Angionesis and the inception of pregnancy

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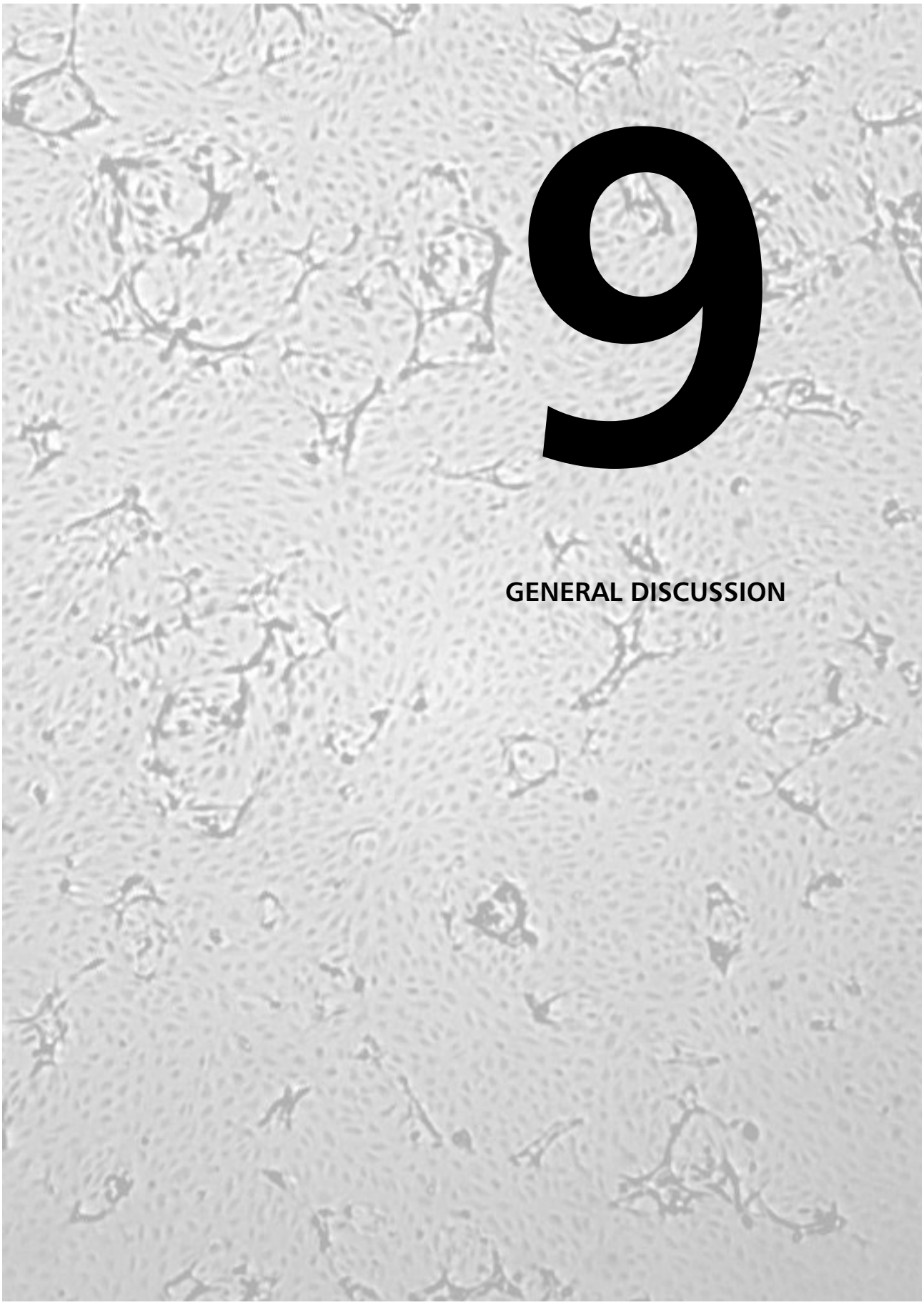

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GENERAL DISCUSSION

1. Results of the studies

In Chapter 2 we demonstrated that women with a low birth weight had a higher risk of myocardial infarction than women with a normal birth weight. In Chapter 3 we found that singleton IVF pregnancies had significantly worse perinatal outcomes than spontaneously conceived pregnancies in subfertile women.

In Chapters 4 - 8 the isolation of human endometrial microvascular endothelial cells (hEMVEC) is described together with their high angiogenic capacity, which was likely due to their high response to VEGF-A and their high expression of u-PA. Besides the u-PA/plasmin system, MMPs, in particular MT3-MMP, contributed to hEMVEC tube formation. Furthermore, we demonstrated that human endometrial stromal cells (hESC) expressed ERα, ERβ and PR and responded to the ovarian steroids by an increase in VEGF-A expression. In contrast, hEMVEC expressed only ERβ and showed at best a marginal angiogenic response to E_2. HEMVEC cultured in close contact with hESC survived better, probably due to paracrine VEGF production by hESC.

The data presented in Chapter 8 demonstrate that conditioned media of human embryos contained VEGF-A and stimulated *in vitro* endometrial angiogenesis, an effect which was counteracted by sVEGFR-1. Among the factors expressed by human embryonic tissue, VEGF-A was obviously the most potent in stimulating hEMVEC proliferation and tube formation.

With these results we elucidate more of the processes involved in angiogenesis, which was our main goal.

2. From clinical questions to implantation and angiogenesis

Our clinical studies confirm the "Barker hypothesis" that low birth weight is a serious risk factor for cardio-vascular disease in later life and, furthermore, indicate that controlled ovarian hyperstimulation (COHS) in assisted procreation is related with low birth weight. In order to prevent fetal growth retardation, which might be induced by assisted procreation, we must understand more about the mechanisms involved.

For a healthy fetal development, an optimal environment needs to be created early in life, during the process of implantation. When the implantation site is located in the uterus, the preparations for an optimal environment already start before fertilization, during the proliferative and secretory phase of the menstrual cycle. Optimalization of the implantation site is subsequently established by the embryo itself, which interacts with its implantation site. Also in an unprepared extra-uterine milieu the

embryo is able to accommodate its implantation site as proven by advanced ectopic pregnancies.

Angiogenesis is considered to be one of the most critical adaptive changes during implantation and placentation. It is essential to establish the vascular structures involved in maternal-fetal exchange. Adaptation of the maternal vasculature to the rising needs of the embryo and fetus occurs, in addition to angiogenesis, through vasodilation, increased permeability, and maturation[1-3]. Although direct embryonic contact with the maternal circulation is not well established until the beginning of the second trimester of pregnancy[4], an extensive vascular network needs to be established to support the endometrial cells (e.g. the epithelial cells) that supply the embryo with nutrition until then. Furthermore, the extending vascular network is necessary for the process of decidualization and placentation. Contact between the embryo and the maternal circulation that occurs too early might lead to pregnancy loss, as excessive entry of maternal blood at a very early stage inside the developing embryonic placenta results in oxidative stress and subsequent degeneration of villous tissue[59,60].

Several studies, both in rodents and in the human, elucidate the importance of a well established endometrial vasculature in implantation;

1) In rodents:
   - administration of an angiogenesis inhibitor (AGM-1470) to pregnant mice resulted in complete failure of embryonic growth due to interference with, among others, decidualization and placental development. When non-pregnant mice were treated with AGM-1470, inhibition of endometrial maturation was observed[5].
   - Sibug *et al.*[6] showed that COHS, more specifically urinary gonadotrophins, negatively affects angiogenic factors (VEGF and VEGF-R expression) in the mouse uterus during implantation. This led to a delay in embryo implantation and smaller size of the implantation site, which might be caused by the decrease in angiogenic factors as other studies have shown that the number of implantation sites was significantly reduced after VEGF antibody treatment[7,8].
2) In the human:
   - uterine perfusion appears to be involved in human endometrial receptivity as a high intra-uterine blood flow resistance is associated with unexplained recurrent miscarriages[9].
   - morphological studies[10,11] show poor placental vascular development in intra uterine growth retardation (IUGR).
   - inadequate transformation of the maternal vasculature is associated with early pregnancy loss, and a higher perinatal morbidity and mortality caused by preterm delivery, pre-eclampsia, and/or intra-uterine growth restriction[12-22].

3. Endometrial angiogenesis

Most of the findings in Chapter 4 - 8 are the result of *in vitro* experiments. They provide us with more knowledge about the regulation, production and physiological responses of the endometrial vasculature. But can we transpose these results to the *in vivo* situation?

3.1 Endometrial endothelial cells

Endothelial cells are heterogeneous and differ in structure, function, antigen composition, metabolic properties and response to growth factors. In this way, the cells can adapt to different (micro-) environmental needs. The endometrial vasculature enhances during preimplantation (proliferative and secretory phase), decidualization, implantation, and placentation[23,24]. For this purpose, the endometrial endothelial cells must be able to react adequately to angiogenic factors in order to prepare the endometrium for implantation and placentation. Endometrial endothelial cells in culture obviously demonstrated rapid responses, particularly when compared with endothelial cells originating from other tissues.

VEGF plays an important role in a variety of angiogenic processes[25]. Hence, it was to be expected that the endometrial endothelial cells are highly sensitive to VEGF as well. What did surprise us was, beside the fact that the cells already formed tubes under control conditions, the huge enhancement of tube formation after the addition of VEGF. This could be explained by the high expression of VEGFR-2 on these cells. *In vivo*, the presence of VEGF and its receptors in the human endometrium is manifest[26-30]. The sub-epithelial-capillary plexus in the endometrium, with which the embryo comes into first contact during implantation, consists of endothelial cells that are not associated with pericytes or a vascular smooth muscle cells. It was shown that such endothelial cells are more susceptible to variations in local levels of VEGF and undergo apoptosis (programmed cell death) upon withdrawal of VEGF more readily than endothelial cells that were stabilized by contact with pericytes or smooth muscle cells[31].

An explanation for the rapid ingrowth in the 3-D fibrin matrix is the relative high expression of u-PA by hEMVEC. A relatively high u-PA expression was found *in vivo* as well.

HEMVEC displayed their enhanced angiogenic capacity *in vitro* in 3-D fibrin and collagen matrices. The endometrial ECM consists of several proteins, among which collagens, and its composition and fibrin contents vary during the menstrual cycle. The *in vitro* angiogenesis model that we used in our experiments has its limitations, as it only

consists of fibrin (which is actually a model for wound healing), collagen or a combination of both. It would be desirable to mimic the cyclic changes of the matrix as occur in vivo, but this will be extremely difficult. Nevertheless, we believe that our *in vitro* model reflects the *in vivo* situation adequately.

3.2 The role of MMP's in endometrial angiogenesis

To be able to respond quickly to an angiogenic stimulant, the endometrial endothelial cells need adequate proteolytic enzymes for pericellular proteolysis, as this is essential in endothelial cell migration, invasion and tube formation. The endometrial endothelial cells make use of both the u-PA/plasmin system and the MMP's for this purpose. The expression of MMPs by human endometrial endothelial cells *in vitro* was in accordance with the observations found *in vivo*[32-36]. Apparently, the endothelial cells maintain this ability when brought into culture and therefore our results may apply to the *in vivo* situation.

The experiments with TIMP-1 and 3, which are also expressed by the endothelial cells *in vivo*[32,36,37], proved that human endometrial endothelial cells express a quite unique pattern of (MT-)MMP's compared with endothelial cell originating from other tissues[38-41]. Our results suggest an important role for MT3-MMP in endometrial endothelial cell tube formation which may apply for the *in vivo* situation as well, as an increase in MT3-MMP is found in endometrium in the proliferative phase of the menstrual cycle, during which angiogenesis definitely takes place[34].

In combination with the high expression of u-PA, these results might further explain the specific angiogenic behavior of the endometrial endothelial cells compared to other endothelial cells. It is very likely that the unique environmental cyclic changes in the endometrium (the ECM) challenge the cells to adjust and express specific proteolytic enzymes necessary for angiogenesis, tissue desquamation and repair.

3.3 Regulation by the ovarian steroids

Development of the endometrium to a receptive state is primarily dependent on the coordinated effects of the ovarian steroids. Therefore it is to be expected that angiogenesis, essential in this process, should also be controlled by an overall regulation of these steroids.

Experiments with steroids require adequate precautions, as steroids, due to their structure, pass the cell membrane very easily and have a high turnover rate. We tried to

approach the *in vivo* situation as much as possible by creating a steroid free environment (charcoal-treated serum) and adding the steroids in the experiments daily to maintain a steady steroid concentration available for the cells.

In our *in vitro* and *in vivo* experiments we found that the endometrial cells do not loose their steroid receptors when kept in culture, except for hESC, which lost ERα at higher passages. From our results, an indirect regulation of endometrial angiogenesis by the ovarian steroids appears most likely. Krikun *et al*.[42] found no effects of E_2 or progesterone on endometrial endothelial expression of angiogenic factors, which supports the idea of an indirect action of the steroids on endometrial angiogenesis.

The endometrial stromal cells, which are not defined in most studies, but which we have identified by immunocytological staining as fibroblasts, appear to play an important role as intermediate cells between the ovarian steroids and the endothelial cells. Other studies support this role for stromal cells. Matsui *et al*.[23] demonstrated that VEGF production by endometrial stromal cells increases in association with decidualization of the cells, a process first induced by steroids. Nayak *et al*.[43], studying the Rhesus Macaque, found that the midproliferative peak in stromal VEGF expression, which did not occur in the absence of estradiol, coincided with the peak in endothelial cell proliferation and that VEGF expression in the stroma, not in the epithelium, was significantly correlated with vascular proliferation. They also doubted whether epithelium derived VEGF plays a role in endometrial angiogenesis. In the endometrial stroma, other cell types are present in addition to the stromal cells we have characterized. These include granulocytes, neutrophils and natural killer cells. These leucocytes may also act as intermediate cells between the ovarian steroids and the endothelial cells, as these cells produce VEGF, come in contact with the endothelial cells, and are attracted to the endometrium via chemokines. The expression of these chemokines is induced by ovarian steroids[28,44-46].

Nayak *et al*.[43] suggested that progesterone, besides stimulating VEGF expression, plays a role in vascular remodeling during implantation and early pregnancy. In our *in vitro* experiments we found no indication for this phenomenon, as we did not see morphological differences in tube formation after stimulation with progesterone compared with estradiol or control conditions

3.4 Interaction between embryo and endometrium; the role of VEGF

Adequate interaction and synchronization between the developing embryo and the endometrium is essential for successful implantation and placentation. In animal studies it was shown that the endometrial vasculature undergoes expansion during preimplantation stages and, even more prominently, after implantation[24,47]. This suggests that the

embryo itself is responsible for further enhancement of angiogenesis at its implantation site. Already during the pre-implantation phase, before there is any physical contact between the embryo and endometrium, pregnancy-related endometrial vascular changes are seen throughout the whole endometrium. When the embryo approaches the endometrium, more localized changes occur[48,49].

Inadequate vascular transformation can have embryonic and/or maternal causes. Cross *et al*.[50] observed an inadequate vascular transformation in pregnancies in which the trophoblast failed to invade. Impaired early stage vascular remodeling in case of an ectopic pregnancy suggests that there could be primary maternal causes[51].

In agreement with our findings, several studies indicate that the embryo prepares its implantation site by stimulating local angiogenesis via the production of VEGF-A. Das *et al.*[47] showed in their rabbit studies a pronounced in situ hybridization signal of VEGF transcripts present in the trophoblast that attached and invaded the endometrium. Furthermore, they detected high levels of VEGF receptor-2 (VEGFR-2) mRNAs on blood vessels during implantation. More indications that VEGF plays a crucial role during this phase are given by the study of Vuorela *et al*.[52], who examined cases of recurrent miscarriage and found a diminished expression of VEGF in trophoblastic tissue and a weaker expression of its receptors in maternal decidual endothelium. Furthermore, Krussel *et al.*[53] showed that the VEGF gene is one of the earliest genes activated during human preimplantation embryo development.

Soluble VEGFR-1 (flt-1), important in modulating the actions of VEGF in angiogenesis, is secreted by the placenta and expected to function as a VEGF antagonist. He *et al*.[54] demonstrated in mice that an increase in the ratio of VEGF to sVEGFR-1 results in an increase in the number of resorption sites. High concentrations of VEGF can harm to the process of angiogenesis during implantation. This emphasizes that a balance in angiogenesis promoters and inhibitors is crucial in angiogenesis.

Taken together, it is very likely that VEGF plays a key role in the interaction between the embryo and the endometrium at the time of implantation. The way it is expressed strongly suggests that it is involved in angiogenesis on both maternal and fetal sides of the placenta[12]. And as the embryo appears to be able to stimulate local angiogenesis in the endometrium, it might very well stimulate angiogenesis in ectopic sites, thereby preparing an alternative implantation site.

4. Future research and clinical implications

Knowing all this, one questions how we can relate and intervene our results to the physiological and moreover, the pathological *in vivo* situation.

To widen our view concerning the physiological situation, one could further optimize the *in vitro* model and study the factors involved in extended detail. To this end, in future research one might use a three dimensional angiogenesis model consisting of a mixture of endometrial ECM components, and further elucidate the role of MT(3)-MMP and the ERβ-mediated signaling pathway in the process of endometrial angiogenesis. Furthermore, one might seek for other angiogenic factors which might be up regulated in endometrial cells by ovarian or pregnancy induced hormones.

In pathological processes in which in endometrial angiogenesis is involved, one would like to be able to therapeutically intervene, this way improving perinatal outcome by optimizing the implantation site and fetal intrauterine environment. Important additional information could be obtained from tissue samples of failed implantations (spontaneous abortions) and of abortus provocatus. In these cases, the determination of angiogenic factors in tissue samples of decidua basalis compared with decidua parietalis and secretory endometrium might enhance our understanding in the role of (disrupted) angiogenesis at this time and why it should contribute to the failure of implantation.

Moreover, studying the influence of the human blastocyst and its signaling factors on other types of endothelium and stromal cells, like those of the mesothelium could deepen our insight into ectopic implantation. More research is necessary to determine which additional early embryonic factors are able to control the process of angiogenesis. A comparison between the factors produced by embryo's which, in retrospect, did and did not implant could be of interest and might be beneficial for IVF/ICSI strategies in the future.

One way of optimizing the embryo implantation site in case of IVF/ICSI seems to be embryo transfer in a natural cycle. So far, the direct effect of COHS on endometrial angiogenesis was studied in animals but not in humans. It would be informative to test different COHS in our *in vitro* angiogenesis model and determine whether this might negatively influence the endometrial environment.

5. Conclusions

Endometrial vascular maladaptation prior and during implantation may lead to serious complications (pregnancy loss, IUGR, pre-term delivery, pre-eclampsia) which may have consequences during pregnancy, perinatally, but also later in the neonates' life. The consequences in later life often appear to be related to an endothelial dysfunction, which is in agreement with our findings. This endothelial dysfunction appears to exist already at a young age[55-57]. Endometrial vascular maladaptation has an embryonic cause, a maternal cause or a combination of both. A less well developed embryo might be impaired

in signaling the endometrial endothelial cells leading to inadequate adaptation of the vessels. On the other side, (pre-existing) maternal endothelial dysfunction may also lead to defective maternal vascular remodeling. Such endothelial dysfunction might be either autogenously (e.g. in diabetes, auto-immune diseases, pre-eclampsia?) or caused by exogenous factors (e.g. smoking, COHS?)[51,58]. We have shown that COHS adversely affects pregnancy outcome, whether COHS negatively affects the endometrial vasculature needs further study.

The experiments have shown that the isolated human endometrial endothelial cells display a high angiogenic capacity, which is further enhanced upon exposition to VEGF-A. The u-PA/plasmin system and MT3-MMP play an important in this process and the ovarian steroids overall regulate this process indirectly, via the endometrial stromal cells. During implantation, the embryo takes over as the main (local) regulator by inducing angiogenesis locally at its implantation site through the expression of VEGF. With the results described in this thesis, we provide more insight in the (patho-)physiology of endometrial angiogenesis, and in the role of the embryo in these phenomena.

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