TROPHOBLAST INFILTRATION

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INTRODUCTION

Sexual reproduction in the ocean necessitates only the combination of gametes, followed by absorption of nutrients and oxygen from the surrounding watery medium. As life moved from the sea to the land, reproductive strategies required compensation for the loss of this aquatic environment. For the mammals, and scattered other animals, the solution to this problem was the development of the placenta, the means by which the fetus extracts nutrients from its environment. As the animals that utilized the placenta evolved from small rodent-like creatures with short gestations to larger animals with prolonged gestations, the demands of the developing fetus grew. Whereas the placenta of the fetal pig, with a gestational period of a little less than four months, can extract sufficient nutrients from the mother by simple diffusion across the uterus to the placenta, the human fetus needs a far more complex utero-placental relationship.

Several solutions to the increased demands of the developing human fetus can be observed (Benirschke and Kaufmann, 1990). One approach was to simply make a larger placenta. For example the Chinchilla has a neonatal:placental weight ratio of 30:1 while the human has a 6:1 ratio. Another means to greater nutritional support for the fetus was to increase the surface area of contact between fetal circulation in the placenta and maternal circulation. Again looking at the pig, this fetus has a diffuse placenta that makes contact with the mother's uterus by a simple folded contact. The human placenta on the other hand has a complex villous structure—similar to the sea anemone's tentacles waving in the sea—that greatly increases the contact surface area between the mother's blood space and the fetal circulation. In spite of this increased fetal-maternal contact, this system is still rather inefficient. We can quantitate this by considering the amount of oxygen in the maternal blood that enters the human placenta and the amount of oxygen in the fetal blood that leaves the placenta to travel to the fetus. Maternal blood has a pO_2 of around 100, while the pO_2 of umbilical vein blood is around 35-40. This represents an efficiency of only 35-40%. It therefore also became necessary to greatly increase the flow of maternal blood into the intervillous space during pregnancy. Without this increased maternal blood flow, fetal loss and preterm birth occurs (Naeye, 1989). One of two mechanisms can increase maternal flow: increased total body blood flow, or increased blood flow to the placental bed through the uterine spiral arteries. Evolution has selected the latter to limit the overall systemic effects that increased total body blood flow would produce. Increased blood flow to the placenta is achieved by increasing the diameter of the arteries that supply blood to the intervillous space. One can appreciate the efficiency of this strategy by recalling that the flow through a tube is related to the radius to the fourth power (Vander et al, 1970). This means that if a vessel's radius increases by ten-fold (Fig. 1), the blood flow will increase 10,000 times! Not surprisingly, therefore, at term up to 40% of the maternal cardiac output can flow through the

placental bed. What causes the maternal spiral arteries to increase their diameters? As is often the case in changes necessary for successful pregnancy, the trophoblast accomplishes this process.

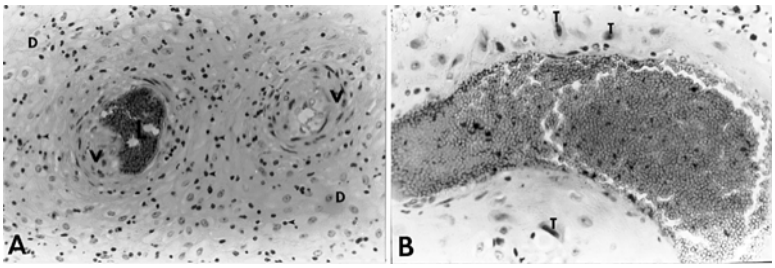

Figure 1. Uterine spiral arteries (264x). A) Decidual spiral artery cross sections from a non-pregnant uterus. Smooth muscle layers are easily identified in the vessel walls (v). Lumen (L). In addition to the decidualized stromal cells (D), many large granulated lymphocytes (arrow heads) can be seen. **B)** Decidual spiral artery from a placental bed at approximately 8 weeks of gestation. Note the increased diameter of the vessel and the numerous trophoblasts around and in the vessel wall (T). Also note that smooth muscle cells can not be identified in the vessel wall.

This article will review what is known about the differentiation of the three major classes of trophoblasts, how the invasive trophoblast performs its function, and what happens when there are defects in the invasive process.

TROPHOBLAST DIFFERENTIATION PATHWAYS

Trophoblasts are unique cells derived from the outer cell layer of the blastocyst which mediate implantation and placentation (Hertig and Rock, 1956). Depending on their subsequent function *in vivo*, undifferentiated cytotrophoblasts can develop into 1) hormonally active villous syncytiotrophoblasts, 2) extravillous anchoring trophoblastic cell columns, or 3) invasive intermediate trophoblasts (Fig. 2). Within the villi of the human placenta—at all gestational ages—there always exists a population of cytotrophoblasts which remain undifferentiated, apparently available for differentiation as necessary.

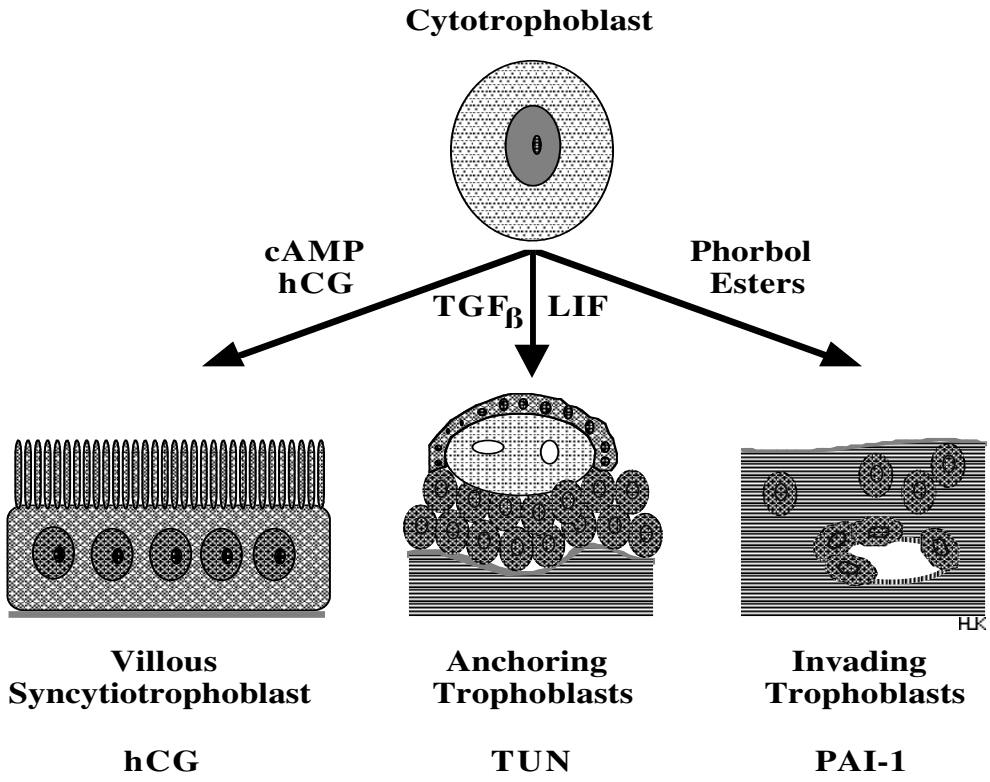

Figure 2. Pathways of trophoblast differentiation . Just as the undifferentiated basal layer of the skin gives rise to differentiated keratinocytes, the cytotrophoblast—the stem cell of the placenta—gives rise to the differentiated forms of trophoblasts. **Left)** Within the chorionic villi, cytotrophoblasts fuse to form the overlying syncytiotrophoblast. The villous syncytiotrophoblast makes the majority of the placental hormones, the most studied being hCG. Cyclic AMP and its analogs, and more recently hCG itself, have been shown to direct cytotrophoblast differentiation towards a hormonally active syncytiotrophoblast phenotype. **Center)** At the point where chorionic villi make contact with external extracellular matrix (decidual stromal ECM in the case of intrauterine pregnancies), a population of trophoblasts proliferates from the cytotrophoblast layer to form the second type of trophoblast—the junctional trophoblast. These cells form the anchoring cell columns that can be seen at the junction of the placenta and endometrium throughout gestation. Similar trophoblasts can be seen at the junction of the chorion layer of the external membranes and the decidua. The junctional trophoblasts make a unique fibronectin—trophouteronectin (TUN)—that appears to mediate the attachment of the placenta to the uterus. TGFß, and more recently, leukemia inhibitory factor (LIF), have been shown to downregulate hCG synthesis and upregulate TUN secretion. **Right)** Finally, a third type of trophoblast differentiates towards an invasive phenotype and leaves the placenta entirely—the invasive intermediate trophoblast. In addition to making human placental lactogen, these cells also make urokinase-type plasminogen activator (u-PA) and type 1 plasminogen activator inhibitor (PAI-1). Phorbol esters have been shown to increase trophoblast invasiveness in *in vitro* model systems and to upregulate PAI-1 in cultured trophoblasts.

Villous syncytiotrophoblast

The hormones secreted by the villous syncytiotrophoblast are critical for maintaining pregnancy (Conley and Mason, 1990; Petraglia et al. 1990). Early in gestation, human chorionic gonadotropin (hCG) is essential to maintain corpus luteum progesterone production. Near the end of the first trimester, the mass of villous syncytiotrophoblast is large enough to make sufficient progesterone and estrogen to maintain the pregnancy. During the third trimester, large quantities of placental lactogen are produced, a hormone purported to have a role as a regulator of lipid and carbohydrate metabolism in the mother. Other syncytiotrophoblast products include: pregnancy specific ß$_1$-glycoprotein (Kliman et al. 1986), plasminogen activator inhibitor type 2 (Feinberg et al. 1989), growth hormone (Jara et al. 1989), collagenases (Moll and Lane, 1990), thrombomodulin (Maruyama et al. 1985; Ohtani et al. 1989), and growth factor receptors (Kawagoe et al. 1990; Posner, 1974; Uzumaki et al. 1989).

Anchoring trophoblasts

The premature loss of attachment of the developing conceptus or placenta to the uterus can terminate the gestation. Therefore, the anchoring trophoblast cell columns and the extracellular matrix proteins which promote this attachment are critical to the developing pregnancy. It has been generally accepted that some form of cell-extracellular matrix interaction takes place at the attachment interface between these trophoblasts and the uterus. Some have considered Nitabuch's layer related to this function. In addition to the anchoring cell columns of the placenta, the extravillous trophoblasts of the external membranes (chorion laeve), play a critical role in maintaining attachment of the external membranes to the endometrial surface. Recently, a specific type of fibronectin—*trophouteronectin (TUN)*—has been implicated as the protein responsible for the attachment of anchoring, extravillous trophoblasts to the uterus throughout gestation (Feinberg et al. 1991).

Invading trophoblasts

The act of implantation is the first clear expression of the trophoblasts' ability to be invasive and has proven to be one of the major hurdles for the recently fertilized embryo. Because of the clinical significance of human infertility, a great deal of research has been performed to understand the basic biology of implantation. Unfortunately, studying human implantation directly is not often possible (Ohlsson, 1989; Lindenberg, 1991), necessitating the use of animal and model systems.

Studies using human specimens three to four weeks after implantation show that as gestation progresses, invasive populations of extravillous trophoblasts attach to and interdigitate through the extracellular spaces of the endo— and myometrium (Kurman, 1991a; Kurman et al, 1984;

Fig. 2). The endpoint for this invasive behavior is penetration of maternal spiral arteries within the uterus (Pijnenborg, 1990). Histologically, trophoblast invasion of maternal blood vessels results in disruption of extracellular matrix components and development of dilated capacitance vessels within the uteroplacental vasculature. Biologically, trophoblast-mediated vascular remodeling within the placental bed allows for marked distensibility of the uteroplacental vessels, thus accommodating the increased blood flow needed during gestation. In addition to the presence of markers of ECM interactions and proteases needed for cell movement and invasion, these trophoblasts also appear to express a unique monomorphic histocompatibility antigen: HLA-G (Kovats et al, 1990). Abnormalities in this invasive process have been correlated with early and mid-trimester pregnancy loss, preeclampsia and eclampsia, and intrauterine growth retardation (Pijnenborg et al. 1981; Pijnenborg, 1990).

HISTOLOGIC OBSERVATIONS OF INVADING TROPHOBLASTS

The morphologic aspects of human trophoblast invasion has been examined in great detail over the last ten years (Pijnenborg, 1990; Robertson et al, 1984). Since it is difficult to reliably obtain human material prior to 4 weeks of gestation, much of our morphologic understanding of the earliest phases of trophoblast invasion has been extrapolated from monkey material (Enders, 1989; Enders and King, 1991; Blankenship et al, 1992). Examination of monkey implantation sites has revealed that trophoblasts begin to migrate down into the maternal spiral arteries as early as 10 days after fertilization, and at 14 days, many of the spiral arteries beneath the conceptus are totally occluded. The specificity of this vascular interaction is revealed by the fact that no such invasion takes place in the veins. The reason for this arteriolar occlusion is not know, although Rodesch et al (1992) have recently suggested that it is critical that maternal blood flow to the embryo be limited very early in gestation to protect the conceptus from excessively high oxygen levels during critical, early stages of differentiation. Beyond 4 weeks, ectopic pregnancies, abortion material, placental bed biopsies and hysterectomy specimens have aided our understanding of human trophoblast invasion (Fig. 3).

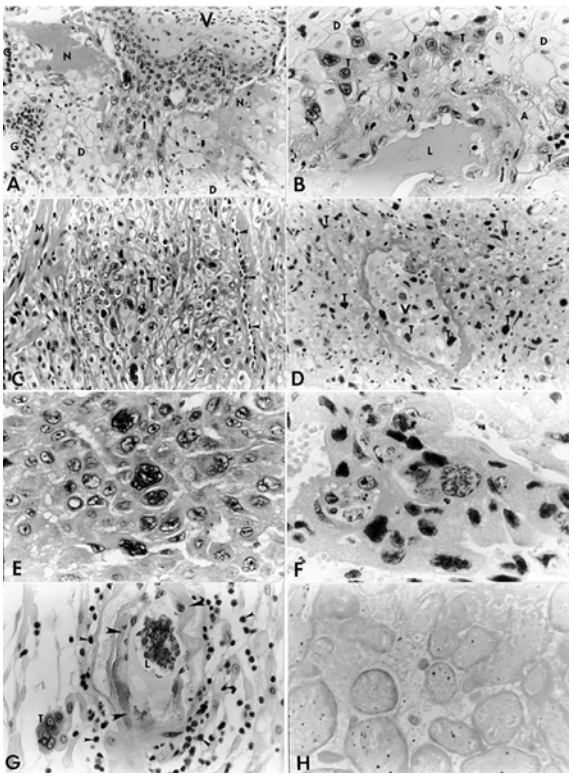

Figure 3. Histology of normal and abnormal invading trophoblasts. A) Junction of anchoring villous (V) and endometrium in a 4-5 week gestation. Note junctional trophoblasts (j) and invasive trophoblasts (I) penetrating through Nitabuch's layer (N) and infiltrating into the decidualized endometrium (D). Endometrial glands can be seen to the left (G). 264x. **B)** Deeper portion of same specimen as shown in A. Spiral arteriole (A) is surrounded by infiltrating trophoblasts (T). Decidual cells (D), vessel lumen (L). 528x. **C)** Dense infiltrate of intermediate trophoblasts (T) in the myometrium from a missed abortion. The differential in this case was between a placental site trophoblastic tumor and an exaggerated placental site. Note smooth muscle fibers (M) and endothelial cells (arrow heads) of a compressed vessel. The patient was followed with no recurrent trophoblastic disease. 264x **D)** Endometrial vessel (v) is filled and surrounded by abnormal, hyperchromatic trophoblasts (T). Chorionic villi in other parts of this spontaneous abortion specimen were diagnostic of a compete hydatidiform mole. The patient's chorionic gonadotropin levels were followed with no further evidence of trophoblastic disease. 264x. **E)** Extremely abnormal focus of trophoblasts found deep in the myometrium of a hysterectomy specimen after MRI diagnosed fundal mass following diagnosis of complete mole in an elective abortion specimen. The patient was found to have lung metastases and was treated with six courses of chemotherapy until her chorionic gonadotropin levels were undetectable. No evidence of trophoblastic disease after two years. 528x. **F)** Anaplastic focus of trophoblasts found in curetted specimen from uterus following four weeks of postpartum bleeding. Patient already had lung and brain metastases at this time. 528x. **G)** Decidual spiral artery from a 28 week gestation with clinical symptoms of severe preeclampsia. The spiral artery wall shows fibrinoid necrosis (arrow heads). Vessel lumen (L). While a few trophoblasts are near the vessel (T), no trophoblasts have entered the vessel wall, possibly because of the lymphocytes that surround the vessel (small arrow heads). 528x. **H)** Infarcted chorionic villi from placenta of the same 28 week gestation. The placenta was less than the 10th percentile for gestational age and had a total infarct volume of greater than 3 cubic centimeters.

The fact that the patient had a history of four previous second trimester losses suggests a primary immunologic reaction against paternal antigens on the invasive trophoblasts.

Pijnenborg has recently reviewed our current knowledge of trophoblast invasion based on histologic examination of human material (Pijnenborg, 1990). Following epithelial penetration of the endometrial surface epithelium, a phase of endometrial interstitial and endovascular trophoblast invasion occurs during the first month of development. The degree of interstitial versus endovascular trophoblast penetration during this early phase may vary between the human and monkey species. Interstitial trophoblasts can be identified in the human myometrium at about 8 weeks of development. Finally, Pijnenborg (1990) has proposed that a second wave of endovascular trophoblast invasion occurs from 14-15 weeks of gestation.

Although these studies have been crucial to identify the presence of trophoblasts in the endo— and myometrium during pregnancy, by their very nature these studies can not tell us about the dynamic process of trophoblast invasion. If one identifies an invasive extraplacental trophoblast in a tissue section, how long that particular trophoblast has been there is not clear (Fig. 3). At the present time we are completely ignorant about the rate of movement of each particular trophoblast and the longevity of these trophoblasts once they reach their destinations. For that matter, we can not determine if trophoblasts move in one direction and then stop, or move from place to place, sometimes present in the lumen of a vessel, sometimes in the wall of a vessel and sometimes outside of the vessels—migrating throughout the endo— and myometrium. At this time, there are no ways to study these problems, at least in the human. For the human process, the best we can hope is to piece together a model of trophoblast invasion using serial specimens and to characterize the cytoplasmic and membrane-associated components of the invasive trophoblasts in an attempt to decipher their interactions with the extracellular matrix and with other cells and to follow their invasive, migratory nature. Finally, we can employ model systems to learn something about cultured trophoblasts, which may shed some light on the *in vivo* processes.

MODEL SYSTEMS

Many different model systems have been employed to study implantation (Table 1) and trophoblast invasion. Implantation model systems have utilized animal blastocysts cultured on

extracellular matrix surfaces (Carson et al, 1988), blastocysts cultured on pieces of uteri (Glenister et al, 1961), blastocysts cultured on whole uteri (Grant et al, 1975), and more recently, blastocysts cultured on Matrigel–polarized endometrial epithelium (Glasser and Mulholland, 1993). Matrigel—a mixture of solubilized basement membrane components containing laminin, type IV collagen, heparan sulfate proteoglycan, and entactin from mouse Engelbreth-Holm-Swarm tumor—has been shown to support the differentiation of a number of cell types (Kleinman et al, 1986). A few workers have been able to utilize human blastocysts in co-culture models (Bulletti et al, 1988; Lindenberg, 1986), but more often cultured human trophoblasts have been utilized in a variety of model systems (Kliman et al, 1989a; Kliman et al, 1989b). The trophoblast invasion model systems have included dispersed trophoblasts cultured on amnion membranes (Yagel et al, 1988), filters containing small (usually 8 μm) holes, and filters with holes covered with ECM material, usually Matrigel (Librach et al, 1991), and slopes of Matrigel (Kliman and Feinberg, 1990).

Although each of these model systems has its limitations and can not truly recapitulate *in vivo* biology, they have all contributed to our understanding of trophoblast invasion. Through them, we have begun to appreciate the many complex proteases, protease inhibitors, hormones, growth factors and structural components that play a role in trophoblast invasion.

ROLE OF PROTEASES IN TROPHOBLAST INVASION

Trophoblast invasion appears to involve multiple steps (Fig. 4): penetration of the endometrial epithelium, rupture of the endometrial basement membrane, infiltration through the endo— and myometrium, penetration and conversion of the maternal spiral arteries, and finally, cessation of invasion. It is reasonable to conclude, therefore, that trophoblasts employ a variety of tools to perform these many functions. It is also not surprising that a variety of protease and protease inhibitor activities have been attributed to trophoblasts (Queenan et al, 1987; Yagel et al, 1988; Feinberg et al, 1989a; Jensen et al, 1989; Bischof et al, 1991; Librach et al, 1991; Herz et al 1992; Fernandez et al, 1992)—no doubt reflecting the different tasks that these cells must perform (Lala and Graham, 1990).

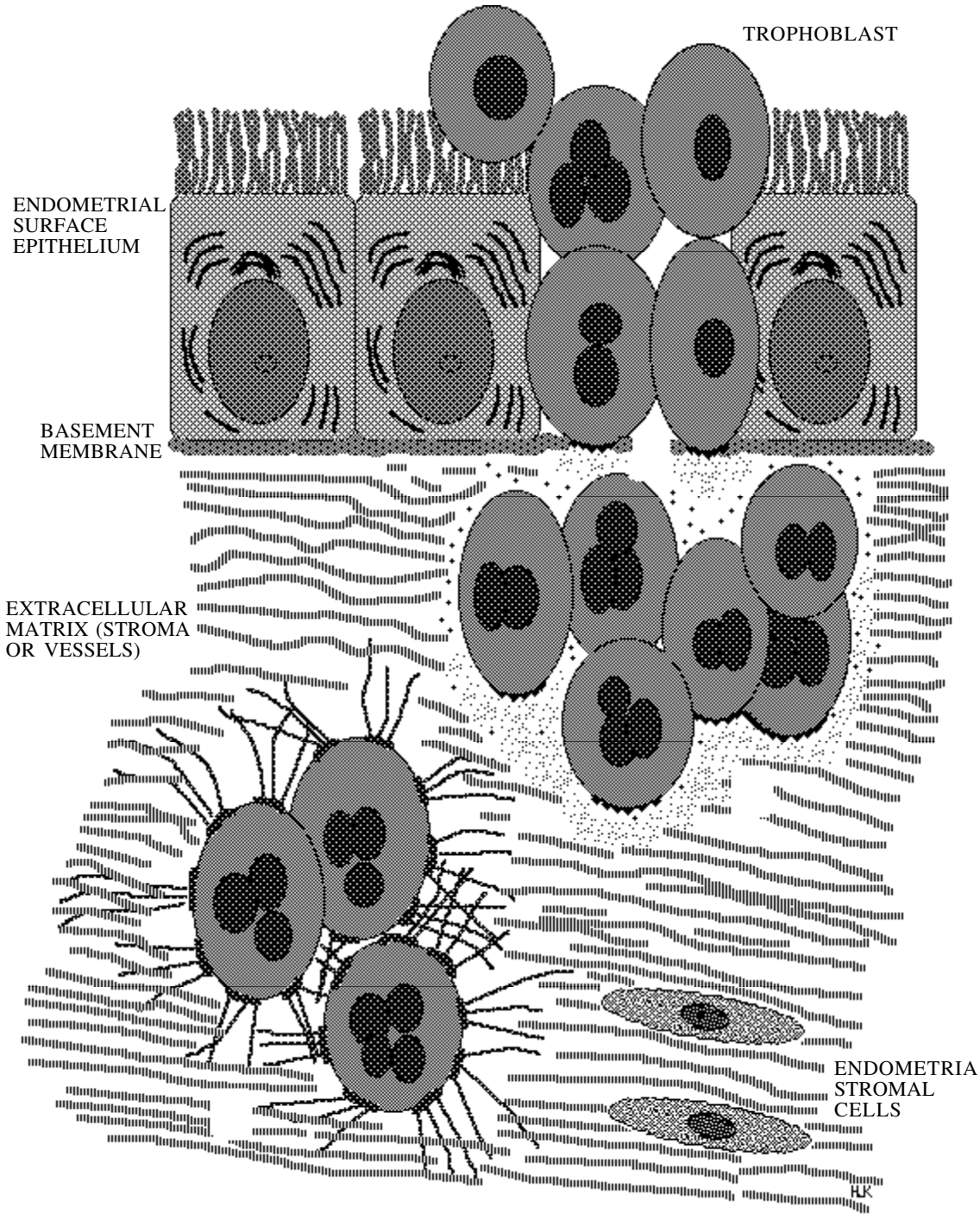

Figure 4. Model of human implantation. (1) Trophoblasts attach to the endometrial surface epithelium through cell adhesion molecules (CAMs) which are hormonally regulated. (2) The trophoblasts interdigitate between the epithelial cells, displacing some in the process. Eventually the trophoblasts make contact with the basement membrane, initiating the secretion of proteases (3). The extent of proteolysis is regulated by membrane associated ($_t$) and secreted (**+**) protease inhibitors. (4) Once the trophoblasts have reached their final destination (e.g., a

maternal spiral artery), they synthesize ECM proteins that firmly attach them to their surroundings.

The plasminogen activator (PA) system

Studies in non-human systems have proposed a critical role for trophoblast-secreted plasminogen activator (PA) during implantation and placentation. In mice, trophoblast production of PA correlates temporally with blastocyst invasion (Strickland et al, 1976; Sherman et al, 1976), and implantation-defective mouse embryos elaborate diminished amounts of PA (Axelrod, 1985). A role for PA in human nidation is suggested by the work of several investigators (Martin and Arias, 1982; Queenan et al, 1987; Zini et al, 1992), who demonstrated that trophoblasts in culture produce active urokinase-type PA (u-PA). In fact, the results of Cajot et al (1989) lend support to the concept that any cell type which produces active u-PA can harbor an invasive phenotype. These workers transfected non-invasive mouse L cells with a cosmid containing the complete human u-PA gene. Those cells which expressed human u-PA could both degrade and invade the ECM, suggesting that u-PA expression alone is sufficient to initiate these processes.

More recently it has been appreciated that trophoblasts do not simply secrete u-PA into the extracellular space to invade, but they must also localize this PA on the cell surface at the invasion front (Ellis et al, 1992; Estreicher et al, 1990). The key components described up to this point (Fig. 5) include (u-PA, the u-PA receptor (uPAR; Zini et al, 1992), plasminogen and plasmin (Hall et al, 1991; Plow et al, 1991; Jensen et al, 1989), type 1 plasminogen activator inhibitor (PAI-1; Feinberg et al, 1989a), LDL receptor-related protein (LRP; Herz et al, 1992), α2-macroglobulin (Strickland et al, 1990), and receptor-associated protein (RAP; Williams et al, 1992). It appears that trophoblast invasion, like tumor cell invasion, not only depends on the synthesis and secretion of plasminogen activators and their inhibitors, but the three dimensional relationships among these proteins, the cell surface and the extracellular matrix.

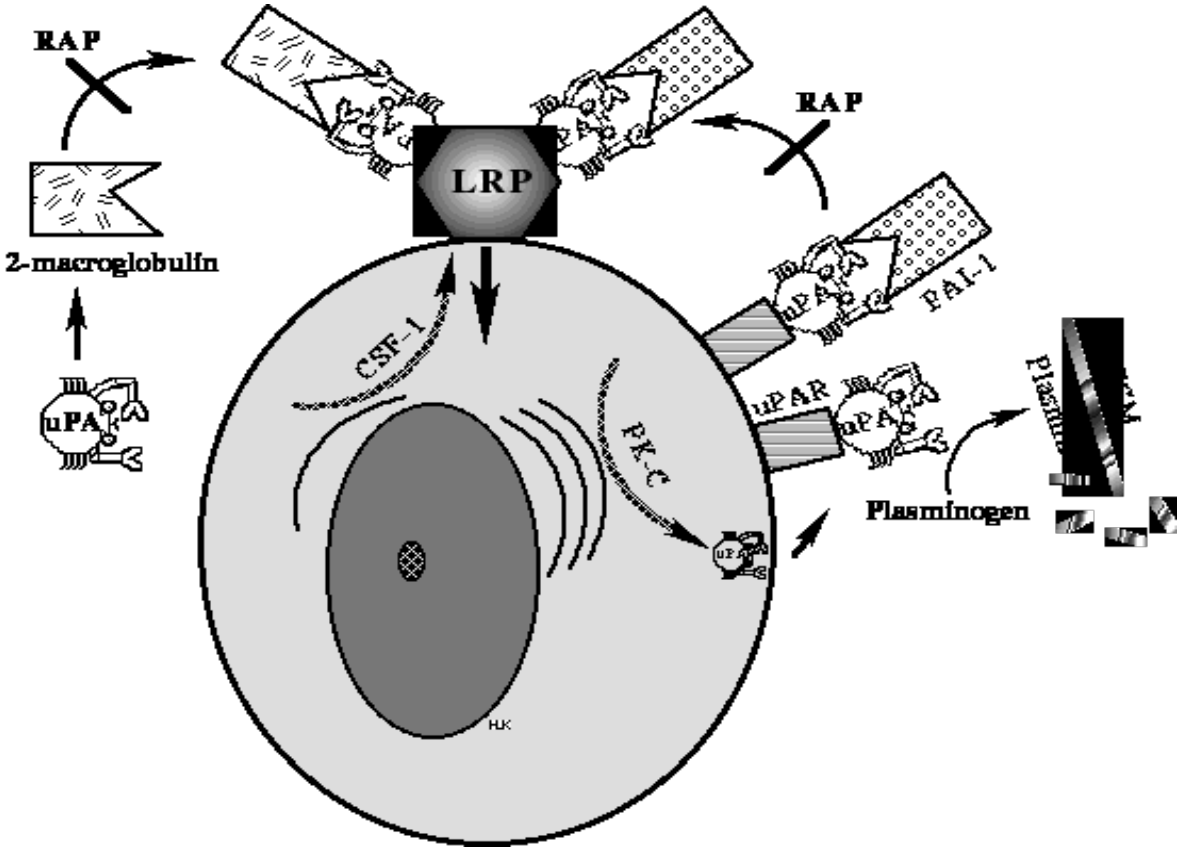

Figure 5. uPA, uPAR, PAI-1, LRP, α2-macroglobulin and RAP interactions. Urokinase-type plasminogen activator (u-PA) is synthesized and secreted by the trophoblast. Phorbol esters can increase trophoblast invasiveness, possibly by increasing u-PA synthesis. At the invasion front, the u-PA binds to its cell surface receptor, uPAR. This binding activates the u-PA and it converts plasminogen, which is present in the extracellular fluid, to plasmin, a potent protease that can either directly or indirectly through other proteases degrade extracellular matrix components. u-PA can also activate collagenases directly. Plasminogen activator inhibitor 1 (PAI-1)—which is also made by the invasive trophoblasts—can covalently bind to and inhibit u-PA. Once coupled to its inhibitor, the u-PA–PAI-1 complex associates with the LDL-receptor-related protein (LRP) and is taken up into the cell. u-PA can also be sequestered and taken into the cell via association with α2-macroglobulin and LRP. LRP synthesis is upregulated by CSF-1, a product of the decidualized endometrium. Finally, researchers have described an inhibitor of u-PA–PAI-1 and u-PA–α2-macroglobulin complex association with LRP: receptor-associated protein (RAP). RAP can regulate the amount of active u-PA that is on the cell surface by preventing u-PA–PAI-1 complexes from being cleared away. See text for details.

PA activity in vascular and extracellular spaces is modulated by PA inhibitors (PAIs), glycoproteins of the SERPIN (serine protease inhibitor) family that covalently bind to and inhibit

u-PA (Loskutoff et al, 1986; Wun and Riech, 1987). Therefore, it is likely that PA-PAI interactions modulate trophoblast invasion *in vivo* and control fibrinolysis within the intervillous spaces of the placenta. Until recently, evidence for trophoblast elaboration of PAIs during pregnancy has been indirect. Two well characterized PAIs, PAI-1 and PAI-2, were isolated from total placental extracts (Kruithof et al, 1987; Wun and Riech, 1987; Ye et al, 1987). In addition, plasma PAI-1 and PAI-2 levels increase with advancing gestation, but decrease dramatically soon after delivery (Kruithof et al, 1987). Altered plasma levels of PAIs have been reported in preeclampsia of pregnancy (DeBoer et al, 1988; Gore et al, 1987), a disease in which abnormalities in fibrinolysis and trophoblast function often occur. Studies to examine the synthesis and regulation of PAI-1 and PAI-2 in normal human cytotrophoblasts revealed that PAI -1 and PAI-2 mRNA and protein are produced by cultured cytotrophoblasts, whereas only PAI-1 is found in JEG-3 cells, a malignant trophoblast cell line (Feinberg et al, 1989b; Feinberg et al, 1990).

These clinical and *in vitro* data suggest a physiological role for the plasminogen activators and their inhibitors, but *in vivo* confirmation of these observations is critical. A number of studies have supplied this confirmation. Invading trophoblasts contain u-PA mRNA (Sappino et al, 1989), are immunoreactive for u-PA (Kliman, unpublished results), and more recently have been shown to express uPAR by immunohistochemistry (McCrae et al, 1993). As for the inhibitors, PAI-2 was localized by immunocytochemistry to villous syncytiotrophoblasts whereas PAI-1 was present primarily in invasive trophoblasts of implantation sites (Feinberg et al, 1989a). These findings imply an important physiological role for these proteins *in vivo*, and suggest that u-PA and PAI-1 are specifically required for the trophoblast invasive process (Fig. 6).

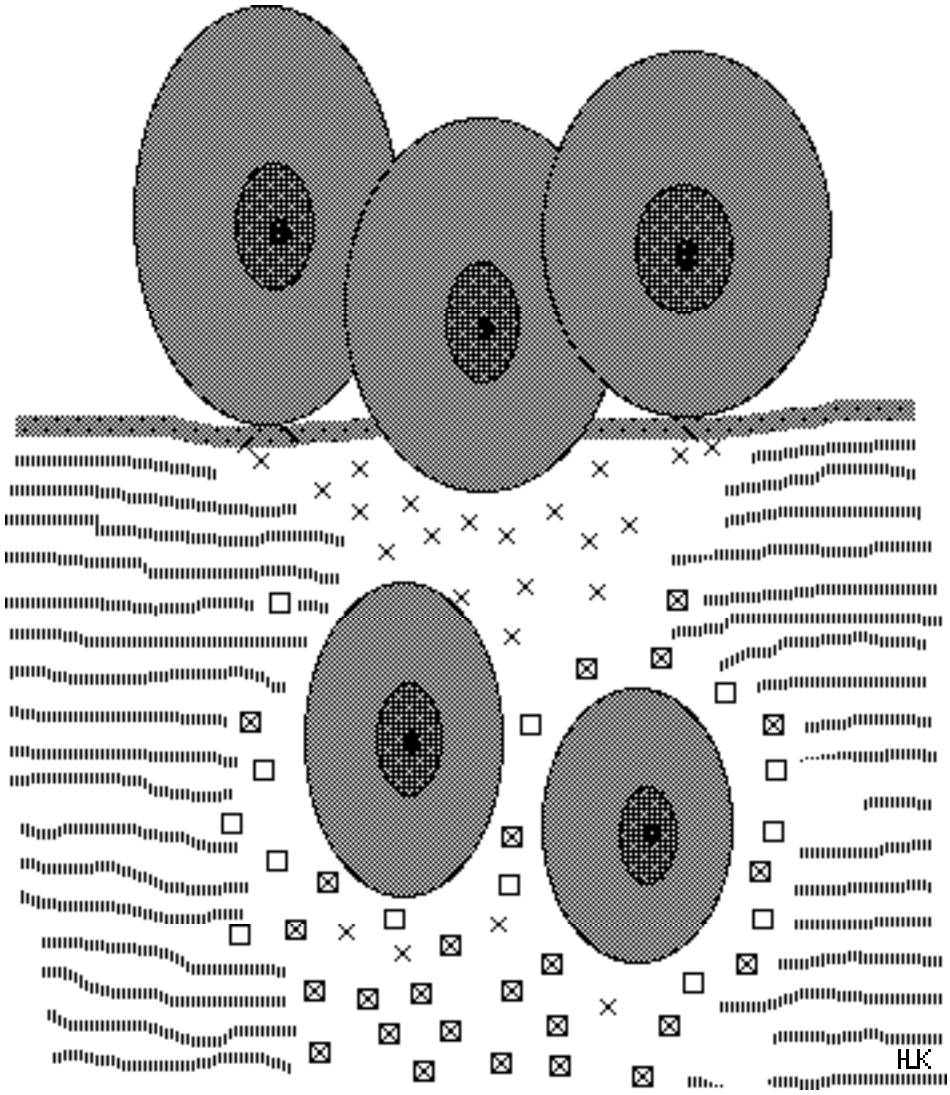

Figure 6. Model for roles of uPA and PAI-1 in trophoblast invasion. ❶ Trophoblasts make contact with basement membrane and begin to synthesize and secrete uPA. ❷ Dominant secreted product is uPA (×), resulting in degradation of ECM components either by direct protease action or by initiating other proteases (e.g., collagenases) in a cascade (see Fig. 7). ❸ PAI-1 (□), a potent inhibitor of uPA, begins to be synthesized and secreted as cells approach their destination. Cells switch over to mainly PAI-1 secretion, causing uPA to become progressively complexed with PAI-1 and inactivated (⊠), allowing cells to begin to synthesize new ECM components and become firmly attached in their new location.

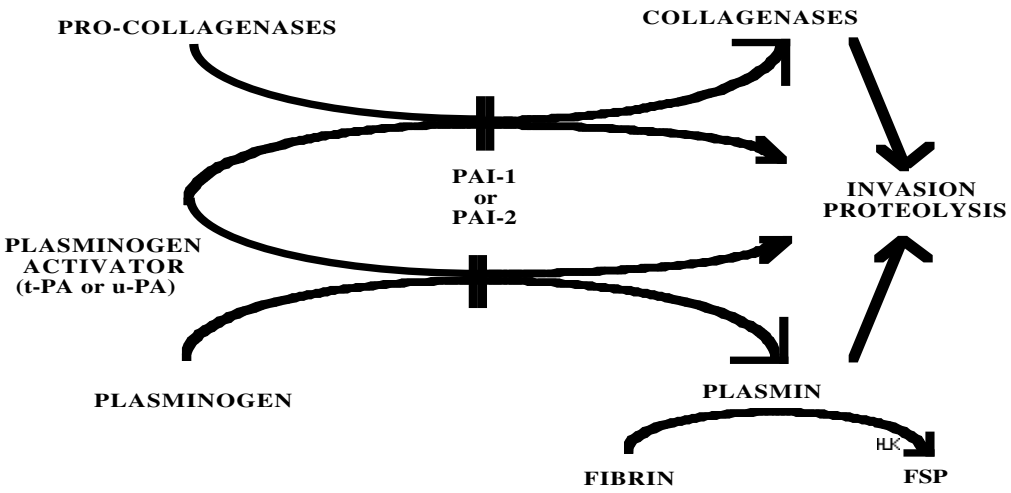

Figure 7. Plasminogen activator/collagenase cascade. Plasminogen activators —tissue type (t-PA) and urokinase-type (u-PA)—are able to convert plasminogen to plasmin. Plasmin is an extremely potent protease that can by itself degrade virtually all extracellular matrix proteins, as well as break down fibrin into fibrin split products (FSP). The plasminogen activators (Mignatti et al. 1986; Reich et al. 1988), as well as plasmin (Gavrilovic and Murphy, 1989), may also activate procollagenases to their active forms. Both plasmin and collagenases appear to participate in trophoblast invasion. The entire cascade can be blocked by the plasminogen activator inhibitors (PAI 1 and 2), which complex with the plasminogen activators. The inactive PA-PAI complexes are taken up by cell membrane receptors, which are then recycled back to the cell surface to bind more active PA (see Fig. 5).

HCG gradient formation: regulation and consequences for trophoblast invasion

Why don't all trophoblasts produce u-PA and PAI-1? Recent research suggests that hCG may play a critical role in determining whether a trophoblast will be a hormonally active cell or an invasive cell. As is typical of most hormones, chorionic gonadotropin synthesis is regulated by positive and negative factors (Kliman, 1994). GnRH and activin—which are made by the cytotrophoblasts—and EGF and hCG itself—which act as autocrine factors—all act, via adenylate cyclase and cAMP, to stimulate hCG secretion. TGF_{α} and inhibin—which are also made by the cytotrophoblasts—act to inhibit hCG secretion. hCG itself—which is secreted in large quantities into the intervillous space—also acts as a paracrine factor on the cytotrophoblasts (Shi et al, 1993), inducing them to differentiate towards a hormonally active syncytiotrophoblast phenotype (Fig. 8). The high concentrations of hCG in the intervillous space keeps the cytotrophoblasts differentiating down the syncytiotrophoblast pathway and inhibits their ability to make proteases (Milwidsky et al, 1993). In addition, cyclic AMP, the intracellular second messenger of hCG stimulation, has been shown to block trophoblast invasion (Kliman and Feinberg, 1990). The villi near the decidua are exposed to less hCG and are also affected by

other paracrines, including TGFß (Lysiak et al, 1992) and LIF (leukemia inhibitory factor; Stewart, 1994), which are made by the neighboring decidua. These factors switch the differentiation pathway (Fig. 2) of the cytotrophoblast away from becoming a villous syncytiotrophoblast to become an anchoring trophoblast which makes trophouteronectin—the fibronectin responsible for attaching the placenta to the decidua (Feinberg et al, 1991). The anchoring trophoblasts also make TGFß (Lysiak et al, 1992), which in an autocrine fashion also keeps these trophoblasts in the attachment pathway. Some trophoblasts escape the junctional zone, possibly by down regulating certain integrins (Damsky et al, 1992), and are free to migrate into the endo— and myometrium. Once free of the inhibitory effects of the hCG gradient, the invasive trophoblasts can make high levels of urokinase-type plasminogen activator and collagenases. By creating a TGFß gradient, the decidua protects itself and can limit trophoblast invasiveness by inducing the trophoblasts to make the inhibitors of these proteases: PAI-1 for the PA system (Feinberg et al, 1989a) and TIMP (tissue inhibitor of metaloproteinases) for collagenases (Graham and Lala, 1991; Lala and Graham, 1990).

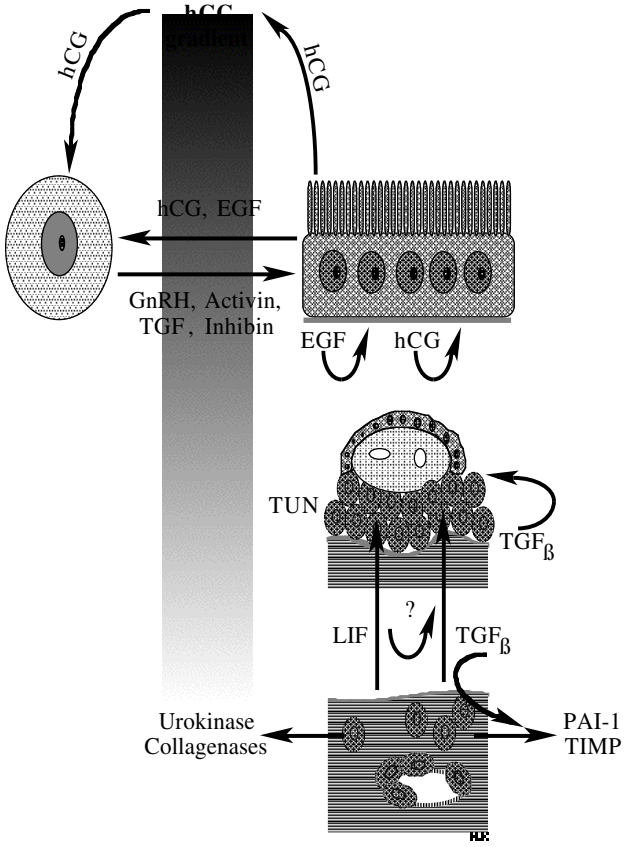

Figure 8. hCG gradient formation: regulation and consequences for trophoblast invasion. The chorionic villous syncytiotrophoblasts secrete high levels of hCG into the intervillous space, creating an hCG gradient. While bathed in high hCG levels, cytotrophoblasts are pushed towards a villous differentiation pathway and are inhibited from making proteases. The decidua secretes both TGF_{β} and LIF, forming a second gradient. At the placental-decidual junction, cytotrophoblasts are exposed to less hCG and are stimulated by TGF_{β} and LIF to make TUN, which anchors these trophoblasts to the endometrium. Some trophoblasts escape from the junction and migrate into the endometrium. The factors that control this step, and the subsequent migration of the invasive trophoblasts to the maternal spiral arteries are not known. Released from the effects of hCG and possibly stimulated by as yet unknown factors in the decidua, these invasive trophoblasts make plasminogen activators, collagenases, and the specific inhibitors for these proteases, all to coordinate the regulated trophoblast invasion of the endo— and myometrium (see Fig. 6)

Collagenases

Cultured trophoblasts produce a number of collagenases (Fernandez et al, 1992; Bischof et al, 1991; Librach et al, 1991; Emonard et al, 1990; Moll and Lane, 1990) and collagenase inhibitors (Graham and Lala, 1991; Librach et al, 1991). Although *in vitro* model systems have demonstrated collagenase dependent invasion (Librach et al, 1991; Yagel et al, 1988)—unlike the situation for the plasminogen activator system—only limited data exist to prove *in situ* a role for collagenases in implantation and trophoblast invasion (Fernandez et al, 1992).

In vitro work with normal and malignant trophoblasts have clearly demonstrated the expression of collagenases by these cells. In addition to simply being expressed, collagenase production is modulated by the matrix in which the trophoblasts find themselves. Emonard et al (1990) discovered that laminin caused normal trophoblasts to increase secretion of type IV, but not type I, collagenase, while type I collagen induced these trophoblasts to make increased quantities of both types I and IV collagenases. Bischof et al (1991) found that trophoblasts grown on Matrigel produced a 59 kD gelatinase that was not seen in the supernatants of cells grown on other matrices. It seems reasonable to hypothesize that trophoblasts make different proteases and inhibitors depending on where they are located in the invasion pathway (Fig. 4). Furthermore, the types and quantities of proteases and protease inhibitors may be modulated by cytokines found in different locations of the implantation site (Fig. 8).

As suggested above, trophoblast invasion requires a complex interaction of many enzymes, inhibitors and substrates. Not surprisingly, therefore, the activity and activation of the plasminogen activator system and collagenases appear to be intertwined (Fig. 7). In breast cancer lines, plasminogen via plasminogen activator has been shown to activate interstitial collagenases (Paranjpe et al, 1980; O'Grady et al, 1981), while in other cell lines, PA and

plasminogen are required for type IV collagenase activity (Salo et al, 1982; Gavrilovic and Murphy, 1989). Evidence also suggests that plasminogen activators can activate collagenase cascades (Mignatti et al. 1986; Reich et al. 1988), as well as evidence that u-PA can directly activate the 72 kD collagenase (Keski-Oja et al, 1992). It appears likely, therefore, that just as the movement of other invasive cells is facilitated by these enzymes, both the PA and collagenase systems coordinately facilitate trophoblast movement into the endo— and myometrium.

EXTRACELLULAR MATRIX (ECM) INTERACTIONS

Trophoblasts do not invade or migrate in a vacuum. As for any motile cell, interactions with the ECM are as important as degradation of matrix proteins. In fact, motility—and the requisite interaction with ECM proteins that motility demands—may be more important than ECM degradation (Liotta et al, 1986; Terranova et al, 1986). For this reason, a complete discussion of trophoblast invasion must also include binding interactions with the ECM. The elucidation of the fundamental role of integrins in cell-ECM interactions has opened another phase of trophoblast investigation. This work has led to a new understanding of trophoblast invasion, which may be more appropriately considered trophoblast *infiltration*.

Trophoblasts in culture avidly stick to ECM proteins, including laminin, type 1 collagen and fibronectin (Kao et al, 1988), as well as Matrigel (Kliman and Feinberg, 1990). Whole blastocysts also appear to have a propensity for laminin (Armant, 1991). The affinity of invasive extravillous trophoblasts for laminin appears to be specific characteristic of these cells (Romagnano and Babiarz, 1993; Loke et al, 1989a), and even has been exploited as a way of purifying this cell type (Loke et al, 1989b). This interaction is made more probable when one considers the large amount of laminin that is made by decidualized stromal cells of the luteal and pregnant endometrium (Loke et al, 1989a).

Trophoblasts, as is the case for virtually all cell types, interact with the ECM through integrins. Collectively, this work (Damsky et al, 1992; Aplin, 1993; Burrows et al, 1993; Zhou et al, 1993) has shown that as trophoblasts leave the cell columns and enter the maternal space they lose integrins (laminin specific) for basement membrane interactions and gain integrins for fibronectin and type I collagen interactions. In addition to integrins, specific cell-associated carbohydrates may play an important role in how tightly trophoblasts interact with the ECM (Yagel et al, 1990).

In addition to the integrins and the ECM proteins involved in these interactions, the thickness—or possible deformability—of the ECM appears to play a role in trophoblast biology and behavior. When trophoblasts from first or third trimester placentas were grown on glass or a slope of Matrigel with a thickness of less than 1 μm to over 60 μm, the cells behaved very differently depending on where they were growing (Kliman and Feinberg, 1990). After 48 h, the trophoblasts on the glass surface had flattened, aggregated, and begun to form syncytia, as has been described previously (Kliman et al, 1986). On the thin part of the beach—zone 1 (defined as the Matrigel extending from the visible edge to 4 μm in thickness)–the cells flattened out in a fashion very similar to the flattening on glass. On zone 2 (defined as the Matrigel from 4 to 14 μm in thickness), the cells caused marked clearing of the Matrigel by creating pericellular zones of lysis around the trophoblast aggregates. SEM revealed that the trophoblast groups on zone 2 progressively eroded the Matrigel until the glass surface was exposed. Zone 2 lysis was particularly pronounced after the Matribeaches were immunocytochemically stained for mouse laminin, which resulted in multiple areas of negative staining around the cells. The same effect was observed when first trimester trophoblasts were used. The cells on zone 3—Matrigel thicker than 14 μm—remained spherical, mostly single, and exhibited little or no zones of lysis. Immunohistochemical staining revealed that trophoblasts on all zones exhibited cytoplasmic staining for u-PA, PAI-1, and PAI-2 (Fig. 9). The cells on zone 2 were surrounded by a haze of u-PA and PAI-1, but not PAI-2, staining. In addition, u-PA staining appeared to involve the fine cytoplasmic processes seen to emanate from the cells of zone 2 (Fig. 9E).

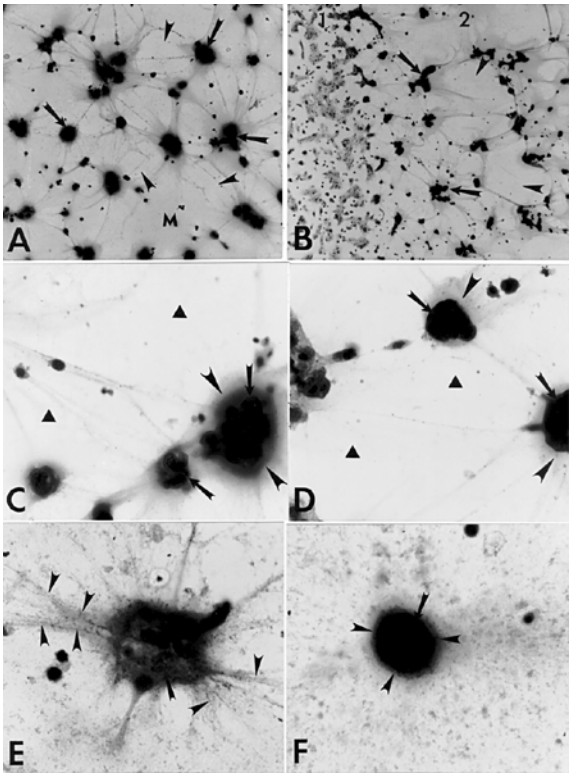

Figure 9. Matribeach immunocytochemistry for PAI-1, PAI-2 and u-PA. Normal human trophoblasts were cultured on Matribeach slopes for 48 h, fixed with Bouin's solution and immunocytochemically stained with specific polyclonal antibodies. Control cultures utilizing non-immune rabbit sera as primary antibody showed no staining. A. PAI-1 staining of zone 2 cells. After 48 h, the trophoblasts have aggregated into multicellular masses which stain intensely for PAI-1 (arrows). These zone 2 cells have induced marked clearing of the Matrigel (arrow heads). Note the long intercellular processes which extend into the Matrigel and connect many of the cell groups. Also note a portion of undigested Matrigel away from the trophoblast aggregates (M). B. PAI-2 staining of zone 1 and 2 cells. Light cytoplasmic staining of the flat zone 1 cells can be appreciated. The zone 2 cells show some cytoplasmic staining, but do not show an intense haze of PAI-2 staining around the cell groups (arrows). As in panel A, the zone 2 cells are surrounded by areas of Matrigel clearing (arrow heads). C. PAI-1 staining of zone 2 cells, higher power. The individual cells of the trophoblast aggregates can now be seen (arrows). In addition, the distinct PAI-1 pericellular ECM staining can be seen. Note the zones of Matrigel clearing (▲), and the fine intercellular processes. D. PAI-2 staining of zone 2 cells. Although the trophoblast aggregates are staining for PAI-2 (arrows), the pericellular area around the cells is unstained (arrow heads). Again, note the Matrigel clearing typical of zone 2 cells (▲) and the fine intercellular processes. E. u-PA staining of zone 2 trophoblasts, high power. Like the PAI-1 stained cells, this trophoblasts aggregate (arrow) exhibits distinct pericellular staining for u-PA. Unlike the PAI-1 stained cells, however, this cell reveals fine u-PA staining of the emanating cytoplasmic processes (arrow heads). See Fig. 10 for description of possible significance of this observation. F. u-PA staining of zone 3 trophoblasts, high power. Tightly massed group of trophoblasts (arrow) showing strong staining for u-PA. The staining appears to extend into the immediate pericellular space (arrow heads), but does not reveal evidence of u-PA stained cytoplasmic processes. Note the undigested Matrigel around the trophoblasts.

How can trophoblasts "know" how thick the Matrigel is under them? One hypothesis relates to the extension of u-PA rich cell processes beyond the reach of cell secreted PAI-1 (Fig. 10). A second possibility is that the matrix itself absorbs motility factors, making the cells on the thickest part of the Matribeach slope less able to move. Finally, recent work on integrin-ECM-cell transduction models (Ingber and Folkman, 1989; Ingber, 1991) has suggested the possibility that cell behavior—and hence cell differentiation and biochemistry—may be affected by the deformability of the surrounding ECM.

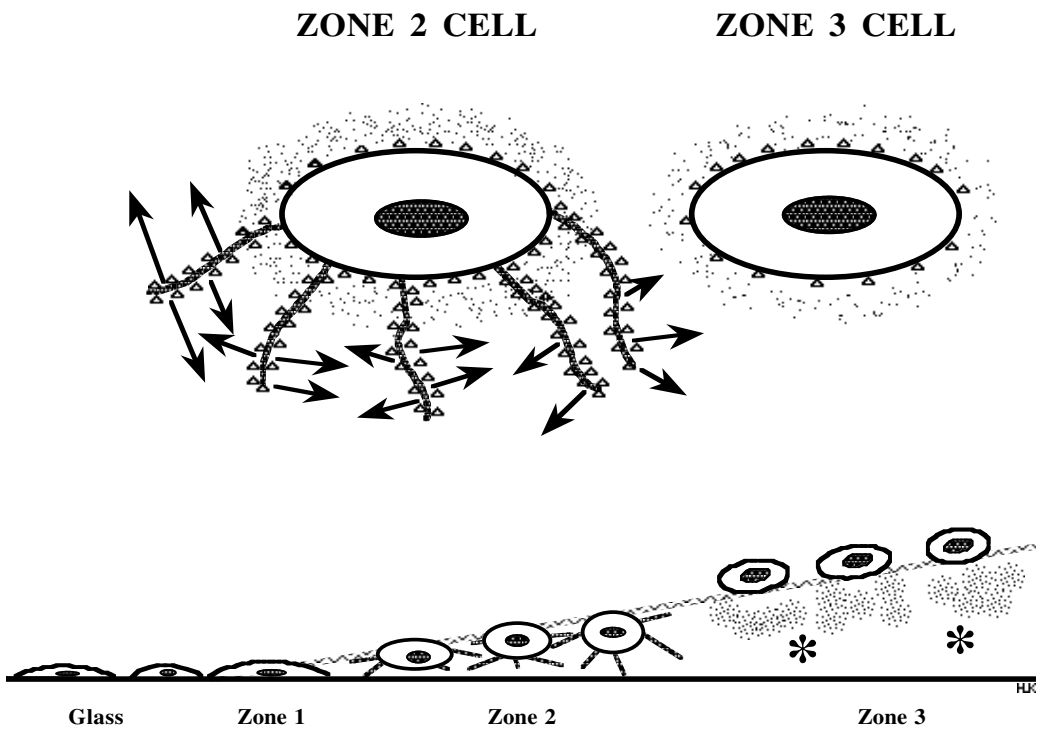

Figure 10. How does ECM thickness modulate trophoblast behavior on Matribeach? A model: The cells on zone 2, by random cell extensions, make contact with the glass, triggering extension of u-PA ( ) rich lamellipodia and microspikes (Pöllänen et al, 1987). These cell processes (**ZONE 2 CELL**) extend beyond the area of PAI inhibition (**dots**), allowing the membrane-associated u-PA to activate ECM resident collagenases and/or plasminogen, beginning ECM degradation (**arrows**). In zone 3, the cells are not able to extend processes through the >14 μm layer of Matrigel to the glass, and are thus not stimulated to extend u-PA rich lamellipodia or microspikes (**ZONE 3 CELL**). Alternatively, a factor (or factors) could be adsorbed by the thick Matrigel (*) of zone 3, thus removing stimulation for invasion by u-PA rich processes.

Once a migrating–invading trophoblast reaches its destination, it must reestablish bonds to the surrounding ECM (Fig. 2). In addition to being able to attach to a many ECM proteins, trophoblasts are also able to synthesize a wide variety of ECM proteins (Blankenship et al, 1992; Autio-Harmainen et al, 1991; Feinberg et al, 1991; OShea et al, 1990). It is in this way that the trophoblast solves the problem of reattachment to the matrix.

Which ECM proteins trophoblasts make depends on the local environment of the trophoblast. In addition to having a specific pattern of integrin distribution from the villi, through the anchoring cell columns on to the invading trophoblasts (Damsky et al, 1992), trophoblasts make unique ECM proteins at each of these different locations. Within the villi, laminin is the major ECM protein, while fibronectin is the major ECM protein around invasive intermediate trophoblasts (Damsky et al, 1992). When trophoblasts penetrate the spiral arteries of the placental bed, they synthesize type IV collagen and laminin (Blankenship et al, 1992). At the junction of the placenta and decidua, a unique fibronectin is made—trophouteronectin (TUN; Feinberg et al, 1991). This fibronectin, related to onco-fetal fibronectins, appears wherever trophoblasts make first contact with a foreign tissue (Feinberg et al, 1991). What factors regulate the synthesis of trophoblast ECM proteins in the villi and spiral arteries is not known at this time. However, we have noted that both TGFβ and LIF—which are made in high concentrations by the decidua (Figs. 2 and 8)—stimulate the production of trophoblast TUN (Feinberg et al, 1993; Nachtigall et al, 1994).

CYTOKINES AND GROWTH FACTORS

Growth factors and cytokines control more than just whether a trophoblast will or will not make a particular ECM protein. Evidence suggests that these factors control an entire repertoire of cell behaviors, coming together in concert to control implantation and placentation as a whole (Strickland and Richards, 1992).

Transforming growth factors α and ß (TGF_{α}, TGF_{β}), and epidermal growth factor (EGF) have been identified in trophoblasts, both *in vitro* and *in vivo*. TGF_{β} has been identified by immunohistochemistry in first and third trimester human placenta (Vuckovic et al, 1992), especially in the syncytial trophoblasts and the cell columns of first trimester anchoring villi. EGF and the EGF receptor have been localized to the syncytiotrophoblast in intrauterine and ectopic pregnancies (Hofmann et al, 1992), suggesting a potential autocrine role for EGF in placental growth. TGF_{α}, an EGF-like hormone, has also been identified in the placenta throughout gestation, but in the cytotrophoblasts of the chorionic villi (Filla et al, 1993). Both

EGF and TGFα were able to stimulate cultured cytotrophoblasts to increase their mitotic rate (Filla et al, 1993). The factors that may be responsible for activating trophoblast TUN production include TGFβ (Feinberg et al, 1993) and LIF (Nachtigall et al, 1994). TGFβ has been identified in the region of the utero-placental junction, possibly made by both decidual cells in that area and by the trophoblasts themselves (Lysiak et al, 1992). LIF has been identified in human endometrium (Stewart, 1994), but has not been shown to be made by trophoblasts. Interestingly, both TGFβ and LIF have been shown to upregulate TUN secretion from cultured trophoblasts while down-regulating hCG secretion (Feinberg et al, 1993; Nachtigall et al, 1994). In addition to switching the differentiation pathway of trophoblasts from becoming a villous trophoblast to becoming an anchoring trophoblast (Fig. 2), TGFβ also, as evidence shows, down regulates trophoblast invasiveness (Lala and Graham, 1990), and may even upregulate TIMP and PAI-1 in this cell type—as it does in other invasive cells (Laiho and Keski-Oja, 1989).

DISEASES OF TROPHOBLAST INVASION: PREECLAMPSIA AND GESTATIONAL TROPHOBLASTIC NEOPLASIA

Preeclampsia, the clinical state prior to full blown eclampsia (seizures), is one of the 'toxemias' of pregnancy. The basic clinical definition is a "pregnancy-specific condition of increased blood pressure accompanied by proteinuria, edema, or both" (Smith, 1993). In spite of simplicity of the clinical signs and symptoms, the etiology of the disease has remained elusive. Many phenomena have been investigated, but the recurring theme appears to be an abnormally low blood flow into the placenta (Naeye, 1989). One of the difficulties has been to distinguish between primary cause and secondary effects (Khong et al, 1992; Roberts et al, 1989; Chua et al, 1991; Bishop et al, 1990; Zeeman et al, 1992; Toppozada, 1990; Hsu et al, 1993). Part of this may be attributable to the fact that the common end result of low uteroplacental blood flow may be caused by many primary defects. Possibly, therefore, preeclampsia/eclampsia is not a disease, but a syndrome with many causes (Fay, 1990; Purcell, 1992; Zeeman and Dekker, 1992; Krege and Katz, 1990; Tuohy and James, 1992; Feinberg et al, 1991; Boyd et al, 1987). Significantly, one of the most frequent findings in preeclampsia is decreased or absent trophoblast invasion of the maternal spiral arteries (Pijnenborg et al. 1981; Pijnenborg, 1990; Robertson et al, 1984).

Decreased or absent trophoblast invasion may be a consequence of primary defects in the invasive trophoblasts or in the environment that the trophoblasts are attempting to invade. Studies show that in preeclampsia there is abnormal trophoblast integrin expression (Zhou et al, 1993), abnormal trophoblast glycogen metabolism (Arkwright et al, 1993), and decreased

trophoblast galactose alpha 1-3 galactose (Christiane et al, 1992). In addition, preeclampsia has been associated with trisomy 13 (Tuohy and James, 1992), the chromosome that carries the gene for type IV collagen. Placental bed biopsy in a case of preeclampsia in a multiparous woman carrying a trisomy 13 fetus showed lack of trophoblast invasion of maternal spiral arteries (Feinberg et al, 1991). These trophoblasts may have had difficulty invading through the maternal ECM because of increased type IV collagen production. In addition to primary trophoblast defects, many cases of preeclampsia appear to be related to maternal immunologic reaction against the invading trophoblasts. A common clinical finding in these cases is that the spiral arteries are not converted and instead are surrounded by lymphocytes (see Fig. 3G), presumably attacking the foreign-appearing invasive trophoblasts.

In contrast to the clinical syndrome of decreased trophoblast invasion, gestational trophoblastic disease (GTD) represents increased and uncontrolled trophoblast invasion. Expanded trophoblast invasion ranges from an exaggerated placental site with increased numbers of benign intermediate trophoblasts (Kurman, 1991a), to placental site trophoblastic tumors (Young et al, 1988), to invasive moles, to frank choriocarcinoma (Kurman, 1991b). Morphologic distinction between these forms of trophoblast proliferation can be difficult (see Fig. 3; Sasagawa et al, 1988), but we have been able to show that the normal mechanisms that stop trophoblast invasion are defective in choriocarcinoma cell lines (Kliman and Feinberg, 1990). Normal cytotrophoblast differentiation can be shifted towards a villous syncytiotrophoblast and away from an invasive trophoblast phenotype by cAMP analogues (Kliman, 1994), while this treatment does not affect choriocarcinoma invasiveness (Kliman and Feinberg, 1990), suggesting a primary defect in differentiation-signalling in the malignantly invasive trophoblast.

SURVIVAL ADVANTAGE

The advent of trophoblast invasiveness allowed humans to gestate long enough to have a survival advantage over those creatures that did not acquire this ability. With this enhancement in the placental-uterine relationship came new demands. Signals were required to trigger trophoblasts to attach firmly to the uterus, to leave the placenta and invade into the endo— and myometrium, to modify the maternal spiral arteries without causing hemorrhage and thrombosis, and finally to limit invasiveness. Our increased understanding of trophoblast biology is beginning to clarify the many aspects of trophoblast differentiation and invasiveness that lead to a healthy pregnancy; or when defects in these control mechanisms occur, to understand how trophoblast pathology can cause serious, even life threatening, complications of pregnancy. Our

challenge now is to apply these new insights to promote healthy pregnancies for all who want them.

REFERENCES

Aplin JD. (1993) Expression of integrin alpha 6 beta 4 in human trophoblast and its loss from extravillous cells. Placenta 14:203-15

Arkwright PD, Rademacher TW, Dwek RA, Redman CW. (1993) Pre-eclampsia is associated with an increase in trophoblast glycogen content and glycogen synthase activity, similar to that found in hydatidiform moles. J Clin Invest 91:2744-53

Armant DR, Kaplan HA, Lennarz WJ. (1986) Fibronectin and laminin promote *in vitro* attachment and outgrowth of mouse blastocysts. Dev Biol 116:519-523

Armant DR. (1991) Cell interactions with laminin and its proteolytic fragments during outgrowth of mouse primary trophoblast cells. Biol Reprod 45:664-72

Autio HH, Sandberg M, Pihlajaniemi T, Vuorio E. (1991) Synthesis of laminin and type IV collagen by trophoblastic cells and fibroblastic stromal cells in the early human placenta. Lab Invest 64:483-91

Axelrod HR. (1985) Altered trophoblast functions in implantation-defective mouse embryos. Dev Biol 108:185-90

Benirschke K, Kaufmann P. (1990) Pathology of the human placenta. New York, NY, Springer-Verlag, pp 585-613

Bischof P, Friedli E, Martelli M, Campana A. (1991) Expression of extracellular matrix-degrading metalloproteinases by cultured human cytotrophoblast cells: effects of cell adhesion and immunopurification. Am J Obstet Gynecol 165(6 t 1)

Bishop PW, Malam JE, Morris JA, Fox H. (1990) Accelerated expression of Ca antigen by placental villous trophoblast in pre-eclampsia. Placenta 11:487-92

Blankenship TN, Enders AC, King BF. (1992) Distribution of laminin, type IV collagen, and fibronectin in the cell columns and trophoblastic shell of early macaque placentas. Cell Tissue Res 270:241-8

Boyd PA, Lindenbaum RH, Redman C. (1987) Pre-eclampsia and trisomy 13: a possible association. Lancet 2:425-7

Bulletti C, Jasonni VM, Tabanelli S, Gianaroli L, Ciotti PM, Ferraretti AP, et al. (1988) Early human pregnancy *in vitro* utilizing an artificially perfused uterus. Fertil Steril 49: 991-996

Burrows TD, King A, Loke YW. (1993) Expression of integrins by human trophoblast and differential adhesion to laminin or fibronectin. Hum Reprod 8:475-84

Cajot JF, Schleuning WD, Medcalf RL, Bamat J, Testuz J, Liebermann L, Sordat B. (1989) Mouse L cells expressing human prourokinase-type plasminogen activator: effects on extracellular matrix degradation and invasion. J Cell Biol 109:915-25

Cammarata PR, Oakford L, Canta-Crouch D, Wordinger R. (1987) Attachment of blastocysts to lens capsule: A model system for trophoblast-epithelial cell interactions on a natural basement membrane. Cell Tissue Res 250: 633-640

Carson DD, Tang JP, Gay S. ((1988) Collagens support embryo attachment and outgrowth *in vitro*: effects of the Arg-Gly-Asp sequence. Dev Biol 127: 368-375

Christiane Y, Aghayan M, Emonard H, Lallemand A, Mahieu P, Foidart JM. (1992) Galactose alpha 1-3 galactose and anti-alpha galactose antibody in normal and pathological pregnancies. Placenta 13:475-87

Chua S, Wilkins T, Sargent I, Redman C. (1991) Trophoblast deportation in pre-eclamptic pregnancy. Br J Obstet Gynaecol 98:973-9

Conley AJ, Mason JI. (1990) Placental steroid hormones. Baillieres Clin Endo Met 4:249-272

Damsky CH, Fitzgerald ML, Fisher SJ. (1992) Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, *in vivo*. J Clin Invest 89:210-22

DeBoer K, Lecander I, ten Cate JW, Borm JJJ, Treffers PE. (1988) Placental-type plasminogen activator inhibitor in preeclampsia. Am J Obstet Gynecol 158:518

Ellis V, Pyke C, Eriksen J, Solberg H, Dano K. (1992) The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. Ann N Y Acad Sci 667:13-31

Emonard H, Christiane Y, Smet M, Grimaud JA, Foidart JM. (1990) Type IV and interstitial collagenolytic activities in normal and malignant trophoblast cells are specifically regulated by the extracellular matrix. Invasion Metastasis 10:170-7

Enders AC. (1989) Trophoblast differentiation during the transition from trophoblastic plate to lacunar stage of implantation in the rhesus monkey and human. Am J Anat 186:85-98

Enders AC, King BF. (1991) Early stages of trophoblastic invasion of the maternal vascular system during implantation in the macaque and baboon. Am J Anat 192:329-46

Estreicher A, Mullhauser J, Carpentier J-L, Orci L, Vassalli J-D. (1990) The receptor for urokinase type plasminogen activator polarizes expression of the protease to the leading edge of migrating monocytes and promotes degradation of enzyme inhibitor complexes. J Cell Biol 111:783-792

Farach MC, Tang JP, Decker GL, Carson DD. (1987) Heparin/heparan sulfate is involved in attachment and spreading of mouse embryos *in vitro*. Dev Biol 123:401-10

Fay RA. (1990) Uric acid in pregnancy and preeclampsia: an alternative hypothesis. Aust N Z J Obstet Gynaecol 30:141-2

Feinberg RF, Kao LC, Haimowitz JE, Queenan JTJ, Wun TC, Strauss JF3, Kliman HJ. (1989a) Plasminogen activator inhibitor types 1 and 2 in human trophoblasts. PAI-1 is an immunocytochemical marker of invading trophoblasts. Lab Invest 61:20-6

Feinberg RF, Kao LC, Haimowitz JE, Queenan JTJ, Wun TC, Strauss JF3, Kliman HJ. (1989a) Plasminogen activator inhibitor types 1 and 2 in human trophoblasts. PAI-1 is an immunocytochemical marker of invading trophoblasts. Lab Invest 61:20-6

Feinberg RF, Strauss JF3, Wun T-C, Kliman HJ. (1989b) Plasminogen activators (PAs) and plasminogen activator inhibitors (PAIs) in human trophoblasts: markers of trophoblast invasion (Abst). Proc Soc Gyn Invest 36:487

Feinberg RF, Kao LC, Wang C-L, Bui L, Kliman HJ, Strauss JF3. (1990) Plasminogen activator inhibitor (PAI) expression in normal and malignant human trophoblasts: regulation by 8-bromo-camp and phorbol esters. Presented at the 37th Annual Meeting of the Society for Gynecologic Investigation.

Feinberg RF, Kliman HJ, Lockwood CJ. (1991) Oncofetal fibronectin: A trophoblast "glue" for human implantation? Am J Pathology, 138:537-543

Feinberg RF, Kliman HJ, Wang CL. (1993) Transforming growth factor beta (TGFß) is a critical modulator of tropho-uteronectin (TUN) synthesis *in vitro*: Implications for trophoblast implantation. Presented at the 40th Annual Meeting of the Society for Gynecologic Investigation.

Fernandez PL, Merino MJ, Nogales FF, Charonis AS, Stetler-Stevenson W, Liotta L. (1992) Immunohistochemical profile of basement membrane proteins and 72 kilodalton type IV collagenase in the implantation placental site. An integrated view. Lab Invest 66:572-9

Filla MS, Zhang CX, Kaul KL. (1993) A potential transforming growth factor alpha/epidermal growth factor receptor autocrine circuit in placental cytotrophoblasts. Cell Growth Differ 4:387-93

Gavrilovic J, Murphy G. (1989) The role of plasminogen in cell-mediated collagen degradation. Cell Biol Int Rep 13:367-375

Glass RH, Spindle AI, Pedersen RA. (1979) Mouse embryo attachment to substratum and interaction of trophoblast with cultured cells. J Exp Zool 208: 327

Glasser SR, Mulholland J. (1993) Receptivity is a polarity dependent special function of hormonally regulated uterine epithelial cells. Microsc Res Tech 25:106-20

Glenister TW. (1961) Organ culture as a new method for studying the implantation of mammalian blastocysts. Proc Royal Soc B 154: 428-431

Gore M, Eldon S, Trofatter KF, Soong S-J, Pizzo SV. (1987) Pregnancy-induced changes in the fibrinolytic balance: evidence for defective release of tissue plasminogen activator and increased levels of the fast-acting tissue plasminogen activator inhibitor. Am J Obstet Gynecol 156:674

Graham CH, Lala PK. (1991) Mechanism of control of trophoblast invasion *in situ*. J Cell Physiol 148:228-234

Grant PS, Ljungkvist I, Nilsson O. (1975) The hormonal control and morphology of blastocyst invasion in the mouse uterus *in vitro*. J Embryol Exp Morphol 34: 310

Hall SW, Humphries JE, Gonias SL. (1991) Inhibition of cell surface receptor-bound plasmin by α2-antiplasmin and α2-macroglobulin. J Biol Chem 266:12329-12336

Hertig AT, Rock J. (1956) A description of 34 human ova within the first 17 days of development. Am J Anat 98:435-494

Herz J, Clouthier DE, Hammer RE. (1992) LDL receptor-related protein internalizes and degrades uPA-PAI-1 complexes and is essential for embryo implantation. Cell 71:411-21

Hofmann GE, Drews MR, Scott RTJ, Navot D, Heller D, Deligdisch L. (1992) Epidermal growth factor and its receptor in human implantation trophoblast: immunohistochemical evidence for autocrine/paracrine function. J Clin Endocrinol Metab 74:981-8

Hsu CD, Iriye B, Johnson TR, Witter FR, Hong SF, Chan DW. (1993) Elevated circulating thrombomodulin in severe preeclampsia. Am J Obstet Gynecol 169:148-9

Ingber DE, Folkman J. (1989) Mechanochemical switching between growth and differentiation during fibroblast growth factor-stimulated angiogenesis *in vitro*: role of extracellular matrix. J Cell Biol 109:317-30

Ingber D. (1991) Integrins as mechanochemical transducers. Curr Opin Cell Biol 3:841-8

Jara CS, Salud AT, Bryantgreenwood GD, Pirens G, Hennen G, Frankenne F. (1989) Immunocytochemical localization of the human growth hormone variant in the human Placenta. J Clin Endocrinol Metab 69:1069-1072

Jenkinson EJ. (1978) The *in vitro* blastocyst outgrowth system as a model for the analysis of peri-implantation development. In: Johnson M, ed. Development in Mammals, Vol. 2. Amsterdam, North-Holland, p157

Jensen HE, Poulsen OM, Hau J. (1989) Localization of plasmin on the surface of the human trophoblast. Biomed Biochim Acta 48:437-40

Kao LC, Caltabiano S, Wu S, Strauss JF3, Kliman HJ. (1988) The human villous cytotrophoblast: interactions with extracellular matrix proteins, endocrine function, and cytoplasmic differentiation in the absence of syncytium formation. Dev Biol 130:693-702

Kawagoe K, Akiyama J, Kawamoto T, Morishita Y, Mori S. (1990) Immunohistochemical demonstration of epidermal growth factor (EGF) receptors in normal human placental villi. Placenta 11:7-15

Keski-Oja J, Lohi J, Tuuttila A, Tryggvason K, Vartio T. (1992) Proteolytic processing of the 72,000-Da type IV collagenase by urokinase plasminogen activator. Exp Cell Res 202:471-476

Khong TY, Sawyer IH, Heryet AR. (1992) An immunohistologic study of endothelialization of uteroplacental vessels in human pregnancy-evidence that endothelium is focally disrupted by trophoblast in preeclampsia. Am J Obstet Gynecol 167:751-6

Kishimoto Y, Tominaga T, Aso T, Kinoshita M, Mori T. (1987) Human trophoblast and endometrial interactions *in vitro*. Acta Obst. Gynaec Jpn 39: 463

Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR. (1986) Basement membrane complexes with biological activity. Biochemistry 25:312-8

Kliman HJ, Nestler JE, Sermasi E, Sanger JM, Strauss JF3. (1986) Purification, characterization, and *in vitro* differentiation of cytotrophoblasts from human term placentae. Endocrinology 118:1567-82

Kliman HJ, Coutifaris C, Babalola GO, Soto EA, Kao LC, Queenan JTJ, Feinberg RF, Strauss JF3. (1989a) The human trophoblast: homotypic and heterotypic cell-cell interactions. Prog Clin Biol Res 294:425-34

Kliman HJ, Coutifaris C, Feinberg RF, Strauss JF III, & Haimowitz JE. (1989b) Implantation: *in vitro* models utilizing human tissues. In Blastocyst Implantation, ed. Yoshinaga, K. (Adams, Bost, MA), pp. 83-91

Kliman HJ, Feinberg RF. (1990) Human trophoblast-extracellular matrix (ECM) interactions *in vitro*: ECM thickness modulates morphology and proteolytic activity. Proc Natl Acad Sci U S A 87:3057-61

Kovats S, Main EK, Librach C, Stubblebine M, Fisher SJ, DeMars R: A class I antigen, HLA-G, expressed in human trophoblasts. Science 1990, 248:220-3

Krege JH, Katz VL. (1990) A proposed relationship between vasopressinase altered vasopressin and preeclampsia. Med Hypotheses 31:283-7

Kruithof EKO, Tran-Thang C, Gudinchet A, Hauert J, Nicoloso G, Genton C, Welti H, Bachmann F. (1987) Fibrinolysis in Pregnancy: A Study of Plasminogen Activator Inhibitors. Blood 69:460

Kurman RJ, Main CS, Chen H-C. (1984) Intermediate trophoblast: a distinctive form of trophoblast with specific morphological, biochemical and functional features. Placenta 5:349-370

Kurman RJ. (1991a) The morphology, biology, and pathology of intermediate trophoblast: A look back to the present. Human Pathol 22:847-855

Kurman RJ. (1991b) Pathology of trophoblast. Monogr Pathol 33:195-227

Laiho M, Keski-Oja J. (1989) Growth factors in the regulation of pericellular proteolysis: a review. Cancer Res 49:2533-2553

Lala PK, Graham CH. (1990) Mechanisms of trophoblast invasiveness and their control: the role of proteases and protease inhibitors. Cancer Metastasis Rev 9:369-79

Librach CL, Werb Z, Fitzgerald ML, Chiu K, Corwin NM, Esteves RA, Grobelny D, Galardy R, Damsky CH, Fisher SJ. (1991) 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. J Cell Biol 113:437-49

Lindenberg S, Hyttel P, Lenz S, Holmes PV. (1986) Ultrastructure of the early human implantation *in vitro*. Hum Reprod 1: 533-538

Lindenberg S. (1991) Ultrastructure in human implantation: transmission and scanning electron microscopy. Baillieres Clin Obstet Gynaecol 5:1-14

Liotta LA, Murano G, Katz DA, Gordon RK, Chiang PK, Schiffmann E. (1986) Tumor cell autocrine motility factor. Proc Natl Acad Sci U S A 83:3302-6

Loke YW, Gardner L, Burland K, King A. (1989a) Laminin in human trophoblast--decidua interaction. Hum Reprod 4:457-63

Loke YW, Gardner L, Grabowska A. (1989b) Isolation of human extravillous trophoblast cells by attachment to laminin-coated magnetic beads. Placenta 10:407-15

Loskutoff DJ, Ny T, Sawdey M, Lawrence D. (1986) Fibrinolytic system of cultured endothelial cells: regulation by plasminogen activator inhibitor. J Cell Biochem 32:273

Lysiak JJ, McCrae KR, Lala PK. (1992) Localization of transforming growth factor-beta at the human fetal-maternal interface: role in trophoblast growth and differentiation. Biology of Reproduction 46:561-72

Martin O, Arias F. (1982) Plasminogen activator production by trophoblast cells *in vitro*: effect of steroid hormones and protein synthesis inhibitors. Am J Obstet Gynecol 142: 402-9

Maruyama I, Bell CE, Majerus PW. (1985) Thrombomodulin is found on endothelium of arteries, veins, capillaries, and lymphatics, and on syncytiotrophoblast of human placenta. J Cell Biol 101:363-71

McCrae K, Multhaupt H, Cines D, Warhol M. (1993) Expression of urokinase receptors by human trophoblast: a histochemical and ultrastructural analysis. Presented at the Molecular and Cellular Biology of Plasminogen Activation Meeting, Cold Spring Harbor, September 1993

Mignatti P, Robbins E, Rifkin DB. (1986) Tumor invasion through the human amniotic membrane: requirement for a proteinase cascade. Cell 47:487-98

Milwidsky A, Finci YZ, Yagel S, Mayer M. (1993) Gonadotropin-mediated inhibition of proteolytic enzymes produced by human trophoblast in culture. J Clin Endocrinol Metab 76:1101-5

Moll UM, Lane BL. (1990) Proteolytic activity of 1st trimester human placenta - localization of interstitial collagenase in villous and extravillous trophoblast. Histochemistry 94:555-560

Nachtigall MJ, Kliman HJ, Feinberg RF, Meaddough EL, Arici A. (1994) Potential role of leukemia inhibitory factor (LIF) in human implantation. Presented at the 41st Annual Meeting of the Society for Gynecologic Investigation

Naeye RL. (1989) Pregnancy hypertension, placental evidences for low uteroplacental blood flow, and spontaneous premature delivery. Hum Pathol 20:441-444

O'Grady RL, Upfold LI, Stephens RW. (1981) Rat mammary carcinoma cells secrete active collagenase and activate latent enzyme in the stroma via plasminogen activator. Int J Cancer 28:509-515

Ohlsson R, Larsson E, Nilsson O, Wahlstrom T, Sundstrom P. (1989) Blastocyst implantation precedes induction of insulin-like growth factor II gene expression in human trophoblasts. Development 106:555-9

Ohtani H, Maruyama I, Yonezawa S. (1989) Ultrastructural immunolocalization of thrombomodulin in human placenta with microwave fixation. Act Hist Cy 22:393-5

OShea KS, Liu LH, Kinnunen LH, Dixit VM. (1990) Role of the extracellular matrix protein thrombospondin in the early development of the mouse embryo. J Cell Biol 111(6 t 1)

Paranjpe M, Engel L, Youg N, Liotta LA. (1980) Activation of human breast carcinoma collagenase through plasminogen activator. Life Sci 26:1223-1231

Petraglia F, Calza L, Garuti GC, Giardino L, De RB, Angioni S. (1990) New aspects of placental endocrinology. J Endocrinol Invest 13:353-371

Pijnenborg, R., W. B. Robertson, I. Brosens, G. Dixon. (1981) Trophoblast invasion and the establishment of hemochorial placentation in man and laboratory animals. Placenta 2: 71-92

Pijnenborg R. (1990) Trophoblast invasion and placentation in the human—morphological aspects. Troph Res 4:33-47

Plow EF, Felez J, Miles LA. (1991) Cellular regulation of fibrinolysis. Thromb Hemost 66:32-36

Pöllänen J, Saksela O, Salonen E-M, Andreasen P, Nielsen L, Dano K, Vaheri A. (1987) Distinct localizations of Urokinase-type Plasminogen Activator and Its Type 1 Inhibitor under Cultured Human Fibroblasts and Sarcoma Cells. J Cell Biol 104:1085-96

Posner BI. (1974) Insulin receptors in human and animal placental tissue. Diabetes 23:209-217

Purcell WM. (1992) Human placental mast cells: a role in pre-eclampsia? Med Hypotheses 39:281-3

Queenan JT Jr, Kao L-C, Arboleda CE, Ulloa-Aguirre A, Golos TG, Cines DB, Strauss JF III. (1987) Regulation of urokinase-type plasminogen activator production by cultured human cytotrophoblasts. J Biol Chem 262:10903-6

Reich R, Thompson EW, Iwamoto Y, Martin GR, Deason JR, Fuller GC, Miskin R. (1988) Effects of inhibitors of plasminogen activator, serine proteinases, and collagenase IV on the invasion of basement membranes by metastatic cells. Cancer Res 48:3307-12

Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, Mclaughlin MK. (1989) Preeclampsia - An Endothelial Cell Disorder. American Journal of Obstetrics and Gynecology 161:1200-1204

Robertson WB, Brosens I, Pijnenborg R, DeWolf F. (1984) The making of the placental bed. Europ J Obstet Gynec Reprod Biol 18:255-266

Rodesch F, Simon P, Donner C, Jauniaux E. (1992) Oxygen measurements in endometrial and trophoblastic tissues during early pregnancy. Ob Gyn 80:283-5

Romagnano L, Babiarz B. (1993) Mechanisms of murine trophoblast interaction with laminin. Biol Reprod 49:374-80

Salo T, Liotta LA, Keski-Oja J, Turpeenniemi-Hujanen T, Tryggvason K. (1982) Secretion of basement membrane collagen degrading enzyme and plasminogen activator by transformed cells—role in metastasis. Int J Cancer 30:669-673

Sappino AP, Huarte J, Belin D, Vassalli JD. (1989) Plasminogen activators in tissue remodeling and invasion: mRNA localization in mouse ovaries and implanting embryos. J Cell Biol 109:2471-9

Sasagawa M, Sasaki T, Yasuda M, Yamazaki T, Kurose T, Tanaka K, Higuchi M, Hando T, Kanazawa K, Takeuchi S. (1988) Morphological and immunohistochemical study on specimens of trophoblastic diseases in which the presence of a villous formation could not be established. Nippon Sanka Fujinka Gakkai Zasshi 40:761-8

Sengupta J, Given RL, Carey JB, Weitlauf HM. (1986) Primary culture of mouse endometrium on floating collagen gels: a potential *in vitro* model for implantation. Ann NY Acad Sci 976:75-94

Sherman MI, Strickland S, Reich E. (1976) Differentiation of early mouse embryonic and teratocarcinoma cells *in vitro*: plasminogen activator production. Canc Res 36: 4208-4216

Shi QJ, Lei ZM, Rao CV, Lin J. (1993) Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts. Endocrinology 132:1387-95

Smith MA. (1993) Preeclampsia. Prim Care 20:655-64

Stewart CL. (1994) A cytokine regulating embryo implantation. NY Acad Sci, in press

Strickland S, Reich E, Sherman MI. (1976) Plasminogen activator in early embryogenesis: enzyme production by trophoblast and parietal endoderm. Cell 9:231-40

Strickland S, Richards WG. (1992) Invasion of the trophoblasts. Cell 71:355-7

Terranova VP, DiFlorio R, Hujanen ES, Lyall RM, Liotta LA, Thorgeirsson U, Siegal GP, Schiffmann E. (1986) Laminin promotes rabbit neutrophil motility and attachment. J Clin Invest 77:1180-6

Toppozada MK. (1990) Role of prostaglandins in pre-eclampsia. Acta Obstet Gynecol Scand 69:375-7

Tuohy JF, James DK. (1992) Pre-eclampsia and trisomy 13. Br J Obstet Gynaecol 99:891-4

Uzumaki H, Okabe T, Sasaki N, Hagiwara K, Takaku F, Tobita M, Yasukawa K, Ito S, Umezawa Y. (1989) Identification and characterization of receptors for granulocyte colony-stimulating factor on human placenta and trophoblastic cells. Proc Natl Acad Sci U S A 86:9323-6

Vander AJ, Sherman JH, Luciano DS. (1970) Human physiology: the mechanisms of body function. McGraw-Hill, New York, p 264

Vuckovic M, Genbacev O, Kumar S. (1992) Immunohistochemical localisation of transforming growth factor-beta in first and third trimester human placenta. Pathobiology 60:149-51

Williams SE, Ashcom JD, Argraves WS, Strickland DK. (1992) J Biol Chem 267:9035-9040

Wun TC, Reich E. (1987) An inhibitor of plasminogen activation from human placenta. Purification and characterization. J Biol Chem 262:3646-53

Yagel S, Kerbel R, Lala P, Eldar GT, Dennis JW. (1990) Basement membrane invasion by first trimester human trophoblast: requirement for branched complex-type Asn-linked oligosaccharides. Clin Exp Metastasis 8:305-17

Yagel S, Parhar RS, Jeffrey JJ, Lala PK. (1988) Normal nonmetastatic human trophoblast cells share *in vitro* invasive properties of malignant cells. J Cell Physiol 136:455-62

Ye RD, Wun TC, Sadler JE. (1987) cDNA cloning and expression in Escherichia coli of a plasminogen activator inhibitor from human placenta. J Biol Chem 262:3718-25

Young RH, Kurman RJ, Scully RE. (1988) Proliferations and tumors of intermediate trophoblast of the placental site. Semin Diagn Pathol 5:223-37

Zeeman GG, Dekker GA, van GHP, Kraayenbrink AA. (1992) Endothelial function in normal and pre-eclamptic pregnancy: a hypothesis. Eur J Obstet Gynecol Reprod Biol 43:113-22

Zeeman GG, Dekker GA. (1992) Pathogenesis of preeclampsia: a hypothesis. Clin Obstet Gynecol 35:317-37

Zhou Y, Damsky CH, Chiu K, Roberts JM, Fisher SJ. (1993) Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts. J Clin Invest 91:950-60

Zini JM, Murray SC, Graham CH, Lala PK, Barnathan ES, Mazar A, Henkin J, Cines DB, McCrae KR. (1992) characterization of urokinase receptor expression by human placental trophoblasts. 79:2917-2929