Angiogenesis, proteases and angiogenic factors during the inception of pregnancy. Crucial contributors or trivial bystanders?

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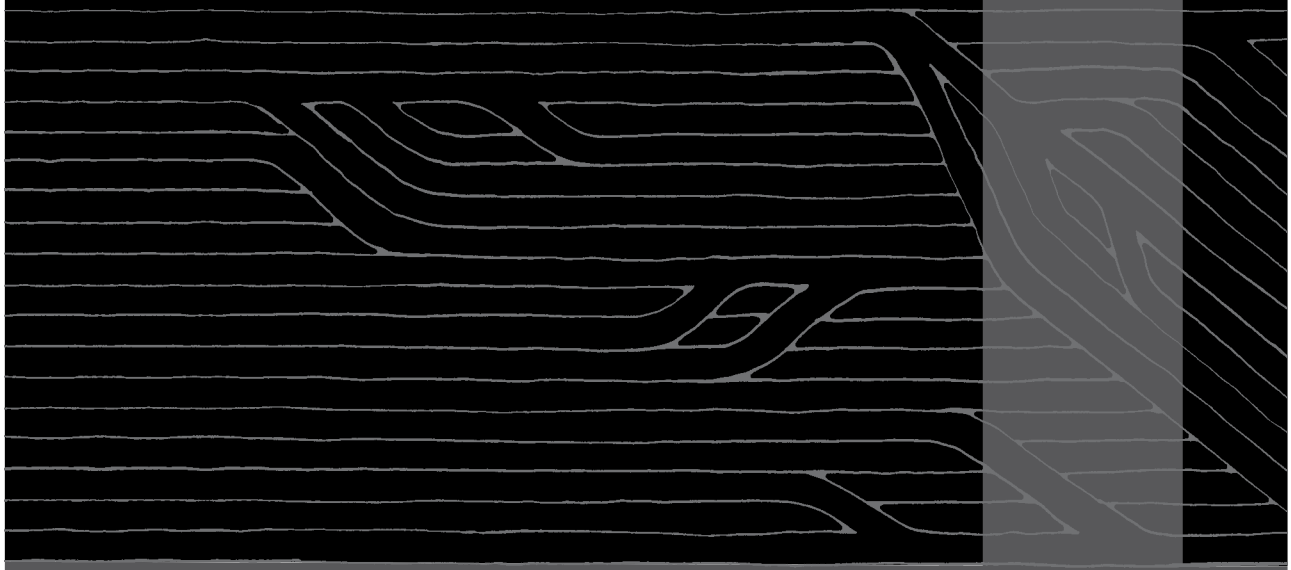
Chapter 1

General Introduction



BACKGROUND

The maximum chance of a clinically recognised pregnancy given a menstrual cycle is only 10-15%, even when conditions are optimal. Increasing evidence points to implantation failure rather than conception failure as the reason for the relatively low fecundity observed in human species [Coulham 1991; Dickey *et al.,* 1994; Macklon *et al.,* 2002; Simpson *et al.,* 1994; Wang *et al.,* 2003; Wilcox *et al.,* 1988]. The success of implantation and of the embryo-maternal interaction depends on a well vascularised, receptive endometrium [Zygmunt *et al.,* 2003]. The generation of receptive endometrium starts during the secretory phase of the menstrual cycle, continues throughout the first-trimester and includes decidualisation, angiogenesis, and immune cell invasion. [Bulmer *et al.,* 1991; Giudice 1999; Lessey 2000; Salamonsen *et al.,*2002/2003; Smith 2000]. The invasion of immune cells is enormous: from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. [Bulmer *et al.,*1991]. Vascular adaptation includes (pseudo-) vasculogenesis, arterial remodelling, and angiogenesis, the formation of new vessels out of existing ones [Burton *et al.,* 1999, Pijnenborg *et al.,* 1983].
Disturbances in vascular development may play a role in the pathogenesis of pregnancy complications, such as miscarriage, pre-eclampsia, intrauterine growth restriction, and even during adulthood, i.e. cardiovascular disease [Barker *et al.,* 1993; Torry *et al.,* 2004; Vailhe *et al.,* 1999; Vuorela *et* 2000; Zygmunt *et al.,* 2003]. This thesis assessed the role of angiogenesis in cycling endometrium and during human implantation.

THE INCEPTION OF PREGNANCY

Endometrium

The uterine mucosa, or endometrium, goes through cyclic breakdown and regeneration throughout reproductive life. It is composed of two distinct layers; the functionalis layer, the upper two third layer which is shed and renewed monthly during reproductive life, and the basalis layer, the lower one third layer representing the germinal layer from which renewal occurs [Li *et al.,* 1994].
The cyclical process is regulated by changes in circulating levels of oestradiol and progesterone. Morphologic alterations are particularly evident in the functionalis layer and only minimal in the basalis layer [Mutter, Ferenczy 2002]. After menstruation, the functionalis layer is regenerated by proliferation of endometrial glands, stromal and endothelial cells in response to oestradiol. This phase is called the proliferation phase and will last for approximately 14 days. Once the ovarian follicle has matured, ovulation occurs and this preludes the post ovulation period or secretory phase. Progesterone, mainly produced by the corpus luteum, will cause precisely controlled changes in the oestradiol -primed

endometrium in preparation for blastocyst implantation. For instance, the glands display increased lumen and change secrete production. In addition, the stromal compartment becomes more prominent and is characterised by oedema, invasion of leucocytes, and cuffing of vessels. These morphological changes are known as pre-decidualisation and even occur in the absence of fertilisation [Aplin 2000; Lessey 2003]. When fertilisation fails, the corpus luteum degenerates and progesterone levels diminish. This causes breakdown and shedding of the functionalis layer during the menstrual phase [Li *et al.,* 1994; Mutter, Ferenczy 2002].

Decidua

Additional decidualisation occurs in the presence of pregnancy and slowly converts secretory endometrium into decidua. Several subtypes of decidua are described in the first-trimester. Decidual secretory endometrium (DSE) is only pre-decidualised and will develop into decidua parietalis (DP) under influence of pregnancy-induced hormones, i.e. progesterone, oestradiol and hCG. Decidua basalis (DB) will arise in the additional presence of the extravillous trophoblast (EVT, Figure 1).

The additional decidualisation results in further tissue remodelling, increased vascular permeability, oedema, proliferation and differentiation of stromal cells, invasion of leukocytes, and vascular remodelling [Aplin 2000; Salamonsen *et al.,* 2003]. Stromal cells transform from small spindle-shaped cells into large decidual round cells and display an increased production of secreted proteins and extra cellular matrix proteins. This may function in facilitating migration of EVTs towards spiral arteries [King 2000; Trundley,Moffett 2004]. Epithelial glands decrease in density, generate smaller amounts of secrete and have a more silent appearance. Furthermore, arteries become extensively remodelled and the length and size increase because of proliferation of endothelial and elongation rather then conventional sprouting angiogenesis [King 2000; Trundley, Moffett 2004].

Implantation and trophoblast invasion

Implantation is only facilitated during the narrow window of 7 to 10 days after ovulation, the "implantation window" . Initiation of implantation is due to an active biochemical process that requires interaction between the implanting blastocyst and the endometrial epithelium [Aplin 2000]. A variety of different molecules, e.g. prostaglandins, proteases, cytokines and growth factors, secreted by human trophoblast as well as endometrial cells regulate this "crosstalk" and allow apposition, attachment and invasion of the blastocyst [Guidice 1999; Krussel *et al.,* 2003; Nardo *et al.,* 2003; Salamonsen 2002; van der Weiden *et al.,* 1991]. Moreover, hCG production by the conceptus announces the presence of fertilisation to the maternal system [Reshef *et al.,* 1990].

Several days after fertilisation the embryo differentiates into a blastocyst, which contains an inner cell mass that will from the embryo and an outer trophectoderm that will become placenta and chorion. Attachment of the blastocyst to the uterine wall triggers the differentiation of trophectoderm into two layers: an inner cytotrophoblast layer and an outer syncytiotrophoblast layer. After attachment, the cytotrophoblast proliferates into buds which protrude through the syncytium. These protruding cytotrophoblasts become either villous or extra-villous trophoblasts (EVT). The first covers the chorionic villi and interacts with maternal blood in the intervillous space thus providing an exchange barrier between mother and foetus. The later invades into the decidua. Some EVT's, called endovascular trophoblasts, migrate into maternal capillaries to replace the endothelium [Burrows *et al.,* 1996; Norwitz *et al.,* 2001; Red-Horse *et al.,* 2004]. EVTs invade up to the myometrium; far enough to access a viable maternal blood supply but not so far that the mother is endangered.

Immune cell invasion

Immune cells infiltrate post-ovulatory endometrium. In the absence of pregnancy their number declines during menstruation, whereas in the presence of fertilisation, their number increases up to the 20th week of gestation [van den Heuvel *et al.,* 2005a]. In the

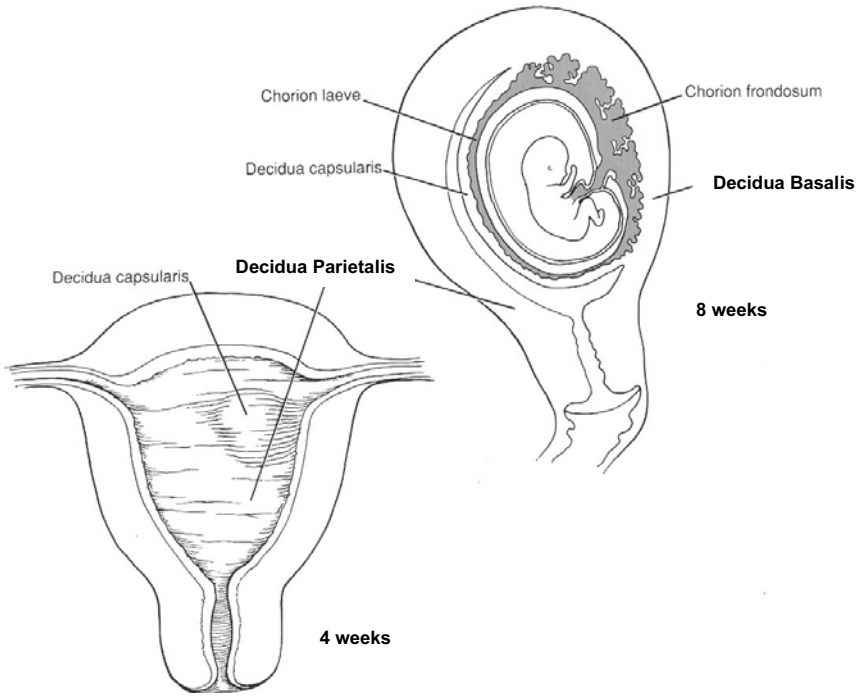


Figure 1. Schematic representation of the different types of decidua in first-trimester pregnancy.

first-trimester 30% of stromal cells are leucocytes; 75% of these leucocytes are uterine natural killer (uNK) cells and 10% are macrophages [Bulmer *et al.,* 1991].
Migration of uterine natural killer (uNK) cells is thought to be regulated both directly and indirectly by hormones, via oestrogen receptor ERβ1 and via chemo-attractants produced by hormone-stimulated stromal cells [Henderson 2003; King *et al.*,1996]. Various growth factors and cytokines are expressed by uterine natural killer (uNK) cells, including angiogenic factors and proteases [Al-Atrash *et al.,* 2001; Albertsson *et al.,* 2000; Hanna *et al.,* 2006; Lash *et al.,* 2006; Li *et al.,* 2001a].
Possible functions of uterine natural killer (uNK) cells include controlling the invasion of trophoblast cells, regulate vessel stability (via IFNγ), decidualisation of endometrium, induce EVT apoptosis, regulate angiogenesis and immunomodulation [Bulmer, Lash 2005; Dosiou, Giudice 2005; Hanna *et al.,* 2006; King *et al.,* 1998; Quenby, Farquharson 2006]. A correlation with failure of implantation is inconsistently described. Women with recurrent miscarriages were shown to have elevated numbers of uterine natural killer (uNK) cells in peri-implantation endometrium compared to controls [Clifford *et al.,* 1999; Quenby *et al.,* 1999; Tuckerman *et al.,* 2007]. However, others have shown comparable numbers of uterine natural killer (uNK) cells in the same setting and in decidua of missed abortions compared to controls [Michimata *et al.,* 2002; Shimada *et al.,* 2004/2006].
Macrophages are immunosuppressive cells in human first-trimester decidua, probably mediated by prostaglandin E2 production [Parhar *et al.,* 1989] and play a role in controlling placenta growth. Their content of lysosomal enzymes could indicate phagocytic functions [Bulmer *et al.,* 1988].

Miscarriage

Only 30-50% of conceptions result in the birth of a child. Most pregnancies fail even before the next menstrual date is due [Macklon *et al.,* 2002; Rai, Regan 2006]. About 10-15% of the recognised pregnancies end in a miscarriage, 90-95% of which will occur before foetal cardiac activity has been detected [Kavalier 2005; Wang *et al.,* 2003]. Approximately half of all miscarriages will evacuate spontaneously as a spontaneous abortion. The other miscarriages remain in utero until noticed by ultra sound, the so-called missed abortions. Aetiological categories of miscarriages can be divided in embryo-related and maternal-related causes. The most likely cause of the first category are the chromosomal abnormalities. Maternal-related causes include uterine, endocrinological, immunological or thrombotic disorders [Coulham 1991; Kutteh 1999]. Few studies have addressed decidual and placental vascularisation in relation to miscarriage. Deficient villous vascularisation, differential decidual vascularisation, increased blood flow, and differential VEGF-A expression have been shown to be correlated with miscarriage [Greenwold *et al.,* 2003; Jauniaux, Burton 2005; Lisman *et al.,* 2004; Meegdes *et*

al., 1988; Vailhe *et al.,* 1999; Vuorela *et al.,* 2000]. Even abnormal uterine circulation in receptive phase of non-conception cycles has been shown in recurrent abortion patients [Habara *et al.,* 2002].

ANGIOGENESIS

General Angiogenesis

Under certain circumstances, the vascular network needs to adapt, expand and remodel to adjust to changing conditions. To that end, angiogenesis is induced. This process occurs only in few physiological conditions, i.e. the ovary, endometrium and placenta, and in various pathological conditions, such as tumour growth. Angiogenesis involves activation and proliferation of endothelial cells, degradation of their basal membrane, migration through the surrounding extracellular matrix (ECM), and finally stabilisation and maturation of vessels. Angiogenesis is a complex process, which is tightly regulated by angiogenic promoters and inhibitors. In quiescent tissue, promoters and inhibitors are in balance. During an episode of vessel growth, the balance tips in favour of the promoters.

Endometrial angiogenesis

Uterine blood supply is facilitated by the uterine arteries, which give rise to arcuate and radial arteries supplying the basal layer of the endometrium. Endometrial angiogenesis is mandatory to support the reconstruction of the endometrium after menstruation and to provide a vascularised, receptive endometrium for implantation and placentation [Gargett, Rogers 2001; Weston, Rogers 2000].

Endometrial angiogenesis appears spatially and transiently regulated during the cycle. Endothelium in the superficial layer of the endometrium shows cyclical variation in proliferation and angiogenic activity. However, due to the lack of correlation between vascular events, e.g. endothelial cell migration and proliferation, the timing of angiogenesis during the cycle remains unclear [Goodger, Rogers 1995]. Despite these reservations, previous studies have described the existence of three angiogenic episodes during the cycle.

The first episode occurs during the early proliferative phase representing post-menstrual repair; the second occurs during the late-proliferative phase under the influence of oestrogen; and the third during the secretory phase under the influence of progesterone [Ferenczy *et al.,* 1979; Goodger, Rogers 1995; Maas *et al.,* 2001; Rogers, Gargett 1998; Smith 2000]. However, no significant differences in endothelial cell proliferation were detected throughout the cycle [Goodger, Rogers 1994]. Furthermore, proliferating endothelial cells were mainly present in existing vessels rather than in vascular sprouts.

These findings suggest that endometrial angiogenesis is a continuing process throughout the cycle and that it proceeds by elongation and intussusceptions rather than by classical angiogenesis via sprout formation (Figure 2) [Gambino *et al.,* 2002; Rogers, Gargett 1998].
Although the overall control of endometrial growth and regression is regulated primarily by oestrogen and progesterone, the role of sex steroids in endometrial angiogenesis is less clear. Several reports describe the expression of progesterone and oestrogen receptors in endometrial cells, including endothelial cells, but their conclusions have not been decisive [Critchley *et al.,* 2001; Iruela-Arispe *et al.,* 1999; Krikun *et al.,* 2005; Rey *et al.,* 1998]. The general idea is that oestradiol and progesterone are able to regulate endometrial angiogenesis probably both directly and indirectly via locally produced angiogenic factors [Bausero *et al.,* 1998; Iruela-Arispe *et al.,* 1999; Kapiteijn *et al.,* 2001; Kayisli *et al.,* 2004; Perrot-Applanat *et al.,* 2000; Salamonsen 1994; Shifren *et al.,* 1996].
Overall, neither the timing of vascular growth during the menstrual cycle nor the mechanisms by which endometrial vessels are formed are currently understood, thus placing major limitations on our understanding of how angiogenesis promoters and inhibitors may act in the endometrium [Rogers, Gargett 1998].

Decidual angiogenesis

Successful pregnancy requires the development of a complex network that facilitates the maternal-foetal exchange. Human maternal vascular adaptation to implantation starts during the secretory phase and continues throughout the first-trimester. This process includes the induction of angiogenesis, vasculogenesis, vascular permeability, and arterial remodelling [Burton *et al.,* 1999; Pijnenborg *et al.,* 1983; Smith 2000]. These complex processes involve various cell types, including immune cells and stromal fibroblasts, which locally regulate the expression of mitogenic and angiogenic growth factors and cytokines [Sherer, Abulafia 2001]. Furthermore, oestradiol, progesterone and hCG also play a role. For example, hCG produced by the blastocyst has been shown to be an angiogenic factor itself [Zygmunt *et al.,* 2002].
Arterial remodelling involves swelling of endothelial cells, arterial dilatation and remodelling of the muscular walls. Regulation of arterial remodelling is poorly understood. Craven *et al.,* described these modifications to be a maternal response to pregnancy, since this remodelling occurred independently of the presence of trophoblast invasion, [Craven *et al.,* 1998]. In contrast, others have stated that the true physiological change, which involves medial necrosis and replacement with fibrinoid material, only occurs in the presence of interstitial trophoblast, thus in the deciduas basalis [Kam *et al.,* 1999; Pijnenborg 1998]. In addition, veins appear to be remodelled as well during early pregnancy, resulting in dilatation and intravenous fibrin depositions in association with trophoblasts [Craven *et al.,* 2002].

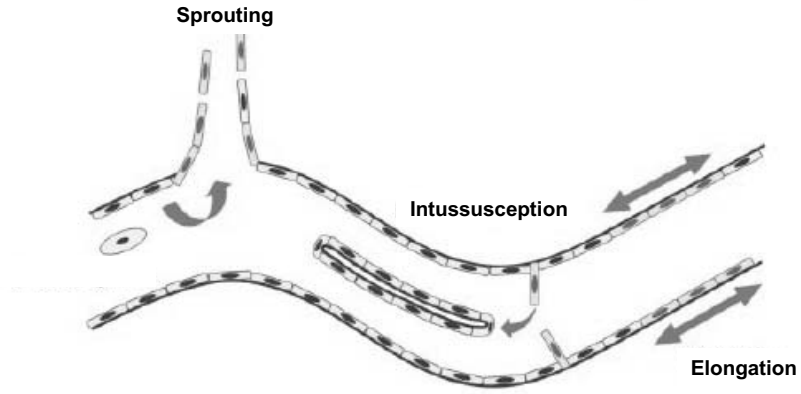


Figure 2. Endometrial angiogenesis proceeds by elongation and intussusception rather than by classical sprout formation. Adapted from Rogers 1998.
See colour figures supplement

An important feature during vascular development in the first-trimester is the invasion of maternal vessels by endovascular trophoblasts. This invasion results in replacement of maternal endothelial cells and plugging of spiral arteries [Pijnenborg *et al.,* 1983]. Partly because of these plugs, the maternal circulation to the placenta is restricted before the 8th week of pregnancy. Maternal blood flow will gradually extend over the next few weeks and by the 12th week blood flow to the inter-villous space is completely established [Burton *et al.,* 1999; Jaffe, Woods 1993; Jauniaux *et al.,* 2000]. Before the 10th week the uterine glands provide nutrition to the embryo. As a result, the human uteroplacental unit development takes place in a relatively low-oxygen environment during most of the first-trimester. Also a burst of oxidative stress occurs after establishment of maternal circulation [Burton *et al.,* 1999; Charnock-Jones *et al.,* 2004; Jauniaux *et al.,* 2000]. Moreover, the arterial plugging may protect the embryo from forceful maternal blood flows and oxygen overload [Burton *et al.,* 1999; Jaffe, Woods 1993; Jauniaux *et al.,* 2000; Kingdom, Kaufmann 1997].
Placental and uterine oxygen levels are spatially regulated as gestation progresses and regulate placental and decidual vascularisation by influencing the production of angiogenic factors [Charnock-Jones *et al.,* 2004; Jauniaux *et al.,* 2000; Sharkey *et al.,* 2000; Shore *et al.,* 1997]. Several reports state that first-trimester is characterised by both vasculogenesis and branching angiogenesis, while the second-trimester mainly displays branching angiogenesis and the third-trimester non-branching angiogenesis and that these series of events are probably regulated by oxygen [Charnock-Jones *et al.,* 2004; Geva *et al.,* 2002].
The low resistance arteriolar system results in pouch-like vessels, which are unresponsive to maternal vasomotor control. The lack of autoregulation of placental blood flow

allows dramatic increase in blood supply required to serve the growing demands of the foetus [Brosens *et al.,* 1967; Greiss *et al.,* 1976]. Limitations of this blood supply may have adverse clinical effects, like intra uterine growth restriction and pre-eclampsia [Khong *et al.,* 1986].

ANGIOGENIC FACTORS

VEGF family

The best known group of angiogenic factors is the vascular endothelial growth factor (VEGF) family, which consists of five mammalian members: placental growth factor (PlGF) and VEGF-A, VEGF-B, VEGF-C, VEGF-D. VEGF-A and PlGF are the most interesting factors of this family with regard to endometrium and decidua (Figure 3).

VEGF-A modulates the expression of many genes, enhances vascular permeability, induces endothelial cell proliferation, regulates apoptosis and plays an important role in the regulation of angiogenesis [Ferrara 2004; Hoeben *et al.,* 2004]. There are two receptors for VEGF-A; VEGF-R1 (flt-1) and VEGF-R2 (KDR) (Figure 3). Binding of VEGF-A to KDR induces proliferation and migration of endothelial whereas binding to flt-1 causes migration but not proliferation. In this way, VEGF-A induced endothelial proliferation and apoptosis can be regulated by changes in endothelial expression levels of KDR and flt-1 [Hoeben *et al.,* 2004].

Regulators of VEGF-A expression are the steroid hormones oestrogen and progesterone [Classen-Linke *et al.,* 2000; Hyder,Stancel 1999; Perrot-Applanat *et al.,* 2000]. Especially VEGF-A mRNA expression by endometrial carcinoma cell lines and stromal cells were found to be sensitive to steroidal stimulation [Charnock-Jones *et al.,* 1993; Shifren *et al.,* 1996]. Another stimulator of VEGF expression is hypoxia [Ferrara 2004; Sharkey *et al.,* 2000].

VEGF-A expression has been studied recurrently in endometrium. Various splice variants were detected, namely $VEGF_{206}$, $VEGF_{189,}$ $VEGF_{183,}$ $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$, of which $VEGF_{165}$, $VEGF_{145}$ and $VEGF_{121}$ are dominantly present in endometrium (Figure 3) [Krikun *et al.,* 2004a; Sherer,Abulafia 2001]. Data regarding the cyclical expression of VEGF-A were not always in agreement [smith 1998]. Several reports describe an increased glandular expression in the secretory phase and an increased stromal expression during the proliferative phase [Charnock-Jones *et al.,* 1993; Moller *et al.,* 2001; Shifren *et al.,* 1996]. Others did not detect variations in epithelial or stromal VEGF expression or in VEGF-A secretion by endometrial explants throughout the cycle [Gargett *et al.,* 1999; Sugino *et al.,* 2002]. Strongest immunoreactivity of VEGF-A on endothelial cells was detected in late proliferative and secretory phases and correlated with the presence of KDR and flt-1 [Bausero *et al.,* 1998].

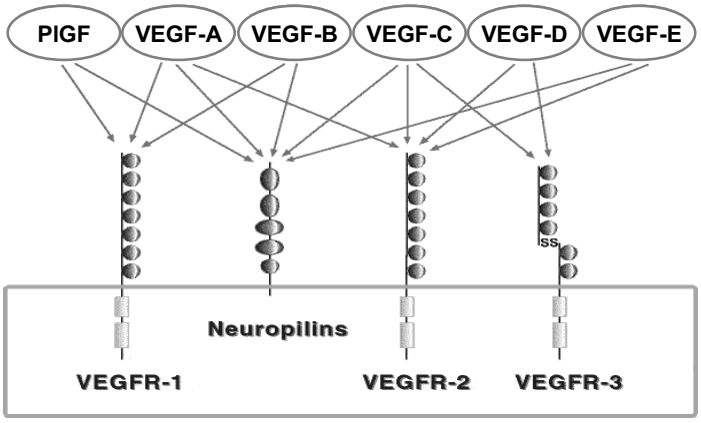


Figure 3. The VEGF family members and their receptors. Adapted from http://ethesis.helsinki.fi/ julkaisut/mat/bioti/vk/jeltsch/14revie7.html.

KDR and flt-1 were mainly found in endometrial endothelial cells and KDR also in glands throughout the cycle [Krussel *et al.,* 1999; Meduri *et al.,* 2000; Moller *et al.,* 2001; Sugino *et al.,* 2002]. Although the abundant VEGF-A expression and the endothelial expression of VEGF-A receptors suggest a role in endometrial angiogenesis, the relation between VEGF, receptor activation and endothelial cell proliferation during the cycle is poorly understood.

Several studies have reported expression of VEGF-A and its receptors in early pregnancy decidua. Abundant levels of VEGF mRNA were detected at the site of implantation and lower levels elsewhere in the decidua [Clark *et al.,* 1996; Sharkey *et al.,* 1993]. VEGF-A, flt-1 and KDR proteins have been detected in maternal decidual, epithelial and endothelial cells [Clark *et al.,* 1996/1998a; Sharkey *et al.,* 1993; Sugino *et al.,* 2002]. VEGF-A proteins were also expressed by maternal macrophages, (syn)cytotrophoblast, and extravillous trophoblasts [Clark *et al.,* 1996; Cooper *et al.,* 1995; Jackson *et al.,* 1994]. VEGF, via its receptor flt-1, appears to play an active role in trophoblast invasion and angiogenesis during human and rhesus monkey implantation [Ahmed *et al.,* 1995; Clark *et al.,* 1996; Cooper *et al.,* 1995; Krikun *et al.,* 2004a; Sharkey *et al.,* 1993; Sengupta *et al.,* 2007; Sugino *et al.,* 2002; Torry,Torry 1997].

PlGF shares biochemical and functional features with VEGF and interacts with VEGFR-1 (Flt-1). PlGF and VEGF-A have synergistic effects regarding angiogenesis, but PlGF-induced vessels are more mature and stable than VEGF-induced vessels [Carmeliet *et al.,* 2001; Luttun *et al.,* 2002]. In contrast with VEGF, low oxygen tension results in reduced PlGF expression in trophoblasts *in vitro* [Shore *et al.,* 1997].

PlGF is abundantly expressed in human placenta, rising from the first-trimester to the late second-trimester and subsequently declining from 30 weeks of gestation to delivery

[Torry *et al.,* 1998]. PlGF mRNA is expressed in villous and extra-villous trophoblast cells while the protein is detected in vascular endothelium of term placental tissue [Clark *et al.,* 1998a; Jackson *et al.,* 1994; Vuorela *et al.,* 1997]. In addition, intense staining for PlGF antigens is detected in decidual stromal cells [Khalig *et al.,* 1996]. The receptor of PlGF, flt-1, is expressed on endothelial cells, perivascular smooth muscle cells and (extravillous) trophoblast during pregnancy [Athanassiades, Lala 1998; Torry *et al.,* 2004; Vuorela *et al.,* 1997]. PlGF may, via flt-1, act as a regulator of decidual angiogenesis and an autocrine mediator of trophoblast function [Khalig *et al.,* 1996; Sherer,Abulafia 2001; Torry *et al.,* 2004].

Angiopoietin family

Angiopoietin-1 (Ang-1), Angiopoitein-2 (Ang-2) and their receptor TIE-2 are known for their involvement in angiogenesis. The two ligands bind with equal affinity to TIE-2 but have different functions. Ang-1maintains vessel integrity, decreases vascular permeability and plays a role in endothelial and vascular maturation after VEGF-induced neovascularisation [Geva,Jaffe 2000]. Transgenic overexpression of Ang-1 in mice results in the development of more complex vascular networks [Suri *et al.,* 1998]. Ang-2 is a functional antagonist of Ang-1 and is only expressed at sites of vascular remodelling. Ang-2 leads to loosening of cell/cell interactions and allows access to angiogenic inducers like VEGF [Maisonpierre *et al.,* 1997]. Co-expression of VEGF and Ang-2 induces angiogenesis and increased vascular permeability, but Ang-2 results in vascular regression in the absence of angiogenic signals [Asahara *et al.,* 1998]. Hypoxia regulates both Ang-1 and Ang-2, i.e upregulates Ang-2 and destabilises Ang-1 [Geva,Jaffe 2000; Zhang *et al.,* 2001].

Ang-1 is widely expressed in the adult, whereas Ang-2 is selectively expressed at sites of active angiogenesis, like the uterus and placenta [Maisonpierre *et al.,* 1997]. In endometrium, both Ang-1 and Ang-2 were detected in glands, stromal cells, and endothelium [Hewett *et al.,* 2002; Krikun *et al.,* 2000]. A significant upregulation in the late secretory phase has been described for Ang-1, whereas Ang-2 and TIE-2 showed only minor variations during the cycle [Hirchenhain *et al.,* 2003]. TIE-2 was mainly detected in endothelium and glands and only small amounts were found in stromal cells [Hewett *et al.,* 2002; Krikun *et al.,* 2000].

Ang-1 and -2 and TIE-2 are detected in human first-trimester decidua; TIE-2 mainly in maternal endothelial cells, endovascular trophoblasts, and (syn-) cytotrophoblasts and Ang-1 and -2 mainly in the latter. These findings suggest an additional role for angiopoietins, besides their role in angiogenesis, in regulating trophoblast behaviour in the development of uteroplacental circulation [Dunk *et al.,* 2000; Goldman-Wohl *et al.,* 2000; Zhou *et al.,* 2003]. The angiopoietins are regulated as gestation progresses: Ang-2 is maximally present in the first-trimester and declines thereafter, whereas Ang-1 increases from first- to third-trimester. This suggests that Ang-2 is mainly involved in first-trimester

vasculogenesis and branching angiogenesis and Ang-1 in third-trimester non-branching angiogenesis [Geva *et al.,* 2002]. An association of the angiopoietins with miscarriage has not been described but reduced endothelial TIE-2 expression has been linked to the occurrence of miscarriage [Vuorela *et al.,* 2000].

PERICELLULAR PROTEASES

Proteolysis plays a pivotal role in the regulation of angiogenesis and placental development [Heymans *et al.,* 1999; Pepper 2001a/2001b; Salamonsen 1999; Solberg *et al.,* 2003; Stetler-Stevenson 1999]. Key players in pericellular proteolysis are the uPA/plasminogen system and the membrane-type matrix metalloproteinases (MT-MMPs) [Alfano *et al.,* 2005; Kindzelskii *et al.,* 2004; Koolwijk *et al.,* 2001; Reuning *et al.,* 2003].

uPA/plasminogen system

The plasminogen activator (PA) system is based on the protease plasmin, which cleaves most ECM components. The circulating plasminogen is converted into the active protease plasmin by either tissue-type plasminogen activator (tPA) or urokinase-type plasminogen activator (uPA). tPA is mainly involved in clot dissolution, whereas uPA mediates pericellular proteolysis during cell migration, tissue remodelling and angiogenesis [van Hinsbergh *et al.,* 2006]. uPA binds a specific cell-surface receptor, uPAR, which restricts the uPA-activity to the cell environment and enables activation of plasmin directly on the cell surface.

The activity of uPA is regulated by at least two specific serine proteinase inhibitors, plasminogen activator inhibitor type-1 and -2 (PAI-1 and 2), of which PAI-1 is expressed on endothelium and PAI-2 on monocytes and trophoblasts [Spengers, Kluft 1987]. Furthermore, the presence of uPA is not only determined by the ability of the cells to produce uPA, but also by their content of uPAR, which binds and internalises uPA in complex with its inhibitor PAI-1 [Blasi, Carmeliet 2002; Kroon *et al.,* 1999].

The role of uPA mediated plasminogen activation in cell migration has been studied for a variety of cells and for endothelial cells, leucocytes, and trophoblasts in particular [Blasi *et al.,*1987; Heymans *et al.,* 1999; Hu *et al.,* 1999; Pepper 2001a; Reuning *et al.,* 2003; Salamonsen *et al.,* 2003]. The uPA expression is low in resting endothelial cells, whereas its expression is more abundant during angiogenesis and inflammation. This may explain the rich expression of uPA in endometrium endothelial cells [Koolwijk *et al.,* 2001]. Furthermore, also stromal cells contained uPA antigen, but glandular epithelium did not [Koolwijk *et al.,* 2001]. Both uPA and uPAR expression has been detected in the invasive trophoblast cells, which indicates a role for uPA and uPAR play in trophoblast

invasion [Floridon *et al.,* 1999; Hofmann *et al.,*1994; Hu *et al.,* 1999; Multhaupt *et al.,* 1994; Pierleoni *et al.,* 1998; Salamonsen 1999].

Membrane-type Matrix metalloproteinases

The matrix metalloproteinases are a still expanding family of zinc-requiring enzymes that play a role in matrix remodelling and cell-matrix interactions. The membrane-type MMPs consist of six proteolytic enzymes which are particularly suited to function in pericellular proteolysis because of their membrane-associated localization [Hotary *et al.,* 2000; Visse,Nagase 2003]. The MT-MMPs incorporated unique domains which anchor them into the cell membrane. Four MT-MMPs are transmembrane proteins, namely MT1-, MT2-, MT3- and MT5-MMP (MMP-14, -15, -16 and 24) and two are GPI-anchored, MT4- and MT6-MMP (MMP-17 and -25) [Visse,Nagase 2003]. We studied the transmembrane-spanning MT-MMPs, MT1- (MMP-14), MT2- (MMP-15), MT3- (MMP-16) and MT5-MMP (MMP-24), since these MT-MMPs induce capillary tube formation and their GPI-anchored counterpart MT4- and MT6-MMP was unable to do so [Hotary *et al.,* 2000].

MT1-MMP is the best known MT-MMP and is involved in degradation of ECM components, cell migration, and generating bioavailability of growth factors [Collen *et al.,* 2003; Galvez *et al.,* 2001; Hiraoka *et al.,* 1998]. MT1-MMP facilitates MMP activity by activation of pro-MMP-2 and pro-MMP-13. MT1-MMP has received considerable attention as being involved in tumour angiogenesis and has been shown to be able to induce angiogenesis [Hotary *et al.,* 2000; Seiki,Yana 2003; Sounni *et al.,* 2002]. MT2-MMP and MT3-MMP are also involved in cell migration and invasion. Furthermore, their overexpression in endothelial cells can induce capillary-tube formation, similar to MT1-MMP. MT1-, MT2- and MT3-MMP mRNA levels increase during tube formation *in vitro* [Hotary *et al.,* 2000; Lafleur *et al.,* 2002; Shofuda *et al.,* 2001]. The function of MT5-MMP is less well studied. MT5-MMP has a gelatinolytic effect and influences axonal growth and embryonic brain development [Llano *et al.,* 1999; Pei *et al.,* 1999].

MMP activity is modulated by growth factors, cytokines, plasmin, steroid hormones and several activated MMPs [Visse,Nagase 2003]. Specific inhibitors are four tissue inhibitors of metalloproteinases (TIMPs) [Gomez *et al.,* 1997; Greene *et al.,* 1996]. TIMP-1 inhibits the activity of all MMPs except MT1-MMP. TIMP-2 acts specifically on MMP-2 and its expression usually follows that of MMP-2. TIMP-3 is anchored in the matrix and inhibits all MMPs, including MT1-MMP, and has additional properties of stimulating cell growth and inducing apoptosis [Will *et al.,* 1996; Woessner 2001]. Finally, TIMP-4 functions in a more tissue specific manner as it is highly expressed in cardiac tissue [Greene *et al.,* 1996]. TIMP-1, -2 and -3 mRNA has been detected in humane endometrium, cytotrophoblasts and decidual endothelium and glandular epithelium during the first-trimester [Hurskainen *et al.,* 1996; Maatta *et al.,* 2000].

Members of the MMP family are widely expressed in the cycling endometrium and their expression and activity are sensitive to regulation by cytokines and to downregulation by progesterone [Freitas *et al.,* 1999; Lockwood *et al.,* 1998; Salamonsen *et al.,* 1997; Singer *et al.,* 1999; Zhang *et al.,* 2000]. Progesterone withdrawal during the late secretory phase leads to increased MMP levels, focal tissue degradation and menstrual bleeding [Lockwood *et al.,* 1998; Salamonsen *et al.,* 1997; Zhang *et al.,* 2000; Zhang, Salamonsen 2002]. The expression, regulation and function of MT-MMPs are studied in less detail. The expression of MT1-, MT2- and MT3-MMP mRNAs and proteins in endometrial tissue has been described in various cycle phases [Chung *et al.,* 2002; Goffin *et al.,* 2003; Maatta *et al.,* 2000; Nakano *et al.,* 2001; Zhang *et al.,* 2000]. Suggested functions of endometrial MT-MMPs include tissue remodelling during the proliferative phase and tissue degradation during menstruation [Curry,Osteen 2003]. The involvement of MT-MMPs in regulating endometrial angiogenesis is not clear, although the expression of MT2-MMP, and to a lesser extent of MT1-MMP, has been described in endometrium endothelium *in vivo* [Maatta *et al.,* 2000; Zhang *et al.,* 2000].
With regard to MT-MMPs in decidua, only MT1- and MT2-MMP have been studied. MT1- and MT2-MMP RNA and protein expression are described in decidual extracts, stromal cells, and the extra-villous trophoblast [Bjorn *et al.,* 1997/2000; Hurskainen *et al.,* 1998; Nakano *et al.,* 2001; Nawrocki *et al.,* 1996]. These MT-MMPs are assumed to regulate trophoblast invasion during implantation. Whether migration of other cell types, e.g. immune and endothelial cells, is also regulated by MT-MMPs remains to be seen [Salamonsen 1999].

OUTLINE OF THIS THESIS

The principal aim of this thesis is to assess the role of angiogenesis in cycling endometrium and during implantation. We hypothesise that angiogenesis is of paramount importance for the development of a receptive endometrium for implantation and therefore is a crucial contributor to the inception of pregnancy.
Pericellular proteolysis plays an important role in angiogenesis being required for endothelial cell migration, invasion and tube formation. **Chapter 2** focussed on regulation of *in vitro* endometrial angiogenesis by pericellular proteases, in particular MT1-MMP and MT3-MMP because of their demonstrated role in angiogenesis. We studied the expression of proteases by human endometrial microvascular endothelial cells (hEMVEC) and their involvement in the formation of capillary tubes. In **Chapter 3** we analysed the presence of MT-MMPs in human endometrium *in vivo* and their correlation with endometrial neovascularisation.

These first chapters provide data on angiogenesis in human endometrium, whereas the following chapters focus on angiogenesis in human decidua. The involvement of cytokines and angiogenic growth factors in angiogenesis during implantation was studied in **Chapter 4**, by determining the effect of IVF supernatants on angiogenesis by endometrial endothelial cells (hEMVEC) *in vitro*.

The vascularisation pattern and the decidual expression of angiogenic factors in human first-trimester pregnancies *in vivo* are described in **Chapter 5**. This study was performed in three first-trimester decidual tissues: decidual secretory endometrium (DSE), decidua basalis (DB) and parietalis (DP). By comparing these decidual tissues within subjects, the influences on vascularisation and expression of growth factors that occur independently of trophoblast invasion, i.e. induced by pregnancy-induced hormones, were separated from the influence of the invasive extra-villous trophoblast.

The presence of pericellular proteases in human first-trimester decidua was studied in **Chapter 6**. This study, like **Chapter 5**, is performed in three decidual tissues, DSE, DP and DB, to be able to determine the regulation of decidual pericellular proteases as well.

The hypothesized involvement of angiogenesis in the pathogenesis of a miscarriage was addressed in **Chapter 7**. First-trimester decidual tissues of miscarriages and matched controls were compared with regard to vascularisation and the expression of angiogenic growth factors and proteases.

Chapter 8, a preliminary report, focused on the role of first-trimester angiogenesis in the pathogenesis of pre-eclampsia and/or intrauterine growth retardation. First-trimester decidua obtained during chorion villous biopsies. The included patients were followed throughout their pregnancy to be able to relate the expression of the angiogenic factors to the pregnancy outcome, ie uncomplicated, pre-eclampsia or intrauterine growth retardation.

In **Chapter 9**, results from above mentioned studies are discussed and future perspectives presented. **Chapter 10** provides the conclusions of the thesis. Finally, **Chapter 11** summarises all presented studies and provides a dutch summary.