paper I was designed as an attempt to overcome these pitfalls and for generation of cell cultures of satisfactory purity, as determined by immunocytochemical characterisation. All HEEC cultures were positive for the EC surface markers CD31 and/or CD54, reached confluence within one week after passaging and showed no signs of interference from the magnetic beads used for isolating ECs from the endometrial tissue homogenate. However, since CD31 is a pan-endothelial cell marker, cell isolation using the method described in this work does not discriminate between vascular HEECs and lymphatic ECs (LECs). Lymph vessels have been estimated to constitute around 13% of all vessel structures in the functional layer and a majority of endometrial LECs express CD31 (Rogers et al. 2008). On the other hand, lymphangiogenesis is generally assumed to be driven by VEGF-C and VEGF-D, acting on VEGFR-3, and not by VEGF-A which was used for treating the HEEC cultures in this study.

Primary HEECs express ERβ, whereas controversy still exists regarding the expression of ERα and PR at a protein level (Demir et al. 2010). Since the culture medium contains low levels of oestrogen originating from the fetal bovine serum, SHR activity may influence HEEC growth. However, according to the results generated under similar experimental conditions, HEECs obtained from proliferative- and secretory-phase endometria and thus exposed to different oestrogen levels *in vivo*, do not seem to behave differently in cell culture (Helmestam et al. 2010).

With the above reservations, taking into account the low number of passages needed before cell numbers were sufficient for RNA extraction, the four pooled replicates of each culture and the paired study design, the method used in this study can be considered reliable for studying endometrial angiogenesis at a transcript level *in vitro*.

Papers II–IV

The number of patients and controls in the first of the three papers in this thesis based on a collaboration with researchers from the RCOGP was small, only 38 and 18 respectively. The presumed relationship between endometrial αBCr expression and chance of spontaneous pregnancy in the entire group of women with endometriosis compared with controls may thus have evaded detection. Further, studies on self-reported spontaneous pregnancies by necessity involve a number of limiting factors. Frequency of intercourse, length of ovulatory cycles and male subfertility all affect a couple's fecundity, as do BMI, smoking and a history of previous surgery. In Papers II and III, demographic data showed no differences between groups of endometriosis patients and controls regarding BMI, menstrual cycle length or age. They all had normospermic partners according to initial investigations at the RCOGP and women with primary infertility were excluded. Insufficient information on smoking, frequency of sexual intercourse and history of surgery was obtained, which is an obvious limitation. Moreover, the 12-months follow-up period would preferably have been longer if infertility is to be ensured.

The follow-up period was even shorter in Study III, partly because several women in the study chose to discontinue their participation in the study after six months and seek IVF treatment. The total number of women included in this study was larger than in Study II and considered to be at an equal level in comparison with other IHC-based studies. One particular weakness was that only a little over one-third of the patients agreed to undergo a second endometrial biopsy, for post-treatment investigation of LIF/LIFR complex expression. This was also criticised in a recent article reviewing the literature on endometrial receptivity after surgery for benign gynaecological disorders (Celik et al. 2016). However, it is understandable, since a second biopsy would have meant another visit, some physical discomfort and a longer wait

until the woman was allowed to conceive. Nevertheless, it no doubt affected the chance of detecting an improvement in the defective LIF and LIF receptor expression. A quantitative method, in addition to IHC, is generally preferred to reduce investigator bias and increase reproducibility. Unquestionably, an mRNA- or protein-based method should ideally have been included in Studies II and III. However, considering the finding in Study II that sometimes only a few cells in an entire gland express αBCr, there is a risk of false-negative results as a result of an insufficient detection level in, for example, a western blot involving the use of tissue lysates.

To partially overcome the above-mentioned restrictions brought about by the use of only one method, RT-qPCR and western blotting were utilised to complement IHC in Study IV. The differences in eutopic and ectopic endometrial VIP density scores, calculated from IHC data, were largely reflected in the WB data, despite the limited number of snap-frozen biopsy samples available to us. The qPCR results were more difficult to interpret in that VIP mRNA expression confirmed the IHC and WB data in ectopic endometriotic tissue, but not in eutopic biopsy samples. Owing to the small sample sizes in both these quantification analyses, conclusions must be drawn with caution.

Although still relatively small, the number of samples used in ELISA/EIA analyses of IL-1α, IL-6 and VIP levels in PF was equal to or greater than that in similar studies (Kondera-Anasz et al. 2005, Barcz et al. 2012, Wickiewicz et al. 2013). As in Studies II and III, the proportion of smokers in the study population was not known. Since nicotine promotes endothelial cell proliferation and survival *in vitro* and possibly stimulates pathological angiogenesis, it may have an effect on MVD (Lee and Cooke 2012). In the context of this thesis it is worth noting that nicotine recently has been shown to increase levels of VEGF mRNA in cultured primary endometrial stromal cells (Totonchi et al. 2016). Moreover, an agonist to the α7 nicotinic acetylcholine receptor (nAchR), expressed on peritoneal macrophages, suppressed the formation of endometriotic lesions after inoculation of endometrium in a murine endometriosis model (Yamada-Nomoto et al. 2016). The nAchR is also expressed on vascular endothelium where it mediates the angiogenic effects of nicotine (Lee and Cooke 2012). Moreover, smoking compounds have been observed to disturb steroidogenesis, causing impaired E2 and progesterone synthesis (Dechanet et al. 2011). However, unless the distribution of smokers in the study population is severely skewed, it can be assumed that the possible effect on MVD and steroid hormone profiles is more or less the same in all groups of patients and controls. This needs to be clarified if similar studies are to be conducted in the future.

General discussion

Paper I

Given the known functions of VEGF as an EC mitogen and angiogenic factor, it was not surprising that many of the upregulated transcripts were related to cell proliferation and survival, transcription and blood-vessel formation. One key factor in angiogenesis including EC survival, permeability and migration, is the PI3K-AKT signalling pathway (Chen et al. 2005, Somanath et al. 2006). The transcript encoding the protein AKT1 was identified as one of the central nodes in the network of regulatory relationships generated from the VEGF data set in this study. AKT1 exerts its anti-apoptotic effects partly by inactivating the caspase cascade-inducing BAD through phosphorylation. Phosphorylated BAD interacts with 14-3-3β polypeptide, causing BAD to be retained in the cytoplasm and prevented from inducing the mitochondrial apoptotic machinery. The polypeptide 14-3-3β further influences the balance between survival and apoptotic signalling by enhancing the function of the Raf family of pro-survival kinases (Morrison 2009).

Hexokinase 1, another mitochondria-associated enzyme, also interferes with pro-apoptotic BAD and prevents the opening of mitochondrial permeability transition pores and thus the initiation of the caspase cascade leading to apoptotic cell death (Gottlob et al. 2001, Azoulay-Zohar et al. 2004). Both the *YWHAB* and *HK1* transcripts, encoding 14-3-3β and hexokinase 1 respectively, were reciprocally regulated under the two experimental conditions in the present study.

Further evidence of the central role of VEGF in EC survival is the induction of *MIF* transcripts in the HEECs exposed to the growth factor. The cytokine MIF is a direct inducer of the PI3K-AKT pathway (Amin et al. 2003) and the corresponding *MIF* downregulation in response to partial serum withdrawal confirms the negative effect on HEEC survival and proliferation of the experimental conditions.

The concomitant upregulation of a few pro-apoptotic transcripts in the VEGF-treated cells is more difficult to interpret. Furthermore, *BAD* and *PDCD5* fold changes were reduced in the serum starvation data set. For *in vivo* blood vessel formation and remodelling, some ECs must undergo apoptosis. Since this study did not involve tube-formation or apoptosis assays, it is not possible to draw conclusions about whether these seemingly contradictory VEGF effects are unique to HEECs 'imprinted on' physiological angiogenesis or simply caused by circumstances intrinsic to the culture of primary ECs.

Growth factor-induced transcriptome changes can be both transient and delayed. In cells exposed to a growth factor, genes are differentially regulated depending on the time point. Temporal changes in EC transcript regulation induced by VEGF have been demonstrated in cultured primary human uterine microvascular ECs and HUVECs (Weston et al. 2002, Suehiro et al. 2010). In a study involving the use of DNA microarrays to detect early- and late-response genes in HUVECs, VEGF-responsive genes were most highly represented after 18 h (Suehiro et al. 2010). Among the early-response transcripts were *BCL6*, upregulated by VEGF after 24 h in Study I, and *HSPA2*, upregulated in response to serum withdrawal. These findings underline the usefulness of harvesting cells at different time points during exposure to a growth factor in order to obtain more detailed insights into the resulting transcriptome changes. Incubation with VEGF for 24 h, as in Study I, probably means losing information about early-response genes, the majority of which are transcription factors/cofactors (Suehiro et al. 2010), in favour of genes related to cell proliferation, migration and survival.

Reducing serum content in the culture medium is an often-used method for studying cellular stress response and apoptosis. A reduction in serum concentration to 2% inhibits EC growth and induces morphological changes consistent with apoptosis after 28 h (Johnson et al. 2004) and completely depriving ECs of serum (or reducing the serum content to 0.1%) initiates the apoptotic program, through activation of caspase 3, within 16 h (Gama Sosa et al. 2016). Endothelial cell apoptosis is believed to play a role in vascular remodelling and lumen formation (Affara et al. 2007). Comparing transcriptome changes induced by growth factor stimulation and anti-proliferative experimental conditions may therefore bring insights into the complex process of angiogenesis.

In Study I, the culture medium was supplemented with 5% foetal bovine serum, which was subsequently reduced to 2% for 24 h. Microarray analysis revealed mainly downregulation of transcripts whose gene products are known to be involved in cell-cycle progression, viability and migration. Cyclin B1, the small GTPase RalA, survivin and Aurora kinase A, encoded by *CCNB1*, *RALA*, *BIRC5* and *AURKA*, respectively, are some examples (Figure 4). Cyclins promote transition through cell cycle phases while the three latter proteins have oncogenic/anti-apoptotic and pro-migratory properties (Wolgemuth 2008, Jeon et al. 2011, Gabrielli et al. 2015, Garg et al. 2016).

Although no apoptosis-detection assay was carried out in Study I, it is nevertheless rewarding to make a comparison with studies involving EC apoptosis induction. Affara and colleagues cultured primary HUVECs in medium supplemented with EGF, FGF and foetal calf serum (FCS), which was then replaced with basal medium containing only 2% charcoal-stripped FCS (Affara et al. 2007). Total RNA was isolated at different time points during 24 h and transcript expression levels were assessed using gene chip microarrays. Some of the regulated transcripts in this study are closely relat-

ed to, or overlapping, the expression data in Study I. The expected downregulation in cell-cycle-related transcripts was seen, e.g. *CCNA2* (-2.3-fold after partial serum withdrawal in Study I) and *CDC6* (*CDC2* and *CDC20* were downregulated in Study I). However, the regulation of these transcripts was delayed (the lowest mRNA levels seen at 24 h) compared with growth factors, such as *ANGPT2*, which was initially upregulated, more than fourfold decreased at 12 h followed by a slight recovery at 24 h. The importance of these changes in transcript levels to angiogenesis and EC biology can be further investigated using siRNA knock-down and receptor antagonist assays.

Paper II

In this work, it was shown that in the study population of women with minimal to moderate endometriosis, higher mid-secretory endometrial epithelial expression of ERα than healthy women, may impair the chances of becoming pregnant. This confirms the findings in a previous study on infertile women with endometriosis and fertile controls where the authors proposed that local endometrial oestrogen production induced ERα expression (Lessey et al. 2006). Moreover, in the present study, endometrial epithelial immunostaining for total PR and PR-B was impaired in the women with endometriosis who failed to conceive during the one-year observation period. Together with the ERα data this points to a general aberration in steroid hormone receptor expression in a subset of women with endometriosis during the period in the menstrual cycle where the endometrium is receptive. The underlying cause of these observations is still unknown but progesterone resistance has been suggested (Young and Lessey 2010). This is a well-known characteristic of both eutopic and ectopic endometrium in endometriosis, but clinical research tends to focus more on its negative effect on progestin treatment of the disease than its possible impact on fertility.

Comparison with another condition associated with reduced fertility, polycystic ovary syndrome (PCOS), is interesting, since the endometrium in some women with PCOS presents with similar features as in endometriosis, in particular mid-secretory epithelial over-expression of ERα, and progesterone resistance (Quezada et al. 2006, Piltonen 2016). The latter aberrant mechanism seems to exist despite persistence of PR in the epithelial compartment during the secretory phase. Blunted progesterone signalling through impaired expression of progesterone-regulated receptivity markers, such as homeobox A10, integrins and claudin 4, has been suggested (Piltonen 2016).

Since the pioneering study twenty years ago, identifying αBCr as a progesterone-responsive protein expressed in human and baboon endometrium (Gruidl et al. 1997), little further knowledge about its role in mammalian reproduction has been gained. In two recent studies on mice, high expression

levels of *Cryab* mRNA were detected in the decidua at the time of implantation, and progesterone induced Cryab in primary mouse endometrial stromal cells (Tian et al. 2013, Zuo et al. 2014). Moreover, knockdown of *Cryab* increased stromal cell apoptosis and inhibited decidualisation (Zuo et al. 2014). The increased presence of αBCr in the endometrial epithelium during the mid-secretory phase also suggests involvement in human endometrial receptivity, supported by the results of a microarray study showing upregulation of *CRYAB* transcripts at the time of WOI (Mirkin et al. 2005). *CRYAB* was also upregulated after endometrial biopsy-induced injury in a study by Kalma et al. (Kalma et al. 2009). Two groups of women subject to IVF-treatment either underwent two endometrial biopsy samples or no biopsy. In the following cycle, biopsy samples were taken from all women on day 21, followed by embryo transfer. Using microarrays for global gene expression, the authors found 2- to 10-fold upregulation of 183 genes in a subset of women who conceived. *CRYAB* was identified among the transcripts assumed to be involved in the preparation of the endometrium for implantation.

Based on the striking progesterone-regulated increase in αBCr mRNA and protein expression during the WOI, it has been hypothesised that αBCr is a component in the molecular events that make human endometrium receptive to an implanting blastocyst (Satyaswaroop and Tabibzadeh 2000). The report presented in this thesis is the first in which αBCr protein levels in endometria from women with and without endometriosis have been compared. The most important finding is that immunoreactivity for αBCr, expressed as HSCORE, in women with endometriosis on a similar level to that in healthy women was favourable for the chance of becoming spontaneously pregnant. Higher or lower HSCOREs were over-represented in the women who did not become pregnant during the one-year follow-up period.

Paper III

To date, there is no consensus on whether surgical and/or medical endometriosis treatment improves a woman's chances of spontaneous conception. Earlier estimations of the benefits of surgery may have been overvalued and hormonal treatment is no longer indicated for subfertility associated with endometriosis, with the probable exception of pituitary downregulation with GnRH agonists (GnRHa) before IVF. After surgery, including resection of ovarian endometriomas, a 10–25% increase in pregnancy rates in cases of endometriosis has been estimated (Vercellini et al. 2009a). In a recent Cochrane review, two randomised controlled trials (RCTs) revealed an association between increased live-birth or ongoing pregnancy rate at 20 weeks of gestation and laparoscopic ablation or excision of endometriosis lesions compared with diagnostic laparoscopy only (combined odds ratio of 1.94) (Duffy et al. 2014). Women undergoing IVF treatment for endometriosis-associated subfertility constitute a more homogeneous cohort than women trying to conceive spontaneously. Opøien and coworkers demonstrated a significantly increased clinical pregnancy rate after surgical resection of stage I/II endometriosis in a retrospective trial (Opoien et al. 2011). Other investigators have come to somewhat conflicting conclusions regarding the impact of surgery on IVF outcomes (Surrey 2015). However, the protocol for investigation and treatment of endometriosis-related infertility at the RCOGP, at the time when the study presented in Paper III was conducted, included both laparoscopic excision and/or cauterisation of lesions and postoperative GnRHa therapy. This made it possible to design the study for analysis of endometrial biomarkers before and after treatment.

Gonadotrophin releasing hormone is produced in a small subset of hypothalamic neurons and secreted in a pulsatile manner into the hypophyseal portal blood system. It stimulates the synthesis and release of FSH and LH, which in turn regulate ovarian steroidogenesis in females (Maggi et al. 2016). Chronic administration of synthetic GnRHa has been used in the treatment of endometriosis for several years, both for reducing pelvic pain and other symptoms and as a prolonged pre-IVF cycle therapy. Searching the literature does not yield any specific information on the possible benefits of GnRHa for the enhancement of spontaneous pregnancies in women with endometriosis. There are, however, some data on the advantages of GnRHa treatment for improving IVF outcome. A meta-analysis of three RCTs showed that three to six months' administration of GnRHa prior to IVF improved the clinical pregnancy rate fourfold (Sallam et al. 2006). Owing to heterogeneity among the included studies, improvement in live-birth rate could not be determined.

For Study III, investigation of LIF and its cognate receptor complex LIFR/gp130 was done on the basis of their established importance in the cross-talk between the endometrium and the implanting embryo. Evidence of

reduced endometrial LIF expression in infertile endometriosis patients has also been presented (Dimitriadis et al. 2006). These earlier results were reproduced in the present (larger) study and extended to include evidence of reduced expression of LIFR/gp130 in the luminal epithelium in women with endometriosis and no pregnancy during the six-month observation period (E/NP), although the strongest difference in immunostaining between patients who became pregnant during the study (E/P) and E/NP women was seen with the LIF antibody. It was not shown that the impaired expression of these endometrial receptivity biomarkers could be restored by surgical excision of lesions and postoperative GnRHa treatment, either because of the methodological limitations outlined elsewhere or intrinsically reduced expression of the LIF/LIFR complex in the endometrium in these women. Also, the study contained no control group of untreated endometriosis patients. Nevertheless, these results add further to emerging insights that combined surgical and medical treatment is of doubtful value in attempts to enhance spontaneous fecundity in women with minimal to mild endometriosis.

That the LIF/LIFR complex plays an important role in embryo-Fallopian tube-endometrium cross-talk, crucial for embryo transport and implantation, has been demonstrated by a number of investigators. As mentioned previously in this thesis, LIFR is highly expressed in the Fallopian tubes and in the pre-implantation embryo, whereas its presence in the endometrium is more moderate (Wanggren et al. 2007). In a recent paper by Yen and co-workers, endometrial LIF and LIFR expression in women with adenomyosis, a condition closely related to endometriosis and associated with subfertility, was investigated (Yen et al. 2016). The authors demonstrated impaired LIF/LIFR expression levels during the WOI compared with control women and subsequently reduced STAT3/ERK phosphorylation in both endometrium and cultured stromal cells. Their results are in agreement with the findings in Study III regarding LIF/LIFR expression. However, since the study was performed on hysterectomy specimens, there are no data from before and after medical adenomyosis treatment, which would otherwise have been especially interesting in the context of this thesis.

There was a clear relationship between the levels of IL-1α and IL-6 in PF and fecundity among the women in the present study. Endometriosis-free women were a homogeneous group in this respect, with low interleukin concentrations within a narrow range, whereas endometriosis patients displayed both higher levels and a wider spread.

The IL-1 family of cytokines possesses strong pro-inflammatory effects and the biological functions of IL-1β have been extensively described in the literature, including its actions in endometriosis. The biosynthesis and mechanisms of action of IL-1α are less well characterised. It is constitutively expressed in many cell types and can be both membrane-bound and secreted, in contrast to IL-1β, whose expression is induced upon stimuli and is secreted only (Di Paolo and Shayakhmetov 2016). IL-1α binds foremost to the recep-

tor IL-1R1 and it has the ability to induce secretion of IL-6 from various cell types. IL-1-mediated PDGF-α production has mitogenic effects on fibroblasts which, if prolonged, lead to fibrosis in some autoimmune diseases (Di Paolo and Shayakhmetov 2016). This specific pathological process has not been studied in endometriosis, but since increased IL-1α levels in PF from endometriosis patients, probably produced by macrophages and endometriotic epithelial cells, have been observed (Kondera-Anasz et al. 2005), it is a concept well worth investigating. A novel coupling between IL-1α and endometriosis was found in a recent genome-wide association study, where endometriosis risk was associated with single nucleotide polymorphisms within the *IL1A* gene locus (Sapkota et al. 2015). The association was strongest with moderate-to-severe endometriosis and the authors postulated that the results provide supporting evidence for a link between inflammatory responses and the pathogenesis of endometriosis.

The fact that IL-6 levels were clearly elevated in E/NP women compared with the other women in Study III is of particular importance, given the known detrimental effects on sperm motility and ovarian follicular aromatase activity brought about by this cytokine (Gupta et al. 2008). Increased PF levels of IL-6 were also seen in a subgroup of women diagnosed with minimal–mild endometriosis, in a study involving 57 patients undergoing laparoscopy for unexplained infertility, using the endometriosis-free women as controls (Kalu et al. 2007). In conflict with these results and the data presented in Paper III are the findings of high PF levels of IL-6 (110.0 pg/ml) and TNF-α in a subgroup of fertile women with minimal endometriosis compared with infertile women with and without endometriosis (Skrzypczak et al. 2005).

Comparison of the IL-6 results from the present study with those produced in a paper by Wickiewicz and colleagues (Wickiewicz et al. 2013) turned out to be informative. They reported a mean value of 60.6 pg/ml, measured by using a cytometric bead array, in a cohort of 36 women diagnosed with endometriosis during laparoscopy but without data on fertility status. This is just slightly below the mean value of 67.9 pg/ml (ELISA) in all endometriosis patients in Study III and shows the reliability of the different analysis methods used. Contradictory results were reported in a small cross-sectional study in which IL-6 levels were measured with a commercial ELISA kit, in PF from 19 endometriosis patients investigated for pain and/or infertility (Rathore et al. 2014). IL-6 levels were substantially lower (mean 17.7 pg/ml in patients and 18.7 pg/ml in controls) than in the study by Wickiewicz et al. and in Study III. Median levels were even lower in both groups whereas the highest values were seen in controls (72.7 pg/ml). This is somewhat surprising, given that the majority of endometriosis patients had stage III–IV disease and one would expect elevated IL-6 levels in this group.

Taken together, a growing body of evidence links IL-1α and IL-6 to the pelvic inflammatory state encountered in endometriosis and hence its patho-

genesis. Further knowledge about the mechanisms behind their potential roles in endometriosis-associated infertility is needed, however, before these results can be translated into a clinical application.

Paper IV

Chronic pelvic pain (CPP) in the female population is a common disorder with various causes (Vercellini et al. 2009b). A detailed definition of CPP was proposed by the American College of Obstetricians and Gynecologists in ACOG Practice Bulletin #51 (Bulletins--Gynecology 2004):

> ’non-cyclic pain of six or more months duration that localises to the anatomic pelvis, anterior abdominal wall at or below the umbilicus, the lumbosacral back, or the buttocks and is of sufficient severity to cause functional disability or lead to medical care’

One of the most common causes of CPP in women is endometriosis. The prevalence of endometriosis in adolescent girls and women undergoing laparoscopy for CPP varies between one-third to more than two-thirds (Howard 1993, Triolo et al. 2013). The overall incidence of endometriosis in a large retrospective cohort study of more than 9,600 hysterectomies was 15.2%, rising to 21.4% in a subgroup where the preoperative indication was CPP (Mowers et al. 2016).

Neoinnervation of the uterus, endometriosis lesions and the surrounding pelvic peritoneum as a principal mechanism behind pain in endometriosis has attracted increasing interest during the last 20 years or so. In the initial publications, notably those by the Canadian research group of Togas Tulandi, it was concluded that although nerve fibres were identified in pelvic adhesions of different origin, in peritoneum from endometriosis patients and in endometriomas, their presence was not related to the underlying pathology or pain symptoms (Tulandi et al. 1998, Tulandi et al. 2001, Al-Fozan et al. 2004). For these studies, they used a monoclonal antibody to neurofilament and no distinction between neuronal subtypes was made. Later studies have revealed higher densities of nerve fibres of various modalities in pelvic organs and tissues from endometriosis patients when immunostaining for diverse neurotransmitters (Mechsner et al. 2007, Tokushige et al. 2007, Mechsner et al. 2009, Tokushige et al. 2010, Wang et al. 2011). The results of these studies supported an earlier observation by Atwal and coworkers in a work on hysterectomies, where an increased density of neurons and perivascular nerve fibre proliferation were more common in women with CPP and endometriosis than in women without either condition (Atwal et al. 2005). The presence of disrupted nerve bundles with the occurrence of nerve fibre sprouting and microneuromas in the myometrium, together with peri-

vascular nerve growth, led the authors to conclude that endometriosis causes re-/neoinnervation of the tissue in women with CPP. A number of studies have also shown that, besides the findings regarding pain and nerve growth in the uterus and/or superficial peritoneal endometriosis, lesions penetrating deeply under the peritoneal surface are probably even more prone to cause CPP symptoms, regardless of hyperinnervation (Fauconnier and Chapron 2005).

Vasoactive intestinal peptide (VIP) has been identified as one of the neurotransmitters found in small unmyelinated sensory and parasympathetic perivascular peptidergic neurons (Tokushige et al. 2007, Wang et al. 2009b, Barcena de Arellano and Mechsner 2014), providing a link between pelvic pain in these patients and the paradigm of endometriosis-associated pelvic (neuro-)inflammation. Indeed, the ratio of noradrenergic to peptidergic nerve fibres is markedly reduced in the peritoneum in women with endometriosis compared with women without. The decrease in noradrenergic nerve fibres can be seen both within the immediate surrounding of a lesion and in unaffected areas in close vicinity to the endometriotic implants (Barcena de Arellano and Mechsner 2014).

The over-expression of pro-inflammatory factors such as cytokines, prostaglandins and COX-2 and the increased number of activated macrophages in the pelvic environment in cases of endometriosis has been investigated extensively (Howard 2009). There is, however, very little knowledge, except for the repeated demonstrations of VIPergic innervation, about possible mechanisms involving VIP in endometriosis-associated neuroinflammation and neuroangiogenesis. Paper IV presents the first evidence for increased VIP expression in both intrauterine and ectopic endometrial tissues, as well as elevated levels of VIP in cell-free PF from endometriosis patients with CPP. No discrimination as regards nerve fibres was done, instead the total expression of VIP mRNA and peptide was investigated.

Over the years, understanding of VIP as an immunomodulator, systemically and locally produced by lymphocytes and granulocytes and released from sensory and parasympathetic neurons has accumulated (Delgado et al. 2004). It is thus improbable that VIP plays only a minor role as a sensory neurotransmitter in such a complex chronic inflammatory disease as endometriosis.

In Study IV, VIP immunostaining in endometrial and ectopic lesions was seen in blood vessels, nerve fibres and scattered small cells, which may consist of immune cells. A literature search found no evidence for VIP synthesis in the vascular endothelium. However, primary rat microvascular ECs and murine EC cell lines express VPAC1 and VPAC2 receptors (Castorina et al. 2010, Yang et al. 2013). Binding of antibody against VIP seen in blood vessels in tissue samples must therefore have been either to VIP bound to endothelial or perivascular VIP receptors or to VIP produced in nerve fibres in close proximity to vessel walls, or a combination of these possible binding

sites. Production of VIP in endometriotic lesions is not surprising given that inflammation-driven neuroangiogenesis has been demonstrated in endometriosis.

Peritoneal fluid of women with endometriosis contains neurotrophic and angiogenic factors, stimulating neural growth and the formation of a new microvascular network (Asante and Taylor 2011, Barcena de Arellano and Mechsner 2014). Some of the cytokines present, often at elevated levels, in the PF of endometriosis patients have angiogenic properties. IL-6, IL-1β and TNF-α, secreted from activated macrophages, either alone or in synergy, induces VEGF and bFGF production from fibroblasts in rheumatoid arthritis (Elshabrawy et al. 2015). By analysing gene networks in a microarray study on HUVECs, IL-6 was found to potentiate TNF-α-mediated proliferation and vessel formation, providing evidence of a novel form of inflammation-driven angiogenesis (Ogami et al. 2012). In an earlier study, co-administration of interleukin-1α, elevated in the PF of endometriosis patients in Study III, and VIP was found to produce an angiogenic response comparable with high doses of VIP alone in a rat model for quantitative angiogenesis assay (Hu et al. 1996). This neovascular response was inhibited after administration of a selective VIP receptor antagonist.

The increased MVD seen in eutopic and ectopic endometrial biopsy samples from E/CP+ cases may therefore be due to the supposedly higher pelvic inflammatory state in these women. Whether or not the earlier observations of higher MVD in proliferative endometriotic lesions and in secretory-phase eutopic endometria from endometriosis patients *vs*. healthy women (Bourlev et al. 2006) are related to inflammation remains to be elucidated in integrative analysis. The increased levels of VEGF and its receptors VEGFR-1 and -2 in these patients, however, do not contravene this hypothesis.

Further insights into the importance of neoangiogenesis in endometriosis comes from a murine endometriosis model in which mice with endometrial implants were treated with the angiotensin II type I receptor antagonist telmisartan (Nenicu et al. 2014). The suppressive effects of telmisartan on angiogenesis, inflammation and proliferation in the endometriosis-like implants were investigated. The authors demonstrated reduced MVD, using a fluorescent antibody against CD31, decreased immune cell and Ki-67-positive cell counts in the implants, as well as downregulation of angiogenesis-related genes such as *Vegfa*, *Angpt1* and *Flt1*. Interestingly, levels of *Il6* and *Il1b* transcripts, coding for IL-6 and IL-1β, were also more than twofold lower in peritoneal endometriosis-like implants in the telmisartan-treated mice, in line with the reduced numbers of lymphocytes and macrophages in the same tissue. Unfortunately, in this context, *Vip* was not represented in their RT-qPCR array of selected genes, nor was any immunostaining carried out to detect nerve fibres.

Most research and clinical attention in the field of endometriosis is directed towards the pelvic environment and less to the eutopic endometrium.

That the presence of intrauterine endometrial VIPergic nerve fibres contributes to the generation of CPP in women with endometriosis has been shown, among others, by the research group of Ian Fraser [reviewed in (Miller and Fraser 2015)]. Moreover, in a recent study concerning eutopic endometrial nerve fibres in women reporting procedural pain during office hysteroscopy, VIP staining intensity scoring showed the highest correlation with pain of the investigated neuronal markers (Di Spiezio Sardo et al. 2015). This was evident both in the whole study population and in a subset of women diagnosed with adenomyosis/endometriosis. VIP immunoreactivity in the endometrial biopsy samples was seen not only in nerve fibres but also in vessels and stromal cells, partly supporting the IHC findings in Study IV. However, no quantitative method was applied to verify the IHC findings, which must therefore be interpreted with some cautiousness.

In Study IV, increased presence of VIP was verified in eutopic endometria of endometriosis patients who reported chronic pelvic pain compared with women without the disease, using two different techniques based on both mRNA and protein. The results were in line with those from ectopic lesions, pointing to VIP over-expression in women with endometriosis and CPP. The regulation of such a condition and the complex system of pro- and anti-inflammatory responses in the pelvic environment in general need to be further clarified if new therapies are to be developed.

Conclusions

Isolation and culture of primary HEECs by means of the method described in this thesis, involving equal numbers of cell isolates from both menstrual phases and using each cell culture as its own control, provides a reliable tool for the study of endometrial angiogenesis *in vitro*.

Exposure to VEGF-A or partial serum withdrawal for 24 h regulates several transcripts associated with proliferation, migration and cell-fate regulation in cultured primary HEECs. Activation at the transcript level of the PI3K-dependent cell-survival machinery is illustrated by the upregulation of *AKT1*, *MIF* and *YWHAB* following VEGF treatment. Curiously, a number of transcripts encoding proteins with pro-apoptotic properties are also upregulated under these experimental conditions, thus indicating a complex interplay of cell-fate regulation in growth factor-stimulated HEECs *in vitro*. In line with it being a major EC growth factor, VEGF positively regulates transcripts of cell proliferation-associated genes.

Reducing the serum content in the culture medium induces negative regulation of several growth-promoting and cell-cycle progression-related transcripts in primary HEECs. A concomitant increase in some mRNAs encoding apoptosis-inducing factors is also seen.

Comparing the changes in the transcript profile induced by VEGF stimulation and partial serum withdrawal revealed 88 reciprocally regulated mRNAs. Transcripts related to growth, cell fate and cell cycle progression were highly represented.

In subfertile women with rASRM stage I–III endometriosis, expression of ERα is increased in eutopic endometrial epithelium during the mid-secretory menstrual phase compared with that in healthy women. Temporally, this abundance of ERα coincides with the time when the endometrium is receptive to an implanting blastocyst. This has been demonstrated in previous studies and is considered to be part of the inherent progesterone resistance in the endometria of endometriosis patients, probably contributing to their impaired fertility. Epithelial expression of total PR and PR-B is reduced in the same group of endometriosis patients during the mid-secretory phase. These aberrant ER and PR expression patterns are found in both the luminal and glandular epithelium.

The small heat-shock protein αB-crystallin is upregulated in the endometrium during the secretory phase, peaking around the time of the WOI, which suggests regulation by progesterone. Its expression in mid-secretory endo-

metrial glands seems to be related to an improved chance of achieving pregnancy in endometriosis patients, when on a similar level to that in fertile control women. Higher or lower levels of αB-crystallin are associated with lower pregnancy rates.

A combination of endometriosis-reducing surgery and postoperative goserelin acetate therapy does not influence the luminal epithelial expression of the IL-6 superfamily members LIF, LIFR and gp130 in the endometria of women with minimal to mild endometriosis (rASRM stage I–II). The LIF/LIFR complex is associated with endometrial receptivity. Its expression, when on a similar level to that in healthy women, appears to improve the probability of pregnancy in cases with a history of secondary infertility, whereas reduced levels are unfavourable. The opposite is true regarding peritoneal fluid levels of IL-1α and IL-6. In apparently infertile endometriosis patients, cytokine levels are increased compared with both fertile patients and endometriosis-free women. This suggests a higher state of inflammation in the pelvic environment in these patients, which may have a negative influence on fecundity.

Women with low-stage endometriosis and chronic pelvic pain have higher levels of total VIP mRNA and peptide expression in eutopic endometrium and ectopic peritoneal lesions than endometriosis patients without chronic pain. There appears to be a discrepancy between the relative differences in VIP transcript and peptide levels in eutopic endometria, where the progressive rise in VIP peptide from healthy, pain-free controls, to endometriosis patients without chronic pain and further to women with chronic pelvic pain is poorly reflected in the mRNA levels. Whether this is a true discrepancy or a methodological shortcoming cannot be determined as a result of the small sample size.

Neovascularisation is one effect of long-term inflammation. Elevated levels of IL-6 in PF, together with increased microvessel density in both intrauterine endometrium and peritoneal lesions, supports the hypothesis of active local inflammation in endometriosis patients. Among them, patients with CPP have the highest IL-6 levels in PF and the highest microvessel density. Thus, more active and/or long-term inflammation in the pelvic environment is assumed in these women.

Although the main source of and effects exerted by secreted VIP in the endometriotic pelvis are not known, VIP concentrations in peritoneal fluid are clearly related to the disease and to chronic pain in particular. Levels of VIP are more than 20 times higher in CPP patients compared with a control group and almost three times as high as in endometriosis patients without chronic pain.

Future perspectives

Today's medical treatments of uterine bleeding disorders and endometriosis are almost exclusively sex-steroid hormone-based. Negative side-effects on psychological well-being and disturbed bleeding patterns are common in connection with hormonal medicines, which limit their use by many women. For some time, researchers have investigated the possibilities of non-hormonal approaches to control heavy and irregular menstruation and endometriosis. Anti-angiogenic agents are established in oncology and ophthalmology (e.g. bevacizumab, a "humanized" mouse monoclonal antibody against VEGF-A) and have been tried in animal models of endometriosis with varying degrees of success. Although inhibition of angiogenesis has been proposed as a novel way to terminate an early pregnancy, for use in medical abortions and as a treatment of ectopic pregnancies, no human clinical trials have been published. More detailed knowledge about possible unique mechanisms in endometrial vascular biology is needed if anti-angiogenic therapies are to be developed. Blocking VEGF has several severe side-effects and it is not a viable alternative in treatment of benign disorders.

However, constructing gene networks of regulated transcripts following exposure to VEGF and other EC growth factors may demonstrate signalling pathways downstream of growth factor receptors. These pathways may in turn reveal possible candidate proteins for blocking or agonist binding, which are worth investigating in the search for a more selective endometrial angiogenesis inhibitor. Co-cultures of HEECs and endometrial stromal and epithelial cells, preferably in 3D endometrial constructs, together with animal models of uterine vasculogenesis, are likely to yield more detailed insights into these pathways in comparison with EC monocultures. *In vitro* or *in vivo* models, including transcriptome and proteome analyses, may also be used for further understanding of the regulation of endometrial angiogenesis.

Oestrogen receptor β is expressed in HEECs and appears to be the dominant form of ER in endometriotic lesions and endometriomas. It has anti-proliferative effects in breast and prostate cancer, while evidence as regards its involvement in the pathogenesis and progression of endometrial cancers is conflicting. Recent reports have shown that novel selective antagonists to ERα and ERβ inhibit growth of ectopic lesions in murine models of peritoneal endometriosis (Han et al. 2015, Zhao et al. 2015). Conversely, gain of ERβ function stimulates progression of endometriosis and ERβ is involved in evasion of TNF-α-induced apoptosis. Moreover, blocking either ERα or

ERβ results in decreased angiogenesis and microvessel density in ectopic lesions. The role of ERβ in HEEC biology is, however, still unclear. Potentially clinically useful knowledge about ERβ agonist and antagonist action on HEECs could be gained by the use of tube formation assays and 'omics' technologies.

Although endometrial and blastocyst expression levels of LIF may not be absolutely vital for successful human reproduction, downregulation of the LIF/LIFR complex appears to impair female fertility. Whether subfertile women with endometriosis and decreased LIF expression in the luminal epithelium are helped by assisted reproductive techniques is not known. In the study presented in Paper III, some of the women who failed to spontaneously conceive during the period of observation dropped out of the study because they requested IVF treatment. A retrospective follow-up study on these women, with records from fertility centres as a data source, could help in stratifying endometriosis patients according to the chance of achieving pregnancy in relation to LIF expression. If the results are confirmed in larger studies, preferably with a less invasive sampling method, determining endometrial levels of LIF might be a useful tool in fertility counselling.

Overexpression of pro-inflammatory cytokines in peritoneal fluid is related to reduced fertility and pelvic pain in endometriosis patients. IL-1 and IL-6 are among the most studied inflammatory markers in this context, and in autoimmune diseases. Levels of IL-1 in venous blood and peritoneal fluid have been associated with disease severity/inflammatory activity in rheumatoid arthritis (RA). An inhibitor of IL-1α and IL-1β (anakinra), exerting its action by competitive binding to their main receptor IL-1R1, is commercially available for treatment of RA. Patients treated with the IL-1 inhibitor experience a decrease in severity of joint pain and other symptoms of inflammation. In addition, reduced serum levels of IL-6 have been reported, in line with the upregulation of IL-6 induced by IL-1. To what extent the increased PF levels of IL-1 in women with endometriosis contribute to pain symptoms is not known. A pilot clinical trial of anakinra among patients with endometriosis, with relief of pain symptoms as an end-point, could therefore prove to be interesting, not least because current anti-inflammatory therapy, including selective COX-2 inhibitors, is often insufficient.

The upregulation of αB-crystallin during the secretory phase of the menstrual cycle suggests a role in preparing the endometrium for receptivity. In the mouse uterus, αB-crystallin is highly expressed around the implanting blastocyst. The specific processes in the endometrium in which αB-crystallin is involved are as yet unknown. Knock-down of αB-crystallin in mouse or in *in vitro* models for the study of implantation, immunolabelling with anti-αB-crystallin antibody for transmission electron microscopy of implantation sites and exposing endometrial cell cultures to sex-steroid hormones and growth factors are all imaginable future research projects that may increase

our knowledge of the regulation and function of αB-crystallin in the endometrium.

A first necessary step if one wants to understand the role played by VIP in endometriosis is to identify the main source(s) of VIP in eutopic and ectopic endometrium. Besides VIPergic nerve fibres, lymphocytes and neutrophils are likely to release VIP in tissue and into the PF. Constitutive expression of VIP and VPAC1 in an immortalised human endometrial stromal cell line has also been reported. Immunolocalisation of VIP in endometrial samples taken throughout the menstrual cycle, with double-staining for leukocyte, neural and stromal cell markers, will help determine which cell populations contain VIP.

A clinical question worth exploring is whether or not plasma levels of VIP differ between endometriosis patients and healthy control women. If so, endometriosis patients could be treated with anti-inflammatory and/or hormonal medication and systemic VIP could be measured before and after therapy. Thus, an indication of whether VIP can be used as a marker of disease activity could be given.

As yet, VIP receptors are not very well characterised in the female reproductive tract and there is no information in the literature about the function of VPAC1 and 2 in human endometrium or in endometriotic lesions. It is expected that VPAC receptors are expressed in glandular epithelium and in vascular smooth muscle cells, given the known roles of VIP in secretion and vessel dilation. In addition, tissue macrophages are also likely targets of VIP. However, this needs to be confirmed by immunolocalisation. If VPAC expression in endometrial glands is demonstrated, the hypothesis that VIP regulates endometrial secretion could be tested *in vitro*, using cultured epithelial cells as a first step.

Summary in Swedish

Sammanfattning på svenska

Det humana endometriet (livmoderslemhinnan) består av två lager, det övre, funktionella lagret, och det undre, basala lagret. Redan under menstruationen inleds den så kallade tillväxtfasen då epitelet (endometriekörtlarna) och stromat (stödjevävnaden) växer från det basala lagret under inverkan av östrogen. Under den här perioden bildas det också ett stort antal nya blodkärl. Efter ägglossningen ändras den hormonella balansen så att progesteron (gulkroppshormon) är det dominerande könshormonet. Endometriet övergår då i sekretionsfasen, under vilken det förbereds på att ta emot ett befruktat embryo, implantationen. Körtlarna förstoras och sekretionen aktiveras, stromacellerna decidualiseras (ändrar form och börjar frisätta ämnen som möjliggör implantation) och blodkärlen blir större och genomsläppliga för proteiner. Om det inte sker någon implantation stöts det funktionella lagret i livmoderslemhinnan bort (menstruation) och tillväxten startar på nytt från det basala lagret. Ökad kunskap kring hur dessa olika funktioner i endometriet regleras under menscykeln är nödvändig för att kunna utveckla nya behandlingar av t.ex. blödningsrubbningar, infertilitet och endometrios.

En viktig funktion är angiogenesen, nybildningen av blodkärl. Angiogenes förekommer normalt endast sparsamt hos vuxna friska individer. Undantagen är endometriet, äggstockarna och moderkakan, där fysiologisk angiogenes fyller en viktig funktion under de fertila åren. Angiogenes uppstår annars främst i samband med sjukdomar och skador, såsom tumörer, inflammatoriska sjukdomar och sårläkning.

Flera former av angiogenes förekommer men en gemensam central roll spelas av endotelcellerna, blodkärlens innersta cellager. Endotelcellernas tillväxt och delning stimuleras av proteiner som kallas tillväxtfaktorer, där VEGF-A är den viktigaste. VEGF-A har visats skydda odlade endotelceller från programmerad celldöd och öka blodkärlens genomsläpplighet för vätska och proteiner i vävnader. En standardiserad metod för att hämma odlade cellers tillväxt och i vissa fall inducera programmerad celldöd är att minska mängden serum i odlingsmediet. Detta förfarande kan delvis ses som motsatsen till stimulering med tillväxtfaktorer.

Endometrios, definierat som förekomsten av celler från livmoderslemhinnan utanför livmodern, är en vanlig gynekologisk sjukdom som drabbar 6-10% av den kvinnliga befolkningen i reproduktiv ålder. Endometrios uppstår

sannolikt genom flera mekanismer där den äldsta och mest utbredda teorin går ut på att levande celler och fragment från livmoderslemhinnan följer med mensblod genom äggledarna in i bäckenhålan där de växer fast i bukhinnan. På de platser där endometrieceller har vuxit fast (endometrioslesioner) uppstår inflammation och smärtnervfibrer växer in i området, särskilt om endometriosen vuxit djupare ned i den omgivande vävnaden, vilket antas bidra till den bäckensmärta som är vanlig vid endometrios. Även nybildning av blodkärl är en del i processen när endometrioslesioner etableras. I många fall utvecklas en mer generellt inflammatorisk miljö i bäckenet, vars negativa effekter anses bidra till den nedsatta förmågan att bli gravid och/eller risken att utveckla kronisk bäckensmärta som ofta drabbar endometriospatienter.

Livmoderslemhinnan skiljer sig i flera avseenden mellan friska kvinnor och kvinnor med endometrios. Man har kunnat visa att antalet nervfibrer är betydligt högre hos kvinnor med endometrios och att uttrycket av receptorer för östrogen och progesteron, samt biomarkörer viktiga för implantationen, är felaktigt.

Syftet med denna avhandling var dels att studera förändringar i det totala genuttrycket hos isolerade endotelceller från det humana endometriet (HEEC) som resultat av stimulering med VEGF-A och efter minskning av mängden serum i odlingsmediet, dels att undersöka om uttrycket av könshormonreceptorer och ett antal andra proteiner skiljer sig mellan kvinnor med och utan endometrios samt om det i så fall finns en koppling mellan uttrycket av dessa proteiner och chansen att bli spontant gravid eller att ha kronisk bäckensmärta.

Exponering för VEGF-A uppreglerade uttrycket av ett antal gener med betydelse för celldelning och tillväxt samt skydd mot programmerad celldöd hos odlade HEEC. Något förvånande uppreglerades samtidigt ett litet antal gener som kodar för proteiner vilka i tidigare studier har visats inducera programmerad celldöd. En del av dessa gener var också nedreglerade efter att cellerna exponerats för minskad serummängd i odlingsmediet. Detta motsatsförhållande är svårförklarligt och kan antingen bero på de experimentella förhållandena eller på unika inneboende egenskaper i HEEC. I övrigt noterades som förväntat nedreglering av uttrycket av ett flertal gener associerade med främst celldelning och -tillväxt efter serumminskningen.

I den andra delstudien undersöktes vävnadsprover från endometriet från kvinnor med endometrios och från friska kontroller. Studien visade att antikroppsinfärgningen av östrogenreceptor α i epitelet var ökad under den period då endometriet är mottagligt för implantation hos de endometriospatienter som inte blev gravida under en tolvmånaders uppföljningsperiod. Samma kvinnor hade också minskat uttryck av progesteronreceptorer. Dessa resultat är i linje med den minskade känsligheten för progesterons effekter på livmoderslemhinnan som har påvisats hos kvinnor med endometrios i andra studier men har inte tidigare kopplats till graviditetschans på detta sätt. Vidare sågs också flest graviditeter hos de endometriospatienter vars antikroppsin-

färgning i livmoderslemhinnans körtelepitel för αB-crystallin, ett protein med skyddande egenskaper i samband med proteintillverkning inuti celler, var på samma nivå som hos de friska kontrollerna.

Leukemi-inhibitorisk faktor (LIF) och dess receptor, bestående av subenheterna LIFRα och glykoprotein 130, har stor betydelse för implantationen hos flera däggdjursarter. Även hos människa tycks LIF spela en roll även om denna inte är känd i detalj. Med samma metod som beskrivits ovan visades i det tredje delarbetet att minskade nivåer av LIF och dess receptorkomplex i det ytliga endometrieepitelet, jämfört med friska kontroller, medförde färre spontana graviditeter under en sexmånadersperiod efter genomgången kirurgisk och medicinsk endometriosbehandling. Ingen skillnad mellan uttrycksnivåerna av LIF och LIFR före och efter behandlingen kunde ses. Vidare hade de endometriospatienter som inte blev gravida under studien högre nivåer av de inflammatoriska cytokinerna interleukin-1α och -6 i peritonealvätskan, den mindre mängd vätska som vanligen finns längst ner i lilla bäckenet.

Bäckensmärtor är ett vanligt symtom vid endometrios och ligger bakom en stor del av den nedsättning i livskvaliteten som många patienter upplever. Vasoaktiv intestinal peptid (VIP) spelar stor roll som hämmare av inflammation vid autoimmuna sjukdomar och som signalsubstans i en del av de små smärtnervfibrer som påvisats i endometriet och i och omkring endometrioslesioner i bäckenet. Det är inte känt vilken betydelse VIP eventuellt har i den endometriosorsakade bäckeninflammationen. Med antikroppsinfärgning och kvantitativa metoder påvisades högre nivåer av VIP i livmoderslemhinna från endometriospatienter jämfört med friska kontroller. De högsta nivåerna uppmättes i endometrioslesioner från patienter med kronisk bäckensmärta. Samma kvinnor hade också de högsta koncentrationerna av VIP och IL-6 i peritonealvätskan.

Resultaten från den här avhandlingen kan utgöra en grund för fortsatta studier av regulatoriska signalvägar i samband med livmoderslemhinnans blodkärlsnybildning och mottaglighet för embryon. Funktionsstudier inriktade på inflammation och smärta kan också ge svar på frågan om VIP och dess receptorer har betydelse för bäckensmärta hos kvinnor med endometrios och om de kan vara en ingång mot nya terapier av detta ofta svårbehandlade symtom.

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Paper I

VEGF-A and Serum Withdrawal Induced Changes in the Transcript Profile in Human Endometrial Endothelial Cells

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The changes in transcript profile induced by vascular endothelial growth factor (VEGF-A) and serum withdrawal in primary human endometrial endothelial cells (ECs) were investigated using microarrays, gene ontology and pathway analysis. Vascular endothelial growth factor A altered the levels of transcripts involved in angiogenesis, cell survival, and apoptosis, including up-and downregulation of AKT1, BAD, MIF, *and* IGFBP3 *and* ANGPT2, *respectively. Serum deprivation induced downregulation of cell-cycle-related transcripts such as mitosis regulators* CDC20 *and* SPC25. *Of the transcripts regulated by both VEGF-A and partial serum deprivation, remarkably 88 of 89 showed reciprocal regulation ($p < 1 \times 10^{-49}$). These are predominantly cell-fate-associated transcripts and this novel observation suggests that endometrial ECs may be particularly dependant on the levels of these transcripts. Our results show that in addition to the known role of VEGF-A as an EC growth and survival promoter, it also regulates apoptosis-related messenger RNAs (mRNAs), many of which were reciprocally regulated following serum withdrawal.*

Key words: Angiogenesis, apoptosis, cell survival, endometrial endothelial cells, VEGF-A.

INTRODUCTION

Angiogenesis is thought to play a pivotal role in the cycling endometrium and is regulated by a combination of systemic and local factors. These are coordinated by 17β-estradiol and progesterone and probably mediated by the local action of factors including vascular endothelial growth factor (VEGF-A).[1,2] Physiological angiogenesis is part of the endometrial repair and growth process following menstruation as well as at the time of embryo implantation. Endometrial pathologies such as endometriosis, hyperplasia, and malignancy are all dependant on angiogenesis. Defective angiogenesis and altered VEGF-A expression has been suggested as a cause of bleeding disturbances in women, with or without the concomitant use of progestins.[3,4] Vascular endothelial growth factor A belongs to a family of VEGF proteins present in human endometrium and exerts several effects on endothelial cells (ECs); it is a specific endothelial mitogen and promotes microvascular permeability.[1,5] Vascular endothelial growth factor A plays a crucial role in EC survival, protecting ECs from apoptosis induced by various pro-apoptotic agents.[6]

Understanding the regulation of endometrial angiogenesis requires studies of the complex patterns of interacting cell populations and their response to hormones and various growth factors. Numerous endometrial cell types including glandular epithelial and stromal cells, vascular smooth muscle cells, macrophages, intravascular neutrophils, and uterine natural killer cells produce angiogenic regulators.[2,7] Print et al reported that human umbilical vein ECs (HUVECs) proliferated following treatment with the supernatant from cultured endometrium.[8] More detailed knowledge of this complex regulatory network can be gained by investigating the contributions of different endometrial cell types individually, supplementing whole tissue

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studies. We therefore used a model of isolation and culture of primary human endometrial ECs (HEECs) and sought to define the effect of stimulatory and inhibitory stimuli (VEGF-A and partial serum withdrawal, respectively). Changes in the endothelial transcript profile following treatment of the HEECs were assessed by microarray analysis and the important differentially regulated transcripts were further investigated by searching gene ontology (GO) and pathway analysis databases.

MATERIALS AND METHODS

Cell Culture and Characterization

Endometrial tissue samples were obtained from 12 women of reproductive age undergoing hysterectomy for benign indications such as myomas. All participants were nonsmokers of proven fertility, had a history of benign Pap smears prior to surgery, and had not been using hormonal medication or an intrauterine device for at least 3 months. They were not receiving treatment for any other illness, gynecological or otherwise. The study was approved by the Ethics Committee of Uppsala University, and all participating women gave their informed consent.

Six endometrial tissue samples were obtained from during the proliferative and six from the secretory phase of the menstrual cycle (dated by a pathologist and assessed as normal). Endometrial tissue was collected under sterile conditions using a sharp curette immediately after the uterus was removed and placed in ice cold phosphate-buffered saline ([PBS], pH 7.4; Gibco, Invitrogen AB, Sweden) containing 0.5 μg/mL gentamicin (Gibco). The HEECs were isolated using a protocol similar to procedures previously published.[9-11] The tissue specimens were washed twice in iodine disinfectant solution (Jodopax, Cederroth, Sweden) and 4 times in PBS. The specimens were then cut into smaller pieces and disaggregated at 37°C for 2 hours in PBS containing 2.5 mg/mL collagenase type II (Gibco), 50 μg/mL DNase type IV (Sigma-Aldrich Sweden AB), 200 μg/mL hyaluronidase (Sigma-Aldrich), and 100 μg/mL gentamicin (Gibco). The cell suspension was then filtered through a Falcon 100 μm nylon filter (BD Labware, UK) and the filtrate was washed twice with PBS. The cell pellet was resuspended in 1 mL ice cold PBS and 25 μL of CD31-coated Dynabeads (Dynal, Invitrogen AB) were added and incubated on a rocking table for 30 minutes at 4°C.

Bead-coated cells were collected using a Dynal MPC (Dynal) and washed twice before resuspension in EGM-2MV culture medium (Clonetics, Cambrex, In vitro Sweden AB) and seeded in Falcon T-25 uncoated flasks.

For EC characterization, a small proportion of cells from passage 0 to passage 2 (P0-P2) were grown on 4 chamber slides and fixed in 3.7% formaldehyde tris buffered saline (TBS) or in ice cold acetone depending on the antibody. After rinsing twice in TBS, immunostaining was performed as follows. Endogenous peroxidase activity was quenched with 0.3% H_2O_2-TBS for 10 minutes and cells were rinsed twice in TBS. Nonspecific binding was blocked by incubation with 10% horse serum TBS. The primary antibodies were then incubated over night at 4°C, followed by biotinylated rabbit anti-mouse secondary antibody (1/300 in 0.1% bovine serum albumin [BSA]-TBS), streptavidin–horseradish peroxidase [HRP] conjugate (1/400 in TBS) and 3,3′-diaminobenzidine chromogen (DAB) solution (1 drop per 1 mL DAB Buffered Substrate). All incubations, except DAB, were done in a humidified chamber with 3 rinses in TBS between each step. Cells were then counterstained with filtered 30% Meyer hematoxylin and rinsed in tap water. Primary antibodies, diluted in 0.1% BSA-TBS, were monoclonal mouse anti-human antibodies against (formaldehyde fixation) von Willebrand factor (vWF)/Factor VIII-related antigen (clone F8/86, diluted 1/25), CD31/PECAM-1 (clone JC70A, dil. 1/40), CD105/endoglin (clone SN6h, dil. 1/10), (acetone fixation) CD34 (clone QBEnd 10, dil. 1/10), CD54/ICAM-1 (clone 6.5B5, dil. 1/25), cytokeratin (clones AE1 and AE3, dilution 1/25), and prolyl 4-hydroxylase/fibroblast (clone 5B5, dilution 1/25). Negative control staining was performed with mouse immunoglobulin G (IgG) 1 (code no. X 0931, dilution 1/10). All antibodies and DAB reagent were obtained from Dako Sweden AB.

Cells were grown in EGM-2MV medium supplemented with 5% fetal bovine serum (FBS) and EC growth supplements (Bullet Kit) provided by the manufacturer, in a fully humidified atmosphere of 5% CO_2 at 37°C. Confluent cells were passaged no more than 3 times before use.

Each of the 12 cell cultures was divided into 2 subsets of treatment and matched control. To the first subset either VEGF-A_{165} (100 ng/ml, Sigma-Aldrich) in 0.1% BSA-PBS or an equal volume of vehicle control (125 μL 0.1% BSA-PBS) was added. The second subset of cells was exposed to partial serum starvation by reducing the FBS content in the medium from 5% to 2% or changed to fresh 5% FBS medium (controls). All cells were grown under these conditions for 24 hours. Four replicates of each treatment and control were used for

each cell culture and pooled after harvesting with TRIzol reagent (Gibco). This setup design allowed each individual treated cell culture to have its own control, subsequently allowing paired statistical analysis, but no distinction was made between cells isolated from either the proliferative or secretory phase tissue.

Microarray Analysis

Total RNA was extracted according to manufacturer's protocol and quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Labtech International Ltd, UK). RNA integrity was assessed by loading 300 ng of total RNA onto an RNA LabChip and analyzed using the Agilent 2100 Bioanalyzer (Agilent Technologies UK Ltd). All cultures yielded sufficient RNA for hybridization to microarrays.

The SMART (Clontech, UK)- based protocol was used for preparing amplified double-stranded complementary DNA (cDNA) and has previously proved to be reliable in microarray analyses.[12,13] Briefly, total RNA (1 μg) from each individual treatment and control was mixed with SMART CDS primer IIA (5′-AAG CAG TGG TAT CAA CGC AGA GTA $CT_{(30)}N_{(-1)}N$-3′) and template-switching primer (5′-d(AAG CAG TGG TAT CAA CGC AGA GTA CGC)r(GGG)-3′) for double-stranded cDNA synthesis using the PowerScript reverse transcriptase. The AmpliTaq DNA Polymerase II Kit (Applied Biosystems, UK) with PCR primer (5′-AAG CAG TGG TAT CAA CGC AGA GT-3′) was used for cDNA amplification (15 cycles, annealing at 65°C and extension at 68°C). Fluorescence labeling of the amplified cDNA product was performed with the BioPrime DNA labeling system (Invitrogen Ltd, UK) using low-C deoxynucleotide triphosphate (dNTP) mix (5 mmol/L deoxyadenosine triphosphate [dATP], 5 mmol/L deoxyguanosine triphosphate [dGTP], 5 mmol/L deoxythymidine triphosphate [dTTP], and 2 mmol/L deoxycytosine triphosphate [dCTP]), CyDye Cy3-dCTP or Cy5-dCTP (GE Health Care UK Ltd) and 40 U of Klenow fragment (Invitrogen Ltd). Unincorporated fluorescent dCTPs were removed using AutoSeq G-50 columns (GE Health Care UK Ltd) and the cDNA was precipitated and washed with ethanol. The Oligator Human Genome RefSet (Illumina Inc, USA) oligonucleotide library was used for the in-house preparation of the arrays at the microarray core facility of Department of Pathology, University of Cambridge. This library contains 22 740 70mer oligonucleotide probes, including negative controls, targeting 20 726 genes in the human genome including 1310 alternate splice variants of genes expressing multiple mRNA isoforms. The library was suspended in 150 mmol/L $NaPO_4$ pH 8.5/0.0001% Tween 20 and printed onto Corning UltraGAPS Coated Slides (Corning Inc, Lowell, Massachusetts) using a BioRobotics MicroGrid robot (Genomic Solutions Ltd, UK) with a 48-pin tool. Details concerning the methods used in the in-house array production have been published previously.[14]

The microarray slides were then prehybridized for 2 hours at 50°C using a hybridization buffer comprising 40% deionized formamide, 5× Sodium Chloride Sodium Citrate buffer solution (SSC), 1 mmol/L sodium pyrophosphate, 50 mmol/L Tris-HCl pH 7.4 and 0.1% Sodium Dodecyl Sulfate solution (SDS), for 2 hours at 50°C. Ethanol-precipitated fluorescently labeled cDNA probes were then resuspended in 50 μL warm hybridization buffer containing 5× Denhardt solution, 2 μL Human Cot-1 DNA (1 μg/μL; Invitrogen Ltd), 1 μL $poly(A)_{40–60}$ (8 μg/μL; GE Health Care UK Ltd), and 1 μL yeast transfer RNA ([tRNA]; 4μg/μL; Invitrogen Ltd). This mixture was heated to 95°C for 5 minutes immediately before applying to the array slides and hybridized for 16 to 18 hours in a humidified chamber at 50°C. The slides were then washed twice for 5 minutes each in 2× SSC, 0.1× SSC plus 0.1× SDS, and 0.1× SSC. The slides were then rinsed twice in 0.1× SSC, twice in Millipore water (Millipore UK Ltd. Watford, Hertfordshire), and once in isopropanol and spin dried.

Statistics

The slides were scanned on a GenePix Personal 4100A microarray scanner (Axon Instruments, Molecular Devices Ltd, UK) and dual-channel Tagged Image File Format (TIFF) images were captured using GenePix Pro 4.1 software. The images were then imported to BlueFuse for Microarray 3.1 software (BlueGnome Ltd, UK) for removal of uncertain probe signals and noise, yielding numerified data. The hybridization signal values obtained were normalized using the LIMMA package in the "R" programming environment (http://bioinf.wehi.edu.au/limma) and differentially expressed transcripts were identified using the Rank products and Cyber-T algorithms.[15,16] The latter includes both Bayesian *t* test and an estimation of experimentwide false positive and negative levels based on the modelling of *P* value distributions Posterior Probability of Differential Expression (PPDE). Rank products do not assume that the data are normally distributed. Selected transcripts had to pass both

Table 1. Characterization of Antigen Expression in Early Passage Human Endometrial Endothelial Cell Cultures as Detected by Immunostaining With Monoclonal Antibodies

		Antibody					
Culture	**Passage**	**vWf**	**CD31**	**CD34**	**CD54**	**CD105**	**Menstrual Phase[a]**
1	0	+[b]	++	++	−	+	P
2	0	+	+++	−	++	−	S
3	0	++	++	+	++	−	P
4	0	++	+++	++	+++	+++	P
5	1	++	+	−	+++	−	S
6	1	−	−	−	+++	−	S
7	1	++	++	−	−	−	S
8	1	−	++	−	+++	−	P
9	1	−	++	+	++	+	S
10	1	+++	+++	+	+++	−	P
11	0, 1	++	+++	++	++	++	P
12	1, 2	+++	+++	++	+++	+++	S

[a] Menstrual phase in which the endometrial tissue was obtained for endothelial cell isolation (P, proliferative phase; S, secretory phase).
[b] The percentage of stained cells were labeled 0% to 19% (−), 20% to 49% (+), 50% to 74% (++), and 75% to 100% (+++).

the test methods. The overlap between the sets of regulated transcripts was also analyzed in R. Gene ontology analysis was performed using FatiGO, a Web-based data mining tool for comparing gene lists derived from microarrays.[17] Over- and underrepresented GO categories in the gene lists compared to the whole arrays were identified using GOstat (http://gostat.wehi.edu.au). Functional gene networks were obtained by uploading the gene lists to the Ingenuity Pathway Analysis (IPA) software v5.0 (Ingenuity Systems, USA, http://ingenuity.com), searching the Ingenuity Knowledge Base for known relationships in the literature.

RESULTS

Culture and Characterization of Human Endometrial ECs

Growth rate was found to vary between isolates, but all cultures reached confluence within 5 to 7 days. There were no signs of the attached Dynabeads interfering with cell growth, adhesion, or spreading, and beads were lost after the first 2 passages. Although the size and shape of the cells initially varied, with increasing cell number and after the first passage the HEECs began to show the typical cobblestone morphology which was most pronounced when they reached subconfluency.

Table 1 summarizes the results of early passage HEECs immunostained with an array of monoclonal antibodies. Cultured HEECs at all passages examined (P0-P2) expressed one or more of vWF, CD31, CD34, CD54, or CD105 (Figure 1). No staining for the epithelial cell marker cytokeratin or the fibroblast marker prolyl 4-hydroxylase[18] was detected in any of the cultured HEECs at any time (data not shown).

Identifying Transcripts and Functional Networks Regulated by VEGF-A or Partial Serum Deprivation

Analysis of the microarray data identified 593 transcripts displaying significant changes in level ($P < .0001$ [in both tests], PPDE >.999, PFP <.0001) following VEGF-A treatment: 546 transcripts were found to be upregulated and 47 transcripts to be downregulated. Searching the VEGF-A gene list for endothelium-related probes we identified transcripts encoding proteins known to be expressed in vascular ECs (*FLT1, ANGPT2, NOS1, NOSIP, MIF, EDF1*, and *ATPIF1*).

Gene ontology analysis grouped the 593 transcripts according to GO categories. A selection of these is listed in Table 2 and the full list can be found in Supplementary Table 1. When searching for overrepresented GO categories in the list of transcripts regulated by VEGF-A, only the category ''Iron ion binding'' reached significance (Table 3). No significantly underrepresented GO categories were found in the VEGF-A data set. *P* values of <.05 were considered significant.

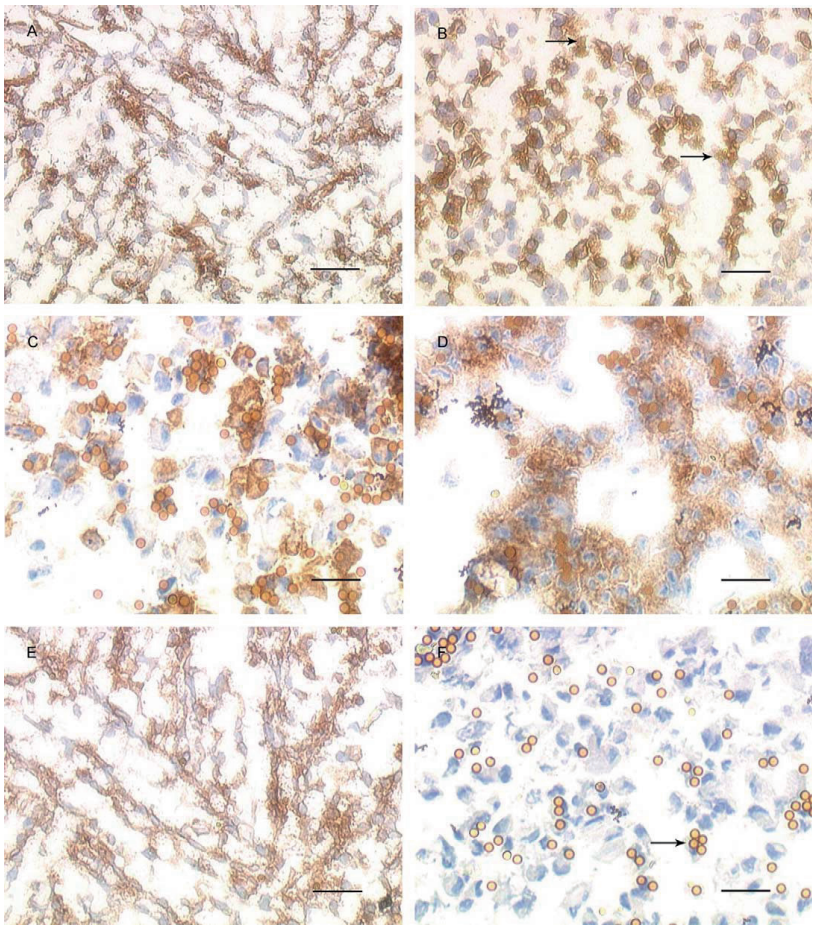

Figure 1. Characterization of early passage primary human endometrial endothelial cells. Photomicrographs of human endometrial endothelial cells (HEECs) immunostained with (A) von Willebrand factor antibody, (B) CD31 antibody, (C) CD34 antibody, (D) CD54 antibody, (E) CD105 antibody, (F) control IgG1. Arrows denote Dynabeads® still attached to cells. Scale bars represent 40 μm in (A), (B) and (E); and 20 μm in (C), (D) and (F).

Analysis of the array data from HEEC cultures exposed to 24 hours partial serum deprivation resulted in 440 transcripts meeting the statistical criteria given above: 177 transcripts were upregulated and 263 transcripts were downregulated. A selection of the GO categories associated with these transcripts is shown in Table 4 and the full list can be found in Supplementary Table 2. GOstat analysis revealed 2 significantly overrepresented GO categories in the gene list from the partial serum withdrawal array data set (Table 5), both related to cell cycle and growth. Transcripts encoding proteins known or believed to promote cell proliferation or to be components of cell cycle checkpoints were generally downregulated. Transcripts whose products have been described as inhibiting cell growth were upregulated. The exceptions were *FGF7*, *AURKAIP1*, *WEE1*, and *TGFB3*. One GO category (cell communication) was found to be significantly underrepresented (Table 6).

After uploading our set of 593 VEGF-A regulated transcripts, the 2 most significant networks in the IPA

Table 2. Transcripts Regulated by Vascular Endothelial Growth Factor A Assigned to Functional Subsets by Gene Ontology Analysis

GO Term	Gene Symbol	FC[a]	Gene Name
Cell proliferation	PTN	1.9	Pleiotrophin
	TNF	1.8	Tumor necrosis factor (TNF superfamily, member 2)
	MIF	3.0	Macrophage migration inhibitory factor
	CDK2	1.9	Cyclin-dependent kinase 2
	CDC14A	−1.4	CDC14 cell division cycle 14 homolog A (*S cerevisiae*)
	NEUROD4	1.9	Neurogenic differentiation 4
	BCL6	2.0	B-cell CLL/lymphoma 6
	FLT1	2.2	Fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor 1)
	EMP3	4.2	Epithelial membrane protein 3
	IGF1	1.8	Insulin-like growth factor 1
	PTHLH	2.0	Parathyroid hormone-like hormone
	GPC3	2.8	Glypican 3
	BAX	2.6	BCL2-associated X protein
	TCIRG1	2.9	T-cell, immune regulator 1
	CDK4	2.1	Cyclin-dependent kinase 4
Death	PDCD5	2.1	Programmed cell death 5
	FASTK	2.3	Fas-activated serine/threonine kinase
	CIB1	3.6	Calcium- and integrin-binding 1 (calmyrin)
	TNFRSF10A	2.1	Tumor necrosis factor receptor superfamily, member 10a
	TNF	1.8	Tumor necrosis factor (TNF superfamily, member 2)
	MIF	3.0	Macrophage migration inhibitory factor
	ARHGDIA	2.9	Rho GDP dissociation inhibitor (GDI) alpha
	CLDN15	2.1	Claudin 15
	MPO	1.9	Myeloperoxidase
	BCL6	2.0	B-cell CLL/lymphoma 6
	EMP3	4.2	Epithelial membrane protein 3
	IGF1	1.8	Insulin-like growth factor 1
	IGFBP3	−1.4	Insulin-like growth factor−binding protein 3
	BAX	2.6	BCL2-associated X protein
	CTSB	3.6	Cathepsin B
	BAD	6.1	BCL2-antagonist of cell death
	AKT1	2.6	V-akt murine thymoma viral oncogene homolog 1
	PPP2R1B	1.9	Protein phosphatase 2, regulatory subunit A, beta isoform
Response to stress	S100A9	−1.5	S100 calcium binding protein A9
	CIB1	3.6	Calcium and integrin binding 1 (calmyrin)
	TNF	1.8	Tumor necrosis factor (TNF superfamily, member 2)
	NTHL1	3.4	Nth endonuclease III-like 1 (*E coli*)
	MIF	3.0	Macrophage migration inhibitory factor
	MPO	1.9	Myeloperoxidase
	BCL6	2.0	B-cell CLL/lymphoma 6
	HDAC5	2.3	Histone deacetylase 5
	MAP4K4	2.3	Mitogen-activated protein kinase kinase kinase kinase 4
	HPSE	2.0	Heparanase
	RAD51	2.8	RAD51 homolog (RecA homolog, *E coli*; *S cerevisiae*)
	GPX4	7.7	Glutathione peroxidase 4
	BAX	2.6	BCL2-associated X protein
	AKT1	2.6	V-akt murine thymoma viral oncogene homolog 1
	HSPA6	−1.5	Heat shock 70 kd protein 6
Ion transport	GABRB1	2.1	GABA A receptor, beta 1
	GABRB2	2.4	GABA A receptor, beta 2
	GRIK5	2.6	Glutamate receptor, ionotropic, kainate 5

(continued)

Table 2. (continued)

GO Term	Gene Symbol	FC[a]	Gene Name
	KCNC2	1.8	Potassium voltage-gated channel, Shaw-related subfamily, member 2
	KCNJ1	1.9	Potassium inwardly rectifying channel, subfamily J, member 1
	KCNK15	2.2	Potassium channel, subfamily K, member 15
	CLCN6	2.8	Chloride channel 6
	CNGB1	3.0	Cyclic nucleotide gated channel beta 1
	TRPC1	2.2	Transient receptor potential cation channel, subfamily C, member 1
Intracellular transport	YWHAB	2.0	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, beta polypeptide (14-3-3ß)
	GABARAPL2	2.4	GABA(A) receptor-associated protein-like 2
	FUSIP1	−1.4	FUS interacting protein (serine/arginine-rich) 1
Blood vessel (vasculature) development	CRHR2	2.2	Corticotropin releasing hormone receptor 2
	FLT1	2.2	Fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor 1)
	ATPIF1	3.4	ATPase inhibitory factor 1
	TBX1	1.9	T-box 1
	ANGPT2	−1.5	Angiopoietin 2
Cell-cell signaling	TNF	1.8	Tumor necrosis factor (TNF superfamily, member 2)
	NOS1	1.8	Nitric oxide synthase 1 (neuronal)
	GRM7	2.0	Glutamate receptor, metabotropic 7
	PTHLH	2.0	Parathyroid hormone-like hormone
	DRD2	2.0	Dopamine receptor D2
	GABARAPL2	2.4	GABA(A) receptor-associated protein-like 2
Cell surface receptor linked signal transduction	GHRHR	4.9	Growth hormone-releasing hormone receptor
	CRHR2	2.2	Corticotropin releasing hormone receptor 2
	WNT8A	1.9	Wingless-type MMTV integration site family, member 8A
	AVPR2	2.0	Arginine vasopressin receptor 2
	FLT1	2.2	Fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor 1)
	AKT1	2.6	V-akt murine thymoma viral oncogene homolog 1beta isoform
Cell adhesion	PCDH10	2.4	Protocadherin 10
	PCDHB6	2.5	Protocadherin beta 6
	CLDN15	2.1	Claudin 15
	ICAM4	2.3	Intercellular adhesion molecule 4
	CNTNAP2	2.8	Contactin associated protein-like 2
	HDAC5	2.3	Histone deacetylase 5
	MAX	2.4	MAX interactor 1
Lipid transport	APOB	−1.6	Apolipoprotein B
	LPA	2.2	Lipoprotein Lp(a)
Branching morphogenesis of a tube	PPP1CA	3.4	Protein phosphatase 1, catalytic subunit, alpha isoform
	FLT1	2.2	Fms-related tyrosine kinase 1 (vascular endothelial growth factor receptor 1)
	GPC3	2.8	Glypican 3
Endothelial cell proliferation	POLD4	2.5	Polymerase (DNA-directed) delta 4
	ATPIF1	3.4	ATPase inhibitory factor 1
Endothelial cell differentiation	EDF1	2.0	Endothelial differentiation-related factor 1

Abbreviations: GO, gene ontology; *S cerevisiae*, *Saccharomyces cerevisiae*; *E coli, Escherichia coli*.
[a] Denotes average fold change.

Table 3. Statistically Overrepresented Gene Ontology Terms Among Transcripts Regulated by Vascular Endothelial Growth Factor-A

GO Term	Gene Symbol	FC[a]	Gene Name	*P* Value
Iron ion binding				.016
	CYP19A1	1.8	Cytochrome P450, family 19, subfamily A, polypeptide 1	
	CYP4F3	2.2	Cytochrome P450, family 4, subfamily F, polypeptide 3	
	CYP17A1	1.9	Cytochrome P450, family 17, subfamily A, polypeptide 1	
	CYP51A1	2.0	Cytochrome P450, family 51, subfamily A, polypeptide 1	
	CYP2C9	2.2	Cytochrome P450, family 2, subfamily C, polypeptide 9	
	CYP2C18	3.0	Cytochrome P450, family 2, subfamily C, polypeptide 18	
	CYP3A4	1.8	Cytochrome P450, family 3, subfamily A, polypeptide 4	
	SDHB	2.1	Succinate dehydrogenase complex, subunit B, iron sulfur (Ip)	
	NDUFS7	5.4	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20 kd	
	NDUFV1	2.9	NADH dehydrogenase (ubiquinone) flavoprotein 1, 51 kd	
	NDUFS8	3.2	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23 kd	
	ASPH	2.0	Aspartate beta-hydroxylase	
	ALOX12	2.0	Arachidonate 12-lipoxygenase	
	ALOXE3	2.4	Arachidonate lipoxygenase 3	
	NTHL1	3.4	Nth endonuclease III-like 1 (*E coli*)	
	SLC25A37	3.2	Solute carrier family 25, member 37	
	TPH2	2.2	Tryptophan hydroxylase 2	
	PPP1CA	3.4	Protein phosphatase 1, catalytic subunit, alpha isoform	
	MPO	1.9	Myeloperoxidase	
	NOS1	1.8	Nitric oxide synthase 1 (neuronal)	
	EGLN3	1.9	Egl nine homolog 3 (*C elegans*)	

Abbreviations: *E coli*, *Escherichia coli*; GO, gene ontology; *C elegans*, *Caenorhabditis elegans*.
[a] Denotes average fold change.

hierarchy, comprising a total of 45 nodes, were selected and merged for more convenient presentation (Figure 2). Several transcripts encoding proteins involved in cell death and survival were regulated by VEGF (serine-threonine protein kinase Akt1 [*AKT1*], BCL2-antagonist of cell death [*BAD*], BCL2-associated X protein [*BAX*], insulin-like growth factor binding protein 3 [*IGFBP3*], tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, 14-3-3beta protein [*YWHAB*], Max protein [*MAX*], Mad1-like protein [*MAD1L1*], macrophage migration inhibitory factor [*MIF*], hexokinase 1 [*HK1*], and tumour necrosis factor [*TNF*]). Furthermore, expression of mRNAs for subunits of the serine-threonine specific protein phosphatases 1 and 2 was also increased (*PPP1CA* and *PPP2R1B*).

From the data set of the 440 up- or downregulated transcripts in the partially serum deprived cells, IPA assigned 40 transcripts to the 2 top networks (Figure 3), showing the interactions of mRNAs encoding proteins primarily involved in cell cycle regulation (eg, cell division cycle 2 [*CDC2*], cyclin-dependent kinase 2 [*CDK2*], cyclin A2 [*CCNA2*], and cyclin B1 [*CCNB1*]).

Comparing the list of transcripts regulated by VEGF-A and by serum withdrawal treatment revealed 89 transcripts in common, of which 88 were reciprocally regulated (ie, they were significantly upregulated by one treatment and down-regulated by the other; Table 7). This degree of overlap is remarkable and extremely unlikely by chance, ($P < 1 \times 10^{-49}$). Among these 89 transcripts, several were cell-cycle-related and others are known to be involved in the regulation of cell survival and apoptosis, for example *BAD, IGFBP3, MIF, HK1, S100A9, PPP2R1B, PPP1CA*, and *YWHAB* (Figure 4). Many of the shared transcripts encode mitochondrial proteins (*NDUFS7, MRP63, MOSC2, NDUFS6, MRPL34, NDUFB10, GPX4, MRPL10, BAD, SLC25A3, Magmas, MRPS12, TSPO, CYP17A1, MRPL39, SLC25A1*, and *CLDN15*; $P = .00027$). Of the pro-apoptotic genes, only *IGFBP3* was downregulated. Vascular endothelial growth factor A was thus found to upregulate both pro- and anti-apoptotic genes in the HEECs.

DISCUSSION

Although isolation of capillary ECs using CD31-coated magnetic beads has been shown to be a reliable method,[10,19] there is some evidence that ECs isolated

Table 4. Transcripts Regulated by Serum Withdrawal Assigned to Functional Subsets by Gene Ontology Analysis

GO Term	Gene Symbol	FC[a]	Gene Name
Cell proliferation	CDKN2C	2.0	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)
	IFITM1	2.0	Interferon induced transmembrane protein 1
	DLGAP5	−2.7	Discs, large homolog 7 (Drosophila)
	TTK	−2.3	TTK protein kinase
	KLF4	−2.5	Kruppel-like factor 4
	GPNMB	2.3	Glycoprotein (transmembrane) nmb
	TOB1	1.9	Transducer of ERBB2
	MIF	−5.6	Macrophage migration inhibitory factor
	CDK2	-2.4	Cyclin-dependent kinase 2
	FGF7	2.5	Fibroblast growth factor 7
	MKI67	−3.2	Antigen identified by monoclonal antibody Ki-67
	PGR	1.9	Progesterone receptor
	TGFB3	2.0	Transforming growth factor, beta 3
	FOSL1	−2.9	FOS-like antigen 1
	CDC25C	−2.8	Cell division cycle 25 homolog C (*S pombe*)
	CSRP2	2.2	Cysteine and glycine-rich protein 2
	FABP3	2.7	Fatty acid binding protein 3, muscle and heart (mammary-derived growth inhibitor)
	CDKN3	−3.0	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)
	BUB1B	−2.3	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)
	PDGFRA	2.0	Platelet-derived growth factor receptor, alpha polypeptide
Death/apoptosis	NME3	−2.5	Nonmetastatic cells 3, protein expressed in
	ITM2B	1.8	Integral membrane protein 2B
	PDCD5	−2.5	Programmed cell death 5
	NME5	1.8	Non-metastatic cells 5, protein expressed in (nucleoside-diphosphate kinase)
	IGFBP3	1.9	Insulin-like growth factor binding protein 3
	BCL2L12	−2.4	BCL2-like 12 (proline rich)
	TNFRSF10A	−3.3	Tumor necrosis factor receptor superfamily, member 10a
	TP53INP1	2.6	Tumor protein p53 inducible nuclear protein 1
	CLU	1.9	Clusterin
	MIF	−5.6	Macrophage migration inhibitory factor
	ARHGDIA	−2.2	Rho GDP dissociation inhibitor (GDI) alpha
	BAD	−4.2	BCL2-antagonist of cell death
	BIRC5	−3.2	Baculoviral IAP repeat-containing 5 (survivin)
	TNFRSF12A	−2.5	Tumor necrosis factor receptor superfamily, member 12A
	GLRX2	−2.6	Glutaredoxin 2
	TUBB	−3.0	Tubulin, beta
	CLDN15	−2.5	Claudin 15
	BUB1B	−2.3	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)
	ESPL1	−2.3	Extra spindle pole bodies homolog 1 (*S cerevisiae*)
	PPP2R1B	−2.3	Protein phosphatase 2, regulatory subunit A, beta isoform
Response to stress	C1S	2.1	Complement component 1, s subcomponent
	S100A9	2.1	S100 calcium binding protein A9
	HDAC9	2.0	Histone deacetylase 9
	SERPINE1	−3.2	Serpin peptidase inhibitor, clade E (plasminogen activator inhibitor type 1), member 1
	TXNIP	4.2	Thioredoxin interacting protein
	PDPK1	−2.4	3-phosphoinositide dependent protein kinase-1
	SEPP1	3.9	Selenoprotein P, plasma, 1
	RAD51	−2.3	RAD51 homolog (RecA homolog, *E coli*; *S cerevisiae)*

(continued)

Table 4. (continued)

GO Term	Gene Symbol	FC[a]	Gene Name
	GPX4	−2.3	Glutathione peroxidase 4
	HSPA2	1.8	Heat shock 70kDa protein 2
	TP53INP1	2.6	Tumor protein p53 inducible nuclear protein 1
	PKN1	−2.8	Protein kinase N1
	CLU	1.9	Clusterin
	MIF	−5.6	Macrophage migration inhibitory factor
	FGF7	2.5	Fibroblast growth factor 7
	CCNA2	−2.3	Cyclin A2
	GLRX2	−2.6	Glutaredoxin 2
	FOXN3	1.9	Forkhead box N3
	HSPB3	1.8	Heat shock 27kDa protein 3
	PLSCR4	2.1	Phospholipid scramblase 4
	TREX2	−2.4	Three prime repair exonuclease 2
	MAP4K4	−2.3	Mitogen-activated protein kinase kinase kinase
	CCL26	1.8	Chemokine (C-C motif) ligand 26
	BDKRB1	−2.7	Bradykinin receptor B1
Anatomical structure development and formation	HTR2B	2.6	5-hydroxytryptamine (serotonin) receptor 2B
	MMP2	1.9	matrix metallopeptidase 2
	HDAC9	2.0	Histone deacetylase 9
	SERPINE1	−3.2	Serpin peptidase inhibitor, clade E (plasminogen activator inhibitor type 1), member 1
	NCAM1	1.8	Neural cell adhesion molecule 1
	DCN	2.0	Decorin
	CRYAB	2.1	Crystallin, alpha B
	CCNF	−2.3	Cyclin F
	SLC40A1	5.1	Solute carrier family 40 (iron-regulated transporter), member 1
	MMP11	4.1	Matrix metallopeptidase 11 (stromelysin 3)
Reproductive process	PGR	1.9	Progesterone receptor
	PDGFRA	2.0	Platelet-derived growth factor receptor, alpha polypeptide
Vasculature development	MMP2	1.9	Matrix metallopeptidase 2
	SERPINE1	−3.2	Serpin peptidase inhibitor, clade E (plasminogen activator inhibitor type 1), member 1
	TNFRSF12A	−2.5	Tumor necrosis factor receptor superfamily, member 12A
Wnt receptor signaling pathway/JAK-STAT cascade	PPP2R1B	−2.3	Protein phosphatase 2, regulatory subunit A, beta isoform
Positive regulation of I-kappaB kinase/NF-kappaB cascade	HTR2B	2.6	5-hydroxytryptamine (serotonin) receptor 2B
	SLC20A1	−2.2	Solute carrier family 20 (phosphate transporter), member 1
Cell motility	ARHGDIA	−2.2	Rho GDP dissociation inhibitor (GDI) alpha
	TNFRSF12A	−2.5	Tumor necrosis factor receptor superfamily, member 12A
	AKAP3	−2.5	A kinase (PRKA) anchor protein 3
Transcription	KLF4	−2.5	Kruppel-like factor 4
	PAPOLA	−3.0	Poly(A) polymerase alpha
	CDK2	−2.4	Cyclin-dependent kinase 2
	CCNA2	−2.3	Cyclin A2
	CCNT2	−2.1	Cyclin T2
	TRIP13	−2.7	Thyroid hormone receptor interactor 13

Abbreviations: GO, gene ontology; *S pombe*, *Schizosaccharomyces pombe*.
[a] Denotes average fold change.

Table 5. Statistically Overrepresented GO Terms Among Transcripts Regulated by Partial Serum Withdrawal

GO Term	Gene Symbol	FC[a]	Gene Name	P Value
Regulation of progression through cell cycle	BUB1B	−2.3	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	2.89e-06
	ESPL1	−2.3	Extra spindle pole bodies homolog 1 (*S cerevisiae*)	
	MAD1L1	−3.6	MAD1 mitotic arrest deficient-like 1 (yeast)	
	MAD2L1	−2.3	MAD2 mitotic arrest deficient-like 1 (yeast)	
	ZWINT	−2.2	ZW10 interactor	
	PLK1	−4.8	Polo-like kinase 1 (Drosophila)	
	PLK4	−2.3	Polo-like kinase 4 (Drosophila)	
	CCNA2	−2.3	Cyclin A2	
	CCNB1	−2.5	Cyclin B1	
	CCNF	−2.3	Cyclin F	
	CCNT2	−2.1	Cyclin T2	
	AURKAIP1	−3.1	Aurora kinase A interacting protein 1	
	CDC2	−3.7	Cell division cycle 2, G1 to S and G2 to M	
	CDC20	−5.8	Cell division cycle 20 homolog (*S cerevisiae*)	
	CDC25C	−2.8	Cell division cycle 25 homolog C (*S pombe*)	
	CDC45L	−2.9	Cell division cycle 45-like (*S cerevisiae*)	
	TTK	−2.3	TTK protein kinase	
	NUSAP1	−2.3	Nucleolar and spindle associated protein 1	
	UBE2C	−3.6	Ubiquitin-conjugating enzyme E2C	
	WEE1	1.7	WEE1 homolog (*S pombe*)	
	NEK2	−2.6	NIMA (never in mitosis gene a)-related kinase 2	
	CDK2	−2.4	Cyclin-dependent kinase 2	
	CDKN2C	2.0	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	
	CDKN3	−3.0	Cyclin-dependent kinase inhibitor 3 (CDK2-associated dual-specificity phosphatase)	
	DLGAP5	−2.7	Discs, large homolog 7 (Drosophila)	
	BIRC5	−3.2	Baculoviral IAP repeat-containing 5 (survivin)	
	G0S2	−5.2	G0/G1switch 2	
	E2F7	−2.5	E2F transcription factor 7	
	DUSP6	−3.0	Dual-specificity phosphatase 6	
	TGFB3	2.0	Transforming growth factor, beta 3	
	FGF7	2.5	Fibroblast growth factor 7	
	HDAC9	2.0	Histone deacetylase 9	
	IFITM1	2.0	Interferon-induced transmembrane protein 1	
	FOXN3	1.9	Forkhead box N3	
	HRASLS3	1.9	HRAS-like suppressor 3	
	MKI67	−3.2	Antigen identified by monoclonal antibody Ki-67	
	PSMD8	−3.3	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8	
	TACC3	−2.4	Transforming, acidic coiled-coil containing protein 3	
Microtubule-based process	BUB1B	−2.3	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	.0044
	ESPL1	−2.3	Extra spindle pole bodies homolog 1 (*S cerevisiae*)	
	MAP1B	−2.6	Microtubule-associated protein 1B	
	KIF2C	−3.1	Kinesin family member 2C	
	KIF20A	−2.2	Kinesin family member 20A	
	ZWINT	−2.2	ZW10 interactor	
	TUBB	−3.0	Tubulin, beta	
	TUBB2A	−2.2	Tubulin, beta 2A	
	DYNLRB2	2.0	Dynein, light chain, roadblock-type 2	
	PRC1	−3.1	Protein regulator of cytokinesis 1	
	AURKA	−2.6	Aurora kinase A	
	TTK	−2.3	TTK protein kinase	
	NUSAP1	−2.3	Nucleolar- and spindle-associated protein 1	
	UBE2C	−3.6	Ubiquitin-conjugating enzyme E2C	

Abbreviations: GO, gene ontology; *Saccharomyces cerevisiae*, *S pombe*, *Schizosaccharomyces pombe*.

[a] Denotes average fold change.

Table 6. Statistically Underrepresented Gene Ontology Terms Among Transcripts Regulated by Partial Serum Withdrawal

GO Term	Gene Symbol	FC[a]	Gene Name	P Value
Cell communication	KCNMB4	1.8	Potassium large conductance calcium-activated channel, subfamily M, beta member 4	.016
	ZWINT	−2.2	ZW10 interactor	
	DUSP6	−3.0	Dual-specificity phosphatase 6	
	STC1	−2.3	Stanniocalcin 1	
	TGFB3	2.0	Transforming growth factor, beta 3	
	AURKA	−2.6	Aurora kinase A	
	SYDE2	1.9	Synapse defective 1, Rho GTPase, homolog 2 (*C elegans*)	
	TOB1	1.9	Transducer of ERBB2, 1	
	FGF7	2.5	Fibroblast growth factor 7	
	IGFBP3	1.9	Insulin-like growth factor–binding protein 3	
	PDE5A	1.8	Phosphodiesterase 5A, cGMP-specific	
	MIF	−5.6	Macrophage migration inhibitory factor (glycosylation-inhibiting factor)	
	TOP2A	−2.7	Topoisomerase (DNA) II alpha 170 kd	
	SLC20A1	−2.2	Solute carrier family 20 (phosphate transporter), member 1	
	OR52A1	−2.5	Olfactory receptor, family 52, subfamily A, member 1	
	RGS2	2.1	Regulator of G-protein signaling 2, 24 kd	
	RAB20	−2.9	RAB20, member RAS oncogene family	
	IFITM1	2.0	Interferon induced transmembrane protein 1	
	EIF4EBP1	−2.9	Eukaryotic translation initiation factor 4E binding protein 1	
	ITGB1BP1	−2.3	Integrin beta 1 binding protein 1	
	IFI6	1.9	Interferon, alpha-inducible protein 6	
	UBE2C	−3.6	Ubiquitin-conjugating enzyme E2C	
	MLN	1.8	Motilin	
	CD274	−2.1	CD274 molecule	
	PDGFRA	2.0	Platelet-derived growth factor receptor, alpha polypeptide	
	DTNA	1.8	Dystrobrevin, alpha	
	PKN1	−2.8	Protein kinase N1	
	PPP1R12B	2.4	Protein phosphatase 1, regulatory (inhibitor) subunit 12B	
	PDPK1	−2.4	3-phosphoinositide-dependent protein kinase-1	
	CRYAB	2.1	Crystallin, alpha B	
	CD4	−3.3	CD4 molecule	
	CCL26	1.8	Chemokine (C-C motif) ligand 26	
	MAP4K4	−2.3	Mitogen-activated protein kinase kinase kinase kinase 4	
	DPYSL2	1.8	Dihydropyrimidinase-like 2	
	SNX5	−2.8	Sorting nexin 5	
	BUB1B	−2.3	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	
	BAD	−4.2	BCL2-antagonist of cell death	
	RALA	−3.4	V-ral simian leukemia viral oncogene homolog A (ras related)	
	FKBP1A	−3.8	FK506-binding protein 1A, 12 kd	
	PGR	1.9	Progesterone receptor	
	CHRM2	2.0	Cholinergic receptor, muscarinic 2	
	ARHGAP22	−2.3	Rho GTPase-activating protein 22	
	ANGPTL2	2.1	Angiopoietin-like 2	
	TNFRSF10A	−3.3	Tumor necrosis factor receptor superfamily, member 10a	
	CAP2	1.8	CAP, adenylate cyclase-associated protein, 2 (yeast)	
	GRIK2	2.0	Glutamate receptor, ionotropic, kainate 2	
	SMAD9	1.9	SMAD family member 9	
	BAIAP2	−2.4	BAI1-associated protein 2	
	GLRX2	−2.6	Glutaredoxin 2	
	S100A9	2.1	S100 calcium-binding protein A9	
	PLXNB3	−2.7	Plexin B3	
	BDKRB1	−2.8	Bradykinin receptor B1	
	C1S	2.1	Complement component 1, s subcomponent	
	PLA2G1B	1.8	Phospholipase A2, group IB (pancreas)	

(continued)

Table 6. (continued)

GO Term	Gene Symbol	FC[a]	Gene Name	P Value
	DEPDC1	−4.1	DEP domain containing 1	
	MAPK10	1.8	Mitogen-activated protein kinase 10	
	HTR2B	2.6	5-hydroxytryptamine (serotonin) receptor 2B	
	LY6E	−3.1	Lymphocyte antigen 6 complex, locus E	
	AKAP3	−2.5	A kinase (PRKA) anchor protein 3	
	ARHGDIA	−2.2	Rho GDP dissociation inhibitor (GDI) alpha	
	PMP22	2.8	Peripheral myelin protein 22	
	MFAP4	2.0	Microfibrillar-associated protein 4	
	NCAM1	1.8	Neural cell adhesion molecule 1	
	DLGAP5	−2.7	Discs, large homolog 7 (Drosophila)	

Abbreviation: GO, gene ontology; *C elegans*, *Caenorhabditis elegans*.
[a] Denotes average fold change.

from various tissues differ in their cell surface receptor expression.[20] To ensure our primary cultures were of sufficient purity and that the cells showed endothelial characteristics, we used a panel of antibodies detecting EC surface markers and 2 negative controls. We found that staining with anti-vWF varied between cultures. Uneven staining for vWf in HEECs and in the endometrial vasculature without obvious relation to vessel size or location in the endometrium has previously been reported.[21,22] In the current study, all cultures were positive for the CD54 and/or CD31, indicating their endothelial phenotype. To minimize the risk of the HEECs losing their endometrial properties, we used the earliest possible passages in our experiments. Taken together, we believe that cultures of primary early passage HEECs offer a tissue-specific model for the studying of endometrial angiogenesis.

Aware of the known sensitivity of microarrays to small variations in experimental conditions and inter- and intraindividual differences in cell cultures,[23] we used a paired design where each individual cell culture was split into 2. We have used a combination of statistical methods that make different assumptions about the underlying data structure. Transcripts selected have passed both of these tests, thus making the selection process more stringent and reliable. The resulting data have been analyzed in a systematic way, making reanalysis of a very small subset of transcripts using quantitative Real-time Polymerase Chain Reaction (RT-PCR) meaningless.

In the VEGF-A arrays, the highest number of upregulated transcripts were found within groups of genes related to transcription, cell surface-linked signal transduction, and anatomical structure development and formation including blood vessel development. This was expected considering the known roles of VEGF-A as a mitogen and major angiogenic factor.

Furthermore, we found evidence of VEGF-A regulating transcripts encoding proteins involved in cell proliferation and survival, response to stress and apoptosis. The serine-threonine protein kinase Akt is encoded by 3 different genes (*AKT1*, *2*, and *3*) and is a key regulator of numerous cellular processes. It is part of the PI3-kinase (PI3K)-Akt signalling cascade involved in several biological activities, including angiogenesis.[24] Akt1 was shown to be the predominantly expressed isoform in ECs in mouse aorta, lungs, and Matrigel implants.[25] Vascular endothelial growth factor signaling leads to Akt activation, which regulates EC migration and mediates the VEGF-A-induced acute permeability.[26,27] The PI3K-Akt signaling pathway is also responsible for the effects of VEGF-A on EC survival and has both pro- and antiangiogenic effects.[24,28,29] The role of Akt as a growth factor-induced anti-apoptotic mediator through phosphorylation and inactivation of the Bcl-2 antagonist of cell death (Bad) protein has been well established.[30,31] The Bcl-2 family contains proteins with both pro- and anti-apoptotic activities. Bad and the Bcl-2 associated X protein (Bax) are pro-apoptotic regulators acting on the mitochondrial outer membrane enabling the release of cytochrome c from mitochondria.[32] This in turn activates the caspase cascade resulting in cellular apoptosis. When phosphorylated by Akt, Bad binds to cytoplasmic 14-3-3 proteins, one of which is encoded by the *YWHAB* gene found to be regulated in our data set, and its pro-apoptotic functions is temporarily inhibited.[33] The release of cytochrome c is also inhibited by mitochondrion-associated hexokinase 1, another downstream effector of Akt-mediated cell survival, which catalyzes the first step in glycolysis and is also associated with the voltage-dependant anion channels at the outer mitochondrial membrane.[34] The role in apoptosis evasion of hexokinase 1 has not been studied in ECs and very little is known about other angiogenesis-related effects. We show

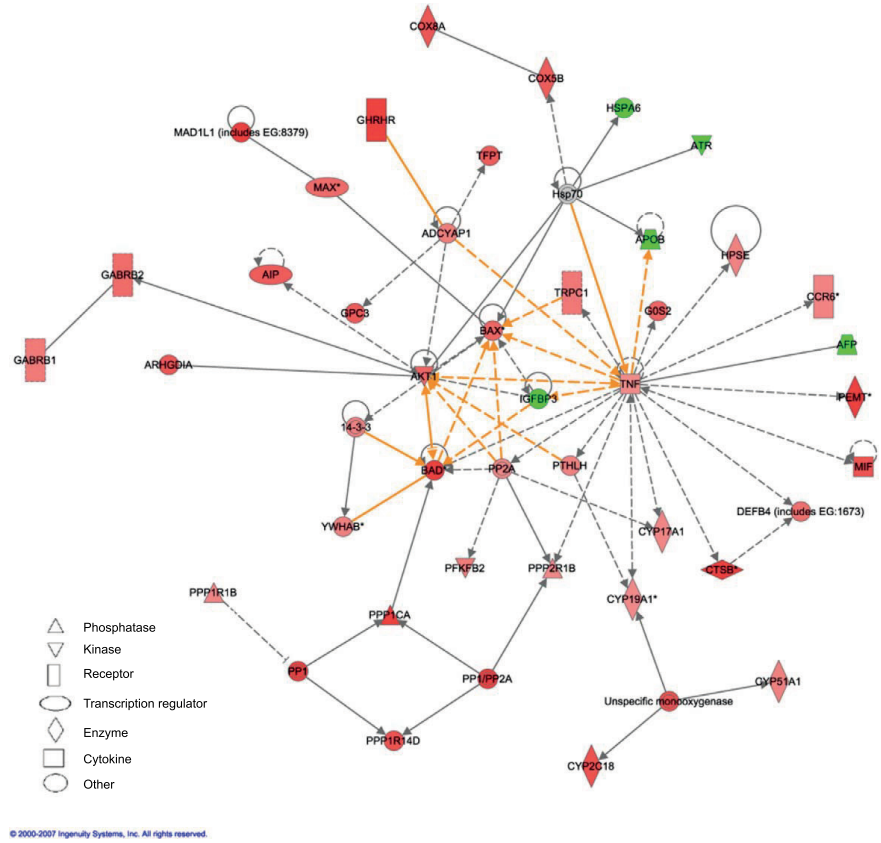

Figure 2. Ingenuity pathway analysis showing regulatory relationships among differentially regulated transcripts in human endometrial endothelial cells following 24 hours vascular endothelial growth factor A stimulation. Nodes marked in red indicate transcripts upregulated in vascular endothelial growth factor-A-stimulated human endometrial endothelial cells and those in green indicate downregulated transcripts. The color intensity reflects the level of regulation. The shape of the symbol denotes the type of protein encoded. Concentric circles indicate transcripts not present on the uploaded gene list but relevant to the network based on the information in the ingenuity pathway analysis (IPA) knowledge base. Transcripts marked with an asterisk were represented by more than one probe on the microarray.

here that the level of *HK1* transcripts is elevated in HEECs in response to VEGF-A and decreased when cells are partially serum starved. It is therefore tempting to speculate that *HK1* is a VEGF-A-responsive pro-survival gene in ECs and further studies are required to confirm this.

The proinflammatory cytokine macrophage MIF directly activates the PI3K/Akt signaling pathway, inducing EC migration, and inhibits apoptosis in fibroblasts and various tumor cells.[35,36] Anti-MIF antibody applied to cultured HUVECs in the presence of VEGF resulted in reduced uptake of [^{3}H]-thymidine compared to VEGF alone.[37] Our results show a 3-fold increase in *MIF* mRNA in cultured HEECs in response to VEGF-A and a more than 5-fold reduction of *MIF* transcripts following serum withdrawal, further strengthening the hypothesis that MIF is closely involved in endothelial fate regulation.

In our study, we have shown that VEGF-A upregulates *AKT1*, *MIF*, and *YWHAB* mRNA in HEECs, showing activation at the transcript level of the PI3K-dependent cell survival machinery.

Interestingly, considering the well-established role of VEGF-A as a pro-survival factor, a concomitant

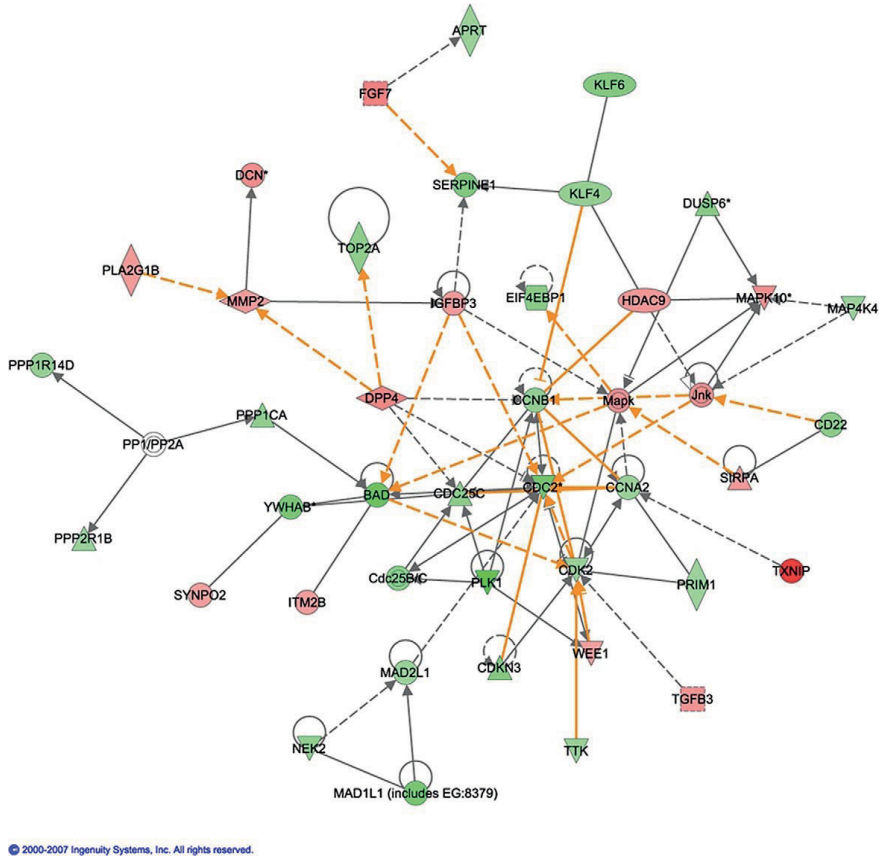

Figure 3. Ingenuity pathway analysis regulatory network of differentially expressed transcripts in human endometrial endothelial cells after 24 hours partial serum deprivation. Nodes designated as in Figure 2.

upregulation of pro-apoptotic genes *BAD*, *BAX*, and *PDCD5* (programmed cell death 5) was also detected, where *BAD* was among the most upregulated transcripts on the whole array. The interplay between pro- and anti-apoptotic genes and proteins in ECs following VEGF-A exposure is complex and remains unclear. Gerritsen et al have investigated the RNA expression pattern in human ECs in response to combinations of VEGF-A and hepatocyte growth factor (HGF) using tube formation assays and standard in vitro cultures.[38,39] Of relevance to our study is the evidence of induction of both promoters and inhibitors of apoptosis despite the presence of 2 major growth factors. Among the upregulated transcripts was *TRAIL*, an apoptosis-inducing member of the TNF family. Complementing this finding we show here the reciprocal regulation of *TNFRSF10A* (encoding TRAIL-receptor 1) following VEGF-A stimulation or serum withdrawal and the upregulation of *TNF* (encoding TNF-α) detected in the HEECs in response to VEGF-A stimulation. Gerritsen et al also found upregulation of other pro-apoptotic transcripts, such as caspases and pro-caspases. These are absent from our list of transcripts. However, we have shown for the first time the upregulation of the BCL2 family apoptosis inducers *BAD* and *BAX* in a subset of ECs in response to VEGF-A stimulation.

In a study investigating the transcript profile in HUVECs exposed to VEGF-A and/or HGF (each at a

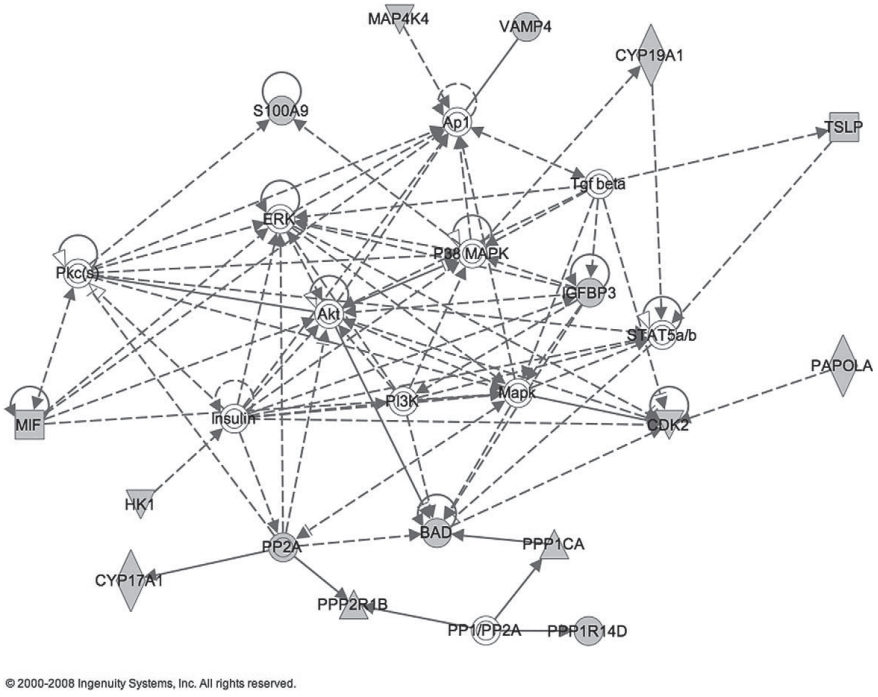

Figure 4. Ingenuity pathway analysis regulatory network of transcripts reciprocally regulated in human endometrial endothelial cells in the 2 study groups (24 hours vascular endothelial growth factor-A stimulation and partial serum deprivation, respectively). Nodes designated as in Figure 2.

concentration of 100 ng/mL) at 4 and 24 hours, the same authors showed a distinct overrepresentation of cell-cycle-related transcripts at 24 hours, a large number of which overlap our results.[39] Furthermore, agglomerative clustering of the different expression profiles showed that the VEGF-A-induced transcripts were predominant at 24 hours when the cells were treated with a combination of the 2 growth factors.

However, VEGF-A_{165} also transiently upregulates transcripts believed to be important for EC biology and angiogenesis. This was shown in a microarray study investigating the in vitro response of human myometrial microvascular ECs (MMECs) to VEGF-A stimulation after 3, 6, and 12 hours, respectively.[40] A total of 110 transcripts displayed a 3-fold or more upregulation compared to unstimulated controls. Of these, 76 transcripts were upregulated at 3 hours (of which 47 transcripts only appeared at the 3-hour time point) and the number of upregulated transcripts decreased rapidly over time. This illustrates one of the difficulties encountered when comparing studies of EC stimulation and could possibly be one explanation as to why studies of similar design sometimes yield conflicting results or at least limited overlap.

Of the 110 upregulated transcripts at any of the studied time points, a large number were related to ECM remodeling, an important endothelial function. Smaller groups of transcripts were associated with sex steroid metabolism and EC proliferation and survival. However, most of the cell fate-associated complex of transcripts identified in our array data was largely absent. This can at least partly be explained by the use of MMEC instead of HEEC, much smaller arrays comprising approximately 4600 transcripts and possibly also the different time points discussed above.

Insulin-like growth factor 1 (IGF-1) signaling, through the type 1 IGF receptor (IGF1R), is known to have a mitogenic effect on various cell types and positively regulates cell proliferation and survival including

Table 7. Transcripts Differentially Expressed in Human Endometrial Endothelial Cells in Response to Vascular Endothelial Growth Factor A Stimulation and Partial Serum Withdrawal

Gene Symbol	FC[a] VEGF	FC[a] Serum withdrawal	Gene Name
ABHD11	2.6	−2.9	Abhydrolase domain containing 11
AKAP3	2.6	−2.5	A kinase (PRKA) anchor protein 3
ALKBH7	2.6	−2.8	AlkB, alkylation repair homolog 7 (*E coli*)
AOC2	1.8	−2.2	Amine oxidase, copper containing 2 (retina-specific)
APRT	2.4	−2.3	Adenine phosphoribosyltransferase
ARHGDIA	2.9	−2.2	Rho GDP dissociation inhibitor (GDI) alpha
ASPH	2.0	−3.4	Aspartate beta-hydroxylase
BAD	6.1	−4.2	BCL2-antagonist of cell death
BET1L	2.0	−2.7	Blocked early in transport 1 homolog (*S cerevisiae*)-like
C9orf16	3.3	−3.5	Chromosome 9 open reading frame 16
C14orf126	2.2	−2.5	Chromosome 14 open reading frame 126
C17orf37	2.1	−2.5	Chromosome 17 open reading frame 37
CCNT2	1.8	−2.1	Cyclin T2
CD22	2.7	−2.8	CD22 molecule
CD37	2.4	−3.1	CD37 molecule
CD4	3.5	−3.3	CD4 molecule
CDK2	1.9	−2.4	Cyclin-dependent kinase 2
CLDN15	2.1	−2.5	Claudin 15
CPNE1	2.4	−2.3	Copine I
CRYBB1	2.1	−2.9	Crystallin, beta B1
CYP17A1	1.9	−2.5	Cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19A1	1.8	−2.5	Cytochrome P450, family 19, subfamily A, polypeptide 1
DDA1	2.8	−2.2	DET1 and DDB1 associated 1
DDT	2.3	−3.2	D-dopachrome tautomerase
DFNB31	2.0	−3.0	Deafness, autosomal recessive 31
EXOSC4	3.4	−5.3	Exosome component 4
FKBP1A	2.6	−3.8	FK506-binding protein 1A, 12 kd
G0S2	2.6	−5.2	G0/G1switch 2
GIMAP2	−1.5	1.9	GTPase, IMAP family member 2
GPX4	7.7	−2.3	Glutathione peroxidase 4
HK1	2.0	−2.8	Hexokinase 1
IGFBP3	−1.5	1.9	Insulin-like growth factor binding protein 3
JOSD2	2.4	−3.7	Josephin domain containing 2
KAZALD1	2.0	−2.3	Kazal-type serine peptidase inhibitor domain 1
FNIP2	−1.4	1.8	Folliculin interacting protein 2
KLK13	2.0	−2.5	Kallikrein-related peptidase 13
L3MBTL2	1.9	−2.6	I(3)mbt-like 2 (Drosophila)
LSM7	2.9	−2.5	LSM7 homolog, U6 small nuclear RNA associated (*S cerevisiae*)
LY6E	2.2	−3.1	Lymphocyte antigen 6 complex, locus E
MAD1L1	3.8	−3.6	MAD1 mitotic arrest deficient-like 1 (yeast)
Magmas	3.6	−3.8	Mitochondria-associated protein involved in granulocyte-macrophage colony-stimulating factor signal transduction
MAP1B	3.6	−2.6	Microtubule-associated protein 1B
MAP4K4	2.3	−2.3	Mitogen-activated protein kinase kinase kinase kinase 4
MOSC2	−1.4	1.9	MOCO sulphurase C-terminal domain containing 2
NOC4L	2.5	−2.8	Nucleolar complex associated 4 homolog (*S cerevisiae*)
NUDT22	2.5	−2.3	Nudix (nucleoside diphosphate linked moiety X)-type motif 22
MIF	3.0	−5.6	Macrophage migration inhibitory factor
MRP63	4.1	−2.8	Mitochondrial ribosomal protein 63
MRPL10	3.0	−2.9	Mitochondrial ribosomal protein L10
MRPL34	3.1	−3.1	Mitochondrial ribosomal protein L34
MRPL39	2.2	−3.1	Mitochondrial ribosomal protein L39

(continued)

Table 7. (continued)

Gene Symbol	FC[a] VEGF	FC[a] Serum withdrawal	Gene Name
MRPS12	2.1	−2.2	Mitochondrial ribosomal protein S12
NDUFB10	2.2	−2.9	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22 kd
NDUFS6	3.6	−3.7	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13 kd
NDUFS7	5.4	−3.3	NADH dehydrogenase (ubiquinone) Fe-S protein 7, 20 kd (NADH-coenzyme Q reductase)
NOSIP	4.2	−2.9	Nitric oxide synthase−interacting protein
PAPOLA	3.0	−3.0	Poly(A) polymerase alpha
PCDH10	2.4	1.9	Protocadherin 10
PDCD5	2.1	−2.5	Programmed cell death 5
PLEKHH1	1.9	−2.4	Pleckstrin homology domain containing, family H (with MyTH4 domain) member 1
PLK1	1.9	−4.8	Polo-like kinase 1
PLXNB3	2.1	−2.7	Plexin B3
POLE4	2.1	−3.5	Polymerase (DNA-directed), epsilon 4 (p12 subunit)
POP7	2.0	−2.7	Processing of precursor 7, ribonuclease P/MRP subunit (*S cerevisiae*)
PPP1CA	3.4	−2.6	Protein phosphatase 1, catalytic subunit, alpha isoform
PPP1R14D	3.0	−2.4	Protein phosphatase 1, regulatory (inhibitor) subunit 14 d
PPP2R1B	1.9	−2.3	Protein phosphatase 2 (formerly 2A), regulatory subunit A, beta isoform
RABAC1	7.9	−2.5	Rab acceptor 1 (prenylated)
RAD51	2.8	−2.3	RAD51 homolog (RecA homolog, *E coli*; *S cerevisiae*)
REG3A	2.1	−2.6	Regenerating islet-derived 3 alpha
RPL39L	2.6	−3.6	Ribosomal protein L39-like
S100A9	−1.5	2.1	S100 calcium-binding protein A9
SELM	7.1	−3.1	Selenoprotein M
SGT	2.3	−2.9	Small glutamine-rich tetratricopeptide repeat (TPR)-containing, alpha
SIVA1	3.5	−2.6	SIVA1, apoptosis-inducing factor
SLC25A1	3.2	−3.1	Solute carrier family 25 (mitochondrial carrier; citrate transporter), member 1
SLC25A3	2.5	−3.5	Solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 3
SMARCC2	2.2	−2.8	SWI-/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily c, member 2
SMTN	3.1	−3.6	Smoothelin
ST6GALNAC3	2.1	−2.4	ST6 (alpha-*N*-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-*N*-acetylgalactosaminide alpha-2,6-sialyltransferase 3
TBCB	2.2	−3.6	Tubulin folding cofactor B
TNFRSF10A	2.1	−3.3	Tumor necrosis factor receptor superfamily, member 10a
TSLP	2.5	−3.1	Thymic stromal lymphopoietin
TSPO	2.3	−3.1	Translocator protein (18 kd)
TSTA3	3.4	−2.3	Tissue-specific transplantation antigen P35B
VAMP4	2.8	−-2.3	Vesicle-associated membrane protein 4
VPS37B	2.7	−2.7	Vacuolar protein sorting 37 homolog B (*S cerevisiae*)
WFDC2	2.1	−2.3	WAP 4-disulfide core domain 2
YWHAB	2.0	−3.4	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, β-polypeptide

Abbreviations: VEGF, vascular endothelial growth factor; *S cerevisiae*, *Saccharomyces cerevisiae*; *E coli*, *Escherichia coli*.
[a] Denotes average fold change.

angiogenesis.[41] In particular, IGF-1 seems to be necessary for VEGF-A-induced Akt activation in ECs.[42]

The binding of IGFs to IGF receptors is modulated by IGF binding proteins (IGFBPs). There is emerging evidence of IGFBP-3 also having IGF-independent pro-apoptotic properties through blocking of Akt phosphorylation[43,44] and a negative regulatory action on angiogenesis.[45,46] *IGFBP-3* mRNA is expressed in the human endometrial endothelium and in the endothelium of the human corpus luteum.[47,48]

Our microarray data show that in HEECs, VEGF-A upregulates both *AKT1* and *IGF-1* mRNA and downregulates *IGFPB3* transcripts, indicating activation of 2 closely related anti-apoptotic pathways at the transcript level. The latter result is in agreement with a previous study showing downregulation of *IGFBP-3* mRNA

following VEGF treatment in cultured bovine aortic ECs.[49] Vascular endothelial growth factor-A stimulation of MMECs, however, resulted in *IGFBP3* being downregulated after 3 hours, upregulated over 3-fold after 6 hours and beginning to decrease again after 12 hours.[40] In contrast, we found that reducing the FBS concentration in the culture medium anticipated to impair cellular proliferation and survival, increased the expression of *IGFBP3* transcripts almost 2-fold.

Angiopoietin 2 (Ang-2), encoded by the *ANGPT2* gene, is a ligand for the Tie-2 receptor, competing with angiopoietin 1 (Ang-1) and having a destabilizing action on mature vessels. Endothelial cells have been identified as the major source of Ang-2, indicating an autocrine mechanism.[50] *ANGPT2* mRNA has been detected in HEECs previously and is induced by hypoxia and slightly downregulated by thrombin.[51,52] In cultured HUVECs, *ANGPT2* mRNA is upregulated by tumor-derived VEGF-A in a dose-dependent manner.[53] In the current study, VEGF-A downregulated *ANGPT2* mRNA by a factor 1.5 after 24 hours, coinciding with the results presented by Weston et al where *ANGPT2* in MMECs was transiently upregulated after 3 hours but roughly halved compared to baseline after 6 and 12 hours, respectively.[40] These findings may indicate a different regulation of *ANGPT2* in uterine microvascular ECs compared to cells from umbilical vessels.

Partially depriving the HEECs of serum resulted in the downregulation of a number of transcripts associated with cell cycle progression and cell growth. In common with other investigators studying HEEC biology,[9,11,54,55] our cells were grown in the presence of nonstripped FBS, which is likely to contain ovarian steroids. It is possible that withdrawal of these may have contributed to the effects we observed.

Compared to an apoptosis study performed on primary HUVECs, there was some overlap in the downregulated cell-cycle-related transcripts.[56] Examples include *CDC2, CDC45, CCNA2, CCNB1,* and *MAD2L1.* Among transcripts associated with phagocytosis of apoptotic cells, both studies show upregulation of clusterin (*CLU*). However, in the HUVEC study, no downregulation of apoptosis-inducing transcripts was detected, whereas our HEEC data present a somewhat more complex picture. The pro-apoptotic transcripts *BAD, SIVA1,* and *PDCD5* were downregulated together with apoptosis-inhibiting *BIRC5* (encoding survivin) and *BCL2L12.* Furthermore, the first 3 transcripts were upregulated by VEGF-A and this seemingly contradictory regulatory pattern has not previously been described and supports our hypothesis of heterogeneity among ECs and unique behavior of HEECs.

Comparing the gene lists derived from the VEGF-A stimulation and serum withdrawal arrays, we identified 88 reciprocally expressed transcripts common to both experiments (Table 7), some of which have been discussed above. Cell-cycle-related genes were highly represented among these overlapping transcripts and growth-promoting transcripts were generally upregulated in response to VEGF-A and downregulated when the culture medium's serum content was reduced. Transcripts related to mitosis inhibition or cell cycle checkpoints were oppositely regulated, for example *FNIP2, G0S2,* and *MAD1L1.*

The possible action of ovarian steroid hormones on HEECs is clearly relevant to this study as low levels of these hormones are present in the serum in the growth media. However, while there is consistent evidence in the literature demonstrating estrogen receptor beta (ERβ) and the absence of ERα in HEECs,[57-59] there is still controversy regarding the presence and action of progesterone receptors. For example, several studies report increased proliferation and tube formation in HEECs by progestogens,[58,59] but Iruela-Arispe reported that progesterone inhibited HEEC proliferation.[21] The partial serum withdrawal may also be regarded a partial withdrawal of steroid hormones, but the specific influence of this on our results is difficult to assess. Krikun et al[59] found differential regulation of more than 300 genes by E_2 and/or medroxyprogesterone acetate, but the only transcripts overlapping our results are *PTHLH* and *NEUROD4* (upregulated by VEGF-A in our study and reported as downregulated by E_2) and *BUB1* (downregulated by partial serum withdrawal and reported as upregulated by E_2). Yasuo and coworkers investigated the effects of E_2 and P, either alone or in combination and at 2 different time points, on the transcript profile in pooled human uterine microvascular cells.[60] Although there is no direct overlap with our results, both regulated data sets contain members of the MMP, FGF, and TNF-receptor (super-) families, suggesting similarities in the effects of VEGF-A and ovarian steroids on HEEC biology.

In summary, we have shown that VEGF-A upregulates several transcripts related to cell proliferation and survival (in particular members of the PI3K-Akt signaling cascade) and angiogenesis in primary cultured HEECs. In addition, the PI3K-dependent pathway can be activated through MIF, and our data show increased *MIF* mRNA. Other survival signals upregulated by VEGF-A include the 14-3-3ß-gene *YWHAB.* The reciprocal regulation

of a subset of transcripts regulated in HEECs under growth-promoting or pro-apoptotic conditions is presented here for the first time, suggesting that these transcripts may be the most important for regulating HEEC fate. These data also highlight the importance of the mitochondrion in these processes.

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ORIGINAL ARTICLE

Levels of oestrogen receptor, progesterone receptor and αB-crystallin in eutopic endometrium in relation to pregnancy in women with endometriosis

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Abstract
Endometriosis affects fertility in many women and may partly be due to decreased endometrial receptivity. Several mechanisms have been suggested, notably, progesterone resistance for which a number of candidate biomarkers have been suggested. Here we demonstrate aberrant levels of steroid hormone receptors and the small heat shock protein αB-crystallin in eutopic endometrial epithelium from 38 women with peritoneal endometriosis diagnosed during investigation for secondary infertility. Spontaneous pregnancies within 1 year after medical and surgical treatment for endometriosis were recorded and semi-quantitative immunohistochemistry data compared between women with endometriosis who did or did not become pregnant and healthy controls. Stronger immunostaining for ER-α was detected in luminal and glandular endometrial epithelium from women with endometriosis who did not become pregnant during the post-treatment observation period versus endometriosis patients who became pregnant and controls. Staining levels of PR and PR-B were lower in patients without subsequent pregnancies than in the two other groups. Endometrial levels of αB-crystallin in endometriosis patients similar to those in controls were strongly correlated with the chance of becoming pregnant, whereas higher or lower levels were not.

Keywords: *Endometriosis, oestrogen receptor, progesterone receptor, αB-crystallin, pregnancy*

Introduction

The eutopic endometrium in women with endometriosis may be less receptive to the implanting blastocyst than that in women without disease (Mahutte & Arici, 2002; Gupta et al., 2008), sometimes contributing to concomitant subfertility. Aberrant expression of implantation-related endometrial factors may be associated with this impaired receptivity (Soares et al., 2008). Additional differences regarding histological structure, regulation of adhesion molecules and cytokines, gene expression and proteolytic enzymes have also been described (Sharpe-Timms, 2001).

Progesterone resistance in endometriosis has major clinical implications, partially due to aberrant expression of oestrogen receptor beta (ER-β) and progesterone receptor (PR) in endometriotic stromal cells compared with eutopic endometrium (Bulun et al., 2010). In this study we investigated whether defective expression of steroid hormone receptors in the eutopic endometrial epithelium is related to a reduced chance of becoming pregnant in women with endometriosis.

The oestrogen receptor is expressed in the endometrium in a cyclic pattern mediating oestrogen-controlled endometrial growth. Of the two ER isoforms, ER-α and ER-β, ER-α is more abundant and undergoes more evident cyclic changes (Matsuzaki et al., 1999, 2000; Lecce et al., 2001).

Progesterone prepares the endometrium for implantation, exerting its action through the PR. Like ER, PR is a nuclear, ligand-activated transcription factor and its endometrial expression is regulated through the menstrual cycle in a similar pattern to ER, although it remains expressed later in the secretory phase (Lessey et al., 1988; Press et al., 1988; Nisolle et al., 1997).

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