Angionesis and the inception of pregnancy

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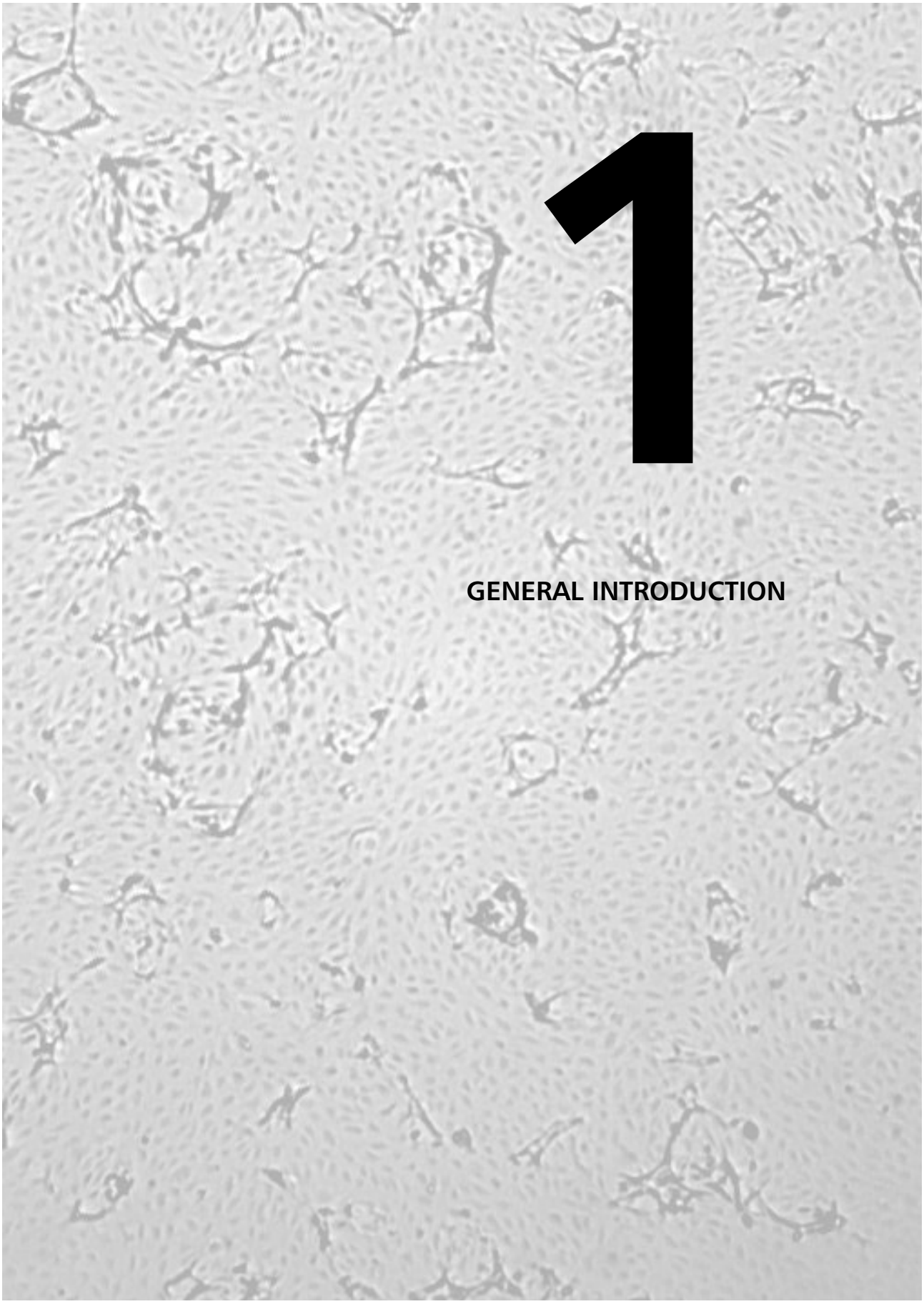

1

GENERAL INTRODUCTION

1. Background

A pregnancy rate of approximately 15% per cycle renders the process of human reproduction inefficient[1]. From assisted procreation studies we have learned that fertilization is not the major problem as this succeeds in about 70-80%. However, the next phase, the implantation, seems to be the biggest challenge. Embryo selection in these procedures can somewhat increase the chance of implantation.

Controlled ovarian hyperstimulation (COHS), used in assisted procreation, adversely affects perinatal outcome. Singleton pregnancies from assisted reproduction have a significantly worse outcome (birth weight and gestational age) compared with spontaneous singleton pregnancies[2] whereas, birth weights of singletons conceived by implanting a cryopreserved embryo tend to be normal or even above average[3]. The difference in these procedures is that embryo transfer of a cryopreserved embryo occurs predominantly in a natural menstrual cycle, whereas embryo transfer after IVF/ICSI occurs directly in an environment that was exposed to COHS.

Whether the factor subfertility confounds the association between COHS and a worse perinatal outcome is still unclear. However, the fact that neonates born after cryopreserved embryo transfer tend to have normal/higher birth weights, suggests that subfertility does not influence perinatal outcome. The adverse effect of COHS on perinatal outcome may be caused by its negative effect on the endometrium[4] and as such on the implantation process. An adverse effect on implantation may lead to worse perinatal outcome, like low birth weight.

Angiogenesis, the formation of new blood vessels from pre-existing ones, is thought to play an important role in the process of implantation. In rodents, it was shown that stimulation with urinary gonadotrophins (a form of COHS), in contrast to recombinant gonadotrophins, negatively affected parameters important in angiogenesis[5,6].

2. Human implantation

Implantation is a series of events, which is initiated when the blastocyst starts to interact with its implantation site, leading to placentation. Of all mammalian physiological processes, implantation involves very species-specific mechanisms, which make comparisons with other species difficult. Therefore research on human implantation has to, at least in part, be performed on our own species by designing *in vitro* assays simulating parts of the *in vivo* process.

Normally, implantation occurs intra-uterine, in the endometrium. However, advanced ectopic pregnancies have been described, for example on the intestinal mucosa. This

shows that the embryo does not solely relies on the endometrium as an implantation site but is able to create its own implantation site even extra-uterine.

2.1 Pre-implantation

As most pregnancies develop intra-uterine, the implantation process in the endometrium is described here.

The endometrium is the mucosa that forms the inner lining of the uterus (Fig. 1). Its growth, differentiation and breakdown is cyclical regulated by the ovarian steroids during the female's fertile life span. Every month the 2/3 upper part of the endometrium, called the functional endometrium, is shed (menstruation). Subsequently, a new functional layer growths from the basal endometrium under the influence of estradiol during the proliferative phase of the cycle. After ovulation, differentiation of the endometrium takes place under the influence of progesterone produced by the corpus luteum (Fig. 2). Both steroids influence processes directly and indirectly via various factors like growth factors and cytokines.

Uterine blood supply is facilitated by the uterine arteries, which give rise to arcuate arteries. From these arteries arise the radial arteries, which divide at the endo-myometrial junction into straight arterioles supplying the basal layer of the endometrium and spiral end-arterioles supplying the functional layer (Fig. 1). Arterioles in the basal layer

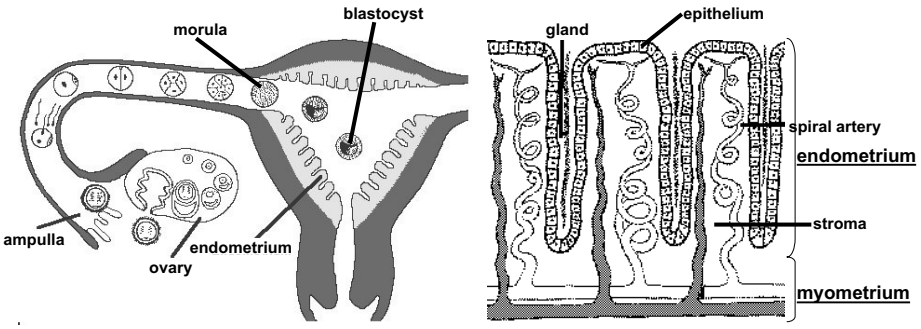

Figure 1. The endometrium.
The inner lining of the uterus, the endometrium, is monthly prepared for the implantation and placentation of a blastocyst. From the basal layer, which is not shed during menstruation, a new functional layer of endometrium develops. Angiogenesis is indispensable for the proliferation and differentiation of the epithelial cells and cells in the stromal compartment. Spiral arteries descend from stumps in the basal layer and from these arteries a new sub-epithelial capillary complex develops.

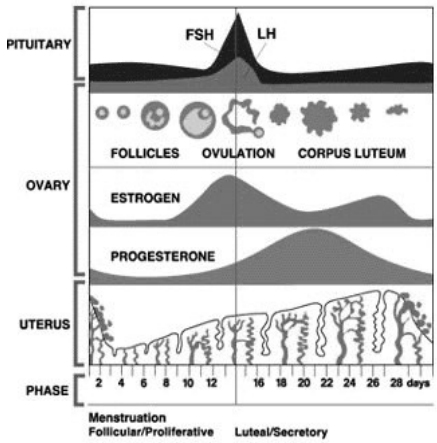

Figure 2. **The menstrual cycle.**
Hormonal, ovarian, and endometrial changes and relations throughout the normal menstrual cycle.

are surrounded by a vascular smooth muscle coat. Smooth muscle and pericytes are reduced in the superficial layer and the most superficial vessels consist only of endothelial cells[7]. The vessels form a capillary plexus under the epithelium. Endothelial cells in the functional layer show cyclical variation in proliferation, while the endothelial cells in the basal layer do not vary with the menstrual cycle[8,9].

Angiogenesis is required to support the proliferation and differentiation of glandular and surface epithelial cells, and stromal cells, of which the endometrium is composed[10,11]. Together with the changes in vascular permeability throughout the menstrual cycle a transformation of a thin, dense endometrium into a thick, highly edematous secretory endometrium takes place[12].

The morphological changes in the endometrial stroma seen after ovulation can be described as pre-decidualization. Decidualization is a reaction of the endometrium to support and regulate implantation and pregnancy. Further decidualization only occurs in the presence of a pregnancy.

Under normal conditions, when fertilization has occurred, the conceptus travels through the oviduct to the uterus proceeding cellular divisions (Fig. 1). Between its 4-8 cell stage it becomes transcriptionally active and genes of the conceptus itself start to contribute to its development. The metabolic activity and growth of the pre-implanted conceptus is stimulated by a number of growth factors for which it has receptors. On its turn the conceptus is able to synthesize several growth factors. These factors likely act as autocrine and/or paracrine factors, to promote its development and implantation.

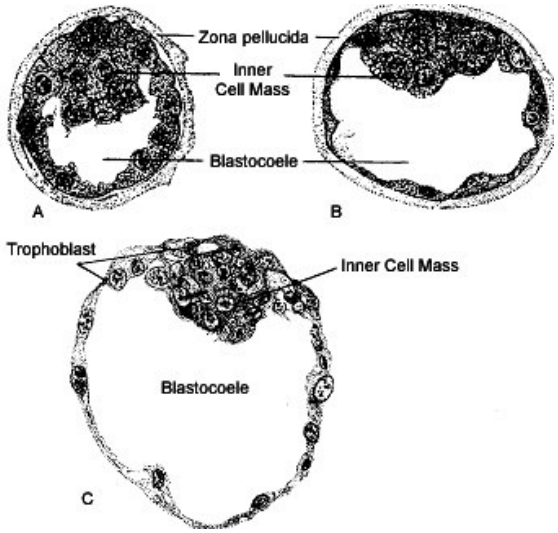

Figure 3. Formation of a blastocyst.
4-5 days after fertilization the embryo has differentiated into two distinct cell types: inner cell mass, which will develop into the fetus, and trophoblasts, which will develop into the placenta. In the blastocyst a cavity has developed called the blastocoele. Around day 6-9 the conceptus loosens its zona pellucida.

3-4 days after fertilization the conceptus enters the uterine cavity and changes from a morula stage (compact 12-16-cell stage) to the blastocyst stage (Fig. 1). The blastocyst contains an outer cell layer called trophoblast which surrounds a cavity called the blastocoele. The extra-embryonic tissue is concerned with the nutrition of the embryo and gives rise to part of the placenta. The group of centrally located cells, know as the inner cell mass, forms the embryo (Fig. 3).

When floating freely in the uterine cavity, the blastocyst derives its nourishment from the secretions of the uterine glands. However, this source becomes inadequate and implantation in highly vascularized endometrium is necessary for its further survival.

Implantation can only take place in a very narrow window of time (48h, 7-10 days after ovulation) during the menstrual cycle, the so-called "implantation window". During this period the endometrial epithelium is receptive to the implanting embryo. Receptive epithelium has specific characteristics that facilitate the conceptus to position and adhere for further implantation (apposition). These characteristics are the expression of small apical protusions called pinopodes and specific cell adhesion molecules called integrins[13-15]. Before and after the receptive period the endometrium resists attachment of the embryo.

For the embryo to survive, its early development and transport must be coordinated

precisely with the changing receptivity of the endometrium. The ovarian steroids play an important role in this coordination.

2.2 Attachment

Implantation involves an initial process of attachment which starts around day 6-9 with the conceptus loosening its zona pellucida (Fig. 3). During attachment close apposition and adherence of the trophoblast cells of the blastocyst to the luminal epithelium of the endometrium occurs.

Evidence derived from *in vitro* experiments and animal studies suggests that successful implantation and placentation depend on the interaction between the conceptus and endometrium[16]. Highly localized signals from the conceptus during apposition, attachment, and later during invasion enhance further decidualization of the endometrium. These processes initiate the development of the maternal part of the placenta. Important features of decidualization are an increase in vascular permeability causing edema, changes in the extracellular matrix (ECM) composition and stromal cell morphology, and angiogenesis. The signaling molecules responsible for decidualization are cytokines, growth factors and hormones[13,17-25]. Some of these signaling molecules and their role(s) are known, others remain unidentified. Studies have shown that the human blastocyst produces activin, colony stimulating factor (CSF)-1, epidermal growth factor (EGF), interferon (IFN) γ, insulin-like growth factor (IGF) I and II, interleukin (IL) 1α and -β, IL-6, IL-10, leukemia inhibitory factor (LIF), platelet-derived growth factor (PDGF), transforming growth factor (TGF) α and β, tumor necrosis factor (TNF) α, vascular endothelial growth factor (VEGF)-A, and hCG[26-33]. The elaborate interaction between the conceptus and the mother has two important distinctive components. First, the conceptus establishes physical and nutritional contact with the maternal endometrium. And second, the conceptus announces its presence to the maternal pituitary-ovarian axis by producing hCG; failure to do so would result in the regression of the corpus luteum, causing progesterone levels to fall, and subsequent loss of the conceptus.

2.3 Invasion

After attachment, controlled invasion takes place. To this end the (syncytio-)trophoblast uses various proteolytic enzymes. By eroding the surface epithelium and larger maternal vessels the trophoblast cells come into contact with maternal blood. This creates a new nutritional source and the basis of placental development (placentation). Decidualization of the endometrium proceeds due to trophoblastic growth factors, cytokines and

steroids. First trimester human trophoblast produces EGF, IGF-II, placental growth factor (PLGF), TGFα, TGFβ, TNFα, hCG, estradiol and progesterone[26-29,34-41 30-33,42].

In these early stages of pregnancy, intact capillaries grow and surround the (syncytio-) trophoblast. These capillaries form a capillary plexus connected to the (syncytio-) trophoblast lacunae and constitute the first very simple vascular system supplying the embryo. A close relationship between embryonic development and the state of vascularization of the chorionic villi has been demonstrated[43]. Nevertheless, the maternal circulation to the human placenta is not well established until the beginning of the second trimester of pregnancy[44]. The main nutritional source remain the uterine glands who deliver secretions into the intervillous space until 10 weeks of gestation.

Implantation is completed two weeks after fertilization. Around this time, the embryo itself synthesizes the hormones required for the continuation of pregnancy and becomes therefore independent of the maternal endocrine condition.

Flaws early in life, during implantation, may result in pregnancy loss or aberrant fetal development, such as intra uterine growth retardation (IUGR), resulting in low birth weight. A low birth weight, on its turn, might have serious consequences later in life as Barker describes in his hypothesis[45,46].

3. Barker hypothesis

In 1989 Barker published the first results from a cohort study of men and women born in Hertfordshire which suggested that cardiovascular disease was inversely related with birth weight[47-49]. Since then, this association has been confirmed by others in different countries[50-54]. Individuals who had low birth weight or were thinner at birth show, besides the increased rate of coronary heart disease, an increased risk for hypertension, (non-insulin-dependent) diabetes, abnormal lipid metabolism, renal disease and coagulation disorders[45,55-59]. Critics doubted the validity of these studies; they were concerned about genetic influences and the influence of socio-economic/environmental confounders on birth weight and cause of death later in life[46,46,60-64]. Several investigators adjusted for socio-economic/environmental factors and they still found, however less strong, an association between birth weight and coronary heart disease. This confirms that socio-economic circumstances at birth and in adult life cannot completely explain the association[51,52,54,65-67].

Out of Barker's observations arose the fetal origin hypothesis or Barker hypothesis, which proposes that several diseases in later life originate in utero from the persistence of physiological, endocrine and metabolic adaptations generated by the fetus (biological programming) when it is undernourished during critical periods of development[45].

These fetal adaptations may be protective in the short term, but may give rise to overt disease later in life. Barker acknowledges that besides the mechanism of programming, genetic and environmental factors play a role in this phenomenon[68]. But, to emphasize the role of biological programming, he believes that what appears to be due to socio-economic or genetic factors may in fact represent a perpetuation of a programming influence through several generations (intergenerational programming)[68].

The underlying biological mechanisms behind the association of low birth weight and adult disease have not been explained yet. Recent studies in animals and man try to elucidate this relationship. Markedly, in most diseases, which have been described to be related to low birth weight, the endothelium is involved. It has been shown that individuals with low birth weight exhibit endothelial dysfunction already at very young ages persisting into childhood and adult life[69-75]. Smith *et al*.[61] found that mothers, who once gave birth to babies with low birth weights, have a higher risk of developing ischemic heart diseases later in life. If this condition of endothelial dysfunction already existed during the time of implantation it might have led to an inadequate placental formation and subsequent IUGR. This suggests that endothelial dysfunction might represent the link between low birth weight and diseases later in life.

4. Angiogenesis

In the process of implantation and placentation, angiogenesis is crucial[1]. It is the result of a delicate balance between stimulators and inhibitors. This balance is influenced by the interaction of endothelial cells with their ECM, by growth factors and cytokines, and by environmental factors such as hypoxia and hormonal status[76-82].

Several steps are involved in angiogenesis. First the endothelial cells need to be activated by angiogenic factors. Secondly, the endothelial cells penetrate their basal membrane and subsequently invade and migrate into the underlying ECM. For this purpose the cells require proteolytic activity, which they obtain by the expression of proteolytic enzymes. Thirdly, the cells proliferate under the influence of angiogenic factors into the underlying interstitial matrix and form new capillary structures. Vessel stabilization is achieved by interaction with pericytes (larger vessels) and reconstitution of the basement membrane (BM)[83,84] (Fig. 4).

4.1 Proteolytic enzymes

Proteolytic enzymes and their inhibitors play an important role in the process of degradation of the BM and ECM and capillary lumen formation[85-88]. They are expressed by the

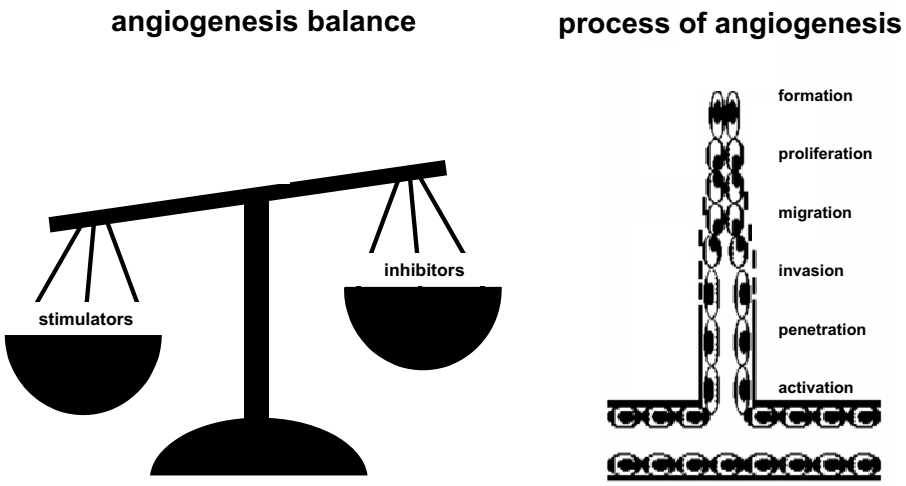

Figure 4. Angiogenesis.
Angiogenesis is a balance between stimulating and inhibiting factors. When resting endothelial cells in an existing capillary get activated by stimulating angiogenic factors they degrade the basement membrane and extracellular matrix (ECM), by expression of proteolytic enzymes. As such, the cells are able to penetrate, invade and migrate into the surrounding interstitium. Subsequently these endothelial cells proliferate, elongate and capillaries are formed.

endothelial cells and act in focal areas at the cell surface, and as such facilitate in a controlled balanced manner cell invasion and migration without loss of the bulk of matrix which is needed as structural support[84]. The proteolytic enzymes can also influence the angiogenic process by generating angiogenesis stimulating or inhibiting ECM fragments and by the activation or release of growth factors.

At least two proteolytic cascades are generally thought to play a major role in cell migration and invasion, namely the urokinase-type plasminogen activator (u-PA)/plasmin cascade and the matrix metalloproteinases (MMPs)[78,79,84,89-92].

4.1-1 The urokinase-type plasminogen activator (u-PA)/plasmin cascade

U-PA converts the inactive plasminogen into the broadly-acting serine protease plasmin. Plasmin is able to cleave fibrin, to degrade several matrix proteins such as thrombospondin and collagens and to activate several MMPs[93-96]. Like plasmin, u-PA is secreted as an inactive single-chain zymogen and can get activated to two-chain u-PA by plasmin or kallikrein to obtain proteolytic activity[97,98]. U-PA is primarily involved in proteolytic processes during cell migration and matrix remodeling. Inhibition of two-chain u-PA occurs by plasminogen activator inhibitors, of which PAI-1 is the predominant physiological inhibitor, secreted, among other cells, by endothelial cells[99,100].

Plasmin(ogen), single-chain u-PA and two-chain u-PA bind with high affinity to their cell surface receptors on endothelial cells. Binding of plasmin(ogen) and two-chain u-PA accelerates the conversion of single-chain u-PA into two-chain u-PA and u-PA-induced plasmin formation[101-108]. The u-PA receptor (u-PAR) acts both as a site for local pericellular proteolysis by u-PA and as a clearance receptor for the u-PA:PAI-1 complex which gets internalized after binding. After internalization the u-PA:PAI-1 complex is degraded and u-PAR is recycled to the cell surface[105,109]. By this process and on a transcriptional level (after stimulation with angiogenic factors) the cell is able to regulate u-PAR density on the cell surface and thus u-PA activity[110,111]. The u-PAR density can also be regulated by the cleavage of u-PAR from the cell membrane and as such generating an soluble form of u-PAR[112-114].

The u-PA expression has been observed to be low in resting endothelial cells[115,116]. The expression is induced in the endothelial cells by e.g. angiogenic factors when migration is induced such is the case during angiogenesis and inflammation[90,117,118].

4.1-2 The MMPs

MMPs are a still expanding, tightly regulated family of zinc-requiring enzymes that play a role in matrix remodeling and many cell-matrix interactions[119]. They have been evidently shown to play a role in angiogenesis both *in vitro* and *in vivo*[84,120-122]. MMPs can also have an inhibitory effect on angiogenesis by cleaving the u-PA, this way disabling its binding to the receptor[123]. Furthermore, MMPs can inactivate plasminogen or cleave plasminogen resulting in the product angiostatin, an angiogenesis inhibitor[124-126]. In the endometrium MMPs are known to play a role in tissue degradation and menstrual bleeding. MMPs have a high affinity for fibronectin, laminins and collagens, which are major ECM components of the endometrium (BM and interstitium). Some MMPs (e.g. MT1-MMP) can, independent of the plasminogen activator pathway, act as a fibrinolysin[127].

MMPs are either secreted from the cell as latent pro-enzymes or they are membrane bound enzymes. Six membrane-type MMPs (MT-MMPs) have been described, 4 transmembrane proteins and 2 GPI-anchored ones. The membrane-associated localization of MT-MMPs makes them particularly suited to function in pericellular proteolysis[128].

Growth factors, cytokines, plasmin but also activated MMPs or MT-MMPS can modulate the expression and activation of MMPs[85,129]. Specific inhibitors are the tissue inhibitors of MMPs (TIMPs) and α-macroglobulins. The TIMP family consists of 4 members, which differ in expression patterns, regulation and ability to interact specifically with latent MMPs[130]. TIMPs are secreted as soluble proteins (e.g. TIMP-1 and -2) or as proteins associated with the matrix components (e.g. TIMP-3)[131].

The relations between the u-PA/plasmin system, MMPs, and their inhibitors is schematically shown in Figure 5.

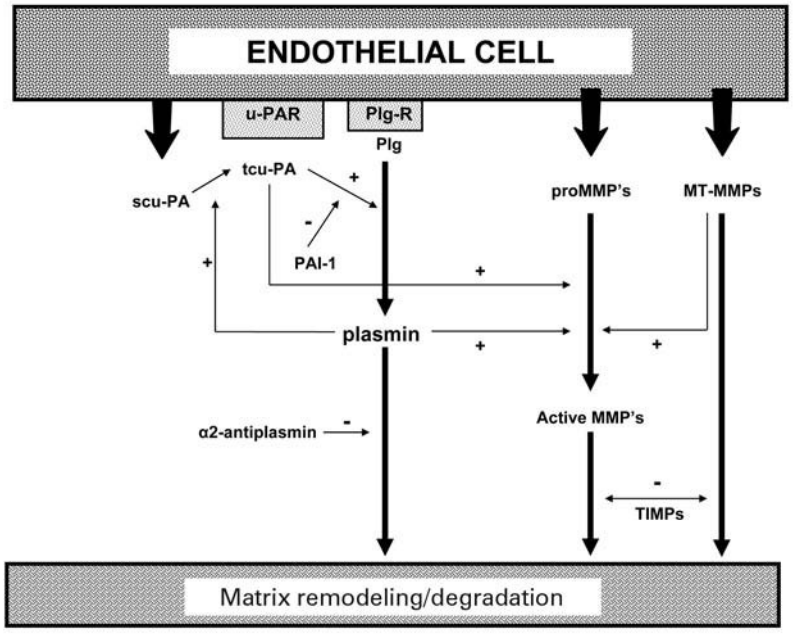

Figure 5. Schematic representation of the relations between the u-PA/plasmin system, MMPs and their inhibitors.
Abbreviations: u-PA: urokinase-type plasminogen activator, sc-u-PA: single-chain u-PA, tc-u-PA: two-chain u-PA, u-PAR: u-PA receptor, Plg: plasminogen, Plg-R: Plg receptor, PAI: PA inhibitor, MT-MMP: membrane-type MMP, TIMP: tissue inhibitor of MMP.

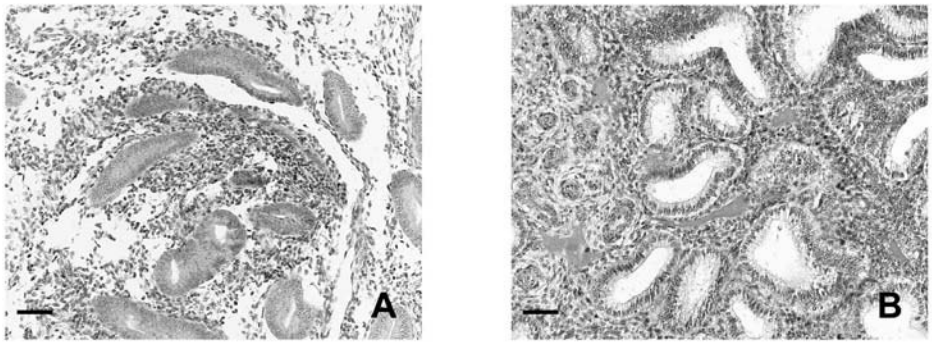

Figure 6. Fibrin staining in secretory endometrium.
In vivo the ECM of the endometrium consists of a number of proteins, such as laminin, fibrin, collagen type I, II, IV and VI, fibronectin and heparan sulphate proteogycan. The composition of the ECM varies during the cycle, as is shown here on paraffin sections of late-proliferative (A) and secretory (B) endometrium of two patients with a Martius Scarlet Blue staining. This staining stains fibrin red, collagen blue and erythrocytes yellow. During the secretory phase an increase of fibrin deposition is seen in the ECM of the endometrium. Bar = 100 μM. *[See appendix: color figures]*

4.2 Extra cellular matrix

In relation to the proteolytic enzymes, the matrix composition also plays an important regulatory role in the process of angiogenesis[132-134]. The composition of the endometrial ECM is subject to cyclic changes. Collagen and fibrin, which have been shown to be a stimulatory factor for endothelial cells and angiogenesis, are components of the endometrial ECM (Fig. 6)[47,135-138,138-140]. Fibrinogen deposition in the endometrium likely results from increased vascular permeability (probably due to VEGF, see below) which is observed during the secretory phase of the cycle and during implantation[138,141].

4.3 Angiogenic growth factors

The growth factor, which is generally assumed to play an important role in both physiological and pathological angiogenesis, is VEGF-A. In addition to inducing endothelial proliferation, VEGF-A modulates the expression of many genes including proteolytic enzymes, it affects endothelial permeability, and it is involved in the maintenance of immature blood vessels[142-145]. It is a homodimeric protein with great homology with placental derived growth factor and the other members of the VEGF family, VEGF-B, C and D[144]. Four forms arise from alternative splicing of the mRNA from a single gene, coding for the proteins of 121, 165, 189 and 206 amino acids (VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, and VEGF-A_{206}). The two larger forms, and VEGF-A_{165} to some extent, apparently stay cell bound via proteoglycans. Less frequent splice variants have also been reported, including VEGF-A_{145}, VEGF-A_{183}, VEGF-A_{162} and VEGF-A_{165b}[146].

In the human endometrium the epithelial and stromal cells produce VEGF-A, with a higher expression in the epithelial cells than in the stroma. The predominant isoforms in the human endometrium are VEGF-A_{121} and VEGF-A_{165}, whereas VEGF-A_{189} and VEGF-A_{145} are only weakly detectable[12,147-149]. Endometrial macrophages and leukocytes also produce VEGF-A[148-155]. By diffusion into the endometrial interstitium, VEGF-A binds to the endometrial endothelial cells. Whether epithelial derived VEGF-A becomes available for the endothelial cells is doubtful, as a mainly apical secretion by epithelial cells has been described[156]. A positive correlation between stromal VEGF immunostaining and endothelial cell density has been found[152].

Several studies have reported a cyclic or a steroid-dependent variation in the expression of VEGF and VEGF receptors in the endometrium[12,151,157-164]. Furthermore, it has been shown that hypoxia, a major driving force for angiogenesis, can regulate the expression of VEGF[80,161,165].

Three VEGF-specific tyrosine kinase receptors are known: VEGFR-1 (flt-1), VEGFR-2 (KDR) and VEGFR-3 (flt-4). Activation of the VEGFR-2 by VEGF results in a mitogenic response as well as migration[166,167]; whereas the VEGFR-1 has been shown to be important for cell migration but not mitogenesis[167,168]. The high affinity receptors VEGFR-1

and VEGFR-2 were mainly found on endothelial cells in the endometrium[141]. Endothelial strands, which have not yet formed a lumen, strongly stained for both receptors. Inhibition of VEGF activity using soluble-VEGFR-1 prevents endometrial maturation[169].

Whereas VEGF-A has been associated with capillary permeability[170] it is suggested to be responsible for the increased endometrial microvascular permeability. This idea is further supported by a high expression of VEGFR-1 and -2 on capillaries during the mid-secretory period. During this period subepithelial microvascular complexes and spiral arteries are formed and hence the VEGF receptors might be expressed for regulation of the microvascular permeability.

VEGFR-3 is thought to be involved in lymphangiogenesis and acts in concert with VEGFR-2. VEGFR-3 binds VEGF-C and VEGF-D, two gene products of the VEGF family.

5. Ovarian steroids

Markee[171] and Abel[172] were the first to shown that the ovarian steroids, 17β-estradiol and progesterone, are the overall regulators of endometrial angiogenesis. In the menstrual cycle, angiogenesis is seen during the early proliferative phase as a process of post-menstrual repair; during the mid-proliferative phase under the influence of estradiol; and during the estradiol and progesterone mediated secretory phase, when the coiled arteries grow and an extensive subepithelial capillary network is formed[173].

17β-estradiol and progesterone can pass through the cell membrane and bind to their specific (nuclear) receptors. These receptors can control the activity of target genes through direct association with specific DNA sequences known as hormone response elements (HREs)[174,175]. Two estrogen receptors (ERs) are known, ERα and ERβ. Both are different in that the receptors are derived from different genes and they have their own tissue distribution and specific functions. They show similarities in the fact that they share a high level of homology in the DNA-binding and ligand-binding domains and that both receptors bind estradiol with high affinity[176-178]. Estrogens may also act via receptors on the cell surface to achieve rapid, non-genomic effects[179,180].

ERα is suggested to be mainly responsible for the uterotrophic response upon estrogen exposure[181]. The precise physiological function and importance of ERβ in the endometrium is still unclear[182]. ERα knockout mice have a uterus that shows a lack of cell proliferation[181], and ERβ knockout mice demonstrate diminished reproductive capacity (small litter size, multiple resorbed fetuses)[182]. It has been suggested that a role of ERβ may be antagonizing and/or modulating ERα mediated actions.

Progesterone receptor (PR) knockout mice develop an inflammatory response to estradiol in the uterus, with no decidual response[181]. Estrogen induces ER and PR during

the proliferative phase; progesterone has therefore mainly an effect on an estrogen-primed endometrium[181]. In addition, progesterone by itself and steroid withdrawal down regulate the PR and ER expression[183,184].

PR reaches highest concentrations around mid-cycle, and ER mid-proliferative, correlating with the plasma peak of estradiol and the maximum mitotic rate of the endometrial cells[184,185]. The receptors decrease during the secretory phase[186].

6. Outline of this thesis

Defects during the process of implantation may lead to pregnancy loss, or aberrant fetal development which may give rise to diseases in later life. To increase the "take-home-baby-rate" in assisted procreation and to be able to prevent possible consequences of defective implantation (due to for example COHS?), it is important to understand more about the physiological process of implantation. As angiogenesis plays a key role in the process of implantation and placentation, and as endothelial (dys-) function might represent a link in fetal programming (Barker hypothesis), we wanted to elucidate more of the processes involved in angiogenesis. Our main focus was the maternal vessels at the endometrial implantation site, as these likely form the basis for successful implantation and subsequent formation of a healthy environment for the developing fetus.

Barker described the relation of low birth weight with diseases in later life in many cohort studies done in different countries. We wanted to know whether his hypothesis also applied to a case control study among Dutch women. In Chapter 2 we investigated the association between low birth weight and myocardial infarction.

COHS is widely used in assisted procreation in subfertile couples. It is of interest to know whether or not COHS might adversely effect the intra-uterine environment leading to a higher risk of low birth weight and/or preterm birth in these patients. We investigated the effect of subfertility and COHS on perinatal outcome. The results are described in Chapter 3.

Endothelial cells in different organs are heterogeneous. Physiologic processes involving the endothelium could therefore be best addressed by studies of endothelial cells derived from the organ of interest. To learn more about the maternal vasculature at the site of implantation, which is most often the endometrium, human endometrial endothelial cells were isolated and examined in an *in vitro* angiogenesis model consisting of a three-dimensional fibrin and/or collagen type I matrix. These studies are described in Chapter 4.

Several MMPs and MT-MMPs are present in endometrial tissue. However, little information is available on the expression and role of specific MMPs and MT-MMPs in

endometrial endothelial cells and their role in endometrial angiogenesis. The role of proteases was studied to obtain more insight in the factors that might act as key regulators in the process of endometrial angiogenesis. The results of these studies are given in Chapter 5.

Chapter 6 describes a literature search on the influence of steroids on factors important in the process of angiogenesis.

It is unknown how ovarian steroids exactly regulate the process of endometrial angiogenesis. They might exert a direct influence on the endometrial endothelial cells or act indirectly via for example the stromal or epithelial cells which are known to express the angiogenic factor VEGF. Crucial in this respect is the expression of steroid receptors by endometrial cells. As overall regulators of endometrial angiogenesis their influence on endometrial endothelial and stromal cells was examined in Chapter 7.

The early embryo (blastocyst, trophoblast) expresses several cytokines, growth factors and hormones by which it can optimize its own implantation site. These factors might induce, directly or indirectly, local angiogenesis at the place of implantation. Chapter 8 describes studies on the influence of the human embryo on human endometrial endothelial cells.

In Chapter 9, the results of the studies are summarized and discussed in a broader perspective.

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