Focus on Vascular Function in Female Reproduction

Regulation of endometrial vascular remodelling: role of the vascular endothelial growth factor family and the angiopoietin–TIE signalling system

Jane E Girling and Peter A W Rogers

*Centre for Women's Health Research, Department of Obstetrics and Gynaecology and Monash Institute of Medical Research, Monash University, Melbourne, Victoria 3168, Australia**Correspondence should be addressed to J E Girling at Monash University Department of Obstetrics and Gynaecology, Monash Medical Centre, Monash University, 246 Clayton Road, Clayton, Victoria 3168, Australia; Email: jane.girling@med.monash.edu.au***Abstract**

Angiogenesis, lymphangiogenesis and vascular maturation occur on a regular, physiological basis in human endometrium. These processes form part of a continuum of vascular remodelling involving numerous regulatory factors. Key factors include vascular endothelial growth factor (VEGF)A, VEGFC and VEGFD, and their associated receptors VEGFR1, VEGFR2 and VEGFR3. A second group of vascular regulatory proteins belongs to the angiopoietin (ANG)–TIE system. Although members of the VEGF family and the ANG–TIE system are represented in the endometrium, our understanding of how these different molecules interact to regulate remodelling of the blood and lymphatic vasculature present in the endometrium is still limited. A review of the current information is provided.

Reproduction (2009) **138** 883–893**Introduction**

The human endometrium contains a dynamic vasculature that grows and remodels each menstrual cycle by processes termed angiogenesis, lymphangiogenesis and vascular maturation. These processes are part of a continuum of vascular development with significant interactions between the mechanisms by which they are regulated. This review will describe the endometrial vascular remodelling that occurs in the human endometrium through the menstrual cycle and outline the role of two key vascular regulatory systems: the vascular endothelial growth factors (VEGFA, VEGFC, VEGFD) and associated receptors, and the angiopoietin (ANG)–TIE system.

Endometrial angiogenesis

Angiogenesis is the formation of new blood vessels from pre-existing vasculature. It can occur by one of several different mechanisms: sprouting, intussusception and elongation (Risau 1997, Burri & Djonov 2002, Peirce & Skalak 2003). Of these, sprouting is the best known,

occurring during the neovascularisation of avascular tissues. Sprouting involves breakdown of the basement membrane, migration and proliferation of endothelial cells, tube formation, basement membrane formation and recruitment of mural or support cells (vascular maturation, outlined below). Alternatively, angiogenesis can occur by intussusception (internal division of vessels resulting in smaller vessels) or elongation (lengthwise growth of vessels without formation of new vessel junctions) and can also involve recruitment of circulating endothelial progenitor cells into the developing vessel (Asahara *et al.* 1999, Khakoo & Finkel 2005). It should be noted that the structure and function of the vascular bed is influenced by the mechanism of angiogenesis that is occurring. For example, while blood flow is continuous through growing vessels during intussusception and elongation, blood flow does not occur until new patent lumen is formed during sprouting angiogenesis.

Angiogenesis is known to occur at all stages of the menstrual cycle (Girling & Rogers 2005). After menstruation, the vessels of the basalis are repaired following shedding of the functionalis. During the proliferative phase, growth of new vessels must match the rapid regrowth of the functionalis. In the secretory phase, the subepithelial capillary plexus matures and the specialised spiral arterioles grow and coil

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(Kaiserman-Abramof & Padykula 1989). Detailed stereological data show that elongative angiogenesis occurs as the functionalis regrows during the proliferative phase (Gambino *et al.* 2002). During the secretory phase, a form of branching angiogenesis is thought to occur. This is believed to be intussusception; however, confirmation of this will require either *in vivo* digital recording of developing human endometrium or use of a specific marker of intussusception, neither of which are currently possible.

There is also evidence illustrating an important role for endothelial progenitor cells in endometrial angiogenesis, with several studies demonstrating the presence of donor-derived endothelial cells within the uterus after bone marrow transplant from either male mice or transgenic mice with endothelial cell-specific mutations (Asahara *et al.* 1999, Masuda *et al.* 2007, Mints *et al.* 2008). In a human study, endothelial cells with an XY genotype were detected in the endometrium of a woman receiving an allogeneic transplant with bone marrow derived from a male (Mints *et al.* 2008). The extent of endothelial progenitor cell incorporation is influenced by circulating hormones. In a study by Masuda *et al.* (2007), ovariectomised mice received a bone marrow transplant from transgenic mice expressing β -galactosidase under the regulation of the endothelial cell-specific promoter TIE2. In these animals, oestrogen, but not progesterone, treatment caused a significant increase in bone marrow-derived endothelial progenitor cells incorporated into the growing uterine vasculature. Further research is required to elucidate the relative contribution of proliferation versus progenitor cell incorporation in the elongative and branching angiogenesis that occurs during the formation of a mature endometrial vasculature.

Endometrial lymphangiogenesis

Lymphangiogenesis is the formation of new lymphatic vessels from pre-existing vasculature. It is a process receiving increasing attention due to its importance in the metastatic spread of cancer (Alitalo *et al.* 2005, Karpanen & Alitalo 2008). It is believed that the mechanisms of lymphangiogenesis will be equivalent to the sprouting, intussusception and elongation occurring in the blood vasculature, although specific details have not yet been examined (Scavelli *et al.* 2004). There is also evidence suggesting that lymphatic endothelial progenitor cells may contribute to the formation of lymphatic vessels (Kerjaschki *et al.* 2006, Karpanen & Alitalo 2008).

Until recently, the distribution of lymphatics within the endometrium has been controversial. Two studies (one in rat and one in human) used functional assays to describe uterine lymphatic vessels. Results from both studies illustrated rapid lymphatic drainage of India ink from the myometrium, but no or minimal drainage when

the ink was delivered to the endometrium (Head & Lande 1983, Ueki 1991). Histological studies examining lymphatic vasculature in the rodent endometrium have also been conducted. Two studies report numerous lymphatic vessels in the myometrium with minimal/absent lymphatics in the endometrium (Head & Seeling 1984, Lauweryns & Cornillie 1984). In contrast, a study using three different morphological/histological techniques found numerous endometrial lymphatics in the rat, particularly in the basal, anti-mesometrial portion of the endometrium during dioestrus (Uchino *et al.* 1987). One study of human endometrium observed lymphatic vessels in the functionalis region in approximately two-thirds of the samples examined (Blackwell & Fraser 1981).

In recent years, specific lymphatic endothelial cell markers have been identified enabling detailed studies of lymphatic growth and function. These markers include D2-40 (antibody against podoplanin) and lymphatic endothelial hyaluronan receptor 1 (LYVE1), both of which have been used to examine the location and density of lymphatic vessels in human uterus across the menstrual cycle. In a study with D2-40, the basalis exhibited the highest lymphatic vessel density, with similar densities to that observed in the myometrium (Donoghue *et al.* 2007). In contrast, the functionalis had the lowest number of lymphatic vessels, although the presence of even a small population of vessels in this region confirms the endometrium as a site of regular, physiological lymphangiogenesis. There was no significant change in lymphatic vessel density between proliferative and secretory phase samples. Interestingly, in studies using LYVE1 as a marker, lymphatic vessels were not observed in the endometrium (Koukourakis *et al.* 2005, Red-Horse *et al.* 2006). LYVE1 is a specific and widely used marker of lymphatic vessels. The heterogeneity of LYVE1 immunostaining in the uterus highlights our limited understanding of the phenotype, function and activity of the endometrial lymphatic vessels.

Endometrial vascular maturation

Vascular maturation describes the process by which vessels recruit support cells (mural cells, including pericytes and vascular smooth muscle cells (VSMC); Jain 2003, Armulik *et al.* 2005). As well as providing mechanical support, the presence of mural cells give vessels the ability to regulate flow. Mural cells also have a key role in regulating the growth and function of vessels. Pericytes and VSMC are thought to belong to the same cell lineage with a continuum of phenotypes present across the vascular tree. Arterioles and venules are surrounded by a coat of VSMC; capillaries are supported by pericytes embedded within the basement membrane. Lymphatic capillaries lack mural cell

support, although collecting lymphatic vessels are covered by a coat of VSMC.

In addition to angiogenesis and lymphangiogenesis, mural cell recruitment and vascular maturation are key to the development of a functional endometrial vasculature (see Rogers & Abberton (2003) for review). One of the earliest differentiation markers of mural cells is α -smooth muscle actin (α -SMA, now known as ACTA2). It is present on both VSMC and pericytes throughout the endometrial vascular tree (Abberton *et al.* 1996, 1999a, Kohnen *et al.* 2000, Rogers & Abberton 2003). Other differentiation markers include myosin heavy chain, smooth muscle myosin and γ -SMA. These markers have a more restricted distribution within the endometrial vasculature, suggesting spatial differences in endometrial mural cell phenotypes (Abberton *et al.* 1999a, Kohnen *et al.* 2000). It is not known whether the phenotypic differences reflect variation in mural cell function, or different levels of mural cell differentiation within the endometrium.

Vascular maturation is known to occur during the secretory phase of the menstrual cycle (Rogers & Abberton 2003). Proliferation of VSMC has been examined using immunohistochemistry with antibodies directed against α -SMA and proliferating cell nuclear antigen (PCNA; Abberton *et al.* 1999b). Total VSMC cell proliferation was low during the early stages of the menstrual cycle (2–2.5% of PCNA-positive VSMC cells), increasing significantly during the mid-late secretory phase (~4%). This increase reflected proliferation of VSMC surrounding the spiral arterioles. No changes in VSMC proliferation were observed between the proliferative or secretory phases for the straight arterioles. The mechanisms responsible for the differential regulation of different arteriole types are not known.

Regulation of endometrial vascular remodelling

The vascular system, comprising both blood and lymphatic vessels, has critical roles in oxygen transport, nutrition, excretion, fluid balance, homeostasis and immune defence. It is a complex system regulated by a large number of hormones, growth factors and cytokines, as well as adhesion, cytoskeletal and extracellular proteins. These molecules have direct and indirect effects at various stages of the vascular remodelling process.

Within the endometrium, growth and remodelling of the vasculature is either a direct or indirect consequence of changes in the circulating concentrations of the ovarian steroids oestrogen and progesterone. Increasing concentrations of oestrogen are associated with the rapid vessel growth occurring during the proliferative stage of the menstrual cycle. During the secretory phase, maturation of the subepithelial capillary plexus and the growth and coiling of spiral arterioles are regulated by progesterone. In contrast, the endometrial repair that

occurs peri-menstruation takes place following the withdrawal of ovarian hormones.

Although endometrial vascular remodelling is under the overall control of oestrogen and progesterone, these hormones act indirectly via numerous other regulators. The current review will focus on three members of the VEGF family (VEGFA, VEGFC, VEGFD) and their receptors, and the ANG–TIE signalling system. Although not addressed in this review, the importance of other regulators, including (but not limited to) fibroblast growth factor, thrombospondin, relaxin, adrenomedullin, the prostaglandins, the prokineticins and the matrix metalloproteinases, cannot be overstated (see Nikitenko *et al.* 2000, Jabbour *et al.* 2006, Maldonado-Perez *et al.* 2007, Parry & Vodstrcil 2007, Chennazhi & Nayak 2009) and references therein for additional information). Considerable effort is still required to understand the complex interactions between these regulators in endometrial vascular remodelling.

Vascular endothelial growth factor-A

Members of the VEGF family and their receptors are key mediators of physiological and pathological vascular remodelling throughout the body. The family includes VEGFA, VEGFB, VEGFC, VEGFD, and placenta growth factor. As outlined in Fig. 1, family members have variable interactions with the VEGF receptors, which include the receptor tyrosine kinases VEGFR1 (FLT1), VEGFR2 (FLK1, KDR) and VEGFR3 (FLT4), as well as co-receptors from the semaphorin family neuropilin 1 (NRP1) and NRP2.

The best known of the VEGF family is VEGFA (also known as vascular permeability factor; Ferrara 2004, Hoeben *et al.* 2004). It is able to stimulate proliferation and migration of endothelial cells, as well as vascular permeability. VEGFA has several common splice variants (VEGFA₁₂₁, VEGFA₁₄₅, VEGFA₁₆₅, VEGFA₁₈₉, VEGFA₂₀₆), each with variable interactions with heparin, the extracellular matrix, and the VEGF receptors (Robinson & Stringer 2001, Yamazaki & Morita 2006, Harper & Bates 2008). The different solubilities of each isoform are related to the presence or absence of two heparin binding domains. These domains are encoded by exons 6 and 7 and confer on an isoform the ability to bind to the extracellular matrix via cell surface heparin-containing proteoglycans. In recent years, the understanding of VEGFA and its activities has been further complicated by the identification of anti-angiogenic isoforms (e.g. VEGFA_{165b}, VEGFA_{189b}; Lodomery *et al.* 2007, Harper & Bates 2008). Although the sister isoforms differ from the pro-angiogenic forms only in the six amino acids of the carboxy-terminal, this is sufficient to cause important differences in structure, function and receptor interactions. For example, VEGFA₁₆₅, but not VEGFA_{165b}, binds with NRP1. It has also been suggested that the VEGFA_b isoforms are the dominant forms in

Figure 1 Selectivity of VEGF ligands to the VEGF receptors. Vascular endothelial growth factor (VEGF) family members bind with overlapping patterns to three VEGF receptor (VEGFR) tyrosine kinases: VEGFR1; VEGFR2; and VEGFR3. Signaling is also modulated by binding with the co-receptors neuropilin 1 (NRP1) and NRP2.

many tissues and it is the balance of isoforms that should be considered when the function of VEGFA is being explored (Harper & Bates 2008).

VEGFA mRNA and protein are expressed by both the epithelium and stroma of the primate and rodent endometrium, with temporal and spatial changes apparent during the menstrual cycle (Charnock-Jones *et al.* 1993, Shifren *et al.* 1996, Torry *et al.* 1996, Sharkey *et al.* 2000, Graubert *et al.* 2001, Lee *et al.* 2008). The expression patterns reported are not consistent and are therefore difficult to interpret. However, several studies do report the highest expression of VEGFA during the menstrual phase of the cycle (Charnock-Jones *et al.* 1993, Sharkey *et al.* 2000, Graubert *et al.* 2001).

Cyclic changes in VEGFA expression are consistent with regulation by ovarian steroids. Oestrogen is known to affect VEGFA mRNA and protein levels *in vitro*; however, inconsistent results have been reported. In several studies, VEGFA mRNA and protein expression is increased in endometrial cells in response to oestradiol-17 β (E₂) treatment (Charnock-Jones *et al.* 1993, Shifren *et al.* 1996, Huang *et al.* 1998, Classen-Linke *et al.* 2000). In contrast, other studies report no effect of E₂ or progesterone treatment on endometrial epithelial or stromal cell VEGFA expression (Sharkey *et al.* 2000, Lockwood *et al.* 2002).

Oestrogen treatment has also been shown to affect both temporal and spatial VEGFA expression in the endometrium *in vivo*. The spatial changes in expression are highlighted by work in ovariectomised, oestrogen-treated mice (Ma *et al.* 2001). *Vegfa* expression increased in endometrial stromal cells within 2 h. By 24 h post-administration, there was no stromal *Vegfa* and expression was restricted to the epithelium. In the baboon and rhesus monkey, endometrial stromal and epithelial VEGFA expression was restored by E₂ treatment following a reduction after ovariectomy (Nayak & Brenner 2002, Niklaus *et al.* 2003). In baboon endometrium, VEGFA mRNA levels were increased in

both stromal and glandular epithelium (measured in samples collected by laser capture microscopy) within 2 h of E₂ administration and remained elevated during the 6 h experiment (Aberdeen *et al.* 2008).

Irrespective of how VEGFA expression is regulated by oestrogen, it is apparent that this growth factor is critical to oestrogen-induced endometrial vascular remodelling. In recent studies with marmosets, proliferative phase endothelial cell proliferation was inhibited if the animals were treated with the VEGFA trap Aflibercept (Fraser *et al.* 2008). As VEGFA is also required for ovarian oestrogen production, it was possible that the endometrial effects were due to the low circulating hormone levels. However, oestrogen replacement in these animals only partially restored endometrial angiogenesis. In addition, endothelial cell proliferation was reduced in ovariectomised marmosets and restored by oestrogen treatment. In mice and baboons also, E₂-induced endothelial cell proliferation can be inhibited if VEGFA is blocked (Heryanto *et al.* 2003, Aberdeen *et al.* 2008).

Considerably less attention has been paid to the interactions between progesterone and VEGFA, despite the important endometrial vascular remodelling occurring during the progesterone-regulated secretory phase. In ovariectomised mice, progesterone-induced endothelial cell proliferation, but not mural cell proliferation, was significantly reduced when mice were concurrently treated with a VEGF antiserum (Walter *et al.* 2005, Girling *et al.* 2007). In comparison to the relatively rapid effects of oestrogen on VEGFA production and endothelial cell proliferation, the effects of progesterone are slower and of a lower magnitude. In the study by Ma *et al.* (2001), *Vegfa* increased steadily within the endometrial stroma over a 24 h period in response to a single progesterone injection.

Menstruation is a result of oestrogen and progesterone withdrawal at the end of the menstrual cycle. It is believed to be initiated by tissue breakdown within the

functionalis, with associated vascular breakdown and bleeding (see Salmonsens (2003) for review). It has been hypothesised that the bleeding events are focal and halted by vasoconstriction of spiral arterioles in the basalis. Repair of the endometrial vasculature begins while menstruation is still in progress. This means that vascular breakdown and repair are occurring concurrently at different sites within the endometrium.

Menstrual angiogenesis is thought to be a hypoxia-related process. Support for this comes from Markee's experiments in which endometrial explants were transplanted into the anterior chamber of the eye in rhesus macaques (Markee 1940). Vasoconstriction followed by vasodilation of the spiral arterioles was observed following progesterone withdrawal. If such vasoconstriction occurred in the endometrium *in situ*, it would provide a possible mechanism for vessel repair through hypoxia-induced upregulation of VEGFA. Several studies have observed the highest expression of VEGFA during the menstrual phase of the menstrual cycle (Charnock-Jones *et al.* 1993, Sharkey *et al.* 2000, Graubert *et al.* 2001). In addition, more SLC2A1 (a glucose transporter and marker of anaerobic metabolism that is induced by ischaemia, previously known as GLUT1) was found in endometrial samples from menstruating women in comparison to proliferative or secretory phase samples (Graubert *et al.* 2001). High levels of VEGFA mRNA were observed in the glands, stroma and necrotic areas of menstrual phase endometrium (Charnock-Jones *et al.* 1993, Sharkey *et al.* 2000). In artificially-cycling macaques, VEGFA mRNA was upregulated in the glands and stroma of superficial endometrial tissues 1–2 days following progesterone withdrawal (Nayak & Brenner 2002). VEGFA expression and secretion is also increased in endometrial epithelial and stromal cells exposed to hypoxic conditions *in vitro* (Popovici *et al.* 1999, Sharkey *et al.* 2000, Lockwood *et al.* 2002).

Mechanistic support for a role of VEGFA in endometrial vascular repair is provided by a recent study using the artificially-cycling macaque model. The macaques were treated with VEGF trap during endometrial regeneration. This inhibited angiogenesis and also slowed re-epithelisation following endometrial breakdown (Fan *et al.* 2008). There was no effect of the VEGF trap on pre-existing vessels. These results suggest that VEGF is only essential for the formation of new vessels, not the survival of mature endometrial vessels, a finding that has important implications for our understanding of VEGF function in the endometrium.

Similar results were also observed in studies with a decidualised mouse model (Fan *et al.* 2008). After hormone withdrawal to stimulate endometrial breakdown, the endometrial tissue undergoing repair (stroma adjacent to the lumen) stained strongly with a marker of hypoxia (Hypoxyprobe-1) as well as VEGFA. Use of a VEGFA trap in these mice significantly inhibited vascularisation of the regenerating stroma. The authors

hypothesise that ischemic hypoxia in the uppermost regions of the endometrium is the local stimulus for VEGFA expression and the subsequent angiogenesis (Fan *et al.* 2008).

However, despite the above observations, there is no direct evidence for vasoconstriction or hypoxia during menstruation in human endometrium (Salmonsens 2003). Peri-menstruation, no significant changes in blood flow were observed and no ischaemia/reperfusion episodes have been detected (Fraser & Peek 1992). Only very low levels of hypoxia-inducible factor 1 α (HIF1A) and HIF2A, early markers of hypoxia, have been observed in the human endometrium (Gannon *et al.* 1997). An alternative theory to vasoconstriction and hypoxia suggests that menstruation is an inflammatory-type process (Salmonsens 2003). Future research will need to carefully address the two potential mechanisms, including examining the role of various inflammatory mediators in endometrial vascular remodelling.

In both primates and rodents, the strongest expression of VEGFA is observed in the endometrial epithelium. However, an *in vitro* study has illustrated that most epithelial VEGFA is secreted into the uterine lumen via the apical cell surface (Hornung *et al.* 1998). Based on these observations, it was hypothesised that epithelial VEGF is unlikely to have a role in endometrial angiogenesis. However, there is also the potential for paracrine interactions between the epithelium and endometrial stroma. Experiments were conducted in which endometrial epithelial and stromal cells were co-cultured with human myometrial microvascular endothelial cells (Albrecht *et al.* 2003). In comparison to culture in media only, myometrial microvascular endothelial cell tube formation increased when endothelial cells were cultured in media supplemented with human recombinant VEGFA or co-cultured with endometrial epithelial cells. A further increase occurred when endothelial cells were treated with oestrogen and co-cultured with endometrial epithelial cells. No such increase was observed when the endothelial cells were co-cultured with endometrial stromal cells (Albrecht *et al.* 2003). These results are consistent with a paracrine role for epithelial VEGFA in endometrial angiogenesis. However, as directional secretion (from the apical surface) does not occur *in vitro*, additional studies will be required to determine whether epithelial VEGFA is normally secreted into the endometrial stroma *in vivo*.

It must also be remembered that in addition to stromal and epithelial cells, the endometrium contains a large population of leucocytes that undergo cyclic-dependent changes in number and type during the menstrual cycle. Leucocytes may contribute to endometrial vascular remodelling by expressing and secreting angiogenic factors, or by simulating production of local factors required for vascular remodelling including VEGFA (recently reviewed by Dunk *et al.* (2008)). Neutrophils have been hypothesised to have a specific role in

endometrial angiogenesis. Foci of intense VEGFA immunostaining correlating temporally and spatially with endothelial cell proliferation in the human endometrium were found to be VEGFA expressing neutrophils (Gargett *et al.* 2001). Studies in mice further support a role for neutrophils in endometrial angiogenesis (Heryanto *et al.* 2004). Induction of neutropenia significantly reduced oestrogen-induced endometrial endothelial cell proliferation by 30–40%. Macrophages also express VEGFA, although whether these cells have a specific role in endometrial vascular remodelling has not yet been examined.

Various studies have now shown that the different VEGFA isoforms are present in the endometrium (Charnock-Jones *et al.* 1993, Halder *et al.* 2000, Sharkey *et al.* 2000, Ancelin *et al.* 2002, Niklaus *et al.* 2002, Sugino *et al.* 2002). As in other tissues, VEGFA₁₆₄ is believed to be the dominant isoform in human endometrium. VEGFA₁₂₁, VEGF₁₄₅ and VEGFA₁₈₉ are also present and are thought to be hormonally regulated. To fully understand the role of VEGFA in endometrial vascular remodelling, studies will need to address the location and specific activity of VEGFA isoforms. This is particularly important now with the recent identification of anti-angiogenic VEGFA_b isoforms.

In addition to different interactions with the VEGF receptors, VEGFA isoforms have differing interactions with the extracellular matrix (Robinson & Stringer 2001, Ferrara 2004). VEGFA₁₂₁ is freely soluble, whereas VEGFA₁₄₅ and VEGFA₁₈₉ are largely bound to the extracellular matrix; VEGFA₁₆₅ has intermediate properties. The bound forms provide a reservoir of VEGFA that can be released by a number of proteases (heparin, heparin sulphate, heparinase, plasmin or urokinase-type plasminogen activator) to provide a bioactive VEGFA fragment that can interact with the VEGFA receptors. As yet, nothing is known about the cyclic or hormone-induced changes in stored VEGFA. Protein analyses able to illustrate the distribution of bound versus unbound VEGFA and the changes in this distribution in response to hormone-treatment would be useful.

Vascular endothelial growth factor-C and -D

VEGFC and VEGFD are known for their key role in lymphangiogenesis, although they are also regulators of angiogenesis (Baldwin *et al.* 2002, Karpanen & Alitalo 2008). Increases in blood and lymph vessel size have previously been observed in muscle treated with adenovirus expressing VEGFC or VEGFD (Rissanen *et al.* 2003, Kholova *et al.* 2007) and in regenerating skin treated with transfected tumour cells expressing VEGFC (Goldman *et al.* 2005). VEGFC and VEGFD bind and activate VEGFR2 and VEGFR3, which in the adult are found predominantly on blood and lymphatic endothelial cells, respectively. These growth factors differ from other members of the mammalian VEGF

family because of the presence of pro-peptides at both the N- and C-termini of the conserved VEGF homology domain. Proteolytic cleavage of the pro-peptides by plasmin modulates bioactivity of both molecules, increasing affinity for both VEGFR2 and 3.

Both VEGFC and VEGFD are expressed by the endometrium. To date, studies have reported variable levels of VEGFC immunostaining in the stroma, glands and blood vessels of normal human endometrium (Mints *et al.* 2002, Moller *et al.* 2002). Low levels of constitutively expressed VEGFC mRNA were observed in endometrial biopsies collected throughout the menstrual cycle (Graubert *et al.* 2001). In our own studies, we found the highest level of VEGFC immunostaining in the glandular epithelial cells, the vascular endothelium and in a proportion of stromal cells (Donoghue *et al.* 2007). Of note, there was no difference in staining intensity between the functionalis and basalis, despite the different density of lymphatic vessels between the two endometrial regions.

VEGFD immunostaining has been reported to be low or negative in all compartments of the endometrium (Yokoyama *et al.* 2003). We observed moderate VEGFD immunostaining throughout the human endometrium and myometrium, with no difference in intensity between the endometrial epithelium and stroma (Donoghue *et al.* 2007). As with VEGFC, there was no variation in staining intensity between the basalis and functionalis across the normal cycle.

Although VEGFC and VEGFD are present in the human endometrium, no studies have determined whether they have a functional role in endometrial angiogenesis and/or lymphangiogenesis. As with VEGFA, future studies will need to consider epithelial versus stromal VEGFC and VEGFD. Studies will need to take into account the regulation and function of specific isoforms. SDS-PAGE analysis has identified 58, 41, 31 and 21 kDa VEGFC peptides and 56, 41, 31 and 21 kDa VEGFD peptides in the human endometrium (Donoghue *et al.* 2007). These represent full length, partially processed and fully processed forms of VEGFC and VEGFD, indicating that these key growth factors are both produced and processed in the endometrium. Which cell types and regions within the endometrium express the different isoforms is not known. This information may help explain the different density of lymphatic vessels between the functionalis and basalis.

VEGF and neuropilin receptors

VEGFA, C and D bind in an overlapping pattern to three receptor tyrosine kinases: VEGFR1, VEGFR2 and VEGFR3 (Ferrara 2004, Hoeben *et al.* 2004, McColl *et al.* 2004, Olsson *et al.* 2006; Fig. 1). There is also binding with the co-receptors NRP1 and NRP2. VEGFR2 mediates VEGFA stimulated blood endothelial cell proliferation, while VEGFR1 may be a negative

regulator of VEGFA activity and appears to signal some of the more subtle effects of VEGFA, such as endothelial cell migration and modulation of immune function. Negative regulation of VEGFA is also provided by a soluble form of VEGFR1 (sFLT1), which binds to VEGFA preventing binding with VEGFR2 (Olsson *et al.* 2006). VEGFR3 is important for lymphatic endothelial cell development and function.

The VEGF receptors are able to form both homodimers and heterodimers, although the specific signalling pathways associated with homo versus heterodimers are not known (Olsson *et al.* 2006). Dimerisation and activation of the receptors leads to autophosphorylation and induces various downstream signalling pathways (VEGFR2: phospholipase C (PLC) γ /protein kinase C (PKC) pathway, C-RAF–MEK–MAP-kinase cascade, phosphoinositide 3-kinase/AKT pathway; VEGFR3: PLC γ /PKC pathway; refer Olsson *et al.* (2006), Shibuya (2008), Lohela *et al.* (2009) and references therein for detailed information) resulting in specific biological responses such as vascular permeability, cell migration or proliferation. Discussion of these signalling pathways is beyond the scope of the current review, except to highlight our lack of understanding of the downstream signalling pathways initiated in response to VEGF receptor activation within the endometrium.

VEGF receptors are primarily expressed in the vasculature and information from various genetic mouse models are consistent with the most important function of these ligand/receptor interactions occurring in the vascular system (Olsson *et al.* 2006). However, studies in endometrium demonstrate that VEGF receptors are expressed in other cell types as well. In the human endometrium, most studies examining VEGFR1 and VEGFR2 observed the highest expression in the endometrial vasculature. Additional staining is also variously reported in the epithelium, stroma and leucocytes with changes noted between proliferative and secretory phase samples (Meduri *et al.* 2000, Sugino *et al.* 2002, Punyadeera *et al.* 2006, Mints *et al.* 2007b, Jee *et al.* 2009). There are also recent publications investigating the immunohistochemical expression of VEGFR3 in human endometrium (Mints *et al.* 2002, Moller *et al.* 2002, Yokoyama *et al.* 2003). Immunostaining was reported in endometrial blood endothelial cells in two of the studies (Mints *et al.* 2002, Moller *et al.* 2002), but the third reported no immunostaining in endometrial tissues (Yokoyama *et al.* 2003). VEGFR3 immunostaining has also been reported in glands of the human endometrium, with weak to moderate immunostaining noted in avascular stroma (Mints *et al.* 2007a). The function of non-vascular VEGF receptors in the endometrium is not known.

VEGF receptor expression is also thought to be regulated by oestrogen and progesterone. In ovariectomised oestrogen-treated mice, *Vegfr2* mRNA increased in endometrial stromal cells within 6 h, but

had declined by 24 h (Ma *et al.* 2001). In contrast, *Vegfr2* increased steadily within the endometrial stroma over a 24 h period in response to a single progesterone injection (Ma *et al.* 2001). In mice treated with E₂ or both E₂ and progesterone for 21 days, there was an increase in the number of VEGFR2 immunostained vessels in comparison to control animals (Herve *et al.* 2006). An oestrogen receptor mediated effect was confirmed using similar experiments in oestrogen receptor α knockout mice. The proportion of vessel structures immunostaining with VEGFR2 was significantly reduced in knockout animals compared to similarly treated wild-type animals.

Consistent with a hypothesised role for VEGFA in perimenstrual vascular repair, hormone-withdrawal is also associated with increasing VEGF receptor expression. In artificially cycling macaques, low levels of *VEGFR2* mRNA were observed in the proliferative and secretory phases (Nayak *et al.* 2000). After withdrawal of progesterone, there was a large increase in *VEGFR2*, but not *VEGFR1*, mRNA expression. During the proliferative and secretory phase, *VEGFR2* expression was confined to the vascular endothelium. After progesterone withdrawal, expression was strongly upregulated in the endometrial stroma with a marked gradient of *VEGFR2* mRNA expression from mid way through the functionalis up to the luminal surface. Similar patterns of expression were also described in human premenstrual samples (Nayak *et al.* 2000).

Graubert *et al.* (2001) report increases in VEGFR1, sFLT and VEGFR2 expression at menstruation in human endometrium. This study also assessed the functional status of the VEGF receptors. Using immunoprecipitation and western blot analysis with anti-VEGFR2 and anti-phosphotyrosine antibodies, respectively, VEGFR2 phosphorylation peaked in the late menstrual phase with lower sustained phosphorylation levels during the proliferative phase. Binding of sFLT to VEGFA was observed in the early, but not late menstrual phase (although high levels of VEGFA mRNA were still present in the endometrium; Graubert *et al.* 2001). This is one of the few studies to begin addressing the activity of VEGF receptors, in addition to their expression. Further studies considering the functional status and downstream signalling from VEGF receptors are required.

In addition to the VEGF receptors, the co-receptors NRP1 and NRP2 are also expressed by the endometrium. They report high expression in the vascular endothelium, with variable expression in the stroma and glandular epithelium (Pavelock *et al.* 2001, Germeyer *et al.* 2005, Punyadeera *et al.* 2006, Hess *et al.* 2009). However, no studies have yet examined the functional interactions between the neuropilins and VEGF tyrosine kinase receptors in endometrial vascular remodelling.

The angiopoietin–TIE system

Another system key to endothelial cell function with links to the VEGF family is the ANG–TIE (Tyr kinase with Ig and epidermal growth factor homology domains, also known as TEK) signalling system (recently reviewed by Augustin *et al.* (2009)). The ANG–TIE system is important in vessel maturation and has a role in the regulation of VSMC recruitment. This system is also involved in maintaining blood vessel quiescence. ANG1 activates the TIE2 receptors, whereas ANG2 is described as a ligand that antagonises the activation of TIE2. The consequences of TIE2 inhibition are contextual. If VEGFA is present, ANG2 will enable endothelial cell migration and proliferation and therefore angiogenesis. If VEGFA is inhibited, ANG2 will lead to endothelial cell death and vessel regression (Gale *et al.* 2002, Augustin *et al.* 2009).

The ANG–TIE system is also believed to have a role in lymphangiogenesis. This is based on observations in ANG2 deficient mice, which develop chylous ascites prior to birth (Gale *et al.* 2002, Augustin *et al.* 2009). While these animals only have mild blood vascular defects, they do have considerable lymphatic abnormalities throughout the body with defective patterning of both small and large lymphatics, as well as poor interactions with and abnormal recruitment of surrounding mural cells. The ANG ligands and TIE2 receptors are expressed by both blood and lymphatic vessels. Whether contextual differences related to ligand presentation occur in the lymphatic vessels, as they do in blood vessels, has not yet been elucidated.

While studies have examined ANG1, ANG2, and TIE2 expression in the human endometrium, the results obtained have differed considerably. Staining has been reported variously in the epithelium, stroma and uterine natural killer cells (reviewed in Rogers & Abberton (2003)). In one study, ANG1 immunostaining was observed in stroma, luminal and glandular epithelium, and endothelial cells, whereas ANG2 was detected in the stroma and glandular epithelium (Hirchenhain *et al.* 2003). TIE2 was observed in glandular epithelium, as well as in endometrial endothelium. ANG1 mRNA expression peaked in the mid-secretory phase of the menstrual cycle, while ANG2 peaked in the late secretory phase (Lee *et al.* 2008). These studies did not examine menstrual phase samples. Research examining the functional role of the ANG–TIE system in menstrual vascular repair would be particularly interesting considering the reported contextual effects of ANG2 depending on the presence/absence of VEGFA and the role of this system in vascular maturation and quiescence.

In a detailed study by Nayak *et al.* (2005), TIE2 and ANG1 expression were examined in the endometrium of rhesus macaques treated sequentially with E₂ and progesterone, followed by hormone withdrawal, to

mimic a menstrual cycle. While TIE2 (mRNA and protein) expression was restricted to the endothelium, with no marked changes in expression during the artificial menstrual cycle, ANG1 mRNA expression varied in a cell and cycle-stage-dependent manner (examined using *in situ* hybridisation). ANG1 mRNA was only expressed in the glands of the basalis during the proliferative phase, but expression was observed in the upper glands and the luminal epithelium during the early-mid secretory phases; expression was largely absent from the glands in the late secretory phase and following hormone withdrawal. Of particular note, ANG1 mRNA expression was observed in the VSMC of the spiral arterioles during the early-late secretory phases; this expression correlated closely with VSMC proliferation. The authors hypothesised that ANG1 may have a central role in the progesterone-induced development of the spiral arterioles in the primate endometrium.

Conclusions

Regular growth and regression of the vasculature is central to the normal menstrual cycle. This incorporates growth of both the blood and the lymphatic vessels, requiring angiogenesis, lymphangiogenesis and vascular maturation. While the processes regulating endometrial angiogenesis have received considerable attention, our understanding of lymphangiogenesis is limited. Unlike the lymphatic vessels in the myometrium and elsewhere in the body, lymphatics in the endometrium express the lymphatic endothelial cell marker D2–40 (podoplanin), but not LYVE1. What this phenotype tells us about the function and activity of endometrial lymphatics is still to be elucidated.

Growth and remodelling of the endometrial vasculature is either a direct or indirect consequence of changes in the circulating concentrations of the ovarian steroids oestrogen and progesterone. Oestrogen and progesterone (or withdrawal of these hormones) act directly or indirectly on the endometrial vasculature via numerous vascular and pleiotrophic regulators. The best known of these regulators belong to the VEGF family, although the mechanisms by which these growth factors and their receptors interact to control remodelling of the blood versus lymphatic vasculature is still not understood. Future studies need to examine how the different isoforms of VEGFA, VEGFC and VEGFD are regulated. This will need to include studies examining the expression and distribution of the inhibitory VEGFA_b isoforms. Research is also required to address the interaction of various VEGF family members with the extracellular matrix during the menstrual cycle and the function of non-vascular endometrial VEGF receptors and co-receptors. The specific mechanisms leading to differential receptor phosphorylation and specific downstream signalling pathways leading to

endometrial endothelial cell permeability, migration and/or proliferation will also need to be elucidated.

A second key group of vascular regulators in the endometrium with interesting interactions with the VEGF family are the ANG–TIE ligands and receptors. These proteins are involved in mural cell recruitment and in the maintenance of vessel quiescence. They also have hypothesised roles in lymphangiogenesis. While ANG1, ANG2 and TIE2 are all expressed by the endometrium, the interactions of the ANG–TIE system with VEGF family members during endometrial angiogenesis, lymphangiogenesis and vascular maturation has not been explored. Continuing research into the detailed activity, function and interaction of both the VEGF family and ANG–TIE system will ultimately enhance our understanding of normal endometrial vascular remodelling and provide information relevant to various disorders and pathologies of the endometrium that have a vascular component.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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