Hum. Reprod. Advance Access published November 10, 2011
Human Reproduction, Vol.0, No.0 pp. 1–13, 2011
doi:10.1093/humrep/der376

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

Localization of angiogenic growth factors and their receptors in the human endometrium throughout the menstrual cycle and in recurrent miscarriage

Gendie E. Lash[1,*], Barbara A. Innes[1], Josephine A. Drury[2], Stephen C. Robson[1], Siobhan Quenby[3], and Judith N. Bulmer[1]

[1]Reproductive and Vascular Biology Group, Institute of Cellular Medicine, Newcastle University, 3rd Floor, William Leech Building, Newcastle upon Tyne NE2 4HH, UK [2]Department of Women's and Children's Health, University of Liverpool, Liverpool, UK [3]Clinical Sciences Research Institute, University of Warwick, Warwick, UK

*Correspondence address. Tel: +44-191-222-8578; Fax: +44-191-222-5066; E-mail: g.e.lash@ncl.ac.uk

Submitted on July 1, 2011; resubmitted on October 3, 2011; accepted on October 10, 2011

BACKGROUND: Angiogenesis is a key feature of endometrial development. Inappropriate endometrial vascular development has been associated with recurrent miscarriage (RM) with increased amounts of perivascular smooth muscle cells surrounding them.

METHODS: In the current study, we have used immunohistochemistry to study temporal and spatial expression of a series of angiogenic growth factors (AGFs) and their receptors; vascular endothelial growth factor (VEGF)-A, VEGF-C, VEGF-D, VEGF-R1, VEGF-R2, VEGF-R3, platelet-derived growth factor (PDGF)-BB, PDGF-Rα, PDGF-Rβ, transforming growth factor (TGF)-β1, TGF-βRI, TGF-βRII, angiopoietin (Ang)-1, Ang-2 and Tie-2, in the proliferative, early secretory and mid-late secretory phase endometrium from control women as well as in the mid-late secretory phase of women with a history of RM. The AGFs and their receptors studied were immunostained and assessed separately in stromal, vascular smooth muscle, endothelial and glandular epithelial cells. Laser capture microdissection and real-time RT–PCR were used to confirm expression patterns observed by immunohistochemistry.

RESULTS: Most AGFs investigated showed both temporal and spatial expression patterns in normal cycling endometrium. In addition, immunostaining intensity for several AGFs was altered in women with a history of RM, particularly in vascular smooth muscle cells (VSMCs). VSMC expression of TGF-β1, VEGF-R1 and VEGF-R2 was increased while expression of PDGF-BB, TGF-βRI, TGF-βRII, Ang-2, VEGF-A and VEGF-C was reduced.

CONCLUSIONS: This study confirms that the cycling endometrium is a highly angiogenic tissue and that this process is likely to be altered in women with a history of RM and may contribute to the aetiology of this condition.

Key words: recurrent miscarriage / vascular development / angiogenic growth factors / endometrium

Introduction

The endometrium undergoes regular cycles of growth and breakdown and is one of the few adult tissues in which significant angiogenesis occurs on an ongoing, physiological basis progressing from vessel stumps in the basal layer of endometrium that remain after menstruation (Rogers and Abberton, 2003). Endometrial vessels acquire more vascular smooth muscle cells (VSMCs) as the menstrual cycle progresses, although the origin of these VSMCs and the molecular triggers for these events are not yet clear (Girling and Rogers, 2005). Inappropriate blood vessel development and blood flow characteristics have been associated with menorrhagia (Rogers and Abberton, 2003), recurrent miscarriage (RM) (Habara *et al.*, 2002; Quenby *et al.*, 2009), recurrent implantation failure (Goswamy *et al.*, 1988; Quenby *et al.*, 2009) and unexplained infertility (Steer *et al.*, 1994).

RM is defined as three or more consecutive pregnancy losses; many cases are idiopathic (Quenby and Farquharson, 1993; Rai and Regan, 2006), although an altered endometrial environment has recently been suggested as a potential contributory factor to these idiopathic cases (Brosens and Gellersen, 2010). We have recently demonstrated

that a subset of women with a history of RM with increased numbers of uterine natural killer (uNK) cells in mid-late secretory phase endometrium also have increased endometrial blood vessel maturity and blood flow (demonstrated by uterine artery Doppler) (Quenby et al., 2009). We propose that this may lead to excess oxygen at the early implantation site resulting in oxidative stress damage to the developing embryo and placenta. Embryo implantation occurs in a low-oxygen environment (2–3% O_2) (Rodesch et al., 1992; Yedwab et al., 1976), with oxygen levels in the intervillous space rising to 6–8% O_2 between 10 and 12 weeks gestation (Jauniaux et al., 2000). Inappropriate maternal blood flow to the intervillous space may be underpinned by several different pathologies and has been proposed to be a final common pathway in both sporadic and RM (Jauniaux et al., 2000).

There are several well-defined families of angiogenic growth factors (AGFs) that play roles in endothelial cell (EC) proliferation, migration and tubule formation as well as recruitment and differentiation of VSMCs for development of mature functioning blood vessels. The best characterized is the vascular endothelial growth factor (VEGF) family comprising VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placental growth factor (PlGF) and the receptors VEGF-R1, VEGF-R2 and VEGF-R3 (Tammela et al., 2005). The different actions of VEGF-A are mediated by VEGF-R1 and VEGF-R2, while VEGF-C and VEGF-D interact with VEGF-R2 and -R3 and are primarily involved in lymphangiogenesis, although VEGF-C also possess some angiogenic properties (Tammela et al., 2005). The angiopoietins, Ang-1, Ang-2 and their receptor Tie-2 play fundamental roles in angiogenesis and remodelling of vessel structure; Ang-1 is chemotactic for human ECs, promotes angiogenesis *in vivo* and has roles in vessel stabilization, while Ang-2 is an antagonist ligand for Tie-2, with roles in vascular dilatation and disruption of vessel integrity (Maisonpierre et al., 1997; Witzenbichler et al., 1998; Zhang et al., 2001). Transforming growth factor (TGF)-β1 and its receptors, TGF-βRI and TGF-βRII, have been proposed to play roles in stabilizing newly formed vessels as well as directing VSMC differentiation (Distler et al., 2003). The platelet-derived growth factor (PDGF) family has four members (PDGF-A, -B, -C and -D) which form homo and heterodimers and act through two tyrosine kinase membrane-bound receptors, PDGF-Rα and -Rβ (Betsholtz, 2004). The homodimer PDGF-BB appears to play a key role in arteriogenesis; mice deficient in PDGF-BB or its preferential receptor PDGF-Rβ have diminished numbers of pericytes and markedly dilated blood vessels, leading to oedema and embryonic lethality (Lindahl et al., 1997). PDGF-BB can also bind PDGF-Rα, PDGF-Rβ and receptor heterodimers. PDGF-BB secreted by ECs attracts pericytes via their expression of PDGF-Rβ (Betsholtz, 2004).

The expression profiles of some AGFs and their receptors have been previously investigated in human endometrium and in RM. The aim of the current study was to investigate the spatial and temporal expression of four families of AGFs and their receptors in endometrium of normal cycling women and women with a history of RM.

Materials and Methods

Tissue samples

Endometrial biopsies ($n = 29$) were obtained after hysterectomy for non-endometrial pathology at the Royal Victoria Infirmary, Newcastle upon Tyne. The study was approved by the Joint Ethics Committee of Newcastle upon Tyne Health Authority and the University of Newcastle upon Tyne (Durham committee). Endometrial pipelle biopsies were obtained at LH + 7 (±2 days) from women with a history of RM ($n = 14$) as previously described (Quenby et al., 2009). Demographic details are shown in Table I.

Table I Patient demographics [median (range)].

	Control ($n = 29$)	Recurrent miscarriage ($n = 14$)
Age	42 (28–51)	38 (29–44)
Parity	2 (0–4)	0 (0–5)
Number of previous miscarriages	0 (0–3)	4 (3–7)

In the RM group women were included in the study if they had unexplained RM (three or more consecutive miscarriages). Women were excluded if one of the following screening investigations revealed a possible contributing factor for their pregnancy losses; antiphospholipid syndrome [lupus anticoagulant tested for using the dilute Russell viper venom time and immunoglobulin G (IgG) and IgM anticardiolipin antibodies], thrombophilia (activated protein C resistance, Leiden factor V mutation, prothrombin gene mutation, protein C and S deficiency and antithrombin III deficiency), uterine anomaly (transvaginal ultrasonography), polycystic ovarian syndrome (transvaginal ultrasonography), diabetes (fasting blood glucose), abnormal thyroid function tests or parental balanced translocations (leucocyte culture).

Endometrial biopsies were either fixed in 10% neutral buffered formalin for 24 h, routinely processed and embedded in paraffin wax or frozen in liquid nitrogen cooled isopentane (Sigma Chemical Co., Poole, UK). All biopsies included in the study were histologically staged according to standard criteria (Noyes et al., 1975) by a pathologist (J.N.B.) and the controls were grouped into proliferative ($n = 12$), early secretory ($n = 5$) and mid-late secretory ($n = 12$) phases.

Immunohistochemistry

For all antibodies, sections were dewaxed in xylene, rehydrated through alcohols and incubated in 1% H_2O_2 in methanol for 10 min to block endogenous peroxidase activity. All washes were performed in 0.15 M Tris-buffered 0.05 M saline, pH 7.6 (TBS). All antibodies were fully evaluated on placental tissue, except for VEGF-A (breast cancer tissue), prior to use in the current study. For any given antibody, all tissue sections were immunostained in the same staining run to avoid any day-to-day variation between staining runs.

Antibodies were detected using an avidin-biotin-peroxidase technique (mouse or rabbit Vectastain Elite ABC kit as appropriate; Vector Laboratories, Peterborough, UK) unless otherwise stated. The immunostaining procedure has been described in detail previously (Schiessl et al., 2009). Details of source, dilution and pretreatment for all primary antibodies are described in Supplementary data, Table SI. The reaction was developed for 2–3 min with 3,3′-diaminobenzidine (Sigma Chemical Co.) containing 0.01% H_2O_2 to give a brown reaction product. The sections were lightly counterstained with Mayer's haematoxylin for 30 s, dehydrated, cleared in xylene and mounted with DPX synthetic resin (Raymond A. Lamb Ltd., London, UK). Negative controls included replacement of the primary antibody by appropriate non-immune serum and were performed for each antibody run.

Tie-2 was detected using a peroxidase-anti-peroxidase method. After incubation of slides for 10 min in 1:10 normal rabbit serum in TBS to

block non-specific binding sites, sections were incubated for 60 min with goat anti-Tie-2 (Supplementary data, Table SI). After washing in TBS, the sections were incubated for 30 min with rabbit anti-goat IgG (1:20, Dako, Ely, UK), washed again and overlain for 30 min with peroxidase-anti-peroxidase complex (1:100, Dako).

Analysis of immunostaining

The whole of each immunostained section was analysed semi-quantitatively using a modified 'Quickscore' method (Schiessl *et al.*, 2009) taking into account both intensity of staining (0 = negative, 1 = weak, 2 = moderate, 3 = strong) and percentage of cells for each staining intensity (1 = 0–25%, 2 ≥ 25–50%, 3 ≥ 50–75%, 4 ≥ 75–100%). The intensity and percentage scores were then multiplied and summed to give a range of possible scores of 0–12. Glandular epithelium, stromal cells, ECs and VSMCs were all scored separately.

Vessels were assessed in sections immunostained for von Willebrand factor (Factor VIII-related antigen; vWF), myosin heavy chain (MyHC) and alpha-smooth muscle actin (α-SMA). The number of vessels positive for each antibody in each of five selected fields (×200) was analysed by one observer (G.E.L.) blinded to the origin of the sample. Only vessels with a visible lumen were analysed. Arteriole maturation was assessed by adapting a method previously described (Rogers and Abberton, 2003; Quenby *et al.*, 2009) on sections immunostained with MyHC and α-SMA. Vessels were assessed as being surrounded by one layer of VSMCs ('partial') or as being surrounded by two or more layers of VSMCs ('complete'). The total number of vessels surrounded by VSMCs was determined by the addition of the number of 'complete' and 'partial' vessels. To determine the extent of vessel maturation (% complete) a calculation of complete/(complete + partial) × 100 was made. Vessels with no VSMCs were not analysed.

Laser capture microdissection

Ten micrometer thick cryosections were cut onto 2 μm PEN-Membrane slides (Leica Microsystems, Wetzlar, Germany) and stained with cresyl violet LCM staining kit (Ambion, TX, USA) according to the manufacturer's instructions. From each tissue sample ($n = 6$) glandular epithelium, stroma and blood vessels (containing both EC and VSMC) were laser capture microdissected using a Leica AS LMD microscope (Leica Microsystems).

Real-time RT–PCR

Total RNA was extracted from laser capture microdissected tissue using RNAqueous Micro (Ambion) and reverse-transcribed to cDNA using Superscript III (InVitrogen, Paisley, UK), both according to the manufacturer's instructions. Real-time RT–PCR was performed using Taqman chemistry, 2 μl cDNA per 20 μl reaction with Taqman Universal PCR MasterMix (Applied Biosystems, CA, USA) and run on an ABI7000 real-time PCR machine (Applied Biosystems). Probe/primer sets were purchased from Assays-on-Demand (Applied Biosystems) for glyceraldehyde-3-phosphatedehydrogenase (housekeeping gene), Ang-1, Ang-2, PDGF-BB, PDGF-Rα, PDGF-Rβ, Tie-2, TGF-β1, TGF-βRI, TGF-βRII, VEGF-A, VEGF-C, VEGF-D, VEGF-R1, VEGF-R2 and VEGF-R3. All probe/primer sets were validated for use with a laboratory standard total RNA extracted from early pregnancy decidua. RT–PCR was used to confirm the presence or absence of mRNA corresponding to the AGFs and their receptors in the cell types of interest as identified by immunostaining.

Statistical analysis

Data are presented as means with standard errors. Statistical calculations were performed using the StatView statistical software package (Abacus Concepts Inc., Berkley, CA, USA). Statistical significance was determined by use of analysis of variance (ANOVA) with *post hoc* Fisher's test (menstrual cycle phase) or unpaired *t*-test (mid-late secretory phase endometrium from controls compared with RM). All statistical tests were two-sided and differences were considered statistically significant at $P < 0.05$.

Results

Blood vessel development throughout the menstrual cycle

The total number of vessels in the endometrium was determined by counting the number of Factor VIII, α-SMA and MyHC positive vessels (Fig. 1A and B). The total number of vessels did not alter between the proliferative ($n = 12$) and mid-late secretory ($n = 12$) phases of the menstrual cycle in control women (Fig. 1C). The percentage of vessels surrounded by two or more layers of VSMCs (% complete vessels) increased from the proliferative to mid-late secretory phase of the menstrual cycle in control women (α-SMA $P < 0.02$; MyHC $P < 0.004$) (Fig. 1D). In addition, in the mid-late secretory phase, there was a greater proportion of mature vessels in the endometrium of women with a history of RM compared with normal controls (α-SMA $P < 0.0008$; MyHC $P < 0.04$) (Fig. 1D).

Localization of AGFs and their receptors across the menstrual cycle and in RM

PDGF-BB and receptors

There was no immunoreactivity for PDGF-BB in the endometrial stroma or glandular epithelium at any menstrual cycle stage in either control women or those with a history of RM (Fig. 2A and B). VSMCs in proliferative and mid-late secretory phase endometrium were weakly positive for PDGF-BB, although this ligand was not detected in VSMCs in the early secretory phase in control women or in endometrium of women with a history of RM (Fig. 2A and B). VSMC immunoreactivity for PDGF-BB was higher in mid-late secretory phase endometrium from controls compared with women with a history of RM ($P = 0.01$). Moderate to strong PDGF-BB immunoreactivity was observed in endometrial ECs across the menstrual cycle in control women, with no cyclic changes (Fig. 2A and B). Immunoreactivity for PDGF-BB was considerably lower in EC from women with a history of RM than in control women (mid-late secretory phase) ($P = 0.0003$; Fig. 2A and B).

Stromal cells showed moderate immunoreactivity for PDGF-Rα across the menstrual cycle in control women, with a reduction in women with a history of RM ($P = 0.002$; Fig. 2C and D). VSMC also showed moderate PDGF-Rα immunoreactivity increasing from early to mid-late secretory phases ($P = 0.02$; Fig. 2C and D). There was strong PDGF-Rα immunoreactivity in EC across the menstrual cycle in controls, that was reduced in women with RM ($P < 0.0001$; Fig. 2C and D). Glandular epithelial cells also showed strong PDGF-Rα immunoreactivity across the menstrual cycle in control women, with a reduction in the mid-late secretory compared with the proliferative phase ($P = 0.03$; Fig. 2C and D).

There was moderate stromal cell immunoreactivity for PDGF-Rβ across the menstrual cycle in control women, with a reduction in women with RM ($P = 0.001$; Fig. 2E and F). VSMCs also showed moderate PDGF-Rβ immunoreactivity across the menstrual cycle, that

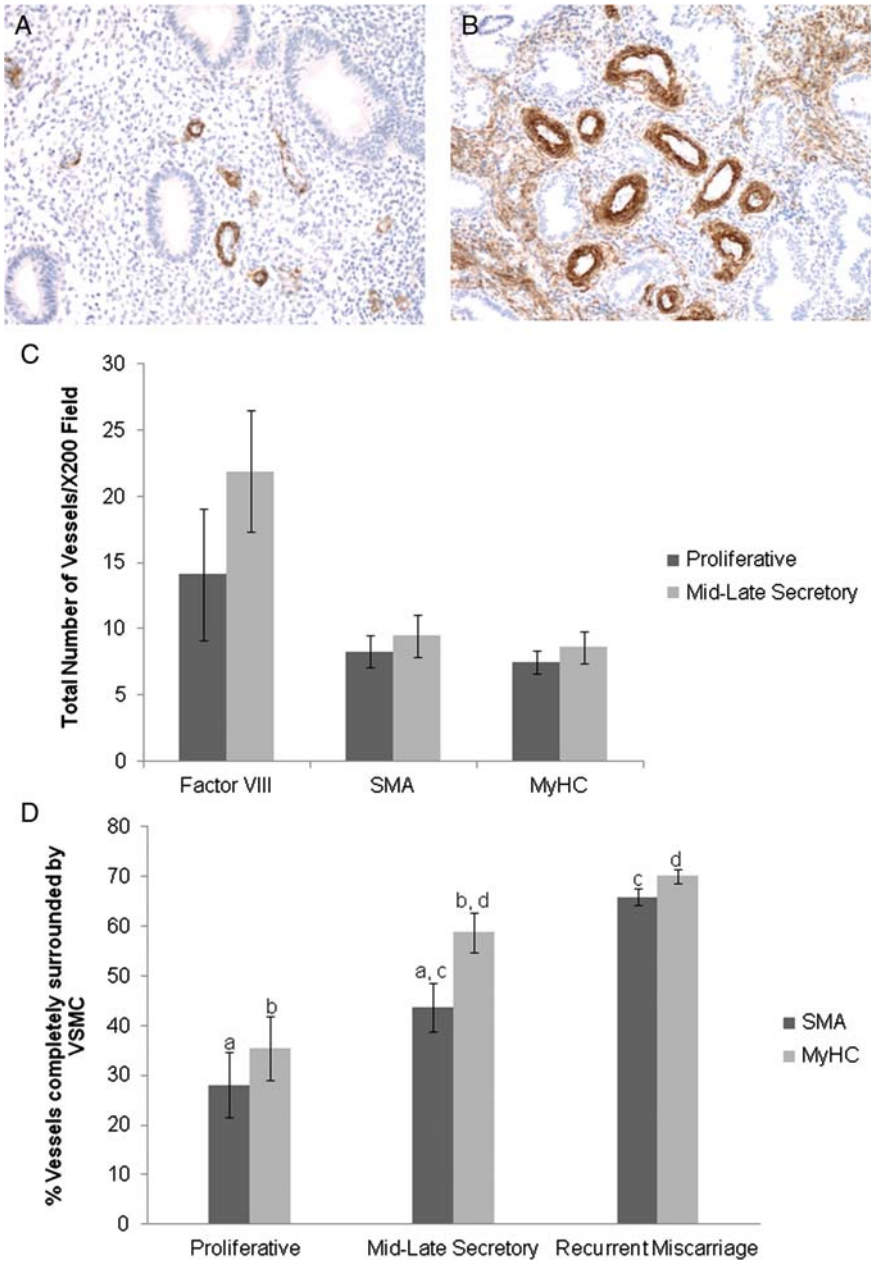

Figure 1 Representative immunohistochemical staining for MyHC on (**A**) proliferative (P) and (**B**) mid-late secretory phase endometrium (MLS). Note that in the MLS phase, the vessel lumens are larger and each vessel profile is surrounded by more layers of VSMCs, original magnification ×200. (**C**) Graphical representation of the total number of vessel profiles in P and MLS phase endometrium as determined by immunostaining for ECs (Factor VIII) and VSMCs (alpha-SMA and MyHC). (**D**) Graphical representation of the percentage of vessels surrounded by two or more layers of VSMCs in Proliferative and MLS phase normal endometrium and MLS phase endometrium from women with a history of RM. Bars with the same letter are significantly different: $^{a}P = 0.02$, $^{b}P = 0.004$, $^{c}P = 0.0008$, $^{d}P = 0.04$; P and MLS $n = 12$, RM $n = 14$. P versus MLS ANOVA and Fishers *post hoc* test, MLS versus RM *t*-test.

increased from proliferative ($P = 0.005$) and early ($P = 0.009$) secretory phases to the mid-late secretory phase (Fig. 2E and F). EC and glandular epithelial cells showed strong PDGF-Rβ immunoreactivity across the menstrual cycle in control women and in women with RM (Fig. 2E and F).

TGF-β1 and its receptors

In general TGF-β1 was weakly expressed in endometrial stromal cells across the menstrual cycle in both control women and those with RM (Fig. 3A and B). However, a small subset of stromal cells showed moderate immunoreactivity for TGF-β1. There was weak to absent

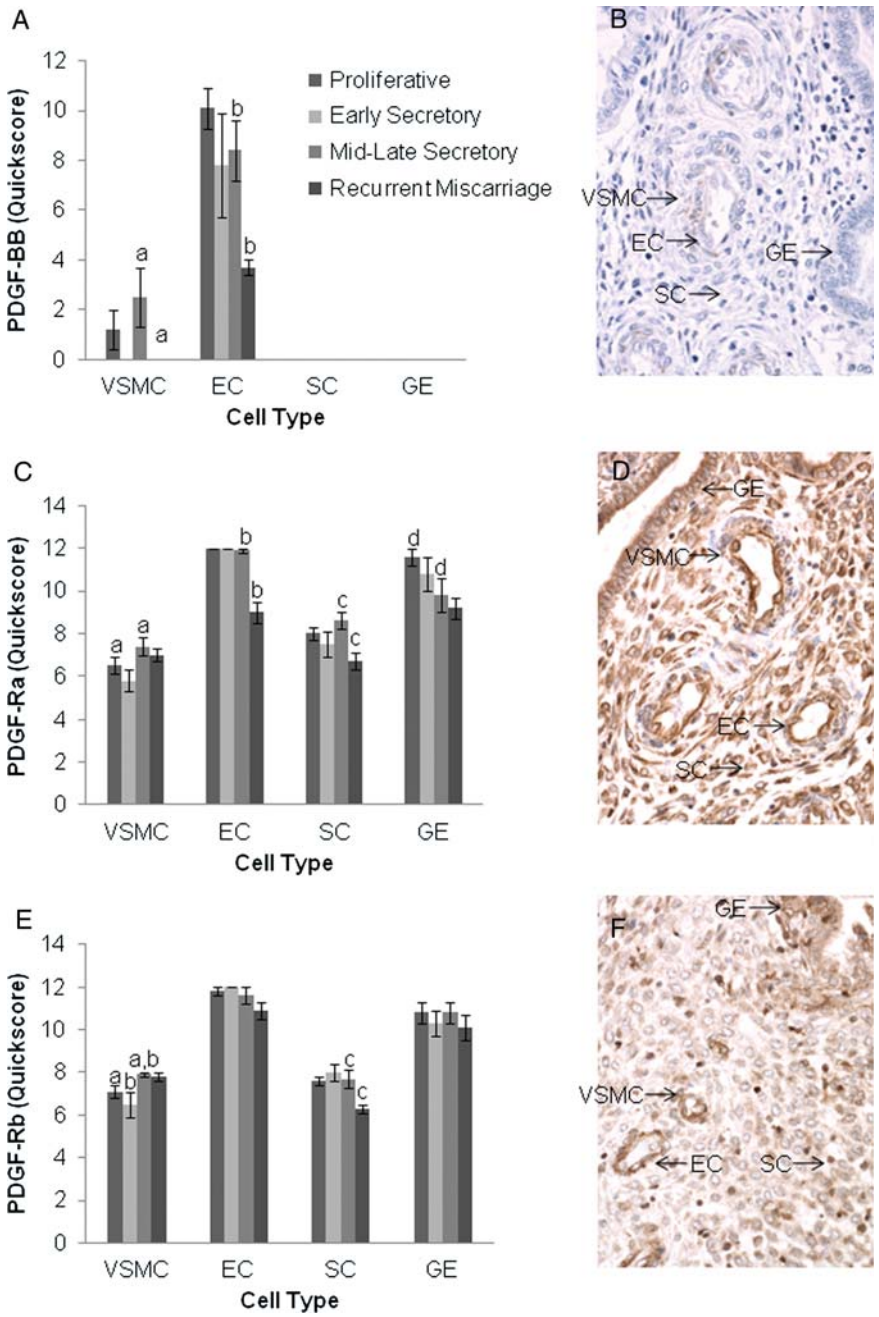

Figure 2 Graphical representation (**A**, **C**, **E**) of Quickscore assessment of immunohistochemical staining of VSMCs, ECs, stromal cells (SCs) and glandular epithelial cells (GEs) in proliferative (P), early secretory (ES), mid-late secretory (MLS) normal endometrium and MLS endometrium from women with a history of RM. Representative immunohistochemical staining (**B**, **D**, **F**) of MLS phase normal endometrium, original magnification ×200. Bars labelled with the same letter are significantly different from each other. (**A**) $^{a}P = 0.01$, $^{b}P = 0.003$; (**C**) $^{a}P = 0.02$, $^{b}P < 0.0001$, $^{c}P = 0.002$, $^{d}P = 0.03$; (**E**) $^{a}P = 0.005$, $^{b}P = 0.009$, $^{c}P = 0.001$. P and MLS $n = 12$, ES $n = 5$, RM $n = 14$; P versus ES versus MLS ANOVA and Fishers *post hoc* test, MLS versus RM *t*-test.

immunostaining for TGF-β1 in both VSMCs and ECs across the menstrual cycle in control women with an increase in women with RM (VSMCs $P = 0.03$; ECs $P = 0.03$; Fig. 3A and B). Immunostaining for TGF-β1 was strongest in glandular epithelium with weak to moderate immunoreactivity that did not alter across the menstrual cycle of control women or in women with RM (Fig. 3A and B).

There was moderate immunostaining for TGF-βR1 in stromal cells, VSMCs and ECs across the menstrual cycle with reduced reactivity in women with RM compared with mid-late secretory phase controls (stromal cells $P < 0.0001$; VSMCs $P = 0.01$; ECs $P = 0.003$; Fig. 3C and D). As for TGF-β1, there was a small subset of stromal cells with strong TGF-βRI immunoreactivity. TGF-βR1 showed strong

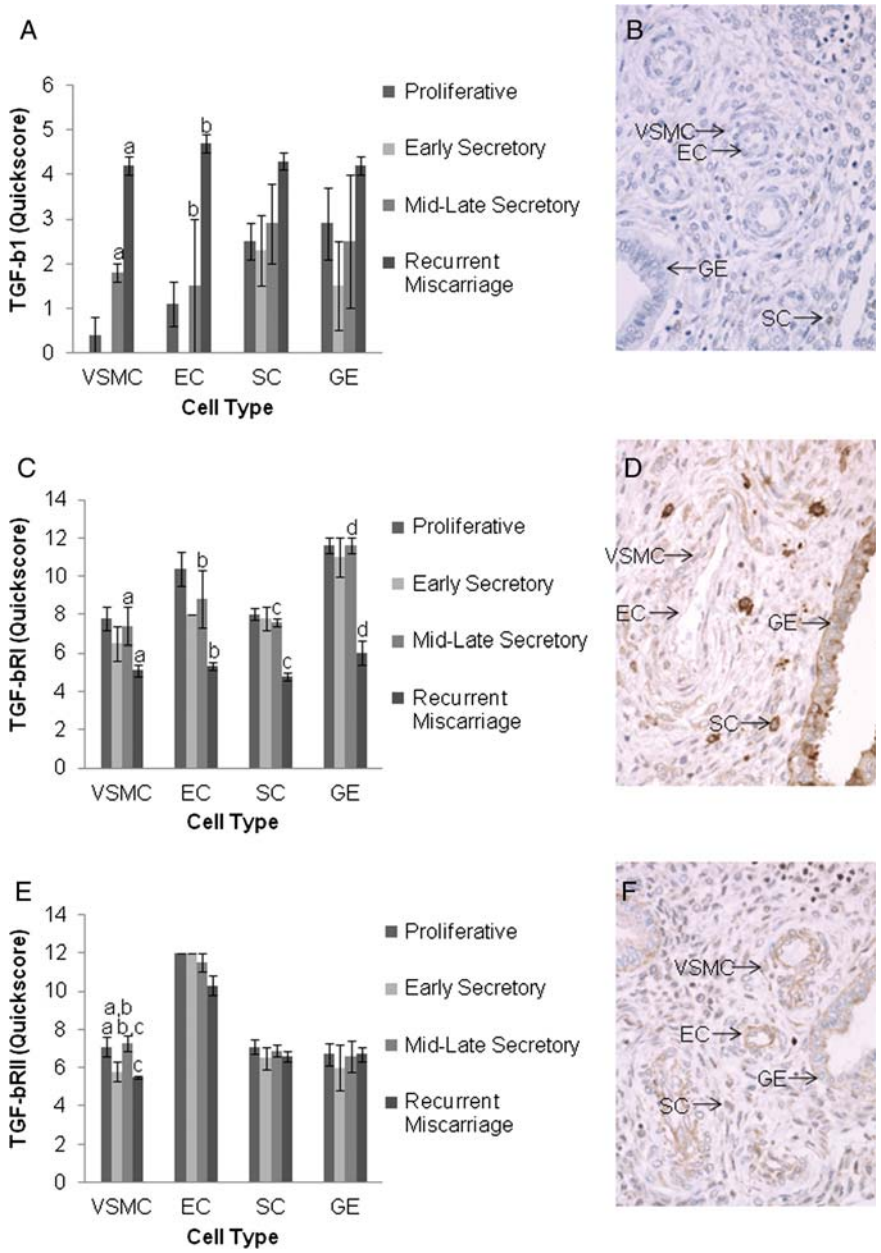

Figure 3 Graphical representation (**A**, **C**, **E**) of Quickscore assessment of immunohistochemical staining of VSMCs, ECs, stromal cells (SCs) and glandular epithelial cells (GEs) in proliferative (P), early secretory (ES), mid-late secretory (MLS) normal endometrium and MLS endometrium from women with a history of RM. Representative immunohistochemical staining (**B**, **D**, **F**) of MLS phase normal endometrium, original magnification ×200. Bars labelled with the same letter are significantly different from each other (**A**) $^{a}P = 0.03$, $^{b}P = 0.03$; (**C**) $^{a}P = 0.01$, $^{b}P = 0.003$, $^{c}P < 0.0001$, $^{d}P < 0.0001$; (**E**) $^{a}P = 0.04$, $^{b}P = 0.03$, $^{c}P = 0.0009$. P and MLS $n = 12$, ES $n = 5$, RM $n = 14$; P versus ES versus MLS ANOVA and Fishers *post hoc* test, MLS versus RM *t*-test.

immunostaining in glandular epithelial cells across the menstrual cycle that was decreased in women with RM ($P < 0.0001$; Fig. 3C and D).

TGF-βRII showed moderate stromal and glandular epithelial cell immunoreactivity that did not alter across the menstrual cycle or in women with RM (Fig. 3E and F). VSMC immunostaining for TGF-βRII was decreased in the early secretory phase compared with both proliferative ($P = 0.04$) and mid-late secretory ($P = 0.03$) phases. In addition, TGF-βRII immunoreactivity was reduced in VSMCs in women with RM compared with mid-late secretory controls ($P = 0.0009$) (Fig. 3E and F). ECs showed strong TGF-βRII immunoreactivity cells that did not alter (Fig. 3E and F).

Angiopoietin family

Ang-1 showed weak immunoreactivity in stromal cells, VSMCs and ECs that did not alter with menstrual cycle phase or in RM (Fig. 4A and B). There was moderate immunoreactivity for Ang-1 in glandular

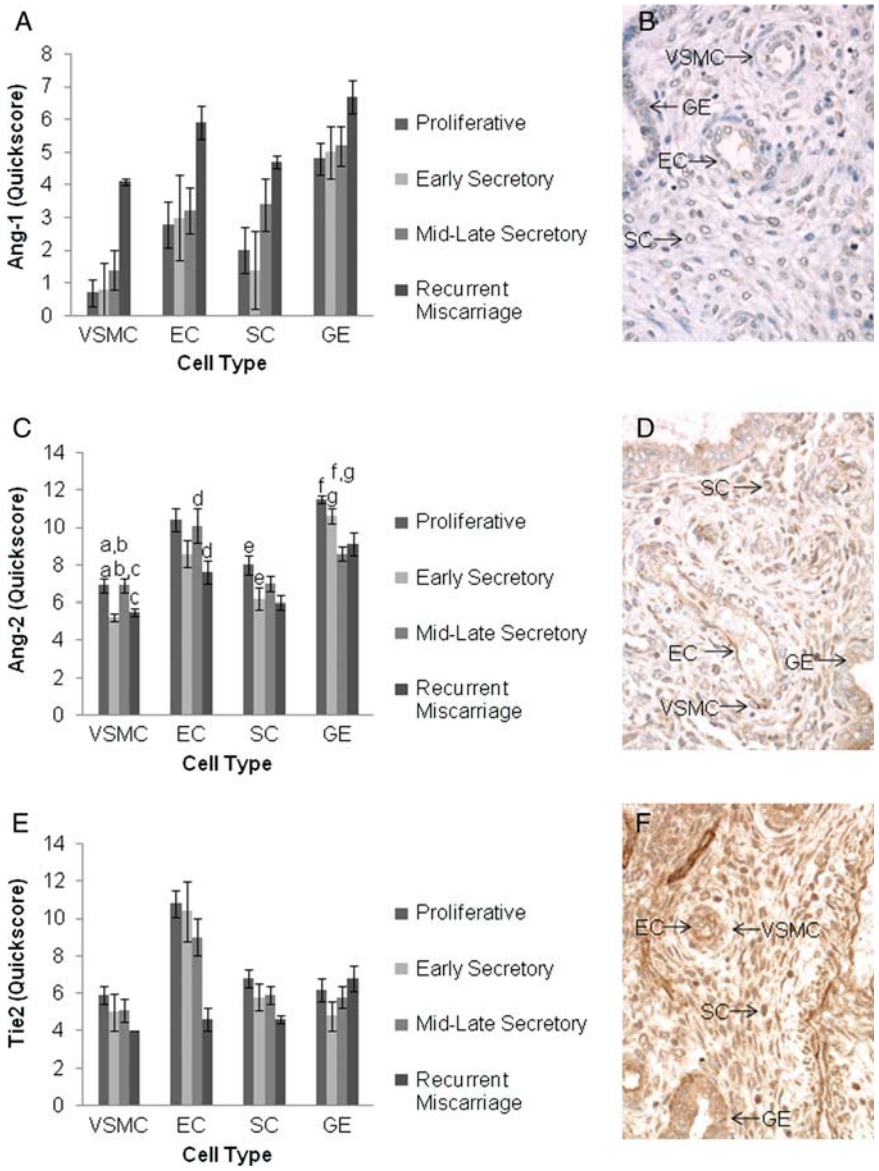

Figure 4 Graphical representation (**A**, **C**, **E**) of Quickscore assessment of immunohistochemical staining of VSMCs, ECs, stromal cells (SCs) and glandular epithelial cells (GEs) in proliferative (P), early secretory (ES), mid-late secretory (MLS) normal endometrium and MLS endometrium from women with a history of RM. Representative immunohistochemical staining (**B**, **D**, **F**) of MLS phase normal endometrium, original magnification ×200. Bars labelled with the same letter are significantly different from each other. $^{a}P = 0.008$, $^{b}P = 0.009$, $^{c}P = 0.005$, $^{d}P = 0.03$, $^{e}P = 0.04$, $^{f}P = 0.0002$, $^{g}P = 0.03$. P and MLS $n = 12$, ES $n = 5$, RM $n = 14$; P versus ES versus MLS ANOVA and Fishers *post hoc* test, MLS versus RM *t*-test.

epithelial cells that did not alter across the menstrual cycle or in RM (Fig. 4A and B).

Ang-2 showed moderate stromal cell immunoreactivity that decreased in intensity from the proliferative to early secretory phase of the menstrual cycle ($P = 0.04$; Fig. 4C and D). VSMC immunostaining for Ang-2 decreased in the early secretory phase compared with both proliferative ($P = 0.008$) and mid-late secretory ($P = 0.009$) phases. Ang-2, immunoreactivity was also reduced in VSMCs in women with RM compared with mid-late secretory controls ($P = 0.005$) (Fig. 4C and D). Ang-2 showed strong EC immunoreactivity throughout the menstrual cycle, with intensity being reduced in RM ($P = 0.03$; Fig. 4C and D). Glandular epithelial cells showed strong Ang-2 immunostaining that was reduced in the mid-late secretory phase compared with both proliferative ($P = 0.0002$) and early secretory ($P = 0.03$) phases (Fig 4C and D).

Immunoreactivity for Tie-2 in stromal cells, VSMCs and glandular epithelial cells was moderate while EC immunoreactivity was strong. There were no alterations in intensity across the menstrual cycle or in RM (Fig. 4E and F).

VEGF family

VEGF-A showed moderate immunoreactivity in stromal cells throughout the menstrual cycle that did not alter in RM (Fig. 5A and B). There was moderate VEGF-A immunoreactivity in VSMCs, ECs and glandular

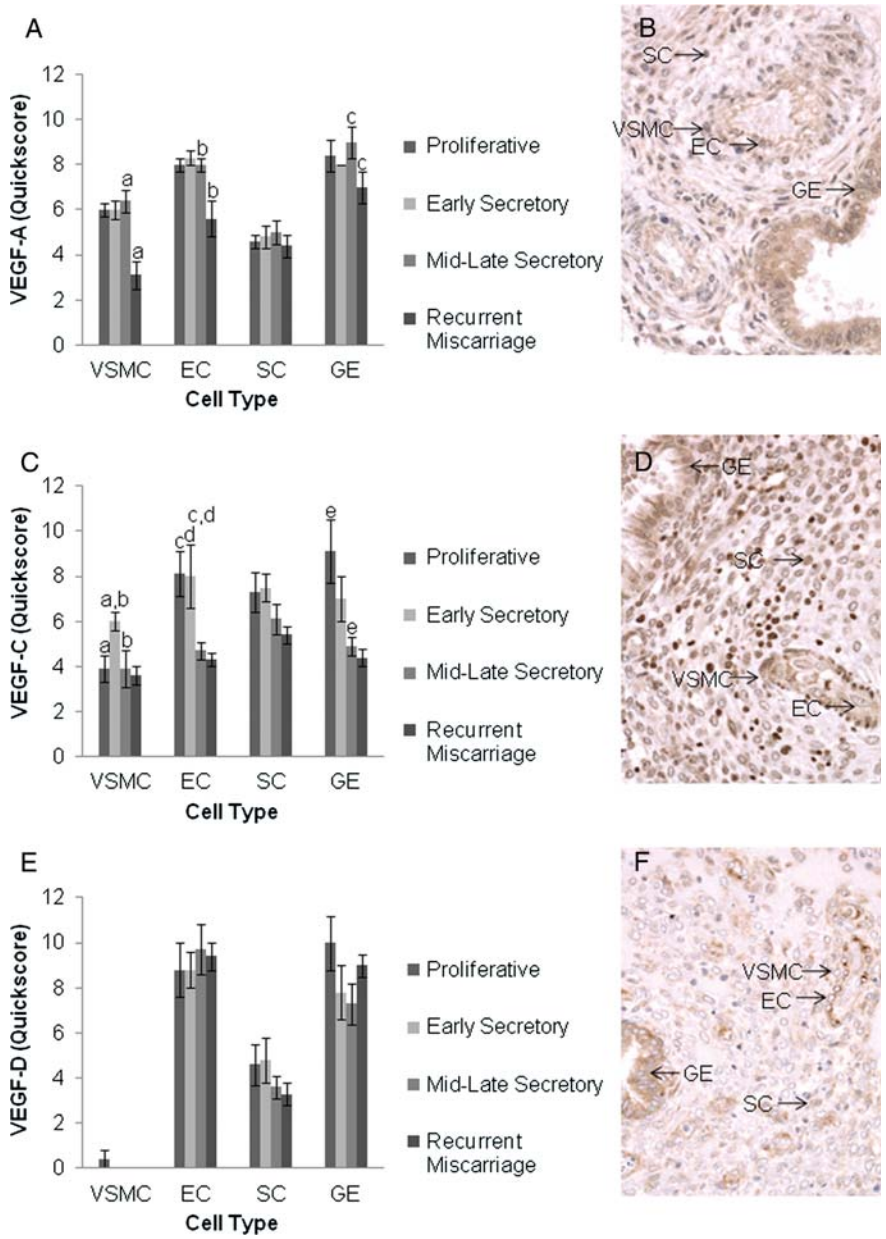

Figure 5 Graphical representation (**A**, **C**, **E**) of Quickscore assessment of immunohistochemical staining of VSMCs, ECs, stromal cells (SCs) and glandular epithelial cells (GEs) in proliferative (P), early secretory (ES), mid-late secretory (MLS) normal endometrium and MLS endometrium from women with a history of RM. Representative immunohistochemical staining (**B**, **D**, **F**) of MLS phase normal endometrium, original magnification ×200. Bars labelled with the same letter are significantly different from each other. (**A**) $^{a}P < 0.0001$, $^{b}P = 0.003$, $^{c}P = 0.05$; (**C**) $^{a}P = 0.04$, $^{b}P = 0.04$, $^{c}P = 0.0009$, $^{d}P = 0.005$, $^{e}P = 0.001$. P and MLS $n = 12$, ES $n = 5$, RM $n = 14$; P versus ES versus MLS ANOVA and Fishers *post hoc* test, MLS versus RM *t*-test.

epithelial cells across the menstrual cycle that was reduced in RM compared with mid-late secretory controls (VSMCs $P < 0.0001$; ECs $P = 0.003$; glandular epithelium $P = 0.05$; Fig. 5A and B).

VEGF-C showed moderate stromal cell immunoreactivity across the menstrual cycle of control women with no change in RM (Fig. 5C and D). VSMC immunoreactivity for VEGF-C was increased in the early secretory phase compared with both proliferative ($P = 0.04$) and mid-late secretory ($P = 0.04$) phases (Fig. 5C and D). ECs showed moderate to strong immunostaining for VEGF-C that was decreased in mid-late secretory phase endometrium compared with proliferative ($P = 0.0009$) and early secretory ($P = 0.005$) phases (Fig. 5C and D). Moderate to strong VEGF-C immunoreactivity in glandular epithelial cells was decreased in the mid-late secretory phase compared with the proliferative phase ($P = 0.001$; Fig. 5C and D). Immunostaining intensity for VEGF-C was not altered in RM in any of the cell types investigated.

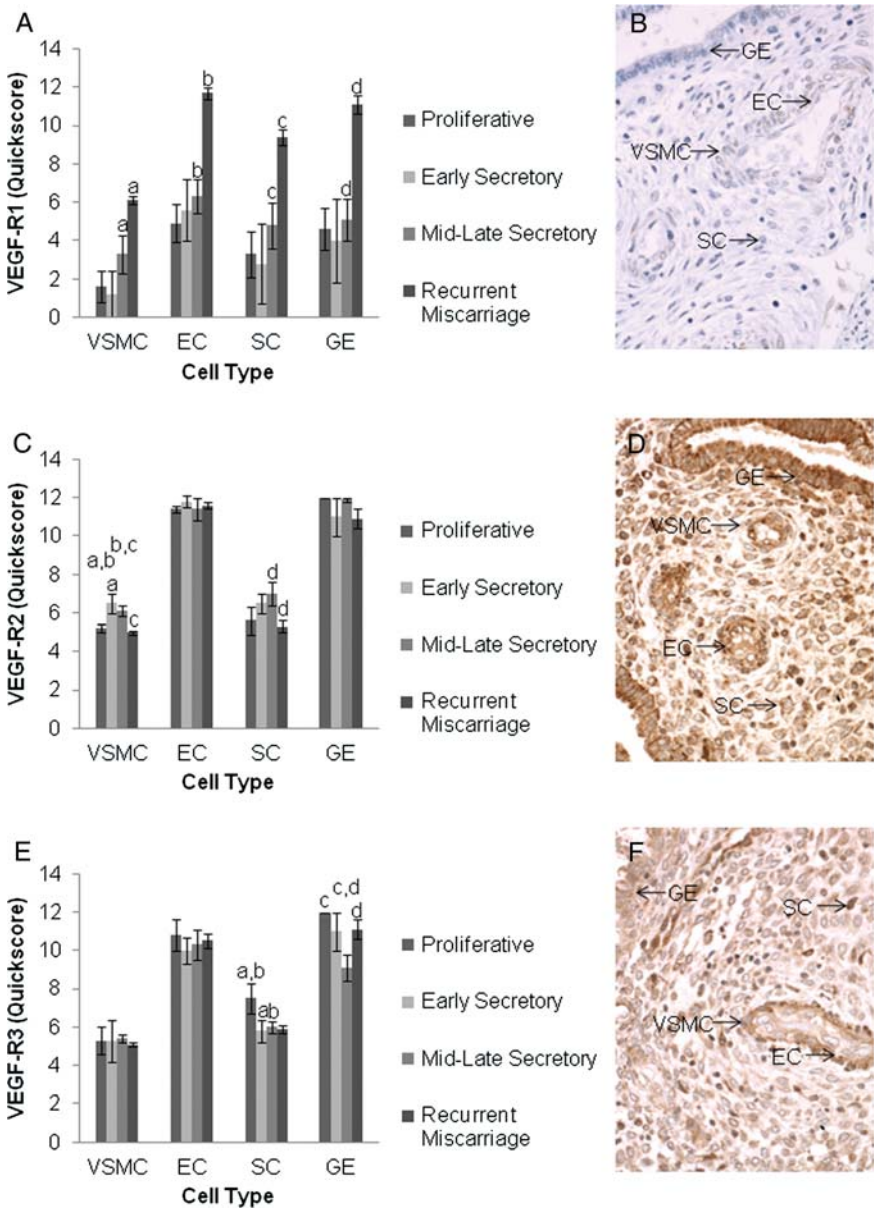

Figure 6 Graphical representation (**A**, **C**, **E**) of Quickscore assessment of immunohistochemical staining of VSMCs, ECs, stromal cells (SCs) and glandular epithelial cells (GEs) in proliferative, early secretory (ES), mid-late secretory (MLS) normal endometrium and MLS endometrium from women with a history of RM. Representative immunohistochemical staining (**B**, **D**, **F**) of MLS phase normal endometrium, original magnification ×200. Bars labelled with the same letter are significantly different from each other. (**A**) $^{a}P = 0.006$, $^{b}P < 0.0001$, $^{c}P = 0.002$, $^{d}P < 0.0001$; (**C**) $^{a}P = 0.003$, $^{b}P = 0.01$, $^{c}P = 0.0003$, $^{d}P = 0.009$; (**E**) $^{a}P = 0.03$, $^{b}P = 0.03$, $^{c}P = 0.004$, $^{d}P = 0.02$. P and MLS $n = 12$, ES $n = 5$, RM $n = 14$; P versus ES versus MLS ANOVA and Fishers *post hoc* test, MLS versus RM *t*-test.

VEGF-D showed moderate to strong immunoreactivity with stromal cells, ECs and glandular epithelial cells throughout the menstrual cycle of control women and in women with RM (Fig. 5E and F). VSMCs showed weak to absent VEGF-D immunoreactivity throughout the menstrual cycle and in RM (Fig. 5E and F). No menstrual cycle alterations in immunostaining were observed and there were no differences in staining intensity in RM.

There was weak to moderate VEGF-R1 immunoreactivity with stromal cells, VSMCs, ECs and glandular epithelial cells with no change across the menstrual cycle. Immunostaining intensity was increased in all four cell types in women with RM compared with mid-late secretory phase endometrium from controls (stromal cells $P = 0.002$; VSMCs $P = 0.006$; ECs $P < 0.0001$; glandular epithelium $P < 0.0001$; Fig. 6A and B).

VEGF-R2 showed moderate stromal cell immunoreactivity across the menstrual cycle of control women that was reduced in RM ($P = 0.009$; Fig. 6C and D). VSMC immunostaining for VEGF-R2 was increased in the early ($P = 0.003$) and mid-late ($P = 0.01$) secretory phases compared

with proliferative phase endometrium. Immunoreactivity was also reduced in VSMCs in women with RM compared with mid-late secretory phase controls ($P = 0.0003$) (Fig. 6C and D). There was strong VEGF-R2 immunoreactivity with ECs and glandular epithelial cells across the menstrual cycle with no alteration in RM (Fig. 6C and D).

Immunostaining for VEGF-R3 in stromal cells was reduced in the early ($P = 0.03$) and mid-late ($P = 0.03$) secretory phases compared with the proliferative phase (Fig. 6E and F). VEGF-R3 showed moderate and strong immunoreactivity in VSMCs and ECs, respectively, across the menstrual cycle with no alterations with menstrual cycle phase or in RM (Fig. 6E and F). Glandular epithelial cells showed strong VEGF-R3 immunostaining that was reduced in mid-late secretory compared with proliferative phase endometrium ($P = 0.004$; Fig. 6E and F). Furthermore, VEGF-R3 immunoreactivity in glandular epithelial cells was increased in RM compared with mid-late secretory controls ($P = 0.02$; Fig. 6E and F).

Laser capture microdissection and RT–PCR

Similar to the immunohistochemical results mRNA for PDGF-Rα, PDGF-Rβ, TGF-β1, TGF-βRI, TGF-βRII, Ang-1, Ang-2, Tie-2, VEGF-A, VEGF-C, VEGF-R1, VEGF-R2 and VEGR-R3 was detected in blood vessels (combination of ECs and VSMCs), stroma and glandular epithelium.

In contrast to the immunohistochemical results mRNA for PDGF-BB was detected by real-time RT–PCR in blood vessels, stroma and glandular epithelium obtained by laser capture microdissection from endometrium. In addition, mRNA for VEGF-D was only detected in endometrial glandular epithelium.

Discussion

Endometrial blood vessels develop via the process of intussusception from vascular stumps remaining in the basal layer of the endometrium after menstruation (Girling and Rogers, 2005; 2009). In the current study, we demonstrate that while the total number of endometrial vessels does not change dramatically as the menstrual cycle proceeds from proliferative to mid-late secretory phase the proportion that is completely surrounded by two or more layers of VSMCs increases. Not all of the vessel profiles identified by immunostaining for Factor VIII had VSMCs associated with them and reflect the presence of a large capillary and vein network within the endometrium. We also demonstrate that the proportion of mature vessels in mid-late secretory phase endometrium is higher in women with RM than in normal controls. These data add further evidence for an endometrial component in the aetiology of RM.

It is interesting to note that in the current study the majority of AGF cyclic expression changes observed were in VSMCs. Expression of VEGF-R2, PDGF-Rα, PDGF-Rβ, TGF-βRII and Ang-2 were all increased in mid-late secretory phase endometrial VSMCs, while VEGF-C was reduced in both EC and VSMCs. PDGF-BB and TGF-β1 have both been associated with VSMC maturation and recruitment to newly forming vessels. Ang-2 plays roles in vessel instability being an antagonist for Ang-1. The role of VEGF-C in VSMC maturation and recruitment to vessels is unclear although it may play a role in EC: VSMC association (unpublished data). The increased Ang-2 and decreased VEGF-C expression may precede the vessel collapse observed in menstruation. Interestingly, VEGF-C, Ang-2, VEGF-R3 and PDGF-Rα were also reduced in glandular epithelium in the mid-late secretory phase although the significance of these results is unclear as the functional role of these ligands and receptors outside of vascular development is not known. However, it is clear from the localization patterns within endometrium throughout the menstrual cycle that these ligands and receptors may play other important roles in endometrial health and development that are still to be elucidated.

RM affects ~1–2% of couples of reproductive age with significant impact on a woman's physical and mental wellbeing. Expression patterns of several AGFs and their receptors investigated in the current study were altered in women with RM compared with mid-late secretory phase controls. Blood vessel (EC and/or VSMC) expression of VEGF-A, VEGF-R2, PDGF-BB, PDGF-Rα, TGF-βRI, TGF-βRII and Ang-2 was reduced and TGF-β1 and VEGF-R1 increased in women with a history of RM. Increased TGF-β1 and reduced Ang-2 may contribute to the advanced vessel maturation observed in RM. However, the contribution of those factors that are decreased cannot be discounted and until the interaction between these growth factors is clarified it is difficult to determine their role in the endometrial changes observed in RM. In addition to the altered vascular expression patterns observed in RM, variations in stromal and/or glandular epithelial cell expression patterns were also observed: VEGF-R2, PDGF-Rα, PDGF-Rβ and TGF-βRI were reduced and VEGF-R1 increased in stromal cells, while VEGF-A and TGF-βRI were reduced and VEGF-R1 and VEGF-R3 increased in glandular epithelial cells. However, until the full functional role of these ligands and receptors in EC:VSMC association and VSMC maturation are elucidated we can only speculate on their potential causal role in RM.

VEGF-A is the best studied AGF in endometrium (Girling and Rogers, 2009) and has been shown to be expressed in glandular epithelium, stromal cells, ECs and VSMCs throughout the menstrual cycle. There are reports of increased (Li *et al.*, 1994; Torry *et al.*, 1996; Zhang *et al.*, 1998; Von Wolff *et al.*, 2000; Sugino *et al.*, 2002; Saito *et al.*, 2007), decreased (Naresh *et al.*, 1999) or unaltered (Gargett *et al.*, 2001; Moller *et al.*, 2001; Takehara *et al.*, 2004; Lee *et al.*, 2008) VEGF-A mRNA (RT–PCR) and protein (immunohistochemistry) levels in secretory phase compared with proliferative phase endometrium. Endometrial VEGF-C has been much less well studied (Girling and Rogers, 2009); two previous studies reported endometrial expression with no menstrual cycle variation (Moller *et al.*, 2002; Takehara *et al.*, 2004). VEGF-D expression has not been previously described in the endometrium. In the current study, VEGF-D was immunodetected on all endometrial ECs and not just those of the lymphatic system. In contrast with the immunohistochemistry results mRNA for VEGF-D was detected only in glandular epithelium microdissected from normal cycling endometrium, suggesting that VEGF-D protein detected by immunohistochemistry in stromal cells, ECs and VSMCs is bound to its receptors, VEGF-R2 or VEGF-R3, potentially playing a biological role in these cell types. Similarly, despite mRNA expression other ligand immunohistochemistry maybe reflective of both expressed and receptor bound protein. VEGF-R1, VEGF-R2 and VEGF-R3 have been reported to be widely expressed in endometrium with expression patterns in general not altering with menstrual cycle phase (Krussel *et al.*, 1999; Meduri *et al.*, 2000; Mints *et al.*, 2007; Moller *et al.*, 2001; 2002; Sugino

et al., 2002); although VEGF-R2 expression in stromal cells, ECs and VSMCs has been reported to increase as the menstrual cycle progresses (Moller et al., 2002; Sugino et al., 2002).

Ang-1 has been immunolocalized to all cell types within endometrium (Blumenthal et al., 2002; Saito et al., 2007) and has been reported to either increase (Hirchenhain et al., 2003; Saito et al., 2007) or not alter (Blumenthal et al., 2002; Hur et al., 2006; Lee et al., 2008) as the menstrual cycle progresses. Ang-2 is also widely expressed in endometrium with levels reported to be decreased (Hirchenhain et al., 2003; Saito et al., 2007), unaltered (Blumenthal et al., 2002; Hewett et al., 2002; Hur et al., 2006) or increased (Lee et al., 2008) as the menstrual cycle progresses. Tie-2 expression is also widespread in the endometrium and has been reported to not alter with the menstrual cycle (Blumenthal et al., 2002; Hewett et al., 2002; Hirchenhain et al., 2003; Hur et al., 2006).

Only a few studies have investigated the expression patterns of TGF-β1 and its receptors TGF-βRI and TGF-βRII in endometrium during the menstrual cycle or in women with RM (Von Wolff et al., 2000; Piestrzeniewicz-Ulanska et al., 2002; Omwandho et al., 2010). Omwandho et al. (2010) reported immunostaining for TGF-β1 in endometrial stromal cells, glandular epithelium and leucocytes (particularly macrophages). We found a small subset of stromal cells that were strongly immunopositive for TGF-β1 and TGF-βRI and these cells may have a leucocyte origin.

There are few studies of PDGF-BB and its receptors, PDGF-Rα and PDGF-Rβ, in normal cycling endometrium. Chegini et al. (1992) reported decreased expression of PDGF-BB in glandular epithelium and stromal cells as the menstrual cycle progressed.

Very few studies have investigated expression of AGFs or their receptors in endometrium of women with a history of RM. One previous study reported no alterations in VEGF-A or TGF-β1 mRNA levels in endometrium of women with a history of RM (Von Wolff et al., 2000).

Differences between the current study and previous reports may arise from several sources including; specificity of antibodies used for immunohistochemistry, investigation of mRNA expression versus protein studies, sample numbers and staging methodology.

High numbers of uNK cells have been associated with idiopathic RM (Clifford et al., 1999; Quenby et al., 1999; Tuckerman et al., 2007) although the functional significance of this is not clear. We have previously demonstrated that in the endometrium of women with high uNK cell numbers the maturation status of the vessels was increased, i.e. there was a higher percentage of blood vessels completely surrounded by two or more layers of VSMCs (Quenby et al., 2009). In addition, this increase in vessel maturation and uNK cell numbers was associated with lowered resistance to flow as determined by uterine artery Doppler studies. We and others have previously demonstrated that uNK cells are a rich source of AGFs in both non-pregnant endometrium (Li et al., 2001) and early pregnancy decidua (Hanna et al., 2006; Lash et al., 2006) although their expression patterns were not specifically investigated in the current study. It was interesting to note strong immunostaining of a subset of stromal cells by TGF-β1 and one of its receptors which may have been a leucocyte cell population, however double-labelling immunohistochemistry would be required to confirm this. It would also be interesting to isolate uNK cells from control and RM endometrium to determine their AGF secretion profiles and potential functional role in angiogenesis.

In summary, we have investigated the expression patterns of seven AGFs and their receptors in endometrium at different menstrual cycle phases as well as mid-luteal phase endometrium in women with a history of RM. The data presented here suggest a significant role for these AGFs in normal endometrial development. In addition, dysregulation of these factors likely contributes to the aetiology of RM in a subset of women. However, the functional role of these ligands and receptors in endometrial blood vessel development needs to be elucidated.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

G.E.L. was involved in study design, data collection and analysis and wrote the manuscript. B.A.I. performed experiments. J.A.D. participated in sample collection. S.C.R. and J.N.B. played a role in study design. S.Q. took part in sample collection and study design. All authors approved the final version of the manuscript.

Acknowledgements

The authors acknowledge the staff at the Royal Victoria Infirmary, Newcastle upon Tyne and Liverpool Women's Hospital, Liverpool for their assistance in sample collection.

Funding

This work was supported by a Research Grant from The Royal Society. G.E.L. was a Faculty of Medicine Research Fellow in Newcastle University.

References

Betsholtz C. Insight into the physiological functions of PDGF through genetic studies in mice. *Cytokine Growth Factor Rev* 2004;**15**:215–228.

Blumenthal RD, Taylor AP, Goldman L, Brown G, Goldenberg DM. Abnormal expression of the angiopoietins and Tie receptors in menorrhagic endometrium. *Fertil Steril* 2002;**78**:1294–1300.

Brosens JJ, Gellersen B. Something new about early pregnancy: decidual biosensoring and natural embryo selection. *Ultrasound Obstet Gynecol* 2010;**36**:1–5.

Chegini N, Rossi MJ, Masterson BJ. Platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and EGF and PDGF beta-receptors in human endometrial tissue: localization and *in vitro* action. *Endocrinology* 1992;**130**:2373–2385.

Clifford K, Flanagan AM, Regan L. Endometrial CD56+ natural killer cells in women with recurrent miscarriage: a histomorphometric study. *Hum Reprod* 1999;**14**:2727–2730.

Distler JH, Hirth A, Kurowska-Stolarska M, Gay RE, Gay S, Distler O. Angiogenic and angiostatic factors in the molecular control of angiogenesis. *Q J Nucl Med* 2003;**47**:149–161.

Gargett CE, Lederman F, Heryanto B, Gambino LS, Rogers PA. Focal vascular endothelial growth factor correlates with angiogenesis in

human endometrium. Role of intravascular neutrophils. *Hum Reprod* 2001;**16**:1065–1075.

Girling JE, Rogers PA. Recent advances in endometrial angiogenesis research. *Angiogenesis* 2005;**8**:89–99.

Girling JE, Rogers PA. Regulation of endometrial vascular remodelling: role of the vascular endothelial growth factor family and the angiopoietin-TIE signalling system. *Reproduction* 2009;**138**:883–893.

Goswamy RK, Williams G, Steptoe PC. Decreased uterine perfusion–a cause of infertility. *Hum Reprod* 1988;**3**:955–959.

Habara T, Nakatsuka M, Konishi H, Asagiri K, Noguchi S, Kudo T. Elevated blood flow resistance in uterine arteries of women with unexplained recurrent pregnancy loss. *Hum Reprod* 2002;**17**:190–194.

Hanna J, Goldman-Wohl D, Hamani Y, Avraham I, Greenfield C, Natanson-Yaron S, Prus D, Cohen-Daniel L, Arnon TI, Manaster I *et al*. Decidual NK cells regulate key developmental processes at the human fetal-maternal interface. *Nat Med* 2006;**12**:1065–1074.

Hewett P, Nijjar S, Shams M, Morgan S, Gupta J, Ahmed A. Down-regulation of angiopoietin-1 expression in menorrhagia. *Am J Pathol* 2002;**160**:773–780.

Hirchenhain J, Huse I, Hess A, Bielfeld P, De Bruyne F, Krüssel JS. Differential expression of angiopoietins 1 and 2 and their receptor Tie-2 in human endometrium. *Mol Hum Reprod* 2003;**9**:663–669.

Hur SE, Lee JY, Moon HS, Chung HW. Angiopoietin-1, angiopoietin-2 and Tie-2 expression in eutopic endometrium in advanced endometriosis. *Mol Hum Reprod* 2006;**12**:421–426.

Jauniaux E, Watson AL, Hempstock J, Bao YP, Skepper JN, Burton GJ. Onset of maternal arterial blood flow and placental oxidative stress. A possible factor in human early pregnancy failure. *Am J Pathol* 2000; **157**:2111–2122.

Krüssel JS, Casañ EM, Raga F, Hirchenhain J, Wen Y, Huang HY, Bielfeld P, Polan ML. Expression of mRNA for vascular endothelial growth factor transmembraneous receptors Flt1 and KDR, and the soluble receptor sflt in cycling human endometrium. *Mol Hum Reprod* 1999; **5**:452–458.

Lash GE, Schiessl B, Kirkley M, Innes BA, Cooper A, Searle RF, Robson SC, Bulmer JN. Expression of angiogenic growth factors by uterine natural killer cells during early pregnancy. *J Leukoc Biol* 2006;**80**:572–580.

Lee YL, Liu Y, Ng PY, Lee KF, Au CL, Ng EH, Ho PC, Yeung WS. Aberrant expression of angiopoietins-1 and -2 and vascular endothelial growth factor-A in peri-implantation endometrium after gonadotrophin stimulation. *Hum Reprod* 2008;**23**:894–903.

Li XF, Gregory J, Ahmed A. Immunolocalisation of vascular endothelial growth factor in human endometrium. *Growth Factors* 1994; **11**:277–282.

Li XF, Charnock-Jones DS, Zhang E, Hiby S, Malik S, Day K, Licence D, Bowen JM, Gardner L, King A *et al*. Angiogenic growth factor messenger ribonucleic acids in uterine natural killer cells. *J Clin Endocrinol Metab* 2001;**86**:1823–1834.

Lindahl P, Johansson BR, Levéen P, Betsholtz C. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science* 1997; **277**:242–245.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N *et al*. Angiopoietin-2, a natural antagonist for Tie2 that disrupts *in vivo* angiogenesis. *Science* 1997;**277**:55–60.

Meduri G, Bausero P, Perrot-Applanat M. Expression of vascular endothelial growth factor receptors in the human endometrium: modulation during the menstrual cycle. *Biol Reprod* 2000;**62**:439–447.

Mints M, Blomgren B, Palmblad J. Expression of vascular endothelial growth factor receptor-3 in the endometrium in menorrhagia. *Int J Mol Med* 2007;**19**:909–913.

Möller B, Rasmussen C, Lindblom B, Olovsson M. Expression of the angiogenic growth factors VEGF, FGF-2, EGF and their receptors in normal human endometrium during the menstrual cycle. *Mol Hum Reprod* 2001;**7**:65–72.

Möller B, Lindblom B, Olovsson M. Expression of the vascular endothelial growth factors B and C and their receptors in human endometrium during the menstrual cycle. *Acta Obstet Gynecol Scand* 2002;**81**:817–824.

Naresh B, Sengupta J, Bhargava V, Kinra G, Hu J, Ghosh D. Immunohistological localisation of vascular endothelial growth factor in human endometrium. *Indian J Physiol Pharmacol* 1999;**43**:165–170.

Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. *Am J Obstet Gynecol* 1975;**122**:262–263.

Omwandho CO, Konrad L, Halis G, Oehmke F, Tinneberg HR. Role of TGF-betas in normal human endometrium and endometriosis. *Hum Reprod* 2010;**25**:101–109.

Piestrzeniewicz-Ulanska D, Brys M, Semczuk A, Jakowicki JA, Krajewska WM. Expression of TGF-beta type I and II receptors in normal and cancerous human endometrium. *Cancer Lett* 2002;**186**:231–239.

Quenby SM, Farquharson RG. Predicting recurring miscarriage: what is important? *Obstet Gynecol* 1993;**82**:132–138.

Quenby S, Bates M, Doig T, Brewster J, Lewis-Jones DI, Johnson PM, Vince G. Pre-implantation endometrial leukocytes in women with recurrent miscarriage. *Human Reproduction* 1999;**14**:2386–2391.

Quenby S, Nik H, Innes B, Lash G, Turner M, Drury J, Bulmer J. Uterine natural killer cells and angiogenesis in recurrent reproductive failure. *Hum Reprod* 2009;**24**:45–54.

Rai R, Regan L. Recurrent miscarriage. *Lancet* 2006;**368**:601–611.

Rodesch F, Simon P, Donner C, Jauniaux E. Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. *Obstet Gynecol* 1992;**80**:283–285.

Rogers PA, Abberton KM. Endometrial arteriogenesis: vascular smooth muscle cell proliferation and differentiation during the menstrual cycle and changes associated with endometrial bleeding disorders. *Microsc Res Tech* 2003;**60**:412–419.

Saito M, Sato Y, Watanabe J, Kuramoto H, Kaba S, Fukuda T. Angiogenic factors in normal endometrium and endometrial adenocarcinoma. *Pathol Int* 2007;**57**:140–147.

Schiessl B, Innes BA, Bulmer JN, Otun HA, Chadwick TJ, Robson SC, Lash GE. Localization of angiogenic growth factors and their receptors in the human placental bed throughout normal human pregnancy. *Placenta* 2009;**30**:79–87.

Steer CV, Tan SL, Mason BA, Campbell S. Midluteal-phase vaginal color Doppler assessment of uterine artery impedance in a subfertile population. *Fertil Steril* 1994;**61**:53–58.

Sugino N, Kashida S, Karube-Harada A, Takiguchi S, Kato H. Expression of vascular endothelial growth factor (VEGF) and its receptors in human endometrium throughout the menstrual cycle and in early pregnancy. *Reproduction* 2002;**123**:379–387.

Takehara M, Ueda M, Yamashita Y, Terai Y, Hung YC, Ueki M. Vascular endothelial growth factor A and C gene expression in endometriosis. *Hum Pathol* 2004;**35**:1369–1375.

Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. *Cardiovasc Res* 2005;**65**:550–563.

Torry DS, Holt VJ, Keenan JA, Harris G, Caudle MR, Torry RJ. Vascular endothelial growth factor expression in cycling human endometrium. *Fertil Steril* 1996;**66**:72–80.

Tuckerman E, Laird SM, Prakash A, Li TC. Prognostic value of the measurement of uterine natural killer cells in the endometrium of women with recurrent miscarriage. *Hum Reprod* 2007;**22**:2208–2213.

Von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, Tabibzadeh S. Regulated expression of cytokines in human endometrium throughout

the menstrual cycle: dysregulation in habitual abortion. *Mol Hum Reprod* 2000;**6**:627–634.

Witzenbichler B, Maisonpierre PC, Jones P, Yancopoulos GD, Isner JM. Chemotactic properties of angiopoietin-1 and -2, ligands for the endothelial-specific receptor tyrosine kinase Tie2. *J Biol Chem* 1998; **273**:18514–18521.

Yedwab GA, Paz G, Homonnai TZ, David MP, Kraicer PF. The temperature, pH, and partial pressure of oxygen in the cervix and uterus of women and uterus of rats during the cycle. *Fertil Steril* 1976;**27**:304–309.

Zhang L, Scott PA, Turley H, Leek R, Lewis CE, Gatter KC, Harris AL, Mackenzie IZ, Rees MC, Bicknell R. Validation of anti-vascular endothelial growth factor (anti-VEGF) antibodies for immunohistochemical localization of VEGF in tissue sections: expression of VEGF in the human endometrium. *J Pathol* 1998;**185**:402–408.

Zhang EG, Smith SK, Baker PN, Charnock-Jones DS. The regulation and localization of angiopoietin-1, -2, and their receptor Tie2 in normal and pathologic human placentae. *Mol Med* 2001;**7**: 624–635.