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

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Chapter 9

General Discussion

Pregnancy demands significant physiologic adaptations, including vascular remodelling, of the uterine environment to facilitate adequate implantation. The timing of angiogenic processes is of paramount importance for the development of a receptive endometrium suitable for implantation. Disturbances in uterine blood supply are associated with first-trimester miscarriages and third trimester perinatal morbidity and mortality caused by pre-eclampsia (PE) and foetal growth restriction (FGR). This thesis assessed the role of angiogenesis in cycling endometrium and during human implantation. Furthermore, we attempted to evaluate the involvement of angiogenesis in the pathogenesis of missed abortions, PE and FGR.

ANGIOGENESIS AND PERICELLULAR PROTEASES IN CYCLING ENDOMETRIUM

Angiogenesis occurs on a regular basis as part of the growth and regression of the human endometrium during the menstrual cycle. There is evidence for multiple mechanisms of endometrial angiogenesis; angiogenesis during post-menstruation repair, elongative angiogenesis during the early and late proliferative phase and intussusception during the secretory phase. However, neither the timing of vascular growth during the menstrual cycle nor the mechanisms by which endometrial vessels are formed are currently completely understood, thus placing major limitations on our understanding of how angiogenesis promoters and inhibitors may act in the endometrium [Rogers,Gargett 1998]. With the development of *in vitro* systems mechanistic questions regarding the regulation of endometrial angiogenesis may become answered.

Previously, an *in vitro* model for endometrial angiogenesis was developed using human endometrial microvascular endothelial cells (hEMVEC) on fibrin and/or collagen 3D matrix. These cells have unique characteristics. They express high amounts of urokinase-type plasminogen activator (uPA), express various matrix metalloproteinases (MMPs) and membrane-type-MMPs (MT-MMPs), and display high angiogenic activity in response to vascular endothelial growth factor (VEGF). These characteristics are demonstrated in their *in vivo* counterparts as well [Chung *et al.,* 2002; Freitas *et al.,* 1999; Goffin *et al.,* 2003; Koolwijk *et al.,* 2001; Maatta *et al.,* 2000; Skinner *et al.,* 1999; Zhang *et al.,* 2000]. The combination of enhanced uPA expression and the high angiogenic activity directed us to determine the contribution of pericellular proteolysis to endometrial angiogenesis *in vitro*.

We demonstrated that both uPA/plasmin and MMPs contribute to the invasion and tubular structure formation of hEMVEC. Besides uPA, these cells express various MMPs, amongst which MT3-MMP (MMP-16) and MT4-MMP (MMP-17) and smaller amounts of MT1-MMP (MMP-14), MT2-MMP (MMP-15), MT5-MMP (MMP-24) and MT6-MMP (MMP-25). These data are in agreement with observations reported from immunohis-

tochemical studies in endometrial tissue [Chung *et al.,* 2002; Freitas *et al.,* 1999; Goffin *et al.,* 2003; Maatta *et al.,* 2000; Skinner *et al.,* 1999; Zhang *et al.,* 2000].
MT1-, MT2-MMP and MT3-MMP are known inducers of capillary-tube formation and their mRNA levels increase during tube formation *in vitro* [Hotary *et al.,* 2000; Lafleur *et al.,* 2002; Shofuda *et al.,* 2001]. Experiments with overexpression of MMP inhibitors (tissue inhibitor of matrix metalloproteinases (TIMP) -1 and -3), which are also expressed by endometrial endothelial cells *in vivo* [Freitas *et al.,* 1999; Maatta *et al.,* 2000; Rodgers *et al.,* 1994], indicated that hEMVEC utilise a unique pattern of MT-MMPs during angiogenesis compared to endothelial cells originating from other tissues [Collen *et al.,* 2003; Galvez *et al.,* 2001; Hotary *et al.,* 2002; Lafleur *et al.,* 2002]. Moreover, the relative high expressions of MT3-MMP mRNAs in hEMVEC, the presence of MT3-MMP protein on endometrial endothelial cells and the inhibition of capillary tube formation by inhibiting MT3-MMP are strongly in favour of a contribution of MT3-MMP in capillary-like tube formation by hEMVEC. This was a striking finding, since up to now MT1-MMP was thought to be the most important MT-MMP involved in angiogenesis [Pepper 2001; Seiki, Yana 2003; Sounni *et al.,* 2002; Stetler-Stevenson 1999].
Certainly, *in vitro* models comprise several limitations. For instance, our model uses fibrin/collagen matrices, whereas the endometrial ECM consists of several proteins, among which collagen, and its composition and the presence of fibrin varies during the menstrual cycle [Aplin *et al.,* 1988; Bulletti *et al.,* 1988; Iwahashi *et al.,* 1996; Okada *et al.,* 2001]. We attempted to mimic endometrial ECM by isolating ECM of cultured endometrial stromal cells (hESC). hEMVEC cultured on fibronectin and on hESC ECM for 24-96 hours displayed differential gene expression. For example, ECM cultured hEMVEC showed markedly induced expression of VE-cadherin, fibronectin, and SPARC (inhibits proliferation of endothelial cells). Alternatively, the MT-MMPs, TIMPs, uPA, uPAR, PAI-1 and CD31 showed less than 2-fold (re)inductions. Thus, the gene expressions of the proteases of interest were not considerably changed by culturing on fibronectin.

Only by a combination of mechanistic *in vitro* and observational *in vivo* or *in situ* studies, an overall picture can emerge. In view of the proposed angiogenic involvement of MT3-MMP, we were interested whether MT3-MMP was expressed in human endometrium *in vivo*. All MT-MMPs were expressed in human endometrium. The elevated expression of MT-MMP antigens during the late secretory and menstrual phase indicates a role in the shedding process during menstruation [Lockwood *et al.,* 1998; Salamonsen, Woolley 1996; Zhang *et al.,* 2000]. Several MT-MMPs also showed a reduced expression during the receptive window, probably under the influence of steroidal hormones, which suggest a contribution to the generation of a stable environment in preparation for embryonic implantation.

With regard to the endothelium, MT2-, MT3-, and MT4-MMP, but not MT1-MMP, were expressed by *in vivo* endothelial cells. The endothelial expression of MT3-MMP and the consistent absence of endothelial MT1-MMP are in accordance with previous *in vivo* and *in vitro* work, but no data regarding the cellular expression of MT3- and MT4-MMP have been reported prior [Zhang *et al.,* 2000; Maatta *et al.,* 2000]. Strikingly, endothelial MT2, and MT3-MMPs were expressed during the proliferative and late secretory phase, which both display high angiogenic activity [Ferenczy *et al.,* 1979; Goodger, Rogers 1995; Morgan *et al.,* 1996; Rogers, Gargett 1998; Smith 2001]. This confirms the suggested contribution of MT3-MMP to angiogenesis *in vitro*. Moreover, MT2-MMP might be an additional angiogenic regulator to consider [Hiraoka *et al.,* 1998; Hotary *et al.,* 2000; Lafleur *et al.,* 2002]. So overall, the pericellular proteases uPA, MT2- and MT3-MMP, but not MT1-MMP, were shown to be involved in endometrial angiogenesis.

CONTRIBUTORS TO THE FORMATION OF FIRST-TRIMESTER DECIDUA

The endometrium transforms during the secretory phase into a well vascularised receptive tissue in response to oestradiol and progesterone. Influx of uterine NK (uNK) cells, decidual remodelling and angiogenesis are dominating features in this stage and continue in the presence of fertilisation and implantation. The differential presence of hormones, extra-villous trophoblasts, and uNK cells results in the generation of different types of decidua; pregnancy-induced hormones are involved in the development of decidua parietalis (DP) from the decidual secretory endometrium (DSE), while the additional presence of the extra-villous trophoblast (EVT) induces the generation of the decidua basalis (DB).

The invasion of immune cells to the implantation site is enormous; from 8% of total stromal cells during the menstrual cycle up to 30% during the first-trimester. Approximately 70 % of these leucocytes are uNK cells and 10% are macrophages [Bulmer *et al.,* 1991]. Various growth factors and cytokines are expressed by uNK cells, including the angiogenic factors and proteases Ang-1, Ang-2, TIE-2, VEGF-c, PlGF, uPA, uPAR and MT1-MMP [Al-Atrash *et al.,* 2001; Albertsson *et al.,* 2000; Hanna *et al.,* 2006; Lash *et al.,* 2006; Li *et al.,* 2001]. Consequently, an influx of uNK cells and macrophages may influence angiogenesis, decidualisation and implantation.

For that reason, we evaluated the presence of these cells in the three types of decidua. uNK cells (CD56+) and macrophages (CD68+) were present in DSE, DP and DB. The percentage of uNK cells showed an increase in DP and DB compared to DSE, while a comparable increase in macrophages did not reach statistical significance.

The foregoing information is summarised in a model, which describes the influences of uNK cells, EVT and pregnancy-induced hormones on DSE, DP and DB (Figure 1). This

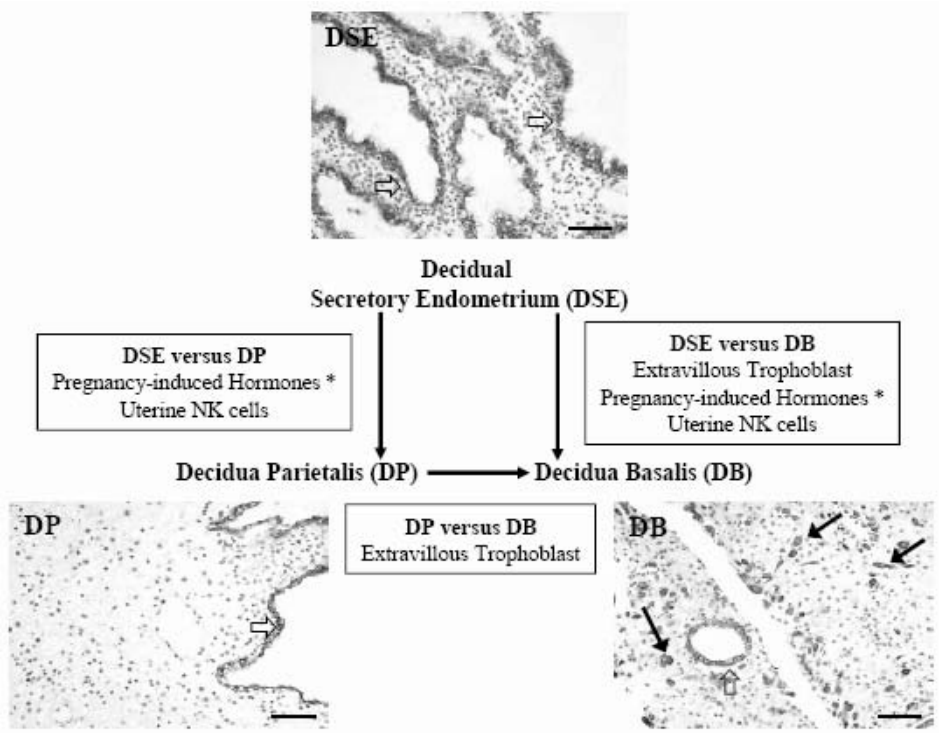

Figure 1. Schematic representation of the contributors to the formation of decidua parietalis and decidua basalis out of decidual secretory endometrium in the first-trimester of pregnancy. *) hCG, oestradiol and progesterone. DSE and DP express cytokeratin in (open arrows), whereas cytokeratin is also expressed in EVT (closed arrows) of DB. Bar =100 μm.
(see figure 5 of chapter 6 in colour figures supplement)

model enables hypothesising about vascular changes that occur independently of trophoblast invasion, and those that are mainly induced by pregnancy-induced hormones and/or uNK cells.

VASCULARISATION PATTERNS IN FIRST-TRIMESTER DECIDUA

Vascular adaptation to pregnancy is not well studied in human tissues and as a consequence, the regulation of decidual vascularisation is not clear. In rats, two separate mechanisms appear to control endothelial cell proliferation during implantation; a maternal control mechanism throughout the endometrium during the first days after fertilisation and a second post-implantation mechanism in the vicinity of the embryo [Goodger, Rogers 1993]. However, whether human decidual vascular adaptation to implantation is regulated by hormones, immune cells and/or the EVT and which mediators are used is unknown. In order to evaluate this, we determined the vascularisation

pattern and expression of angiogenic factors in decidual secretory endometrium (DSE), decidua basalis (DB) and decidua parietalis (DP).
Differences in vascularisation pattern, enhanced total vascular surface and luminal diameter and reduced vessel density, from decidual secretory endometrium (DSE) to decidua parietalis (DP) to decidua basalis (DB). This vascular adaptation appeared to occur under influence of pregnancy-induced hormones, uNK cells, and the EVT. The reduced vascular density at the implantation site has been described before in rats and was suggested to result from oedema [Goodger, Rogers 1993]. However, the vascular density still differed after correction for oedema. The increase in luminal diameter may represent an adaptation to increased blood flow or may be due to vasodilatation reported to occur prior to infiltration of endovascular trophoblasts [Charnock-Jones 2002; Huppertz, Peeters 2005]. Furthermore, increased vessel diameter could also be induced by a relatively low oxygen level, which has been shown previously for pregnancies at high altitude [Zhang *et al.,* 2002]. The enlargement of the total vascular surface in DB and DP has been reported previously and likely is functionally related to the increased blood supply required to serve the growing demands of the foetus. [Gibbons, Dzau 1994; Kam *et al.,* 1999].

ANGIOGENIC FACTORS IN FIRST-TRIMESTER DECIDUA

The differential vascular pattern in various decidual tissues enlarged our interest in vascular regulation. Endometrial vasculature already undergoes expansion during the pre-implantation period. Apparently the embryo prepares its implantation site by stimulating angiogenesis before any physical contact between embryo and endometrium has been made. We demonstrate that the human blastocyst is able to stimulate *in vitro* angiogenesis via the production of significant amounts of vascular endothelial growth factor-A (VEGF-A). Das *et al.,* reported comparable events during rabbit implantation [Das *et al.,* 1997a]. In humans, VEGF has been shown to be one of the earliest genes activated during pre-implantation embryo development [Krussel *et al.,* 2003]. Thus, VEGF-A appears to be a crucial contributor to the inception of pregnancy.
We attempted to further reveal whether VEGF-A and other angiogenic factors are mediators in the regulation of the described differential vascularisation. in the three types of decidua, DSE, DP and DB. VEGF-A was abundantly, but not differentially, expressed in all decidual tissues. KDR and flt-1 and VEGF proteins were expressed on endothelium of both DB and DP. Together, these findings confirm both the role of VEGF in decidual angiogenesis throughout implantation and the hypothesis of VEGF being an important factor during implantation.

Other mediators appeared to play their role more locally at the implantation site. The abundant induction of PlGF mRNA and proteins in decidua basalis in general and of PlGF and Flt-1 proteins on endothelial cells in particular was remarkable. PlGF, via Flt-1, is able to potently induce angiogenesis resulting in mature and stable vessels [Arroyo *et al.,* 2004; Carmeliet *et al.,* 2001; Khaliq *et al.,* 1996; Luttun *et al.,* 2002; Torry *et al.,* 1999]. These are important features in facilitating and resisting the dramatic increase in blood supply at the implantation site to serve the growing demands of the foetus. Furthermore, the Ang-2/Ang-1 ratio was elevated in decidua basalis (DB), which suggests that vessel destabilisation is favoured over vessel maturation in DB. Overall, the angiopoietins, via TIE-2, and PlGF, via Flt-1, appear to be key regulators of the vascular adaptive process and may, partially, account for the vascular changes at the site of embryonic implantation in decidua basalis.

Solely locating numerous angiogenic growth factors in human decidua presents a problem in understanding the coordination of local events in new vessel growth, as most of these factors display differing effects not necessarily related to angiogenesis. It must be remembered that the mere presence of a particular growth factor does not indicate functional availability and that further studies have to be performed to elucidate vascular regulation.

PERICELLULAR PROTEASES IN FIRST-TRIMESTER DECIDUA

Proteolysis is imperative during the adaptation to implantation since trophoblasts, uNK cells and endothelial cells require proteolytic activity to migrate. Key regulators of proteolysis belong to the family of matrix metalloproteinases (MMPs), in particular to the subgroup of membrane-type matrix metalloproteinases (MT-MMPs), and to the plasmin/plasminogen system [Alfano *et al.,* 2005; Kindzelskii *et al.,* 2004; Reuning *et al.,* 2003]. A synergistic effect of both systems on decidual vascularisation in mice has been shown by Solberg *et al*. MMP inhibition altered the differentiation of the decidua, whereas loss of both MMP and uPA/plasmin was necessary to alter decidual vascularisation and induce embryonic lethality [Solberg *et al.,* 2003]. In light of these observations, the PA/plasmin system and MT-MMPs were also evaluated in human decidua.

The expression of various pericellular-acting proteases varied between DSE, DB and DP. This differential expression enabled hypothesising about their functions as well as their regulation (Figure 1). Regarding their regulation, uPAR and MT1-MMP appeared regulated by pregnancy-induced hormones and/or uNK cells, whereas the presence of uPA, MT2-, MT3-, and MT5-MMP appeared regulated by the extra-villous trophoblast.

Possible decidual functions of the proteases may involve trophoblast invasion, immune cell migration, and /or vascularisation. All proteases were expressed by the EVT, which

suggests involvement in trophoblast invasion. Whether the proteases are involved in immune cell migration could not be answered from our data and is neither supported nor contradicted by the equal presence of immune cells in DB and DP. However, the expression of proteases by stromal decidual cells (our finding) and uNK cells enables both autocrine and paracrine influences on immune cell infiltration [Albertsson *et al.,* 2000; Kim *et al.,* 2000].
An interesting observation came into view with regard to their possible role in vascularisation. Endothelial and perivascular smooth muscle cell MT2- and MT3-MMP antigens were less abundantly present in DP and DB as compared to DSE. The endothelial amount of these MT-MMPs correlated well with the vessel density and correlated inversely with the luminal surface of vascular structures. This differential MT2- and MT3-MMP expression, together with the fact that MT2- and MT3-MMP are likely candidates for regulation of angiogenesis, suggests a role for MT2- and MT3-MMP in the regulation of vascularisation at the implantation site *in vivo*. Their reduced endothelial expression might indicate a control mechanism which prevents too vigorous endothelial migration and thereby too fast vascular remodelling. This has not been described previously. Whether MT-MMPs act as crucial contributors, or merely as trivial bystanders, to implantation remains to be elucidated.

ANGIOGENESIS, PROTEASES AND ANGIOGENIC FACTORS IN LATE FIRST-TRIMESTER PREGNANCY

As gestation progresses, vascular adaptation continues and is represented by decreased vessel density and a non-significant increased luminal surface in DB and DP. These vascular changes correlated with decreased total and endothelial expression of MT1-, MT2- and MT3-MMP proteins, whereas endothelial expression of PlGF and VEGF was increased in late first-trimester. The increased levels of growth factor and decreased levels of proteases in endothelial cells at the implantation site appear contradictive. Possibly, the regulatory mechanisms alter as gestation proceeds, perhaps under the influence of changing concentrations of hormones. The observed induced endothelial VEGF and PlGF might be correlated to the enhanced vascular remodelling, whereas the reduced endothelial MT-MMP expression might control vascular infiltration. Furthermore, the reduced overall MT-MMP expression might function in controlling a too destructive infiltration of EVT.

ANGIOGENESIS, PROTEASES, ANGIOGENIC FACTORS AND IMMUNE CELLS IN MISCARRIAGE

Vascularised, decidua is important for implantation and disturbances in vascular development may play a role in the pathogenesis of miscarriages. Few studies have addressed this hypothesis; higher decidual vessel density and differential expression of VEGF and its receptors were correlated with miscarriages [Vailhe *et al.,* 1999; Vuorela *et al.,* 2000]. Additionally, differential MMP and TIMP expression has been detected in receptive endometrium of recurrent miscarriage patients [Inagaki *et al.,* 2003b, Jokimaa *et al.,* 2002].

We demonstrated the importance of vascular remodelling for implantation. The occurrence of miscarriage was associated with altered vascularisation: significant decreased vessel density and increased luminal surface in DB and DP in decidua of miscarriages. The vascular differences correlated with the differential expression of angiogenic factors and proteases at the implantation site of miscarriages. Furthermore, the endothelial antigen expression of Flt1, KDR and MT2- and MT5-MMP was enhanced in DP and DB of miscarriages. Surprisingly, PlGF, the most regulated factor in uncomplicated first-trimester decidua, appeared not to be involved in the pathogenesis of missed abortions. Overall, more angiogenic activity generated by angiogenic growth factors as well as proteases, appeared to be generated in decidua of miscarriages.

The altered vascularisation and expression of angiogenic factors in miscarriages must have an embryonic or a maternal origin. The first category remains fairly hypothetical and might include genetical modifications, like polymorphisms and mutations, which possibly result in changed expression of angiogenic factors. These DNA variations are not detectable by conventional karyotyping techniques and therefore performing karyotyping would not have helped clarify this hypothesis. In addition, previously no differences in the number of leucocytes, in uNK cells and macrophages, and in decidual architecture were previously detected between chromosomally normal and abnormal pregnancies [Greenwold *et al.,* 2003; Quack *et al.,* 2001; Shimada *et al.,* 2006]. Thus, the lack of karyotyping will probably not significantly bias our observations.

Maternal-related causes might include a fundamentally disturbed angiogenic reaction to fertilisation and implantation. Strikingly, this "miscarriage vascularisation" resembles the differences between early and late first-trimester vascularisation, suggesting that too fast maturing vasculature is associated with the pathogenesis of miscarriages. The pre-maturely ripening may allow maternal blood in the intervillous space too early in the development of pregnancy, also demonstrated in vivo by doppler ultrasound imaging [Greenwold *et al.,* 2003; Jauniaux, Burton 2005]. The early-onset of maternal circulation could result in increased oxygen levels with subsequent oxidative stress. This could

modulate the architecture of vasculature and the expression of peri-cellular proteases and angiogenic factors [Burton 1997; Kingdom, Kaufmann 1997; Sharkey *et al.,* 2000].
Another maternal cause might be the altered regulation of oxygen levels, resulting in an even more hypoxic situation then during uncomplicated implantation. The relative "super-hypoxic" situation can induce non-branching angiogenesis and affect the expression of various angiogenic factors. These compensatory mechanisms generate the needed increase in blood supply, but may occasionally induce disproportionate angiogenic activity and set the stage for embryonic loss [Burton 1997, Khaliq *et al.,* 1999; Kingdom, Kaufmann 1997; Regnault *et al.,* 2003; Sharkey *et al.,* 2000; Zhang *et al.,* 2002]. This hypothesis is confirmed by the described increased vascular area and altered VEGF expression and Ang-2/Ang-1 ratio, but not by the unchanged PlGF expression in miscarriage decidua. A shortcoming of this hypothesis is that the effects of oxygen on vascularisation and the expression of angiogenic factors are derived from experiments in term placental tissue and whether these effects are true in first-trimester decidua has not been described yet.
Conclusions from histo-pathological studies of abortion tissue are often questioned since foetal death may occur days before evacuation, allowing post-mortem inflammation or apoptosis during this "retention time". Retention time has been reported to have no effect on vascularisation [Lisman *et al.,* 2004; Meegdes *et al.,* 1988; Nelen *et al.,* 2000], which appears to exclude a contribution of "retention time" to the observed vascularisation differences between miscarriages and controls. To analyse whether our specimens demonstrated differential inflammatory or apoptotic events in response to foetal death, we determined proliferation, apoptosis and the number of immune cells.
Ki67 and active Caspase-3 (aCasp-3) showed similar expression patterns suggesting an increased cellular turnover from DSE, to DP to DB in both cases and controls. The cases and controls showed equal amount of proliferation and apoptosis, thus indicating that increased apoptotic events in the miscarriage samples are unlikely. Comparable numbers of uNK cells were demonstrated in DP and DB of cases, but their number was significantly increased in DSE. Most reports on the relation of uNK cells to miscarriages describe increased uNK cells in receptive endometrium of patients with recurrent miscarriages (RM) [Quenby *et al.,* 1999; Tuckerman *et al.,* 2007]. The abundant presence of NK cells in our DSE samples may reflect these observations. The difference between cases and controls were levelled in DP and DB, possibly by hormone regulated NK cell migration to normal values [van den Heuvel *et al.,* 2005b].

ANGIOGENIC FACTORS IN PRE-ECLAMPSIA AND FOETAL GROWTH RESTRICTION

Both pre-eclampsia (PE) and foetal growth restriction (FGR) are extensively studied in serum/plasma, third-trimester tissues and also, too a smaller extent, by *in vitro* models [Chung *et al.,* 2004; Eriksson Hagen *et al.,* 2005; Geva *et al.,* 2002; Maynard *et al.,* 2003; Sugimoto *et al.,* 2003]. Information about first-trimester molecular and morphological events would be very valuable.

Current knowledge suggests that the pathogenesis of PE starts with defective vascular remodelling of maternal spiral arteries leading to non-invasion of trophoblasts, placental insufficiency and ischemia [Luttun, Carmeliet 2003; Zhou *et al.,* 1997]. The diseased placenta releases soluble anti-angiogenic factors, like soluble flt-1 (sFlt-1) and endoglin (sEng). These factors alter the angiogenic balance, mainly by neutralising VEGF-A and PlGF, induce systemic endothelial dysfunction and finally clinical PE [Ahmad, Ahmed 2004; Clark *et al.,* 1998; Fisher 2004; Hayman *et al.,* 1999; Maynard *et al.,* 2003; Sugimoto *et al.,* 2003; Tjoa *et al.,* 2007; Yuan *et al.,* 2005]. FGR, or Foetal growth restriction (FGR), has been proposed to be induced by relatively high oxygen levels, which cause disturbed expression of angiogenic factors and altered angiogenesis in placental villi [Burton 1997; Kingdom, Kaufmann 1997; Regnault *et al.,* 2003]. These relative hyperoxic conditions may explain the decreased VEGF and increased PlGF expression found in FGR placentas, since both factors are inversely regulated by oxygen [Lyall 1997; Khaliq *et al.,* 1999]. Whether the altered oxygen levels also affect decidual angiogenesis is not documented, but is likely.

We showed that pre-eclampsia and foetal growth restriction were both associated with the induction of angiogenic factors in first-trimester decidua. Unfortunately, the study groups are small because of the rarity of the samples. This results in rather high standard deviations, which prevents significance and makes interpretation of the data difficult. However, the 10 to 100-fold induction of genes in these small groups promises significant differences when groups could be expanded.

The early first-trimester changes in angiogenic factor expression may well occur as a compensatory mechanism, but in turn may unintentionally induce increased non-branching angiogenesis, altered decidual and placental vascularisation and insufficiency that result in pre-eclampsia and/or foetal growth restriction during late gestation.

FUTURE PERSPECTIVES

This thesis includes studies focussing on angiogenesis and its regulation in human endometrium and decidua and provides new insights into the regulation of endometrial and decidual angiogenesis

In human, very little work has been done in relation to decidual angiogenesis apart from few studies focussing on the location of numerous angiogenic growth factors. This approach presents a problem in understanding the coordination of local events in new vessel growth and its controls and limits, as most of these factors display widely differing effects. It must be remembered that the presence of a growth factor does necessarily indicates functional availability. Therefore, observations obtained from these *in situ* studies have to be accompanied by *in vitro* studies in order to confirm and further elucidate the mechanisms controlling decidual vascular development. For example, analysing the involvement of uNK cells and the role of MT2- and MT3-MMP, but also MT5-MMP, in regulating capillary like tube formation *in vitro* will be very interesting. However, it is essential to optimise the *in vitro* tube formation model to the "decidual situation" in order to obtain valuable results.

The interaction between EVT and vascularisation and the regulation of angiogenic factors and proteases by pregnancy-induced hormones, uNK cells and/or the extravillous trophoblast is best studied *in vitro*. The first item can be studied by performing co-cultures of trophoblasts and endothelial cells and for example subsequent gene expression profiling. The latter item is probably best studied by analysing the effect of the three possible regulators on separate cell types. In addition, determining the presence of hormone receptors is imperative as well. Merely the combination of *in vivo* and *in vitro* assays will achieve the understanding of human implantation.

Additionally, the interesting regulatory effects of hypoxia on decidual vascularisation must be further elucidated. The oxygen level in the different types of first-trimester decidua, DSE, DP and DB, has to be established and the regulation of angiogenic factors and proteases in first-trimester tissues by oxygen has to be determined. This information will contribute to a better understanding of oxygen mechanisms in both physiologic and pathologic pregnancies. Moreover, new morphological imaging as well as new ultrasound techniques and measurements hopefully will allow quantification of altered angiogenesis and blood flow in physiologic and complicated first-trimester pregnancies and pregnancies complicated by miscarriage, PE and FGR.

Another promising future research subject is the role of endothelial progenitor cells during pregnancy. Endothelial progenitor cells are mobilised from the bone marrow by oestrogen and are proposed to play an important role in the regulation and maintenance of vasculature during pregnancy. Their increased mobilisation, probably via ischemia, may underlie pre-eclampsia [Robb *et al.,* 2007].

Finally, angiogenesis and its regulation in endometrium and decidua is a fascinating research topic. Angiogenesis and various angiogenic factors and proteases appear crucial contributors to the inception of pregnancy. The obtained knowledge adds to a better understanding of physiologic and pathologic conditions regarding menstruation and pregnancy.