Title

Vascular remodeling at the maternal-fetal interface: Placental interactions with uterine blood vessels and the lymphatic circulation during pregnancy

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VASCULAR REMODELING AT THE MATERNAL-FETAL INTERFACE: PLACENTAL INTERACTIONS WITH UTERINE BLOOD VESSELS AND THE LYMPHATIC CIRCULATION DURING PREGNANCY

by

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DISSERTATION

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VASCULAR REMODELING AT THE MATERNAL-FETAL INTERFACE: PLACENTAL INTERACTIONS WITH UTERINE BLOOD VESSELS AND THE LYMPHATIC CIRCULATION DURING PREGNANCY

By Kristy Red-Horse

Graduate Advisor: Susan J. Fisher, Ph. D.

ABSTRACT: The placenta induces important vascular changes in the uterus during pregnancy that allow the mother to adequately support growth and development of the embryo/fetus. Specifically, the uterine vasculature must incorporate the placenta into the maternal circulation facilitating mechanical attachment and nutrient/waste exchange. As they invade the uterine wall, placental cytotrophoblasts remodel resident blood vessels in a manner that channels maternal blood to and from the placenta. Although both sides of the circulation are involved in this process, remodeling is biased towards uterine spiral arterioles. An ultrastructural (scanning electron microscopy) study that was completed as a part of this thesis dramatically illustrates the unusual nature of this process.

Here, we show that EphB4 and ephrinB2, venous and arterial cell surface receptors, respectively, are involved in patterning cytotrophoblast interactions with maternal blood vessels. As they invade the uterine wall, cytotrophoblasts switch from a venous to and an arterial phenotype downregulating EphB4 expression and sequentially upregulating ephrinB1 and -B2. *In vitro,* the cells avoid EphB4-coated substrates and

decrease their migration in response to 3T3 cells expressing this molecule. EphB4 inhibited migration by regulating chemokine-induced movement rather than a growth factor-stimulated response. These data suggest that, in the uterine wall, EphB4, expressed on maternal veins, restricts cytotrophoblast venular invasion, ultimately resulting in preferential remodeling of arterial vessels.

To further study cytotrophoblast-blood vessel interactions, we developed a novel *in vivo* model of human placentation. Implantation of placental anchoring villous explants into the mammary fat pads or under the kidney capsules of SCID mice gave rise to a cytotrophoblast population that breached murine blood vessels. Histological analyses of these hybrid tissues revealed multiple aspects of vascular remodeling including the impact of the placenta on the arterial and lymphatic vasculature. First, invasive cells induced arterial endothelial and smooth muscle cell apoptosis suggesting that a parallel process is an important part of the mechanism that enables cytotrophoblast endovascular invasion during pregnancy. Secondly, cytotrophoblast invasion was accompanied by extensive lymphangiogenesis, data that was corroborated by *in vitro* experiments that showed that the cells stimulated lymphatic endothelial cell migration. We also show that pregnancy induces an astonishingly robust lymphangiogenic response in the uterine wall since the non-pregnant endometrium normally lacks a lymphatic circulation. Thus, we provide evidence that the placenta establishes an endometrial lymphatic circulation during pregnancy, the functional consequences of which likely involve fluid regulation and maternal-fetal immunity.

Finally, we speculate that this new body of knowledge about placenta-induced remodeling of the maternal vasculature will enable several new research directions. For

example, it will be interesting to determine is any of the aforementioned pathways are disrupted in common pregnancy complications. Perhaps most interestingly, these data provide new insights into mechanisms of antigen presentation at the maternal-fetal interface, a very poorly understood phenomenon.

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Implantation and early development

Embryonic development begins when the oocyte, released during ovulation, is fertilized by a single sperm cell. Then the newly formed zygote initiates a series of cell divisions without increasing in size producing a tightly packed ball of cells called a morula. These early events occur in the fallopian tube where a non-adhesive covering, the zona pellucida, prevents interactions between the morula and tubular epithelium (Figure 1). During this time the first lineage specification occurs. Polarized cells on the outer surface of the morula differentiate into trophoblast cells, which eventually give rise to the placenta, while the non-polarized internal cells are allocated to the embryo proper. It is thought that cell positioning, i.e. polarization vs. non-polarization, directs differentiation by inducing the expression of certain transcription factors (Rossant, 2004). For example, the homeobox gene *cdx2* begins to be expressed in the outer cells at the late morula stage and, in mice, is required for trophoblast differentiation (Strumpf et al., 2005). Conversely, the transcription factor Oct4 (Nichols et al., 1998) and the homeobox gene *nanog* delineate the embryonic lineage, and accordingly, are markers of undifferentiated human and mouse embryonic stem cell lines. Transfection studies have shown that these factors function in part by controlling the expression of mutually exclusive sets of genes (Ralston and Rossant, 2005).

As development progresses, morphological events transform the morula into a blastocyst, which consists of an inner cells mass and a fluid-filled cavity called the

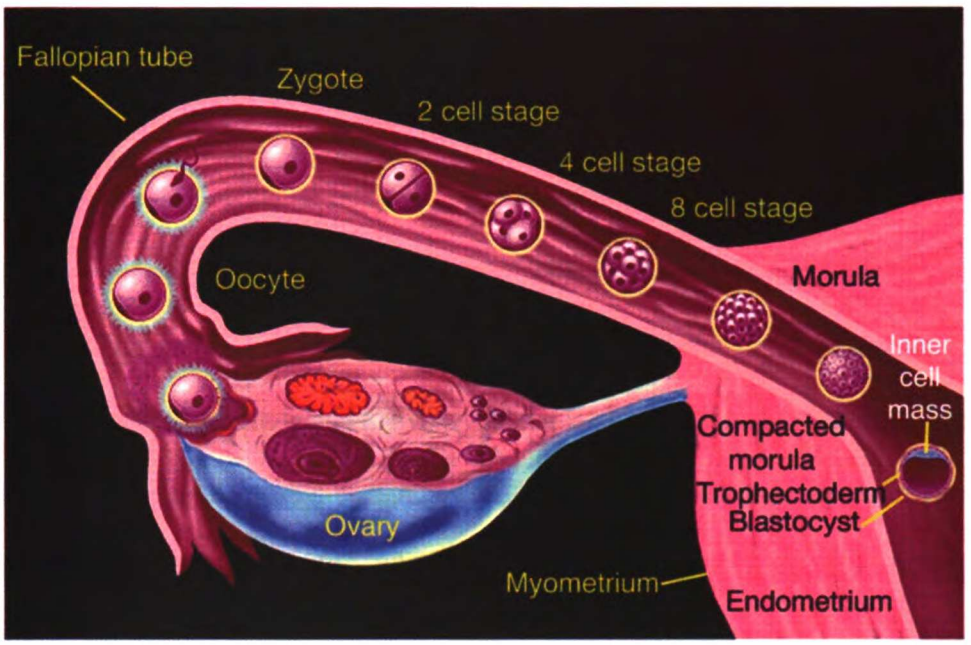


Figure 1. (from Red-Horse et.al., 2004) The early stages of human development from fertilization to blastocyst formation. Fertilization occurs in the fallopian tube within 24 to 48 hours of ovulation.The initial stages of development, from fertilized ovum (zygote) to a solid massof cells (morula), occur as the embryo passes through the fallopian tube encased within a nonadhesive protective shell (the zona pellucida). The morula enters the uterine cavity approximately two to three days after fertilization. The appearance of a fluid-filled inner cavity marks the transition from morula to blastocyst and is accompanied by cellular differentiation: the surface cells become the trophoblast (and give rise to extraembryonic structures, including the placenta) and the inner cell mass gives rise to the embryo. Within 72 hours of entering the uterine cavity, the embryo hatches from the zona, thereby exposing its outer covering of trophectoderm.

blastocoel surrounded by an outer trophectoderm layer. The blastocyst enters the uterine cavity where subsequent maternally- and fetally-derived molecular cues synchronize the production of an implantation-competent blastocyst with the development of uterine receptivity (Paria et al., 2001). This critical time period, referred to as the implantation window, begins approximately 6 days post-fertilization in humans and can last for up to 72 hours. On the embryonic side, the blastocyst must hatch from the zona pellucida, which exposes the adhesive trophoblast layer. This process is part of a continuum termed blastocyst activation (Dey et al., 2004). Concomitantly, uterine epithelial cells become receptive to trophoblast attachment. Many of the molecules involved in different aspects of this process during murine pregnancy have been described using knockout mice. Such studies have identified leukemia inhibitory factor (LIF) as one of the initiating signals required for implantation (Stewart et al., 1992). LIF-deficient mice do not upregulate many of the genes that play a role in uterine preparation including desmin, tenascin, Cox-2, bone morphogenetic protein (BMP)-2 and -7, and Hoxa-10 (Fouladi-Nashta AA, 2005). Animals deficient in phospholipase A2a (Song et al., 2002) and lysophosphatidic acid (Ye et al., 2005), which are upstream of COX-2, have underscored the importance of coupling uterine receptivity with blastocyst maturation/activation. These mice exhibit defective implantation as a result of delayed endometrial preparation.

In humans, endometrial receptivity is associated with an increase in the expression of adhesion molecules that bind receptors present on the hatched blastocyst. Specifically, trophoblasts express L-selectin and its carbohydrate ligand, MECA-79, is upregulated on the uterine wall during the implantation window. Interactions between these two molecules promote blastocyst attachment in a process that may emulate some

of the mechanisms that function during leukocyte trafficking (Figure 2). Leukocyte extravasation from the blood is initiated when selectin-mediated adhesion, which is transient in nature, induces rolling along the endothelium. Subsequently, the cells react to chemokines expressed on the vessel wall that, upon binding their receptors, induce integrin activation and stable adhesion. Finally, the cells migrate across the endothelium in response to signals in the underlying tissue (Butcher and Picker, 1996). Parallels exist between these events and those that occur during implantation. For example, the blastocyst travels along the uterine epithelium transiently adhering to its surface. This type of interaction may be important to ensure proper orientation; implantation occurs with the embryonic pole facing the uterus in humans and vise versa in non-human primates and rodents. *In vitro*, L-selectin mediates trophoblast attachment to uterine epithelium under shear (Genbacev et al., 2003) suggesting it plays a role in establishing initial interactions *in vivo*. Interestingly, complimentary receptor-ligand expression patterns in the blastocyst and uterine wall implicate chemokines and integrins downstream of L-selectin-mediated interactions, possibly by triggering stable adhesion (Caballero-Campo et al., 2002; Dominguez et al., 2003; Nagaoka et al., 2003). Studies in sheep support this theory. In these animals, receptive endometrial cells express the chemokine CXCL10, which induces attachment and migration of caprine trophoblasts (Nagaoka et al., 2003).

Following the establishment of stable interactions between maternal and fetal components, the blastocyst migrates through the uterine epithelial cell layer and embeds completely into the underlying endometrial stroma (Figure 3A-C). Cellular debris and a coagulum of tissue fluid completely cover the implantation site resulting in the interstitial

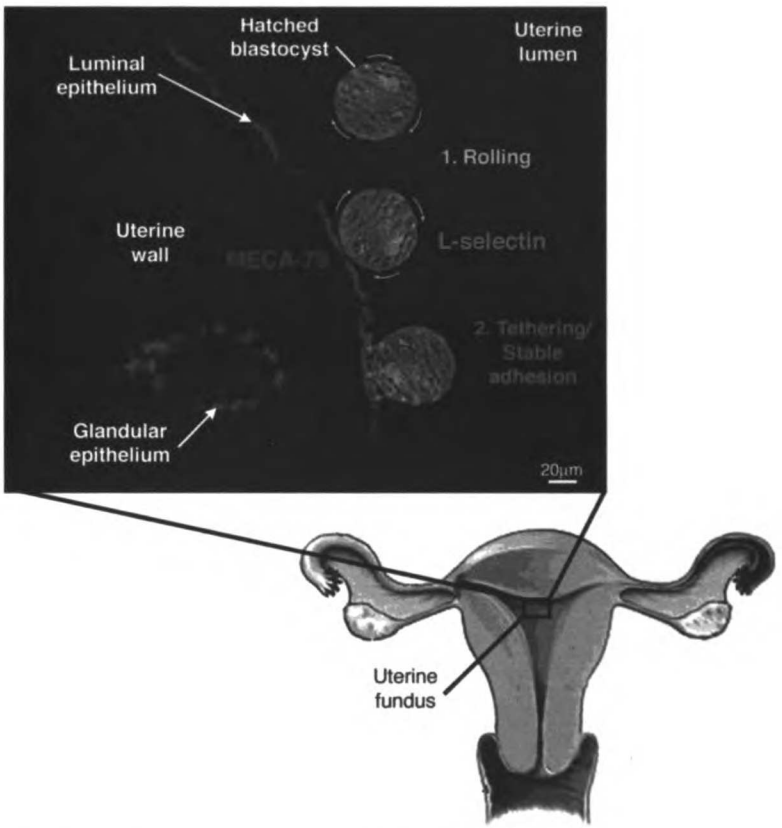


Figure 2. (from Red-Horse et. al., 2004) Implantation in humans involves a number of the molecular mechanisms that mediate leukocyte emigration from the blood to sites of inflammation or injury. The diagram was made from a combination of images: MECA-79 antibody staining of uterine tissue sections and L-selectin antibody staining of cultured embryos. Recently acquired evidence suggests that an implantation-competent human blastocyst expresses L-selectin on its surface (green). This receptor interacts with specialized carbohydrate ligands, including sulfated species, recognized by the MECA-79 antibody, which stains the uterine luminal and glandular epithelium. The specialized nature of these interactions translates into an unusual form of cell adhesion: rolling and tethering. In the uterus, MECA-79 immunoreactivity peaks during the window of receptivity. This finding suggests that apposition, the first step in implantation, includes L-selectin–mediated tethering of the blastocyst to the upper portion of the posterior wall of the uterine fundus.

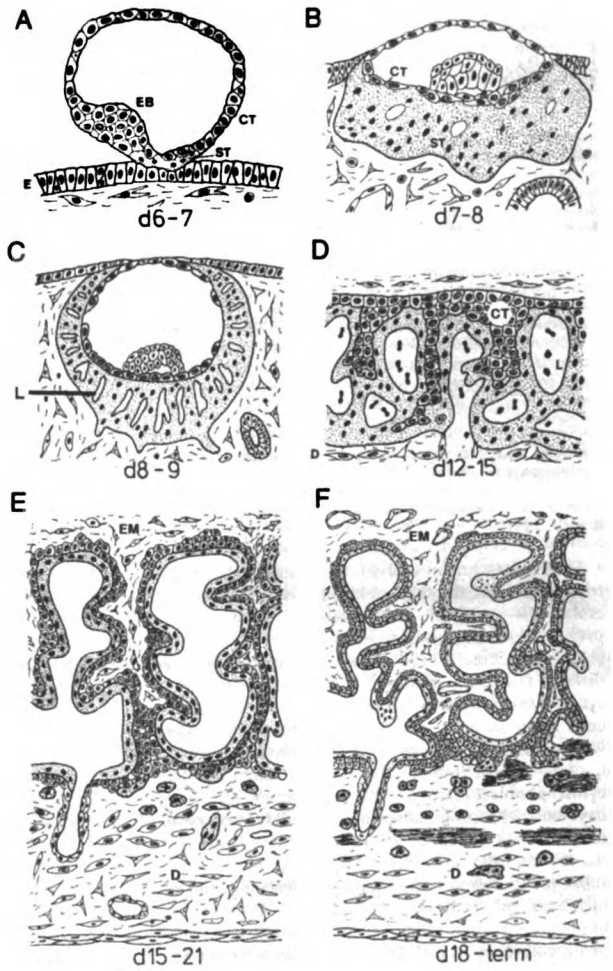


Figure 3. (modified from Pathology of the Human Placenta, 4th ed., Bernirschke and Kaufmann, 2000) Development of the human placenta. A. Placentation is initiated when stable interactions form between trophoblasts and the uterine epithelium. B. Following attachment, cytotrophoblasts at the embryonic pole fuse to form a multinucleated syncytiotrophoblast, which breaches the epithelial layer facilitating blastocyst movement into the uterine stroma. C. Once within the uterine wall, lacunae begin to form within the syncytiotrophoblast leaving cellular pillars called trabeculae. D. Subsequently, cytotrophoblasts located beneath the syncytia prolifereate and migrate through the trabeculae and into the decidua. E. Cytotrophoblast migration is then followed by mesenchymal tissue derived from fetal extraembryonic mesoderm. However, in contrast to cytotrophoblasts, this stromal tissue does not reach the decidua. F. Finally, a network of blood vessels form within the stromal compartment producing a mature villus, which is composed of a fibroblastic core containing blood vessels covered sequentially by a basement membrane, a cytotrophoblast layer and the syncytiotrophoblast. By 10-12 weeks of pregnancy, maternal blood begins to flow through the intervillous space and exchange occurs across trophoblast cell layers. EB, embryo; CT, cytotrophoblasts; ST, syncytiotrophoblast; E, endometrial epithelium; L, lacunae, EM, extraembryonic mesodem; D, decidua.

invasion that is characteristic of human implantation (Figure 3C) (Hamilton, 1972). Very little is known about the molecules involved during this stage, especially in humans. This is due to the fact that implantation and placentation are highly divergent among even closely related species such that data from animal models may not be directly translatable (Benirschke, 2000).

Placental morphogenesis

Placentation begins at the point of contact between the uterus and embryo (Figure 3) (Benirschke, 2000). Once attached, trophoblasts begin to invade while differentiating into two distinct types of cells. Trophoblasts in contact with maternal tissue fuse to form a multinucleated syncytiotrophoblasts, while those adjacent to the inner cell mass remain as a population of single cells termed cytotrophoblasts (Figure3B,C). As implantation progresses, cytotrophoblasts proliferate and fuse increasing the mass of the syncytium, which spreads from the implantation pole to cover the entire blastocyst. Finger-like projections that form at the leading edge of the syncytiotrophoblast allow the embryo to advance into the uterine wall until entirely embedded. Approximately 8 days p.c., vacuoles begin to form within the cytoplasm of the invading syncytiotrophoblasts (Figure3C). These eventually join to create a system of lacunae that will subsequently become the intervillous space. The cellular pillars resulting from lacunae formation, called trabeculae, are the precursors to the floating villi.

Around day 13 p.c, cytotrophoblasts begin to proliferate and migrate into the trabeculae core (Figure3D). A portion of these cells moves through the trabeculae into

the decidua. Here, they form clusters called cell columns as well as a single layer of cells that surround the syncytium. The cytotrophoblast clusters expand longitudinally and become anchoring villi that secure the placenta to the uterine wall. Around day 15, mesenchymal tissue derived from extraembryonic mesoderm follows cytotrophoblast migration moving into the villi. In contrast to cytotrophoblasts, mesenchymal tissue does not invade through to the maternal side. Thus, direct contact between maternal and fetal cells is mediated exclusively by trophoblasts.

These events, which lead to villous formation, do not occur in a radially symmetrical manner. Syncytialization initiates at the embryonic pole before spreading over the entire blastocyst and, as a result, is much thicker in this region. This asymmetry is maintained during early pregnancy, and eventually, the less well-developed portion of the placenta regresses becoming smooth in appearance and fusing with the amnion to produce the chorioamniotic membrane. This membrane and the adjacent decidua that is located between the embryo and the uterine lumen are stretched as the embryo/fetus grows, but retained such that the off-spring remains encapsulated within the uterine wall throughout pregnancy. The final result is the formation of a disc-shaped placenta, where maternal-fetal exchange occurs within a concentrated, disk-shaped area.

Vascularization of the villi first occurs between day 18 and 20 days post coitum when capillaries appear within the villous stroma (Figure3F). It has been proposed that these vessels originate from hemangioblast progenitors that give rise to endothelial and hematopoietic stem cells (Demir et al., 1989). In support of this theory is the recent discovery that the murine placenta is a major site of hematopoiesis during embryonic development, which between embryonic day 10.5 and 13.5 is a source of hematopoietic

stem cells that can repopulate the entire repertoire of blood cells in an irradiated adult (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). Concomitant with vessel formation within the villous core, the allantoic membrane adjacent to the embryo becomes vascularized and fuses with the chorionic plate. The allantoic vessels move into the placenta through the larger villi and fuse with resident villous capillaries completing the placental circulation around the 5th week of gestation, which is connected to the embryo via the umbilical arteries and veins.

Now the placenta begins to grow in size to meet the increasing demands of the developing off-spring. Buds form from preexisting anchoring villi, which grow and give rise to new buds, eventually producing a network of tree-like structures that extend into the intervillous space. Thus, the fully developed placenta is composed of two types of chorionic villi, floating and anchoring (Figure 4). Floating villi are the site of maternal-fetal exchange. They consist of a mesenchymal core that contains resident macrophages and blood vessels, covered sequentially by a basement membrane, a layer of cytotrophoblasts (variably), and the syncytiotrophoblast. The syncytiotrophoblast mediates exchange by selectively transporting nutrients and gases from the maternal blood into the villi and fetal wastes in the opposite direction. Minimally, molecules must past through four layers before reaching the fetal circulation; the syncytium, the underlying cytotrophoblasts, the basal lamina, and the villous endothelium. However, in many places, fetal capillaries form sinusoids that bulge into the syncytial later creating a vasculosyncytial membrane (Fox and Agrafojo-Blanco, 1974) reducing the layers to thinned syncytium and endothelium. This arrangement is especially evident in women

who smoke, suggesting that oxygen demands could regulate the appearance of these structures (Genbacev et al., 2000).

Anchoring villi function in attaching the fetus to the uterus and remodeling uterine arterioles so that maternal blood flow is directed into the intervillous space (Benirschke, 2000; Kliman, 2000). Their structure is identical to floating villi within the intervillous space. However, anchoring villi contain sites of cytotrophoblast proliferation that break through the syncytium. These aggregates attach to the uterine wall where they form columns of non-polarized cells. Cytotrophoblasts from the column begin to invade the uterine wall forming a cellular anchor. A sub-population of these invading cytotrophoblasts homes to the uterine spiral arterioles and replaces a portion of the maternal endothelial lining and tunica media forming a uteroplacental artery. Endovascular invasion is also accompanied by the degeneration of the latter layer of smooth muscle and elastic fibers (Boyd, 1970). These physiological changes result in increased lumen width allowing for an increase in blood flow that is sufficient to perfuse the intervillous space, a requirement that increases as throughout pregnancy (Brosens et al., 1967).

Cytotrophoblast-mediated vascular remodeling

To support fetal growth and development, invasive cytotrophoblasts remodel maternal blood vessels in a manner that incorporates the intervillous space into the uterine vasculature (Figure 4). It is estimated that at term one fifth of a woman's total blood volume is shunted to the intrauterine circulation, which perfuses the placenta at a

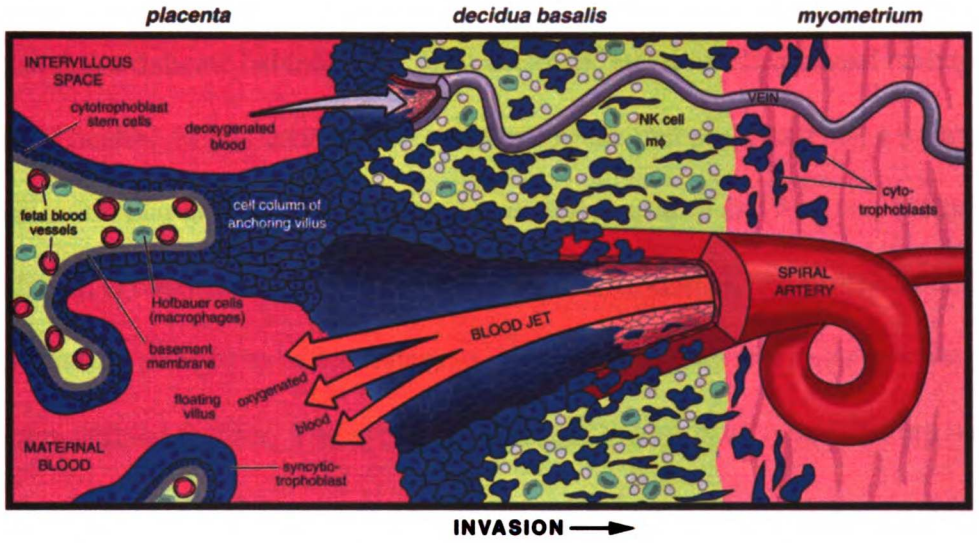


Figure 4. Placental cytotrophoblasts invade the uterine wall where they breach veins and extensively remodel maternal spiral arterioles. The bulk of the placenta is composed of numerous tree-like projections termed floating villi where maternal-fetal exchange occurs. These structures mediate the passage of nutrients, gases, and wastes between fetal blood, which circulates through the villous core, and maternal blood, which circulates through the intervillous space. The uteroplacental circulation is established by cytotrophoblasts that acquire an invasive/endothelial phenotype as they leave the placenta and enter the uterine wall. Differentiation begins when cytotrophoblast progenitors that reside in a single layer surrounding the stromal core of anchoring villi proliferate and form a cell column. These structures attach to the uterine wall and give rise to cells that invade the underlying decidual stroma. Invasive cytotrophoblasts breach uterine blood vessels connecting both the arterial and the venular circulation to the intervillous space. However, once this connection is made, remodeling of the venous side is halted. In contrast, cytotrophoblasts migrate up the lumina of spiral arterioles, eventually replacing the vessels' endothelial lining and a portion of the muscular wall. This process encompasses the decidual and inner third of the myometrial segments of these vessels. NK, natural killer; mϕ, macrophage.

rate of liters per minute (Metcalfe et al., 1955). These dramatic physiological changes suggest that a delicate balance must exist between fetal nourishment and maintenance of maternal health. In this context, it is relevant to note that preeclampsia, a pregnancy complication that is the leading cause of maternal mortality and fetal morbidity worldwide, is characterized by maternal hypertention, edema, and proteinuria. These symptoms are thought to be secondary to widespread endothelial damage induced by placenta–derived factors. The events that precede the onset of symptoms are not completely understood, however, they are most likely related to the drastic reduction in vascular remodeling associated with this complication (Figure 5). Thus, one of the primary goals of understanding the mechanisms involved during normal vascular remodeling is to eventually achieve an understanding of the defects that lead to preeclampsia.

At a morphological level, vascular remodeling is a stepwise process that occurs in parallel with increases in fetal oxygen requirements (Figure 6). The early stages of placental development take place in a relatively hypoxic environment (Jauniaux et al., 2003) that favors cytotrophoblast proliferation rather than differentiation along the invasive pathway (Genbacev et al., 1997). Accordingly, this cell population rapidly increases in number as compared with the embryonic lineages. As development continues, cytotrophoblasts begin to invade the uterine wall and target maternal vessels. Initially, the cells form a luminal plug, a process that helps maintain a state of physiological hypoxia. As the endovascular component of cytotrophoblast invasion progresses, the cells interact with both arterioles and veins, which channel blood to and from the placenta in drastically different ways. The cells barely breach uterine veins, but

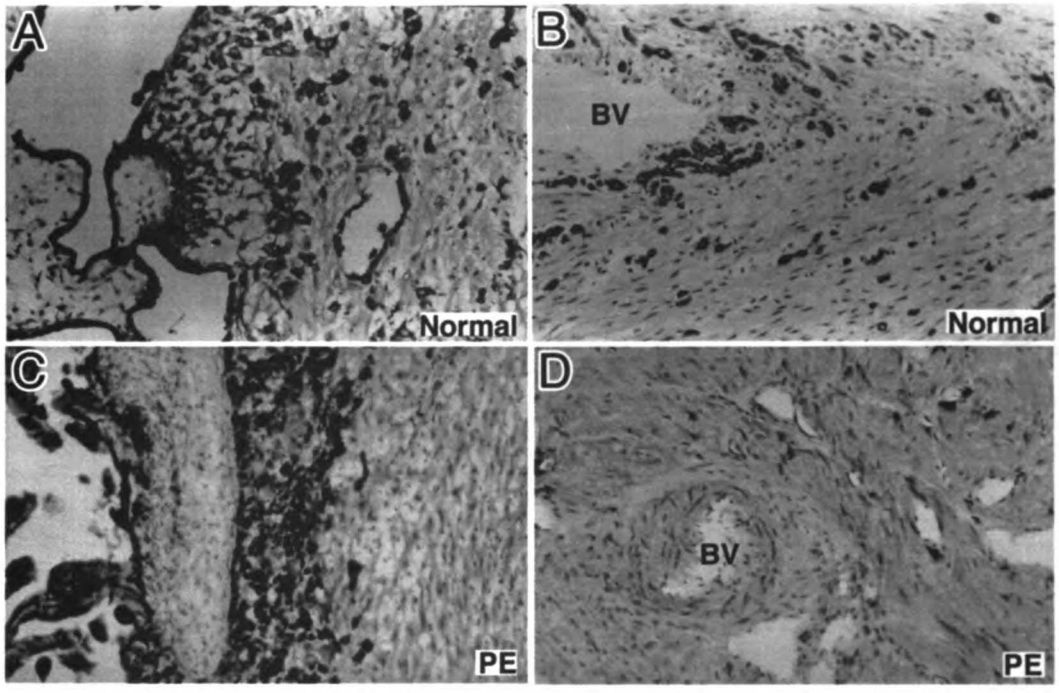


Figure 5. (figure courtesy Yan Zhou) Preeclampsia is associated with deficiencies in cytotrophoblast invasion and defects in remodeling of uterine spiral arterioles. Cytokeratin staining reveals the distribution of cytotrophoblasts within the uterine wall (A-D). A. During normal pregnancy, cytotrophoblasts form a cell column that attaches to maternal tissue and gives rise invasive cells. B. Invasive cytotrophoblasts target arterial blood vessels through to the myometrial layer of the uterus. C, D. Invasion is often shallow in pregnancies complicated with preeclampsia and maternal blood vessels remain unmodified. BV, blood vessel.

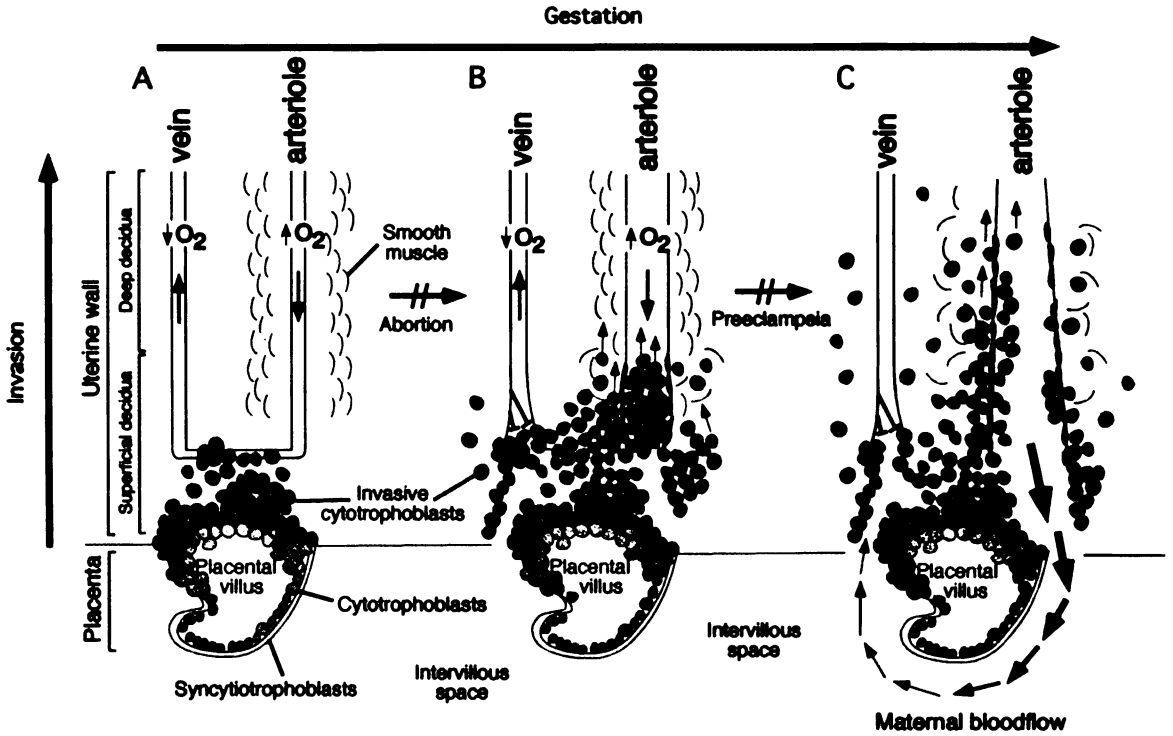


Figure 6. Oxygen tension plays an important role in guiding the differentiation process that leads to cytotrophoblast invasion of the uterus. A. The early stages of placental development take place in a relatively hypoxic environment that favors cytotrophoblast proliferation rather than differentiation along the invasive pathway. Accordingly, this cell population (light green cells) rapidly increases in number as compared with the embryonic lineages. B. As development continues, cytotrophoblasts (dark green cells) invade the uterine wall and plug the maternal vessels, a process that helps maintain a state of physiological hypoxia. As indicated by the blunt arrows, cytotrophoblasts migrate farther up arteries than veins. C. By 10 to 12 weeks of human pregnancy, blood flow to the intervillous space begins. As the endovascular component of cytotrophoblast invasion progresses, the cells migrate along the lumina of spiral arterioles, replacing the maternal endothelial lining. Cytotrophoblasts are also found in the smooth muscle walls of these vessels. In normal pregnancy the process whereby placental cells remodel uterine arterioles involves the decidual and inner third of the myometrial portions of these vessels. As a result, the diameter of the arterioles expands to accommodate the dramatic increase in blood flow that is needed to support rapid fetal growth later in pregnancy. It is likely that failed endovascular invasion leads, in some cases, to abortion, whereas an inability to invade to the appropriate depth is associated with preeclampsia and a subset of pregnancies in which the growth of the fetus is restricted.

migrate along the lumina of spiral arterioles, replacing the maternal endothelial lining and disorganizing surrounding smooth muscle through the inner third of the myometrium. As a result, the diameter of the arterioles expands, and by 10 to 12 weeks of human pregnancy, blood flow to the intervillous space begins. Throughout pregnancy, additional arterioles are recruited in a similar process, which allows the system to continually support the growing fetus' increasing metabolic demands. At term, the surface area of the maternal-fetal interface during normal pregnancy is an average of 22 cm^2.

The ability of cytotrophoblasts to transform the uterine vasculature depends on their differentiation down the invasive pathway where the cells undergo a unique epithelial-to-endothelial transition. Indeed, extensive analysis of cytotrophoblast invasion *in situ* and *in vitro* alongside analyses of pregnancy pathologies such as preeclampsia, where invasion is severely impaired, has established that this switch is vital to successful placental development (Damsky et al., 1994; Lim et al., 1997; Zhou et al., 1993; Zhou et al., 1997a,b; Zhou et al., 2002). Initially, cytotrophoblast progenitors located in floating villi express E-cadherin (epithelial cadherin) and $\alpha 6\beta 4$ integrins. Upon differentiation, the cells downregulate these molecules and upregulate VE-cadherin (vascular endothelial cadherin), $\alpha 5\beta 1$, $\alpha V\beta 3$, PECAM-1, and VCAM-1 (Zhou et al., 1997a,b) and the extracellular matrix (ECM) degrading matrix metalloproteinase MMP-9 (Librach et al., 1994; Librach et al., 1991). This switch is also accompanied by an upregulation of several growth factors and receptors that function during conventional vasculogenesis and angiogenesis including vascular endothelial growth factor (VEGF)-A, placental growth factor (PlGF) and the cognate receptor VEGFR-1 (Zhou et al., 2002) as

well as angiopoietin-2 (Ang-2) (Zhou et al., 2003). Differentiation also induces expression of lymphangiogenic molecules, which include VEGF-C and it receptor VEGFR-3. These molecules continue to define the invasive phenotype as cytotrophoblasts migrate through the uterine wall and its vascular network. However, a portion of the cells enter a terminal differentiation stage when they spread along the vascular wall displaying an endothelial morphology and upregulating distinct molecules including neural cell adhesion molecule (NCAM) (Red-Horse et al., 2004).

The function of several of these molecules during vascular remodeling has been delineated using *in vitro* models of cytotrophoblast differentiation. The observation that cytotrophoblasts express both VEGF family members and their receptors *in vivo* suggested autocrine regulation. This was shown to be the case since depletion of VEGFR-1-ligands, VEGF and PlGF, during cytotrophoblast differentiation in culture increases apoptosis and inhibits β1 intergrin upregulation (Zhou et al., 2002). These data may be relevant *in vivo* as the expression of VEGF-A and its receptor are downregulated in preeclamptic placentas (Zhou et al., 1997b), a condition that is also associated with increased cytotrophoblast apoptosis (DiFederico et al., 1999). These molecules may also function in a paracrine manner. *In vitro,* VEGF-C, PlGF, and Ang-2 within cytotrophoblast-conditioned medium support the growth of uterine microvascular endothelial cells by inhibiting apoptosis. Additionally, these cytotrophoblast-derived factors induce angiogenesis in a chorioallantoic membrane (CAM) assay (Zhou et al., 2003). Thus, extensive *in vitro* analyses suggest powerful functional roles for these molecules during vascular remodeling. It will be interesting to add additional data about

the function of the molecules using a new *in vivo* model of this process that I developed as part of my thesis work.

Chemokines

Chemokines, or chemotactic cytokines, are a family of small inducible molecules first described for their ability to induce the directed migration and activation of leukocytes (Schall and Bacon, 1994). Members of the chemokine family share a similar structure with three anti parallel beta-strands connected by loops with a C terminal helix. Due to conserved cystine residues that participate in disulfide bonds family members usually function as dimers. The exact position of the cystines divides the family into different subgroups. To date, four subgroups have been identified, C, CC, CXC, and CX3C, that are composed of over forty distinct chemokines. The two conserved cystines within the CC subgroup are adjacent while CXC chemokines have a single amino acid separating these two residues. Most chemokines belong to one of these two groups. The remaining two groups have only one member each; XCL1 lost one of the conserved cystine residues and CX3CL1 has three. Finally, CXCL16 and CX3CL1 are structurally unique in that they contain transmembrane regions that anchor them to the cell surface.

Chemokines signal through pertussis toxin sensitive G_i-protein linked seven transmembrane receptors (Rossi and Zlotnik, 2000). The known receptor family is smaller with 16 members. Cells are highly selective in their receptor expression. This permits a single chemokine to act on a defined set of cells. However, they often express receptors for multiple chemokines within a subgroup, a factor that complicates functional

studies. *In vivo,* a complex web of concerted regulation of ligand expression within tissues and complimentary receptors on different leukocyte subsets directs the placement of immune cells in the correct locations, a central component of homeostasis and conversely, of many disease states.

The role of constitutively expressed chemokines in homeostatic leukocyte trafficking has been described for several lymphoid organs. Interplay between CCL17, CCL19, CCL21, CCL22, and CCL25 regulates T cell compartmentalization in the thymus (Rossi and Zlotnik, 2000). Appropriate B-cell development depends on CXCL12 derived from bone marrow dendritic cells (Nagasawa et al., 1996). The mechanism includes retention of precursor cells within the bone marrow in close proximity to dendritic cells that provide essential growth factors (Ma et al., 1999). Chemokines also play an important role in lymphocyte trafficking through secondary lymphoid organs (Cyster, 1999). Here, lymphocytes that express L-selectin roll on high endothelial venules (HEV) that express its carbohydrate ligands. CCL21 expressed on HEVs binds to CCR7 on naïve T cells activating $\alpha 4\beta 7$ and $\beta 2$ integrins. The activated integrins interact with ICAM-1 and MadCAM-1 resulting in arrest and entrance into the tissue. Inside the lymph node, B cells are mainly restricted to the follicle area through expression of CXCL13, which binds CXCR5, a receptor these cells also express. Conversely, formation of the T cell area is guided by the actions of two CCR7 ligands, CCL19 and CCL21. Therefore, chemokine expression within the tissue is important for retaining cells in the appropriate location. Similar mechanisms guide leukocyte trafficking in non-lymphoid tissue during inflammation with an important difference: chemokine expression is regulated according to local immune requirements.

Chemokines in development and angiogenesis

In addition to their long-recognized function of directing leukocyte movement, chemokines also participate in many developmental processes, including those that rely on proliferation, differentiation and targeted migration of nonhaematopoietic cell populations. With regard to mitosis, chemokines commonly stimulate proliferation and protect against apoptosis in targets such as endothelial and neuronal cells, functions that are well defined in the haematopoietic lineage (Bajetto et al., 2001; Schall and Bacon, 1994; Zlotnik et al., 1999). As to cell fate, the receptor CXCR4 and its ligand CXCL12 are expressed in complementary patterns in the developing neuronal, cardiovascular, and haematopoietic systems, as well as in the craniofacial region of the mouse. Null mutations in either gene yield defects in these areas (Nagasawa et al., 1996; Zou et al., 1998). Chemokines also function in nonimmune-cell trafficking, for example in tumor metastasis to selected sites (Muller et al., 2001; Rossi and Zlotnik, 2000) and migration of germ cell and neuronal progenitors in the developing embryo (Doitsidou et al., 2002; Knaut et al., 2003; Zhu et al., 2002).

Chemokines can also regulate blood vessel formation during embryonic development as well as in adult tissues. This activity has been appreciated for members of the CXC subgroup, which are either angiogenic or angiostatic based on whether or not they posses an ELR motif that consists of the three amino acids Glu-Leu-Arg (Strieter et al., 1995). ELR CXC chemokines such as CXCL1, CXCL6, and CXCL8 bind CXCR2 on endothelial cells promoting angiogenesis (Addison et al., 2000). In contrast, the INFγ-

inducible chemokines CXCL9, CXCL10, and CXCL11 lack an ELR motif and have angiostatic properties. An imbalance in chemokine production favoring the angiogenic type is thought to contribute to disease in situations of chronic inflammation (Nickoloff et al., 1994; Walz et al., 1997) and tumor neovascularization (Moore et al., 1999). In fact, in HIF-1α-deficient tumors, which have a reduced level of VEGF expression, angiogenesis is stimulated by compensatory upregulation of CXCL8 (Mizukami et al., 2005). This activity highlights the importance of understanding the role of angiogenic chemokines when considering strategies that target blood vessel growth during cancer progression.

Evidence also exists in support of chemokine functions during angiogenesis in the developing embryo. CXCL12 and CXCR4 are required for vascularization of the gastrointestinal tract (Tachibana et al., 1998). Interestingly, both genes may be master regulators of these events since they are directly upregulated by hypoxia, a feature they share with VEGF (Ceradini et al., 2004; Schioppa et al., 2003; Staller et al., 2003).

Chemokine expression in the placenta

Expression screens aimed at describing the chemokine repertoire at the maternal-fetal interface demonstrated that the decidual environment and placental villi contain an abundance of chemokine mRNAs (Drake et al., 2004; Red-Horse et al., 2001). Interestingly, cytotrophoblasts express a broad repertoire of receptors capable of binding many of these chemokines. Analysis of reciprocal receptor–ligand pairs indicates that chemokines have the capacity to influence cytotrophoblast differentiation and migration

as the progenitors move from the trophoblast basement membrane that surrounds the villous stroma into the interstitial and endovascular compartments of the uterus.

RNAse protection assays using RNA from isolated cytotrophoblasts revealed that these cells consistently express relatively high levels of CCR7, CXCR4 and CXCR6 and intermediate amounts of CCR5 and GPR1 (Figure 7). In addition, invasive cytotrophoblasts within the uterine wall stain with an antibody specific for the receptor CCR1 (Sato et al., 2003). In contrast, chemokine receptor expression by placental fibroblasts, the stromal cells of the fetal compartment, is not detected (Figure 7). This observation most likely reflects the fact that cytotrophoblasts are more dynamic cells that undergo highly specialized differentiation programs that result in their extensive migration.

To date, the expression of 13 chemokines has been localized to placental villi, predominantly in two resident cell types: fibroblasts and Hofbauer cells (placental macrophages) (Figure 8B). In the decidua, chemokine expression is complex (Red-Horse et al., 2001) with several specific patterns involving multiple cell types, including stromal fibroblasts, leukocytes, vascular endothelial cells and invasive cytotrophoblasts (Figure 8C). Generally, the patterns fall into four categories: (1) diffuse expression associated with decidual stromal cells; (2) focal expression by resident leukocytes or fibroblasts; (3) cytotrophoblast expression; or (4) expression that localizes to the cells lining uterine vasculature. Additionally, a few chemokines have more than one expression pattern.

In the pregnant uterus, most of the chemokines are expressed in the first pattern. CXCL6, CXCL11, CCL3, CCL14, CX3CL1, CXCL1 and CCL7 are evenly distributed throughout the decidual stroma. CCL2, CCL3, CXCL10 and CXCL14 have focal *in situ*

hybridization signals. CCL2 and CCL3 are expressed in a punctate pattern that is indicative of resident decidual leukocytes. CXCL14 and CXCL10 mRNAs, which also occur in foci, localize to patches of stromal cells. These patterns differ slightly: CXCL14 is found in smaller, more-numerous patches, often adjacent to clusters of decidual leukocytes, whereas CXCL10 expression is observed in larger area of stroma underlying these leukocyte clusters. Interestingly, CXCL14 expression is gestationally regulated: detection of the corresponding mRNA is confined to the first and second trimesters.

Several chemokines are upregulated by invasive cytotrophoblasts as they differentiate and enter the maternal tissue. Among these are CCL3, CCL14, CXCL6 and CXCL12. Finally, within the uterine wall, chemokine mRNA is also detected in association with cells that line maternal vessels. Both CXCL12 and CXCL21 localize to the uterine vasculature: CXCL12 mRNA is expressed by fetal cytotrophoblasts that breach and colonize the lumina of maternal arteries, whereas CXCL21 appears to be expressed by the maternal endothelial cells that line uterine veins.

Expression patterns suggest sites of cytotrophoblast–chemokine interactions

In the maternal compartment, cytotrophoblasts migrate into the uterine wall to secure attachment of the fetal unit and bring maternal blood to the intervillous space. To achieve the latter function, invading cytotrophoblasts navigate a dense decidual interstitium containing numerous maternal leukocytes before they reach the termini of uterine spiral arterioles. In this location, fetal cells begin replacing the maternal endothelial lining and disrupting the underlying muscular wall of these vessels.

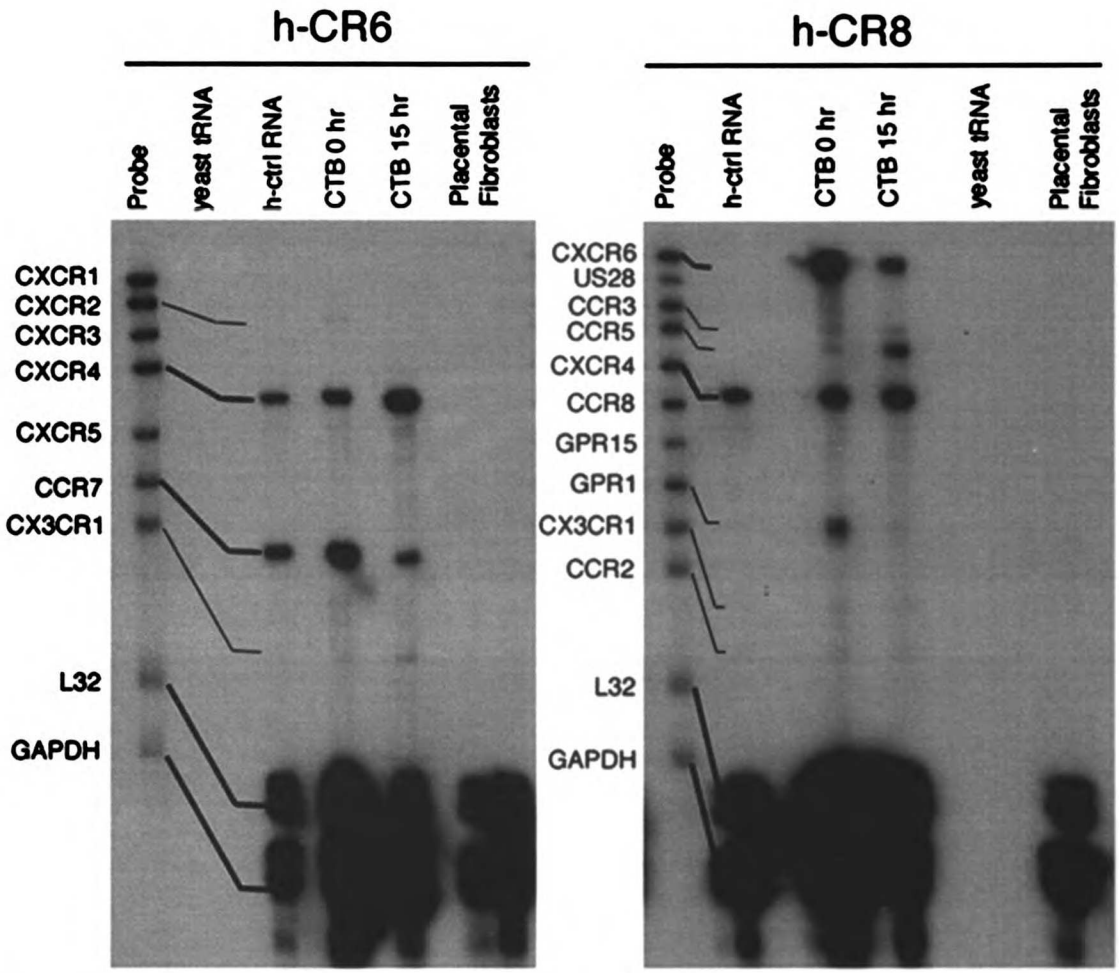


Figure 7. (from Drake et. al., 2004) Cytotrophoblasts, but not placental fibroblasts, express a panel of chemokine receptors. RNase protection assays were used to monitor the expression of chemokine receptor mRNAs. Two probe sets (A, hCR6; B, hCR8) were labeled with 32P and hybridized to RNA extracted from first- or second-trimester cytotrophoblasts either immediately after isolation (0 hr) or after the cells were cultured for 15 hr. A. After digestion and separation on a sequencing gel, the presence of mRNA transcripts was detected as protected bands of the appropriate size. Undigested probe sets were used as reference size markers (left lanes). Protected bands run slightly faster than the undigested probes. Lines connect pairs of undigested and digested probes; dark lines denote relatively higher levels of expression. RNA loading was monitored with probes for L32 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Human leukocyte (h-ctrl) RNA and yeast tRNA served as positive and negative controls, respectively.

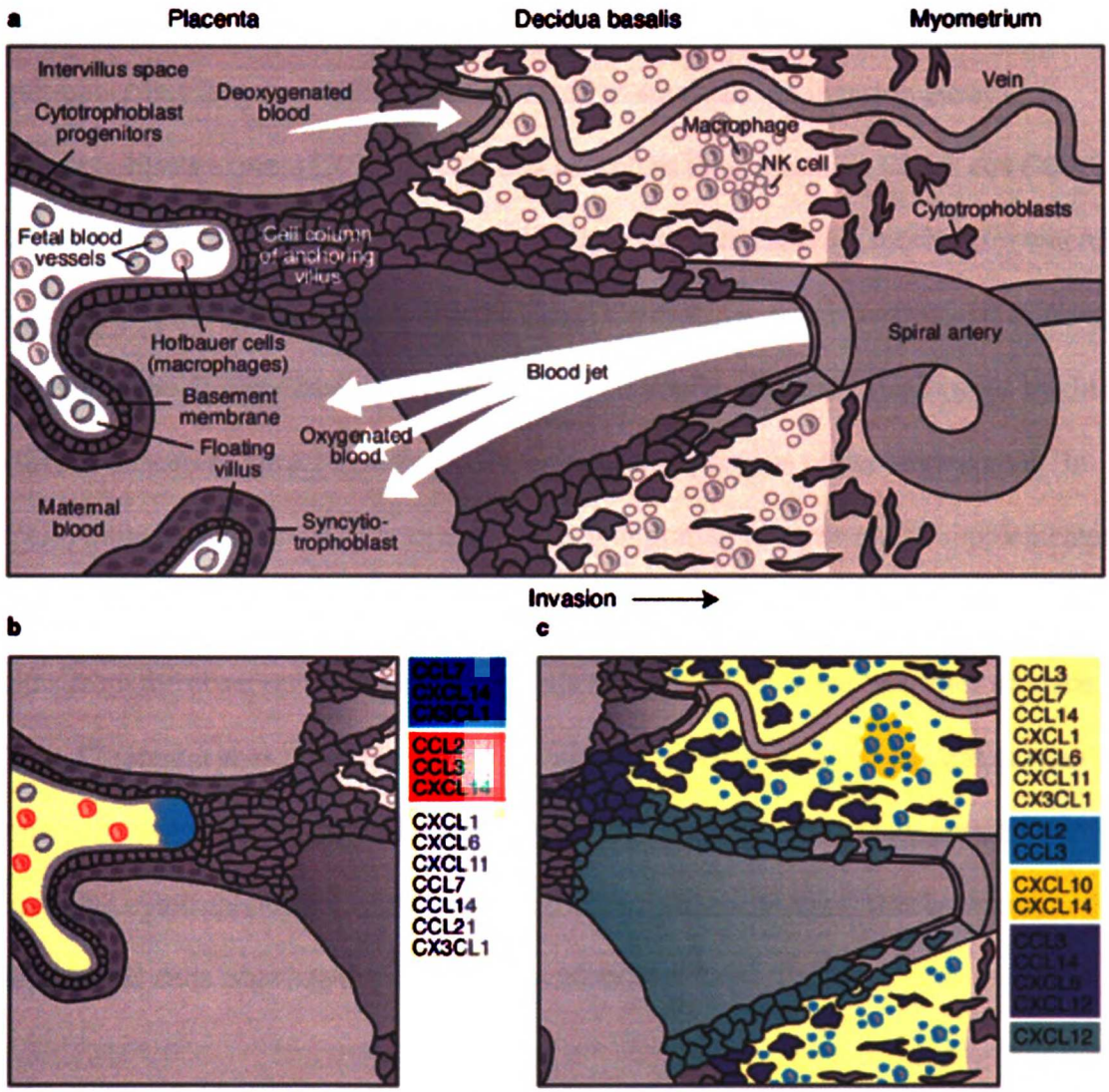


Figure 8. (from Red-Horse et.al., 2004) Chemokine expression at the maternal-fetal interface. A. Anatomy of the placenta as described in Figure 4. B. Chemokine expression by cells located in placental villi. C. Chemokine expression patterns by fetal and maternal cells in the uterine wall. In both B and C, the location of expression of each chemokine is shown by a distinct color, as indicated by the key on the right.

Chemokines and their receptors could regulate several aspects of this process including cytotrophoblast invasion/differentiation as well as survival. As invasion ensues, cytotrophoblasts express CCL3 and CCL14 as well as their receptors CCR1 and CCR5 (Drake et al., 2004; Red-Horse et al., 2001), suggesting an autocrine mechanism whereby cytotrophoblasts regulate their own fate. These chemokines are also expressed diffusely throughout the decidualized uterine stroma, which implies that paracrine signals might be acting in concert to attract invading cells to the deeper portions of the uterine wall. In fact, cytotrophoblast invasion is enhanced when migrating towards media supplemented with CCR1/CCR5 ligands (Sato et al., 2003). One of the most intriguing implications stems from the observation that invading cells that occupy maternal arterioles produce CXCL12 (Hanna et al., 2003; Red-Horse et al., 2001), whereas the equivalent *in vitro* population expresses the cognate receptor CXCR4. Thus, the interesting possibility exists that cytotrophoblasts might use CXCL12 as a targeting signal that becomes amplified as cells populate the maternal vasculature.

The Eph and ephrin family of molecules

In addition to members of the VEGF and Ang protein families, angiogenesis in the developing embryo also depends on a third class of molecules, the Ephs and ephrins. Eph receptors tyrosine kinases and their membrane-bound ligands, ephrins, have important functions during development because they give cells positional information that allows them to make decisions based on their spatial relationships (Figure 9) (Kullander and Klein, 2002). Evolutionarily conserved homologues of these molecules

have been described in worm, flies, fish, chicks, mice, and humans. In higher species, Ephs constitute the largest family of receptor tyrosine kinases with 14 members. They bind a family of ephrins ligands that contains 9 members. Ephs are unusual receptor tyrosine kinases in that their ligands are bound to the cell surface. As a result, these receptor-ligand interactions have two unique characteristics—bi-directional signaling and a requirement for cell-cell contact. Ephrin structure is the basis for subgroup nomenclature, which divides the family into A and B subtypes (EphA1-8, ephrinA1-6, EphB1-6, and ephrinB1-3). EphrinA ligands are attached to the membrane via a GPI-linkage, while ephrinBs are transmembrane proteins. With a few exceptions, Ephs have A/B designations according to which ephrin subgroup they bind.

Eph and ephrin function has been most extensively studied in the context of the nervous system (Wilkinson, 2001). During neuronal development, nerve cells develop a complex circuitry. Neuronal cells from one compartment of the brain extend axons into their target sites within another compartment. Axon targeting is guided through Eph and ephrin interactions. These molecules are expressed in concentration gradients both in the migrating neurons and at their final destinations. The expression levels at both sites are key as they direct cells to their final locations based on precise signaling outcomes that reflect receptor-ligand expression levels (Figure 10). Depending on the cell type and signaling intensity, these signaling outcomes can lend to either repulsion or attraction modulated by cytoskeletal changes as well as effects on integrin and chemokine receptor activity.

The phenotypes of knockout mice generated to study the role of Ephs and ephrins in neural development produced an unexpected outcome. Mice deficient in either

ephrinB2, EphB4, or EphB2/B3 died in utero due to defects in angiogenesis. Also surprisingly, EphB4 and ephrinB2 proved to be early markers of veins and arteries, respectively. The precise contribution of Ephs and ephrins to vessel maturation is not fully understood. Several family members including ephrinA1, ephrinB1, EphB3, and EphB4 induce angiogenesis *in vitro* and *in vivo*. By analogy with neuronal development, these data suggest that Ephs and ephrins direct the organization of the vascular network.

The arterio-venous specificity of EphB4 and ephrinB2 expression has led to the proposal that repulsive receptor-ligand interactions function to restrict arterial and venous components. In support of this theory, plating precursors on ephrinB2-expressing OP9 stromal cells induced vascular network formation, as well as the proliferation of ephrinB2-positive arterial cells and the recruitment of a perivascular population that expressed α-smooth muscle actin. In contrast, plating precursors on EphB4-expressing OP9 cells inhibited all these processes (Zhang et al., 2001). Additionally, ephrinB2-Fc induces endothelial denudation of explanted fragments of umbilical veins (Fuller et al., 2003). Finally, Eph and ephrin interactions play an important role in patterning vessel growth within tissues. For example, ephrinB expressed within somites repels the growth of EphB4-positive vessels, which accounts for their intersomitic distribution (Adams et al., 1999; Gerety et al., 1999; Helbling et al., 2000).

This thesis addresses the question of whether Ephs and ephrins play similar role at the maternal-fetal interface, in particular, whether they are involved in the arterial bias of placenta-mediated vascular remodeling. We tested the hypothesis that cytotrophoblasts within the uterine wall detect ephrinB2 and EphB4 on maternal arterioles and veins, respectively, and that these interactions pattern vascular invasion by regulating the cells

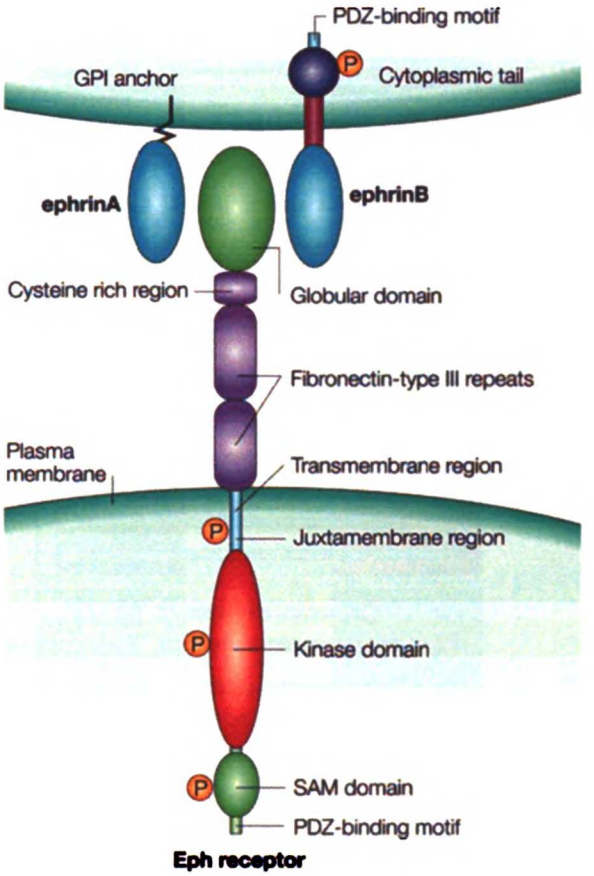


Figure 9. (from Kullander and Klein, 2002). Ephs and ephrins are receptor-ligand pairs that participate in forward and reverse signalling, Both Ephs, tyrosine kinases, and ephrins are tethered to the cell surface such that, upon binding, signals can be transduced within the receptor and ligand expressing cell. This large family of molecules is divided into two subgroups, A and B, based on the method of cell membrane attachment and their binding specificity. EphrinA molecules are bound to the cells surface via a guanidylphosphateinositol (GPI) linkage while ephrinBs are transmembrane proteins. For the most part, EphA receptors bind ephrinAs and EphBs are specific for ephrinBs. However, there are a few exceptions.

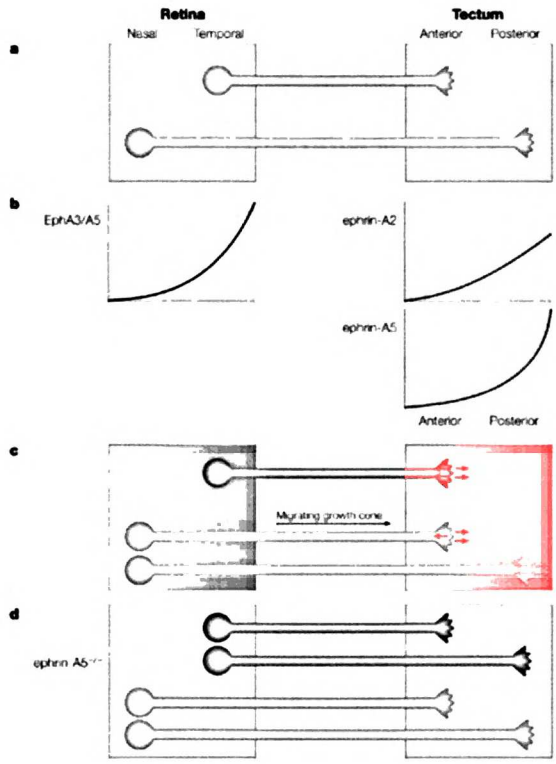


Figure 10. (from Wilkinson, 2001) Retinotectal neuronal pathfinding is guided by Eph and ephrin expression. A. In the developing visual system, axons originating from the temporal region of the retina project into the anterior tectal region of the brain while those from the nasal retina extend into the posterior tectum. Along this axis, they form a gradient such that the more temporal their origin, the more anterior they project and vise versa. B. This map is established by a graded sensitivity of retinal axons to ephrinA2 and ephrinA5. EphA3 and EphA5 (blue line) are expressed in an increasing nasal to temporal gradient and ephrinA (red lines) expression increases in the tectum in an anterior to posterior orientation. C. Because of their higher Eph expression, temporal axons are more sensitive to ephrinA-induced repulsion and do not migrate into the posterior tectum where ephrin expression is highest. In contrast, nasal-derived axons, which are less responsive, are able to populate the posterior region. D, Therefore, axons along this axis project randomly in ephrinA5-deficient mice.

adhesion and migration. In support of this theory, we found that cytotrophoblast commitment to invasion is associated not only with upregulation of endothelial markers but, more specifically, with adoption of an arterial fate—downregulation of EphB4 and sequential upregulation of ephrinB1 and -B2. Functionally, EphB4, but not ephrinB2, had dramatic effects on adhesion and migration. Cytotrophoblasts avoided substrates formed from EphB4 and exhibited dramatically decreased migration in response to EphB4-expressing 3T3 cells. These data suggested a role for EphB4 in negatively regulating cytotrophoblast interactions with maternal veins.

Lymphatic development

The lymphatic circulation is a second vascular network that functions in diverse processes such as interstitial fluid homeostasis, adaptive immunity, and digestion (Oliver, 2004). Lymphatic vessel function compliments the blood vascular system in many ways. For example, during capillary exchange, fluid leaks from the blood into surrounding tissues where it is taken up by post-capillary venules. A portion of this fluid contains high molecular weight proteins and cannot pass freely back into the circulation. Lymphatic capillaries possess structural features that allow them to take up this protein-rich fluid such as a discontinuous basal laminae and specialized, valve-like cell-cell junctions. This protein-rich fluid, termed lymph, passes through the lymphatic network before returning to the blood via the thoracic and right lymphatic ducts. Defects in lymphatic function can result in a special type of swelling called lymphoedema, which

results from either physical damage to the local vessels or inherited genetic deficiencies that impair lymphangiogenesis.

The lymphatic circulation also plays an important role in organizing an adaptive immune response (Cyster, 2005). Lymphocytes congregate in and circulate through lymph nodes, nodule-like structures intermittently spaced within lymphatic vessels. Circulating lymph filters through these nodes allowing lymphocytes to continually survey their environment. Foreign or pathogen-derived products will stimulate an immune response, which involves the various leukocyte populations that traffic through lymph nodes. Dendritic cells are also important players in this activity. These cells, which are present in peripheral tissues, are activated by pathogen-derived products and inflammatory signals, which subsequently upregulate phagocytosis and expression of CCR7, the receptor for CCL21 (Saeki et al., 1999). Many lymphatic vessels constitutively express CCL21 allowing them to attract activated dendritic cells, which then have access to local lymph nodes (Gunn et al., 1998). Therefore, lymphoid tissue is critical in coordinating the cell-cell interactions that are needed to mount an efficient immune response. As a result, individuals with lymphatic defects are highly susceptible to chronic unresolved infections (Karkkainen et al., 2000; Rockson, 2001). In this context, it is interesting to note that lymphatic vessels are excluded from several immune privileged sites including the brain and anterior chamber of the eye. In the latter instance, tolerance can be broken by inducing lymphatic growth and dendritic cell migration (Chen et al., 2004).

Knowledge of lymphatic vessel development and function has exploded over the past several years following the discovery of LYVE-1, a hyaluronic acid receptor

specifically localized to lymphatic endothelial cells (Banerji et al., 1999; Kaipainen et al., 1995; Oliver, 2004). Using this marker, the long-presumed theory that this vasculature arises from veins was confirmed. In mice, development of lymphatic vessels begins between embryonic day 9.0 and 9.5 when certain regions of the cardinal vein begin to express LYVE-1. Subsequently, expression of the transcription factor *Prox1* is induced in a polarized manner and a subset of the LYVE-1/Prox1-positive cells bud off and migrate centrifugally in response to VEGF-C produced by adjacent stromal cells (Karkkainen et al., 2004; Wigle et al., 2002). These cells organize into primary lymph sacs, sprout, and eventually give rise to the mature lymphatic vascular system, which is defined by expression of specific molecules including LYVE-1, Prox-1, VEGFR3, Podoplanin, $\alpha 9\beta 1$ integrins, and the chemokine CCL21.

In the adult, lymphangiogenesis involves sprouting from preexisting vessels and occurs at sites of injury and inflammation (He et al., 2004; Paavonen et al., 2000). Recent evidence also supports an alternative pathway in which vessels form from CD11b+ macrophages that are recruited to specific sites (Maruyama et al., 2005). VEGF-C and Ang-2 are involved: both molecules induce lymphatic endothelial cell hyperplasia and vessel formation when overexpressed in mice where the actions of Ang-2 appear to be upstream of VEGF-C (Gale et al., 2002; Jeltsch et al., 1997; Morisada et al., 2005). Tumors cells can also express VEGF-C and –D and, in mouse models of tumorigenesis, increase lymphangiogenesis and metastasis (Karpanen et al., 2001; Mandriota et al., 2001; Skobe et al., 2001). In addition, in various human tumors, VEGF-C and -D levels are positively correlated with lymphatic invasion and metastasis, and, in some cases, poor prognosis (Stacker et al., 2002). Together, these observations have

stimulated great interest in developing methods of targeting these molecules for clinical use such as cancer treatments that utilize monoclonal antibodies.

In terms of reproductive biology, one of the more striking observations is that, despite its close apposition to the external environment, the uterine endometrium lacks a lymphatic vessel network. This phenomenon has been observed in mice (Saban et al., 2004), rats (Head and Lande, 1983), rabbits (Otsuki et al., 1990), and humans (Koukourakis et al., 2005) where lymphatic vessels are restricted to the deeper myometrial and serosal segments of this organ. Early experiments in rodents showed that dyes and leukocytes are readily taken up from the myometrial region and transported to local lymph nodes, whereas introduction of the same reagents into the lumen or endometrium results in uterine localization (Head and Lande, 1983; Maroni and de Sousa, 1973). Thus far, the reasons for excluding lymphatics from the endometrium remain obscure.

Work described here shows that this situation changes during pregnancy. In fact, the decidua contains numerous lymphatic vessels especially in the capularis region overlying the fetal membranes. These vessels are in contact with cytotrophoblasts, but, similar to veins, their endothelial layer is not remodeled. Decidual lymphatic vessels may develop in response to cytotrophoblast-derived factors as implantation of placental explants into the kidney capsule of SCID mice induced a robust lymphangiogenic response, likely a result of the large amounts of VEGF-C and Ang-2 produced by these cells.

Vascular remodeling in the pregnant uterus

During pregnancy, the uterus has the remarkable ability to collaborate with the placenta in developing a hybrid organ that supports growth and development of the fetus. At this time, uterine tissues undergo dramatic changes that alter their cellular composition, a process that is controlled by complex interactions between maternal- and fetal-derived signals. Included in these are changes in the vascular compartment. Extensive angiogenesis occurs in the endometrium in preparation for implantation. Subsequently, when pregnancy is established, placental cells from the fetus invade the uterine wall and remodel its newly formed vasculature so that blood circulates thru the intervillous space. In other systems, blood vasculature changes are often accompanied by lymphangiogenesis. However, regulation of the uterine lymphatic vasculature had not been previously studied.

Experiments carried out in this thesis significantly advance our knowledge of the interactions that occur between the placenta and both of these vascular networks. We answer the long-standing question of why cytotrophoblasts have an affinity for arteries and not veins, which involves placental recognition of cell-surface proteins specifically expressed on the two sides of the circulation. In addition, we discover that uterine lymphatic vessels are highly regulated; they are excluded from the non-pregnant endometrium, but appear in great numbers during gestation. Using a novel *in vivo* model of cytotrophoblast invasion and vascular remodeling, we were able to provide evidence that the placenta induces decidual lymphangiogenesis. Thus, this important fetal organ not only influences maternal blood vessels during pregnancy, but also uterine lymphatics.

CHAPTER TWO: Materials and methods

Human tissue collection

Informed consent was obtained from all tissue donors. Placental and decidual tissue from elective terminations of pregnancy (6 to 22 weeks) was collected into 10% formalin (for *in situ* hybridization) or 3% paraformaldehyde (for immunofluorescence), or washed repeatedly in PBS containing antibiotics and placed on ice (for cytotrophoblast isolation and implantation).

Endometrial tissue was obtained from normally cycling women, aged 18-49 years after informed consent, under an approved protocol by the Stanford University Committee on the Use of Human Subjects in Medical Research. Endometrium from the proliferative and secretory phases of subjects who were documented not to be pregnant and had no history of endometriosis was fixed in 4% paraformaldehyde. Historectomy specimens were embedded in an orientation that allowed examination of the full thickness of the endometrium.

In situ hybridization

In situ hybridization was carried out using published methods (Red-Horse et al., 2001) and formalin-fixed, paraffin-embedded tissue sections of the maternal-fetal interface (n = 4, 14-21 wks; n = 3, term). Antisense [^{35}S]Sulfur-labeled probes were produced using linearized plasmids encoding cDNAs for ephrinB1 (Beckmann et al., 1994) and ephrinB2 (Cerretti et al., 1995) or gene fragments for EphB4 (nt 412-1253)

and EphB2 (nt 997-1599). Pseudocolor hybridization signals were generated in Photoshop. Tissue sections of heart served as positive controls.

Cytotrophoblast isolation and aggregation

Cells were isolated from pools of first- or second-trimester human placentas by published methods (Fisher et al., 1989; Kliman et al., 1986). Briefly, placentas were subjected to a series of enzymatic digests, which detached cytotrophoblast progenitors from the stromal cores of the chorionic villi. Then the cells were purified over a Percoll gradient and cultured on substrates coated with laminin (Invitrogen) or Matrigel (BD Biosciences) in serum-free medium: Dulbecco's modified Eagle's medium, 4.5 g/L glucose (Sigma Chemical Co.) with 2% Nutridoma (Boehringer Mannheim Biochemicals), 1% penicillin/streptomycin, 1% sodium pyruvate, 1% HEPES, and 1% gentamicin (UCSF Cell Culture Facility).

For aggregation studies, isolated cytotrophoblasts were cultured on Matrigel with or without agents that block chemokine receptor function, including pertussis toxin (100 ng/ml; List Biological Laboratories) or a CXCR4-specific antibody (10 mg/ml; BD Pharmingen). After overnight incubation, aggregates were photographed under bright-field illumination using a Leica inverted CTRMIC microscope fitted with a Hamamatsu camera.

Northern blot hybridization and RT-PCR

Total RNA was extracted from cytotrophoblasts using Trizol reagent (Invitrogen). Samples were obtained immediately after isolation or after 12 or 24 hours in culture on Matrigel-coated tissue culture plates. Two micrograms of poly(A) mRNA, enriched using an Oligotex mRNA midi kit (Qiagen), was separated by formaldehyde–agarose gel electrophoresis, transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech), and analyzed using a QuickHyb Northern hybridization kit (Stratagene). [^{32}P]Phosphorus-labeled probes were prepared using gene fragments cut from the plasmids described above as templates and High Prime DNA labeling mix (Roche). Experiments were performed at least three times. In total, seven first-trimester, eight second-trimester and six term samples were analyzed.

For RT-PCR, AMV reverse transcriptase (Invitrogen) was used to produce cDNA from the RNA samples described above. PCR for PDZ-RGS3 was performed using two primer sets: (1) GGATACCATCCCCGAAGAAT/AGGCACCAGCACACTCTCTT and (2) GGGAGGTGAGAGGTGATTT/GGGTGACGTAGGTGCCATAG. Similar results were obtained with each primer set.

Immunohistochemistry and apoptotic cell labeling

For *in situ* hybridization experiments, tissues were fixed at room temperature for 12-24 hours. The distribution of cytotrophoblasts was determined by staining adjacent sections with a cytokeratin 7-specific antibody (DAKO). Antibody binding was detected by using Vectastain ABC and DAB peroxidase substrate kits (Vector Laboratories).

For immunofluorescence, tissues were fixed for 4 hours on ice in 3% paraformaldehyde before they were snap-frozen in liquid nitrogen and sectioned. Nonspecific binding was blocked by incubating the sections in 3% bovine serum albumin/PBS for 1 hour before addition of a polyclonal (goat) antibody specific for CXCL16 (R&D systems) dissolved in the blocking solution (1:20; vol/vol) and incubation overnight at 4°C. Staining was detected using an Alexa-488–conjugated donkey anti-goat secondary antibody (Molecular Probes) and observed using a Leica CTR5000 upright microscope. As a positive control, lung tissue was processed in parallel. As a negative control, sections were incubated in nonimmune goat serum rather than the primary antibody. Similar staining patterns were observed in all the samples (n = 2, 5-6 wk; n = 3, 14-19 wk; n = 6, term). 3T3 cell lines, produced as previously described (Gao et al., 1999), were fixed with ice-cold methanol for 5 minutes and stained as described above. Primary polyclonal antibodies included goat anti-EphB4 (R&D Systems) and rabbit anti-ephrinB2 (Santa Cruz P-20).

During lymphatic studies, primary antibodies included those specific to human and mouse LYVE-1 (Research Diagnostics) used at a dilution of 1:200 (vol/vol). Cytokeratin 7 (DAKO), HLA-G 48H4 (McMaster et al., 1995), placental lactogen (Chemicon), and CD31, CD41, and CD45 (Pharmingen) were used at a dilution of 1:100. Antibodies that recognize integrin $\alpha 9\beta 1$ (Serotec) and the pan-ephrin antibody (Santa Cruz Biotechnology) were used on freshly frozen tissues that were fixed with methanol before immunolocalization. Primary antibody incubation was 1 h at room temperature. Staining was detected using Alexa-488 and -594–conjugated secondary antibodies (Molecular Probes).

Apoptotic cells were identified by the TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) method, a commercial kit that fluorescein-labels DNA strand breaks (Roche).

Substratum choice assay

Cytotrophoblasts' preference for substrates containing either EphB4 or ephrinB2 was assessed by using published methods (Birgbauer et al., 2001). Briefly, laminin was mixed with either ephrinB2-Fc, EphB4-Fc or (control) human IgG (10 mg/ml) and fluorescein-conjugated goat-anti human Fcg (10 mg/ml), and then spotted in 1 ml aliquots on the bottom of 12-well culture dishes. The spots were allowed to gel for 1 hour by incubation at 37°C, before the entire substrate was coated with laminin (10 mg/ml). Then 1×10^6 cytotrophoblasts per well were plated as a monolayer. After 12 hours, the cells were fixed and stained with anti-human cytokeratin 7. The distribution of cells and protein spots was observed by fluorescence microscopy. The experiment was repeated 10 times, each time showing similar results.

Cytotrophoblast migration in 3T3 co-culture experiments

NIH 3T3 cells were fluorescently labeled for 30 minutes in medium (DME H-21, 4.5 g/L glucose) containing 2 mM Cell Tracker Red CMPTX (Molecular Probes). Freshly isolated cytotrophoblasts and labeled NIH 3T3 cells (1×10^6/ml) were mixed (1:10; vol/vol) and plated in 12-well tissue culture wells coated overnight at 4°C with 10

mg/ml (murine) laminin (Invitrogen) or 100 ml undiluted Matrigel. Cells were allowed to attach for 1 hour, then washed with PBS before being placed in an environmental chamber mounted on a motorized microscope stage (Carl Zeiss MicroImaging, Inc.). Cultures were maintained at 37°C for 15 hours. Time-lapse images were collected every 10 minutes in both the bright light and fluorescence channels using a SPOT-RT CCD camera (Molecular Dynamics). Cytotrophoblast migration was traced by recording the position of 10 randomly chosen unlabeled cells in each successive frame using the Openlab software point counter (Improvision). Positional data were transferred to Excel for calculation of the linear distance traveled by each cell. To normalize the data, the control value from each experiment was set at 100 percent and migration was expressed as a percentage of control values. The experiment was repeated four times on laminin and twice on Matrigel. Similar results were obtained for both first- and second-trimester cytotrophoblasts.

Chemotaxis assays

Modified Boyden-chamber assays were used to assess the effects of chemokines and EphB4 on cytotrophoblast migration. The undersides of Transwell inserts (8 μm pore size, Corning Costar) were incubated overnight at 4°C with 10 mg/ml human plasma fibronectin (Roche) and then washed with PBS. Cytotrophoblasts (2.5×10^5 cells in 250 ml serum-free medium) were added to the upper compartments, and the inserts were placed in 24-well plates that contained 500 ml medium with either vehicle alone (0.1% bovine serum albumin/PBS) or chemokines (10 and 1,000 ng/ml). In some cases, the

medium also contained EphB4-Fc (10 mg/ml). Growth factor-induced migration was stimulated by adding to the lower chamber 125 ml endothelial cell growth medium (Clonetics, EGM-2) containing FCS, hEGF, VEGF, hFGF-B, and IGF-1 at the concentrations provided by the manufacturer to a final volume of 500 ml. The cells were incubated overnight under standard tissue culture conditions, washed in PBS and fixed in 3% paraformaldehyde. Migration was quantified by one of two methods. (1) Cells in the upper chamber were removed with a cotton swab, and those remaining on the underside of the filter were stained for 5 minutes with crystal violet (0.5% in 20% methanol), and then washed with water. Membranes were destained in 1 ml 10% acidic acid. Migration was quantified by determining the A_{600} of the latter solution (Sieg et al., 2000). (2) Cytotrophoblasts that migrated to the underside of the filter were stained with anti-cytokeratin 7. The membrane area covered by cells was quantified in three randomly chosen fields by using the Openlab software region-of-interest tool. To normalize the data, the control value from each experiment was set at 100 percent and migration was expressed as a percentage of control values. Both methods produced the same results. Each experiment was repeated four to seven times, with two to three different chambers per condition.

Method 1 was utilized for lymphatic endothelial cell migration studies. Primary human lymphatic endothelial cells, which were isolated from skin, were provided Dr. Mihaela Skobe (Podgrabinska et al., 2002) and maintained in endothelial cell growth medium (Clonetics). In these experiments, primary lymphatic endothelial cells (2.0×10^5 cells in 200 μl of serum-free medium without growth factors) were added to the upper compartments, and the inserts were placed in 24-well plates that contained 500 μl of

serum-free medium that had been cultured either with Matrigel alone (control) or with cytotrophoblasts at a concentration of 1.0×10^6 cells/ml. Endothelial cell growth medium containing FCS, human epidermal growth factor, VEGF, human epidermal growth factor-B, and insulin-like growth factor-1, at the concentrations provided by the manufacturer, served as a positive control. The cells were incubated for 3 h under standard tissue culture conditions.

In vivo *transplantation*

In all cases, SCID mice were the recipients. For mammary fat pad implantations, placental villi were dissected into 5-mm^2 pieces and placed within a small incision made below this region, which was subsequently sutured. *In toto*, tissue from 4 placentas was transplanted to 11 mice of which 4 had histological evidence of vascular invasion. For kidney capsule implantation, placental villi ($n = 3$; placentas at 6-7 weeks of gestation) were dissected into 2-mm^2 pieces and implanted under the capsular membrane ($n = 15$ mice, all samples implanted for 2 weeks or more had histological evidence of lymphangiogenesis) using surgical methods (McCune et al., 1988; Stoddart et al., 2001). Mice were maintained under pathogen-free conditions for 1 to 3 weeks, at which time the experiment was terminated and either whole kidneys containing implants or implants dissected from a portion of the fat pads were immediately placed in 3% paraformaldehyde. Tissues were fixed for 3 h at 4°C before being snap-frozen in liquid nitrogen and sectioned. Histological analysis allowed examination of the distance between the implanted villi and cells that had invaded murine tissues. Protocols

involving animals and human fetal tissue were approved by the UCSF Committee on Animal Research and the Committee on Human Research, respectively.

Scanning electron microscopy

Scanning electron microscopy was carried out according to published methods (Vrdoljak et al., 2005). Briefly, after fixation in 2% glutaraldehyde, tissue specimens were rinsed with Na-Cacodylate buffer and incubated in 1% aqueous osmium tetroxide. Subsequently, the samples were dehydrated using an ethanol gradient, critical point dried, and sputter coated with 5-6 nm of AuPd. High-resolution images were captured by a Hitachi S5000 SEM using a secondary electron detector at 10 kV.

CHAPTER THREE: EphB4 regulates chemokine-evoked trophoblast responses: a mechanism for incorporating the human placenta into the maternal circulation

Introduction

During pregnancy, the placenta establishes a physical connection to the mother, setting up an exchange system that supports growth and development of the embryo/fetus. At a structural level the mature placenta is divided into two distinct compartments: one mediates maternal-fetal exchange of a myriad of substances, and the other connects the feto-placental unit to the uterine wall and vasculature (Fig. 1). The exchange function is carried out by cells of the extraembryonic lineage that develop into a disk-shaped structure composed of tree-like chorionic villi. The villi, which encase fetal blood vessels, float in a chamber termed the intervillous space, through which maternal blood circulates. Exchange from maternal to fetal blood and vice versa occurs across specialized, multinucleated trophoblast cells of the placenta that cover the surface of the chorionic villi and hence line the intervillous space.

Shunting of maternal blood flow to the chorionic villi, which occurs as a result of trophoblast endovascular invasion, is an integral component of placentation. This process involves remodeling the uterine vasculature and is present in all species with hemochorial placentation, classified as such because the trophoblast cells are in direct contact with maternal blood. However, the pattern of trophoblast interactions with maternal blood vessels spans a wide spectrum. In mice, the endovascular component of trophoblast invasion is limited to the termini of arteries and veins (Adamson et al., 2002). In humans the process is dramatic, with mononuclear trophoblasts, termed

cytotrophoblasts, interacting very differently with the two sides of the uterine circulation (Fig. 11). Endovascular invasion of spiral arterioles encompasses the decidua and the first third of their myometrial segments. As a result, nearly the entire intrauterine course of these vessels is lined by cytotrophoblasts, which also intercalate within and destroy the integrity of their muscular walls. This process redirects the maternal arterial circulation to the intervillous space and expands the luminal diameter of the vessels by as much as 1,000-fold, increasing uterine blood flow to more than 30 liters per hour (Dickey and Hower, 1995; Metcalfe et al., 1955). In contrast, remodeling of veins, which establishes venous return, is limited to their termini.

A portion of the molecules involved in endovascular invasion are known and include adhesion molecules and cell surface receptors important for conventional vasculogenesis/angiogenesis (reviewed in Red-Horse et al., 2004). For example, the cytotrophoblast population that carries out uterine vascular remodeling executes an epithelial-to-endothelial transition. This process, which is synchronized with invasion, involves a switch from molecules characteristic of polarized epithelial cells to those indicative of an endothelial phenotype. With regard to adhesion molecules, the onset of cytotrophoblast differentiation/invasion is accompanied by reduced staining for receptors localized to cytotrophoblast progenitors—e.g., integrin a6β4 and E-cadherin—and the upregulation of adhesion receptors characteristic of endothelium—e.g., VE-cadherin, Ig family members VCAM-1 and PECAM-1, and integrins aVβ3 and a1β1 (Damsky et al., 1992; Zhou et al., 1997a). In addition, cytotrophoblasts begin to produce a wide array of VEGF family members and their receptors, which also regulate important aspects of vascular remodeling (Zhou et al., 2002).

However, due to their pleiotropic expression, the molecules involved in vascular mimicry cannot explain cytotrophoblast tropism for arteries rather than veins. This phenomenon implies that different vessel types possess specific molecular determinants that placental cells decode in terms of adhesion and/or migration. In this regard, members of the Eph/ephrin family could have interesting roles. This family of molecules consists of multiple members that are classified into two subgroups: A and B. In general, EphB receptors bind ephrinB ligands and EphA receptors bind ephrinA ligands. With regard to specific functions, EphB4, a receptor tyrosine kinase, and its ligand, ephrinB2, are expressed at high levels in veins and arteries, respectively (Wang et al., 1998), and interactions between the two are indispensable for angiogenesis in the developing embryo (Gerety and Anderson, 2002; Gerety et al., 1999; Wang et al., 1998). In addition, both the receptors and the ligands are transmembrane molecules. Upon cell-cell contact, their ligation generates bi-directional intracellular signals that can influence cell migration and tissue morphogenesis. In other locations, such as the developing nervous system, these molecules play important roles in boundary formation (reviewed in (Wilkinson, 2001).

Although integral to these processes, members of the Eph/ephrin family are not known to be direct participants in migration. Instead, the signals they generate are upstream of other molecular families that govern cell movement. For example, Eph-ephrin ligation can influence chemokine-induced migration (Lu et al., 2001). This observation may be especially relevant to placentation, as our previous work and that of other investigators shows that, during pregnancy, cells within the uterine wall, including cytotrophoblasts, express a wide array of chemokines (Drake et al., 2001; Drake et al., 2004; Hanna et al., 2003; Red-Horse et al., 2001). Additionally, cytotrophoblasts express

a chemokine receptor repertoire that suggests that they are able to respond to both the autocrine and paracrine chemokine signals they encounter (Drake et al., 2004; Jaleel et al., 2004).

Together, these data suggested the hypothesis that, in humans, Eph and ephrin interactions pattern cytotrophoblast invasion. In accord with this theory, we found that, during pregnancy, maternal endothelial cells of uterine veins and arteries express EphB4 and ephrinB2, respectively. Cytotrophoblast invasion was associated with acquisition of an arterial phenotype—downregulation of EphB4 and sequential upregulation of ephrinB1 and -B2. *In vitro*, cytotrophoblasts avoided substrates formed from EphB4 and exhibited dramatically decreased migration in response to EphB4-expressing 3T3 cells. As to the mechanisms involved, interactions with EphB4 specifically downregulated chemokine-induced responses with little effect on growth factor-stimulated migration. These data support a model in which Eph and ephrin-mediated interactions play critical roles in human placentation at two important junctures, first by generating repulsive signals that initiate cytotrophoblast invasion and later by patterning the cells' interactions with the uterine vasculature.

Results

Chemokines stimulate cytotrophoblast aggregation and migration

During pregnancy, cytotrophoblasts form aggregates that leave the placenta and give rise to migratory cells that invade the decidua as well as uterine blood vessels. Data from our group and other investigators suggest that these fetal cells encounter autocrine

and paracrine chemokine signals during every step of this process. For example, invasive cytotrophoblasts express CXCL12 (Hanna et al., 2003; Red-Horse et al., 2001) and CXCL16 (Fig. 12A), whereas CCL21 is produced by maternal cells (Red-Horse et al., 2001). Additionally, cytotrophoblasts express receptors, including CXCR4, CXCR6, and CCR7, that should enable them to respond to the chemokines they encounter (Drake et al., 2004).

To test this hypothesis, we used a culture model that supports *in vitro* differentiation of cytotrophoblast progenitors isolated from chorionic villi along the pathway that leads to uterine invasion *in vivo* (Librach et al., 1991). At a morphological level, the cells, which are initially plated as a monolayer, rapidly form large aggregates, which in turn give rise to invasive cells. To understand the role of chemokines in this process, we assessed cytotrophoblast aggregation in the presence of agents that block chemokine signaling—pertussis toxin and antibodies that perturb CXCR4 function. Both methods of blocking chemokine actions dramatically decreased aggregate formation (Fig. 12B).

To determine if this phenomenon was attributable to decreased migration, we assayed the individual and collective effects of CXCL12, CXCL16, and CCL21. Each chemokine stimulated migration in a dose-dependent manner, and the effects, which were additive, were nearly comparable to the addition of serum plus a growth factor cocktail (Fig. 12C). The mechanisms involved included increased chemokinesis, as similar effects were observed irrespective of whether the chemokines were added to the upper, lower or both chambers of a transwell filter apparatus (Fig. 12D). Together, these results

suggest that chemokines are powerful inducers of cytotrophoblast migration, which is required for aggregation.

Cytotrophoblasts modulate Eph and ephrin expression during aggregation, migration, and invasion

Next, we investigated mechanisms that could convert random cytotrophoblast migration into specific interactions with uterine arterioles rather than veins. Specifically, we examined the expression patterns of Ephs and ephrins, which are present in the placenta (Goldman-Wohl et al., 2004), using *in situ* hybridization on tissue sections encompassing the maternal-fetal interface. In accordance with other systems, EphB4 (Fig. 13A) and ephrinB2 (Fig. 13B) were expressed by endothelial cells of uterine veins and arteries, respectively. Cytotrophoblasts switched from a venous to an arterial pattern of Eph and ephrin expression as they differentiated along the pathway that leads to uterine invasion. In placental chorionic villi, cytotrophoblast progenitors and syncytiotrophoblasts that line the intervillous space expressed EphB4 (Fig. 13C). At the boundary where progenitors commit to differentiation, they abruptly downregulated EphB4 expression and began to express ephrinB1 (Fig. 13D), which binds EphB4, (Sakano et al., 1996) and EphB2 (Fig. 13E), a receptor for the ephrinB ligands. These molecules continued to be expressed as the cells infiltrated the uterine stroma and occupied maternal arterioles (Fig. 13D,E). Finally, within the uterine wall, many of the interstitial and endovascular cytotrophoblasts upregulated ephrinB2 (Fig. 13F). Based on these expression patterns, we concluded that invasive cytotrophoblasts are equipped to

interact with EphB4, expressed by cytotrophoblast progenitors and maternal veins, via the ligands ephrinB1 and -B2. Additionally, invasive cytotrophoblasts express the EphB2 receptor, a binding partner for ephrinB2 expressed on maternal arteries (Fig. 13G).

Next, we used Northern blot hybridization to determine if our culture model (described above) mimicked the regulated expression of cytotrophoblast Ephs and ephrins that we observed at the maternal-fetal interface *in situ*. In these experiments, RNA samples were isolated from cytotrophoblast progenitors before they were plated on Matrigel (0 hour) and after they were allowed to differentiate for 12 or 24 hours in culture (Fig. 13H). The progenitors expressed high levels of EphB4 mRNA that were lost as the cells differentiated, i.e., acquired an invasive phenotype. Conversely, signals corresponding to ephrinB1, -B2, and EphB2 mRNAs were dramatically upregulated during this same time period. Finally, cytotrophoblasts isolated from first-trimester, second-trimester, or term placentas executed the same general pattern of Eph and ephrin modulation *in vitro*.

Cytotrophoblasts avoid substrates formed from EphB4, but not ephrinB2

To gain mechanistic insights into Eph and ephrin functions in cytotrophoblasts, we used a substratum choice technique that was developed to understand the role of these counter-receptors in the nervous system (Birgbauer et al., 2001). In these experiments, cytotrophoblast progenitors were cultured on laminin substrates that were spotted with either ephrinB2-Fc or EphB4-Fc. The results showed that cytotrophoblasts differentially interpreted signals garnered from ligating ephrinB2 or EphB4. On ephrinB2-spotted

substrates (Fig. 14A,C) and control substrates spotted with human IgG (data not shown), the cells formed aggregates that were equally distributed over the entire surface of the culture dish. In contrast, regions that contained EphB4 had significantly fewer cytotrophoblasts, with the outer edge of the spot appearing to form a boundary (Fig. 14B,D). Video microscopy revealed that the cells were not able to adhere to EphB4 (data not shown). These results suggest that cytotrophoblasts specifically avoid substrates containing EphB4.

Co-culture with cells expressing EphB4 decreases cytotrophoblast migration

Because Eph and ephrin function is highly dependent on the manner of cell surface display, we modeled cytotrophoblast interactions with arteries and veins by co-culturing them with NIH 3T3 cells expressing EphB4 and ephrinB2 (Fig. 15A). In the presence of control (Fig. 15B) or ephrinB2-expressing (data not shown) 3T3 cells, the cytotrophoblasts rapidly migrated toward one another, forming aggregates that included both cell types. In contrast, when cytotrophoblasts were cultured with 3T3 cells that expressed EphB4 (3T3-EphB4), migration was largely inhibited, and the cytotrophoblasts tended to remain as single cells (Fig. 15C). It was also apparent that cytotrophoblasts cultured under control and experimental conditions had very different morphological features, as demonstrated by staining for cytokeratin 7 expression. Cytotrophoblasts co-cultured with the parental (Fig. 15B,D) and ephrinB2-expressing (data not shown) 3T3 cells had a rounded appearance, whereas those co-cultured with 3T3-EphB4 cells spread on the matrix substrate (Fig. 15C,E).

To further characterize this effect, we used time-lapse videomicroscopy to track, during the first 15 hours of culture, cytotrophoblast migration under control or experimental conditions. In each experiment, cytotrophoblasts co-cultured on a laminin substrate with control 3T3 cells (Fig. 15F) migrated on average twice as far as those that were cultured with the 3T3-EphB4 line (Fig. 15G; quantification shown in Fig. 5H). Co-cultures on Matrigel gave very similar results (Fig. 15H). In summary, these data support the hypothesis that cytotrophoblasts' interactions with EphB4-expressing cells, such as those that line uterine veins, restrict their migration, whereas interactions with ephrinB2-expressing cells, such as those that line maternal arteries, do not.

EphB4 specifically regulates chemokine-induced migration

Since invasive cytotrophoblasts respond to both chemokine and Eph/ephrin signals, we investigated the possibility of crosstalk between these two pathways. In the developing nervous system, PDZ-RGS3, a regulator of G-protein-coupled receptors, constitutively associates with the cytoplasmic domain of ephrinB molecules. Upon interactions with Eph receptors, the RGS domain inhibits chemokine receptor signaling by initiating ATPase activity (Lu et al., 2001). Therefore, we used RT-PCR to determine if cytotrophoblasts express this molecule, which links Eph/ephrin and chemokine responses. The results are shown in Fig. 16A. A band of the expected size was observed when RNA samples isolated from either first- or second-trimester cytotrophoblasts were analyzed. A band of the same size was observed when RNA extracted from brain was processed in parallel. In contrast, samples from control cells (peripheral blood mononuclear cells or NIH 3T3 cell lines) lacked PDZ-RGS3 expression.

Accordingly, we looked for evidence of specific effects of ephrin signaling in terms of chemokine-induced cytotrophoblast migration. In these experiments, we tested the effects of EphB4-Fc in the aforementioned transwell migration assay. Under control conditions, the addition of either chemokines (CXCL12, CXCL16, and CCL21) or a growth factor cocktail with serum to the medium significantly increased cytotrophoblast migration (Fig. 16B). In contrast, addition of EphB4-Fc had differential effects on chemokine and growth factor actions (Fig. 16B). Specifically, chemokine-induced migration was inhibited by approximately 60%, whereas growth factor effects were not significantly different from those of the controls (Fig. 16C).

Discussion

During pregnancy, growth and development of the embryo/fetus depend on the unique relationship between the placenta and the uterus. In humans, fetal cytotrophoblasts from the placenta extensively remodel, then occupy uterine spiral arterioles. Paradoxically, cytotrophoblast interactions with uterine veins are by comparison superficial, as the cells merely breach the termini of these vessels. Together, these important processes divert maternal blood flow to the placenta. Establishment and maintenance of the uteroplacental circulation is one of the most critical determinants of pregnancy outcome. Here, we provide insights at a molecular level into the mechanisms involved. Specifically, chemokine-stimulated cytotrophoblast migration is patterned by the interactions of Eph and ephrin family members.

The first indication that interplay between these pathways could play an important role in human placentation came from studies in which we surveyed the expression of both chemokines and Eph/ephrin family members at the maternal-fetal interface. Significantly, cytotrophoblast invasion was associated with modulated expression of molecules from both families, and in some cases overlapping expression suggested the possibility of molecular crosstalk. As to chemokines, invasive cytotrophoblasts express CXCL12 and CXCL16 and their receptors, CXCR4 and CXCR6. In addition, they express CCR7, a receptor for CCL21 expressed by maternal cells. These chemokines were powerful stimulators of cytotrophoblast migration *in vitro*, suggesting a similar role during formation of the maternal-fetal interface. Furthermore, recent evidence suggests that other cell types contribute to the uterine chemokine milieu. For example, cytotrophoblast invasion stimulates platelet deposition within uterine vessels and the latter cells produce a chemokine that attracts cytotrophoblasts *in vitro* (Sato et al., 2005).

Cytotrophoblast invasion is also associated with downregulation of EphB4 and sequential upregulation of ephrinB1 and -B2. Data from our microarray analyses validated by RNase protection assays suggest that these ligand-receptor pairs dominate cytotrophoblast Eph and ephrin expression. With regard to functional analyses, isolated cytotrophoblasts avoided substrates containing EphB4 and decreased migration when they encountered EphB4-expressing cells suggesting that these interactions influence pathways that involve adhesion molecules. Interestingly, subsequent experiments showed that cytotrophoblast interactions with EphB4 specifically downregulated chemokine-induced migration without affecting growth factor-induced movement. These data support a model in which autocrine and paracrine chemokine signals stimulate

cytotrophoblast migration within the uterine wall where they encounter blood vessels. This phenomenon is blunted when the cells interact with EphB4 expressed on the endothelial cells that line maternal veins. As a result, the cells preferentially remodel spiral arterioles.

This theory is consistent with the fact that EphB4-ephrinB2 interactions are required for angiogenesis during development. They function, at least in part, by restricting the mixing of arterial and venous endothelium, but the mechanisms involved are not fully understood (Adams et al., 1999; Fuller et al., 2003; Gerety et al., 1999; Helbling et al., 2000). For example, plating precursors on ephrinB2-expressing OP9 stromal cells induced vascular network formation, as well as the proliferation of ephrinB2-positive arterial cells and the recruitment of a perivascular population that expressed a-smooth muscle actin. In contrast, plating precursors on EphB4-expressing OP9 cells inhibited all these processes (Zhang et al., 2001). Additionally, ephrinB2-Fc induces endothelial denudation of explanted fragments of umbilical veins (Fuller et al., 2003). Finally, Eph and ephrin interactions play an important role in patterning vessel growth within tissues. For example, ephrinB expressed within somites repels the growth of EphB4-positive vessels, which accounts for their intersomitic distribution (Adams et al., 1999; Gerety et al., 1999; Helbling et al., 2000).

We suggest that these receptor-ligand pairs have similar roles at the maternal-fetal interface. Cytotrophoblast commitment to invasion is associated not only with upregulation of endothelial markers (Zhou et al., 1997a) but, more specifically, with adoption of an arterial fate. It is likely that this transition has several important consequences with regard to patterning cytotrophoblast invasion. For example,

cytotrophoblast progenitors that are attached to the trophoblast basement membrane of chorionic villi express EphB4. Cells at the base of columns (see Fig. 11) execute a dramatic downregulation of this transmembrane receptor and a concomitant upregulation of ephrinB1. Our data suggest that interactions between this receptor-ligand pair could form a boundary that orients cytotrophoblast invasion away from the placenta and toward the uterus (Fig. 17A). Our findings also suggest that acquisition of an arterial phenotype restricts cytotrophoblast migration into EphB4-expressing uterine veins, resulting in asymmetrical vascular remodeling (Fig 17B). Despite species differences in vascular invasion, it would be interesting to assess the relationship between cytotrophoblasts and uterine blood vessels in mice deficient in the above molecules. However, ephrinB2 and EphB4 null mice die around the time period when the mature placenta begins to function, and ephrinB1 is not expressed in analogous placental cell types (Sapin et al., 2000).

In this context, ongoing studies are aimed at investigating the molecular basis of cell fate decisions that regulate cytotrophoblast adoption of an arterial phenotype. In the developing embryo, veins appear to form by a default pathway, whereas Notch signaling upstream of ephrinB2 actions induces arterial differentiation (Fischer et al., 2004; Lawson et al., 2001). Our data suggest that a similar situation occurs during human cytotrophoblast differentiation, additional evidence of the fundamental vascular nature of these cells. Specifically, cytotrophoblast progenitors express EphB4. We speculate that differentiation signals induce a switch to an arterial phenotype as evidenced by ephrinB2 expression, which is restricted to invasive cytotrophoblasts.

Given the endothelial characteristics displayed by cytotrophoblasts, a similar interplay between ephrinBs and chemokine receptors may occur during vasculogenesis

and/or angiogenesis. Like EphB4 and ephrinB2, CXCR4 is expressed on endothelial cells and plays important roles in vascular development (Salcedo and Oppenheim, 2003; Tachibana et al., 1998). CXCR4 expression is pro-angiogenic, suggesting that EphB4-ephrinB2 interactions may function by limiting CXCL12-induced migration of arterial endothelial cells into veins and vice versa. Currently, there is a great deal of interest in how the ephrinB2 cytoplasmic domain functions in endothelial cells during angiogenesis (Adams et al., 2001; Cowan et al., 2004; Makinen et al., 2005).

Finally, our findings have interesting implications for other aspects of reproduction. For example, the human blastocyst expresses EphA1, and its ligand, ephrinA1, is present on the uterine epithelium during the implantation window (Fujiwara et al., 2002; Red-Horse). Furthermore, the human blastocyst expresses chemokine receptors (Dominguez et al., 2003), and uterine expression of several chemokines is hormonally regulated (Caballero-Campo et al., 2002). Therefore, it is possible that the pathways described in this report function from the earliest stages of pregnancy onward. With regard to perfusion of the placenta, trophoblast invasion of uterine vessels initiates blood flow to the maternal-fetal interface at ~10-12 weeks of gestation. We speculate that a failure of invasive cytotrophoblasts to upregulate an arterial phenotype would lead to the loss of pregnancy during the late first or early second trimester. In contrast, partial defects in this process could lead to reduced arterial invasion, the hallmark of preeclampsia and a subset of pregnancies complicated by intrauterine growth restriction. Thus, Eph and ephrin actions mediated by chemokines and their receptors could play critical master regulatory roles in the initiation and continuation of human pregnancy.

Figure 11

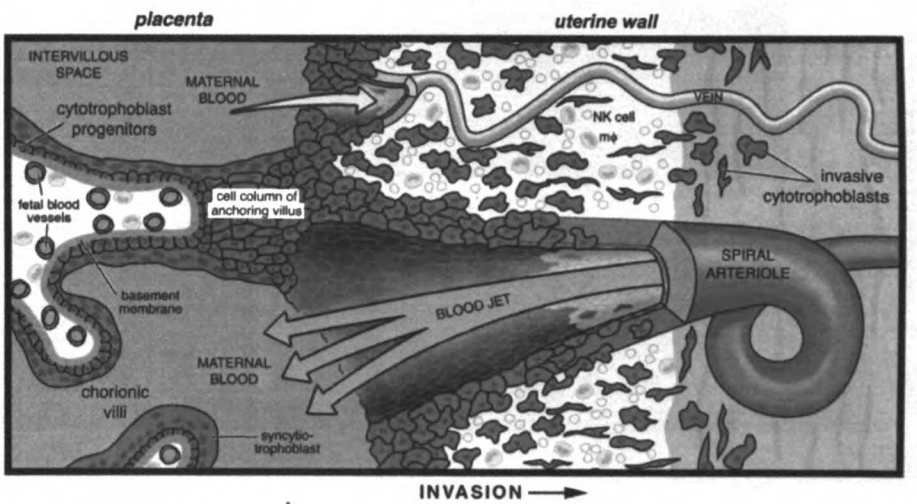

placenta
uterine wall
INTERVILLOUS SPACE
MATERNAL BLOOD
cytotrophoblast progenitors
VEIN
NK cell
mϕ
invasive cytotrophoblasts
fetal blood vessels
cell column of anchoring villus
SPIRAL ARTERIOLE
basement membrane
BLOOD JET
MATERNAL BLOOD
chorionic villi
syncytio-trophoblast
INVASION

Figure 12

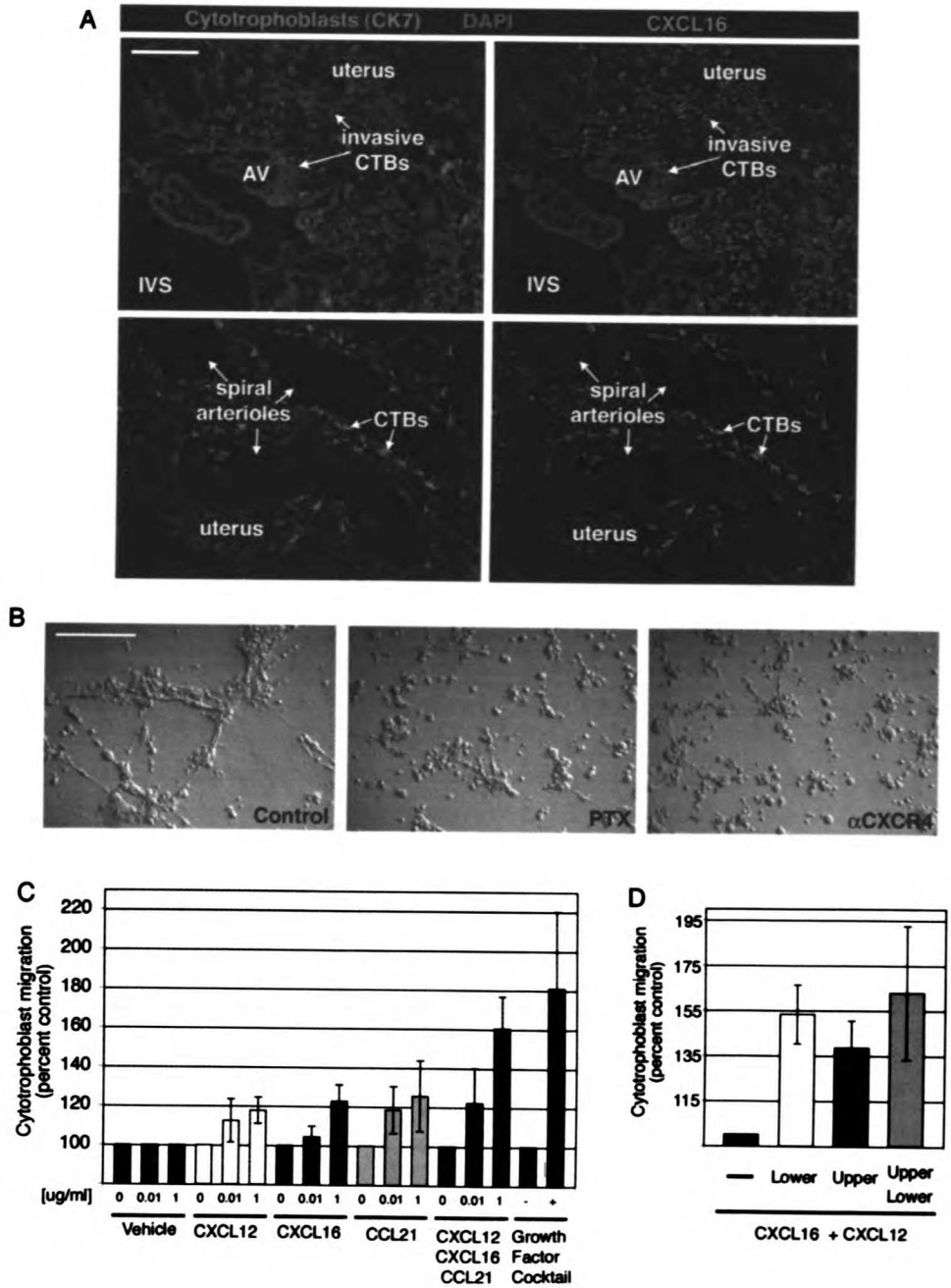

A
Cytotrophoblasts (CK7)
DAPI
CXCL16
uterus
invasive CTBs
AV
IVS
spiral arterioles
CTBs
B
Control
PTX
αCXCR4
C
Cytotrophoblast migration (percent control)
[ug/ml]
Vehicle
CXCL12
CXCL16
CCL21
CXCL12 CXCL16 CCL21
Growth Factor Cocktail
D
Lower
Upper
Upper Lower
CXCL16 + CXCL12

Figure 13

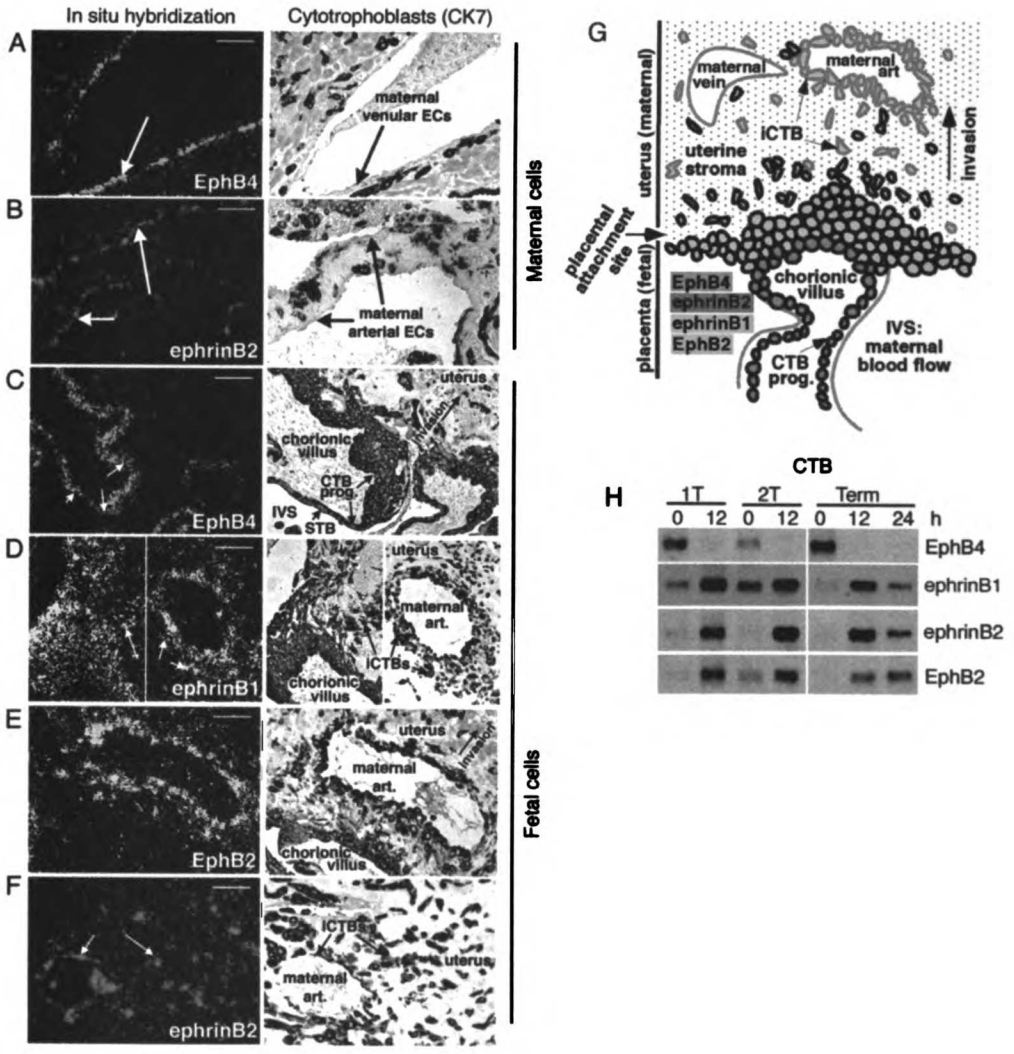

In situ hybridization
Cytotrophoblasts (CK7)
A
EphB4
maternal venular ECs
B
ephrinB2
maternal arterial ECs
Maternal cells
C
EphB4
uterus
vein
chorionic villus
CTB prog.
IVS
STB
D
ephrinB1
uterus
maternal art.
ICTBs
chorionic villus
E
EphB2
uterus
invasion
maternal art.
chorionic villus
Fetal cells
F
ephrinB2
ICTBs
uterus
maternal art.
G
maternal vein
maternal art
uterus (maternal)
iCTB
uterine stroma
invasion
placental attachment site
placenta (fetal)
EphB4
ephrinB2
ephrinB1
EphB2
chorionic villus
IVS: maternal blood flow
CTB prog.
H
CTB
1T
2T
Term
0 12 0 12 0 12 24 h
EphB4
ephrinB1
ephrinB2
EphB2

Figure 14

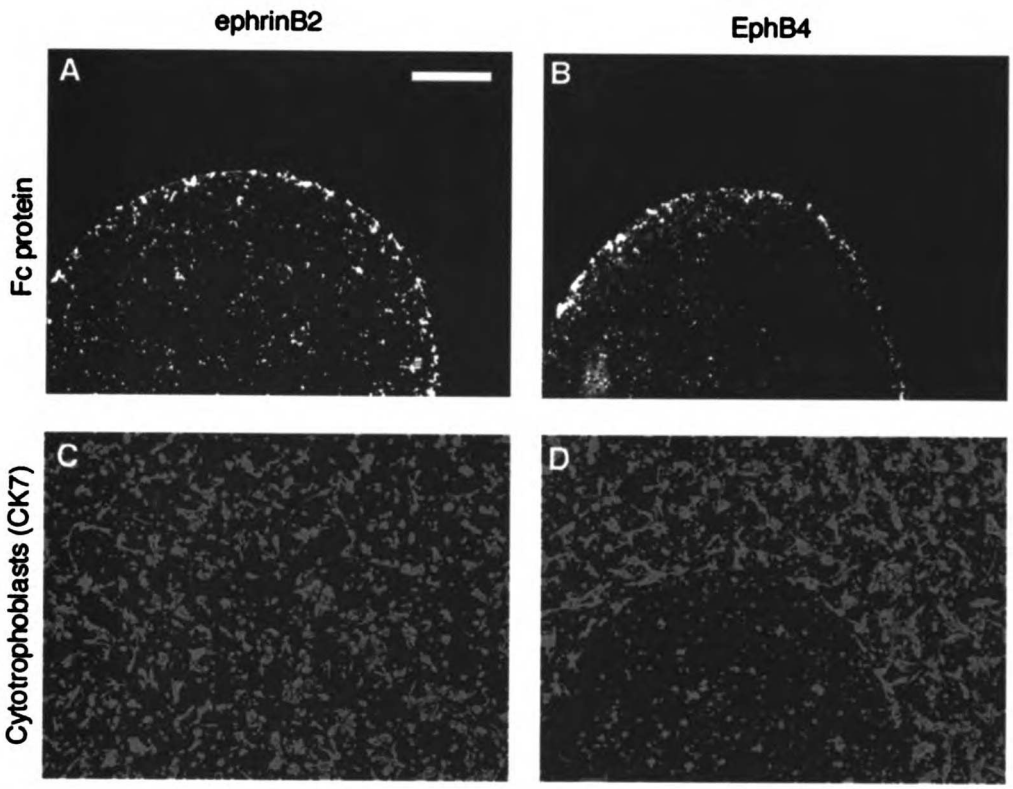


Figure 15

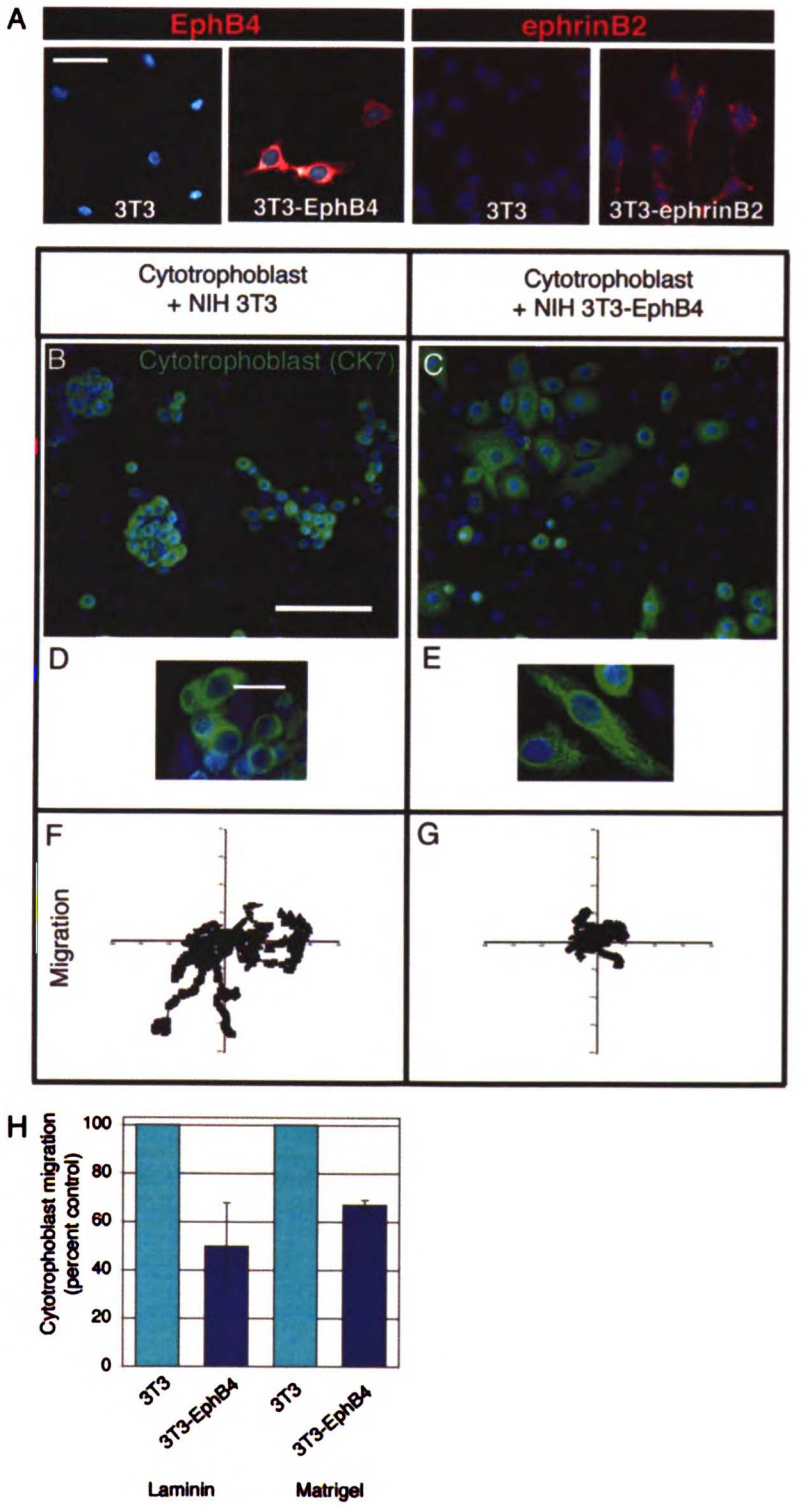

A
EphB4
ephrinB2
3T3
3T3-EphB4
3T3
3T3-ephrinB2
Cytotrophoblast
+ NIH 3T3
Cytotrophoblast
+ NIH 3T3-EphB4
B
Cytotrophoblast (CK7)
C
D
E
F
G
Migration
H
Cytotrophoblast migration
(percent control)
100
80
60
40
20
0
3T3
3T3-EphB4
3T3
3T3-EphB4
Laminin
Matrigel

Figure 16

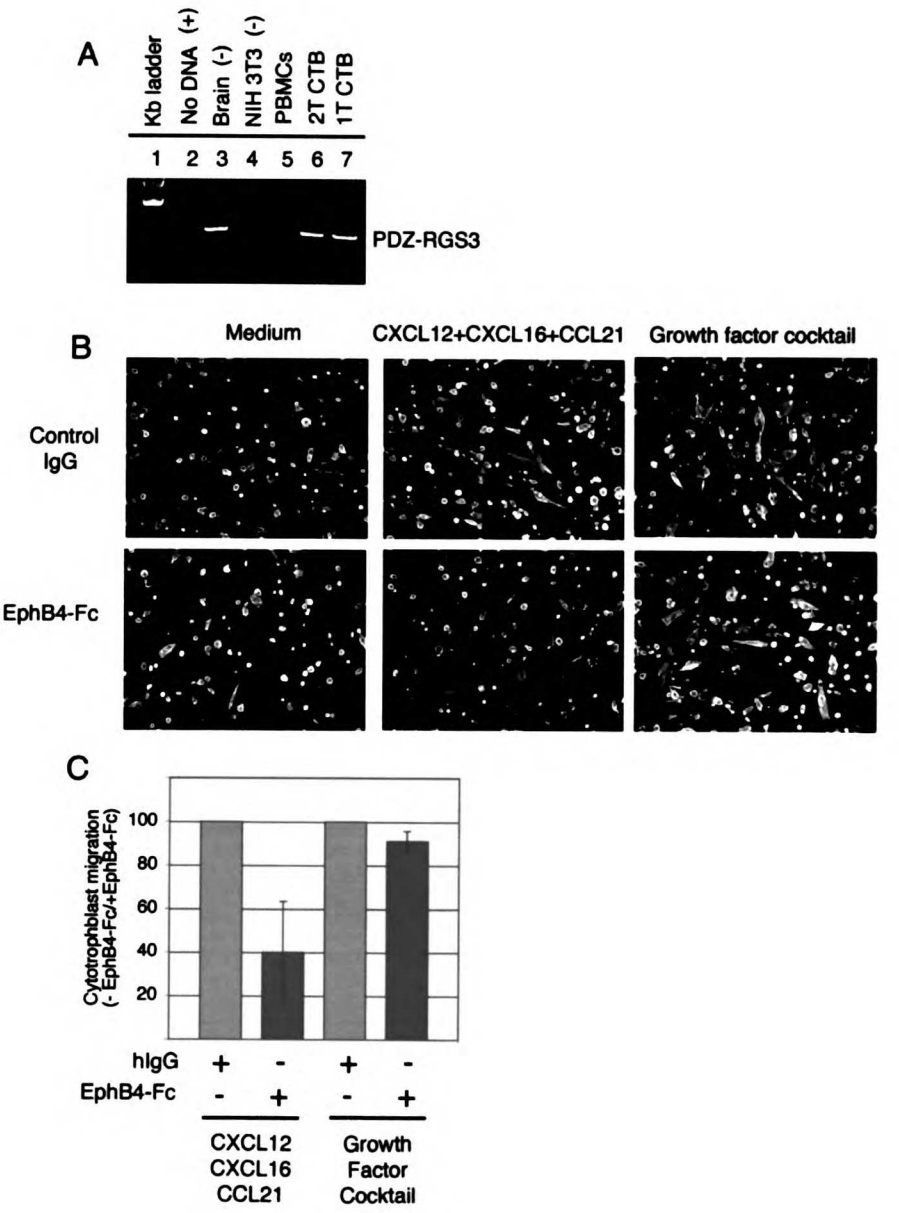

A
Kb ladder
No DNA (+)
Brain (-)
NIH 3T3 (-)
PBMCs
2T CTB
1T CTB
1 2 3 4 5 6 7
PDZ-RGS3
B
Medium
CXCL12+CXCL16+CCL21
Growth factor cocktail
Control IgG
EphB4-Fc
C
Cytotrophblast migration (- EphB4-Fc/+EphB4-Fc)
100
80
60
40
20
hIgG + - + -
EphB4-Fc - + - +
CXCL12
CXCL16
CCL21
Growth
Factor
Cocktail

Figure 17

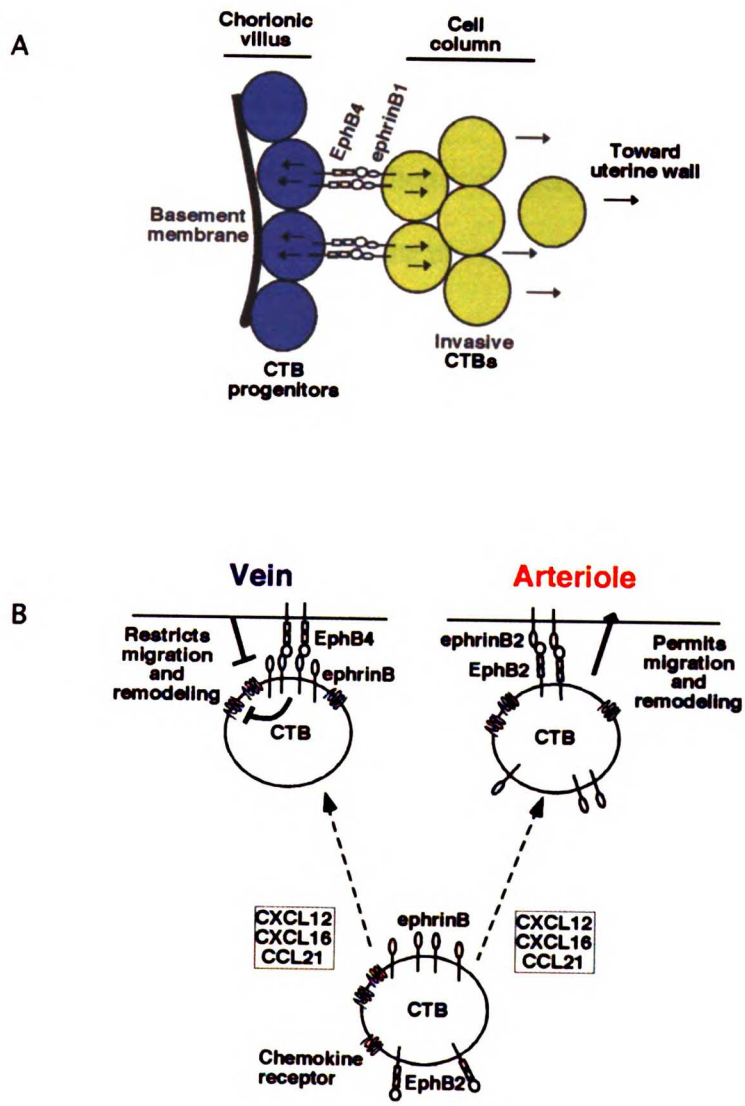

A
Chorionic villus
Cell column
EphB4
ephrinB1
Toward uterine wall
Basement membrane
Invasive CTBs
CTB progenitors
B
Vein
Arteriole
Restricts migration and remodeling
EphB4
ephrinB
CTB
ephrinB2
EphB2
Permits migration and remodeling
CTB
CXCL12
CXCL16
CCL21
ephrinB
CXCL12
CXCL16
CCL21
CTB
Chemokine receptor
EphB2

Figure legends

Figure 11. Placental cytotrophoblasts invade the uterine wall where they breach veins and extensively remodel maternal spiral arterioles. The bulk of the placenta is composed of numerous tree-like projections termed chorionic villi where maternal-fetal exchange occurs. These structures mediate the passage of nutrients, gases, and wastes between fetal blood, which circulates through the villous core, and maternal blood, which circulates through the intervillous space. The uteroplacental circulation is established by cytotrophoblasts that acquire an invasive/endothelial phenotype as they leave the placenta and enter the uterine wall. Differentiation begins when cytotrophoblast progenitors that reside in a single layer surrounding the stromal core of anchoring villi proliferate and form a cell column. These structures attach to the uterine wall and give rise to cells that invade the underlying decidual stroma. Invasive cytotrophoblasts breach uterine blood vessels connecting both the arterial and the venular circulation to the intervillous space. However, once this connection is made, remodeling of the venous side is halted. In contrast, cytotrophoblasts migrate up the lumina of spiral arterioles, eventually replacing the vessels' endothelial lining and a portion of the muscular wall. This process encompasses the decidual and inner third of the myometrial segments of these vessels. NK, natural killer; mϕ, macrophage.

Figure 12. Chemokines stimulate cytotrophoblast migration. (*A*) Cytokeratin 7 (CK7) (red) and CXCL16 (green) expression in the region of the maternal-fetal interface diagramed in Fig. 1. A CXCL16-specific antibody stained invasive and endovascular

cytotrophoblasts (CTBs), identified by their cytokeratin 7 expression. The nuclei of cells, which were stained with DAPI, appear blue. IVS, intervillous space; AV, anchoring villi. (*B*) Pertussis toxin (PTX) or a CXCR4-blocking antibody decreased cytotrophoblast migration required for aggregation *in vitro*. (*C*) In a migration assay, the addition of individual chemokines (CXCL12, CXCL16, and CCL21) stimulated cytotrophoblast migration in a dose-dependent manner, and the effects were additive. Error bars indicate standard deviation. (*D*) Checkerboard analysis in which the chemokines were added to either the upper, lower, or both chambers revealed that these molecules stimulated cytotrophoblast migration by increasing chemokinesis. Bars: *A*, *B* 100 mm.

Figure 13. Coincident with exit from the placental compartment, cytotrophoblasts switch from a venous to an arterial phenotype, as shown by their modulation of Eph and ephrin family members. (*A-F*) *In situ* hybridization; adjacent sections were stained with cytokeratin 7 (CK7), which identified trophoblasts. Venular and arterial endothelial cells (ECs) that lined uterine vessels expressed EphB4 (*A*) and ephrinB2 (*B*). (*C*) Cytotrophoblast progenitors (CTB prog.) and syncytiotrophoblasts (STB) within the placenta expressed EphB4. Commitment to the differentiation pathway that gives rise to invasive cytotrophoblasts (iCTBs) is associated with an abrupt downregulation of EphB4 and a concomitant upregulation of ephrinB1 (*D*) and EphB2 (*E*) mRNAs. Subsequently, within the uterine stroma, ephrinB2 expression is induced (*F*). The endovascular subpopulation of invasive cytotrophoblasts that line maternal arteries also expressed high levels of these molecules (*D-F*). (*G*) Schematic diagram of the human maternal-fetal interface highlighting important aspects of cytotrophoblast interactions with uterine

vessels. The pattern of cytotrophoblast Eph and ephrin expression is shown in the colors indicated. (*H*) Cytotrophoblasts modulated Eph and ephrin expression during differentiation along the invasive pathway *in vitro*. Immediately after isolation from placental chorionic villi, the progenitors expressed EphB4, but upregulated ephrinB1, -B2 and EphB2 after 12 h in culture. IVS, intervillous space; T, trimester. Bars: *A, B, F,* 50 mm; *C, D, E,* 100 mm.

Figure 14. Cytotrophoblasts avoid substrates containing EphB4, but not ephrinB2. Laminin with either ephrinB2-Fc (*A*) or EphB4-Fc (*B*) and fluorescent anti-human Fc was spotted on the plate before the entire surface was covered with laminin alone. (*C* and *D*) Cytokeratin 7 staining showed cytotrophoblast distribution on the tissue culture substrate. Initially, cytotrophoblasts were plated as a monolayer. (*C*) By 12 h, the cells continued to associate with ephrinB2-Fc substrates, but (*D*) few cells were attached to EphB4-Fc spots. Bar: 200 mm.

Figure 15. Cytotrophoblast migration is reduced by co-culture with 3T3 cells that express the venous receptor EphB4. (*A*) Staining of stably transfected 3T3 cell lines with antibodies specific for EphB4 and ephrinB2 showed that they expressed the respective proteins. (*B, D*) Our previous work (Librach et al., 1991) showed that isolated cytotrophoblasts cultured on laminin or Matrigel substrates rapidly migrate toward one another, forming numerous aggregates. Similar behavior was observed when the cytokeratin-positive (CK7) cytotrophoblasts (green) were co-cultured with control 3T3 cells. (*C, E*) In contrast, when cytotrophoblasts were cultured with 3T3 cells expressing

EphB4 (3T3-EphB4), aggregate formation was markedly inhibited, and the cells appeared to spread on the substrate. Tracking cell movement for 15 h showed that, in comparison to controls (*F*), cytotrophoblasts co-cultured with 3T3-EphB4 cells migrated much shorter distances (*G*). (*H*) Quantifying the average linear distance traveled showed that migration was significantly reduced when the cytotrophoblasts were co-cultured with 3T3-EphB4 cells on either laminin or Matrigel substrates. Error bars indicate standard deviation. Scale bars: *A*, 20 mm; *B*, 50 mm; *D*, 10 mm.

Figure 16. EphB4 decreases chemokine-stimulated cytotrophoblast migration. (*A*) RT-PCR detected expression of PDZ-RGS3 in RNA samples isolated from first trimester (1T) and second trimester (2T) cytotrophoblasts (CTB). PBMCs, peripheral blood mononuclear cells. +, positive control; -, negative control. (*B*) Cytokeratin 7 staining of cytotrophoblasts that had migrated to the underside of the transwell filter after overnight culture. EphB4 decreased cytotrophoblast migration toward chemokines while baseline and growth factor-stimulated migration was not significantly affected. (*C*) Quantification of migration assays showed that EphB4 downregulated chemokine-induced migration by an average of 60%. Error bars indicate standard deviation.

Figure 17. Model describing the role of Ephs and ephrins in patterning chemokine-induced cytotrophoblast invasion. (*A*) A rapid switch in Eph and ephrin expression generates repulsive signals that orient cytotrophoblast (CTB) invasion away from the placenta and toward the uterine wall. (*B*) Within the uterine stroma, invasive cytotrophoblasts distinguish veins from arterioles based on their expression of EphB4 and

ephrinB2, respectively. Our data suggest that cytotrophoblast interactions with EphB4-expressing cells inhibit their chemokine-induced migration, one mechanism that could restrict their remodeling of veins. In contrast, interactions with ephrinB2-expressing cells permit migration. As a result, cytotrophoblast remodeling of the uterine vasculature is biased toward the arterial side of the circulation.

CHAPTER FOUR: A new *in vivo* model of human placental development reveals cytotrophoblast-mediated arterial apoptosis and lymphangiogenesis

Introduction

During human pregnancy, placental cytotrophoblasts of fetal origin invade the uterine wall. This process has two components. In the first, cytotrophoblasts invade the uterine parenchyma where they interact with the stromal compartment and a resident immune population that includes primarily NK cells with some dendritic cells, macrophages and T lymphocytes. In the second, a subpopulation of cytotrophoblasts invades uterine blood vessels with subsequent colonization of the arterial side of the circulation. Although some information is known about the molecular basis of the latter process, a great deal remains to be learned because, up to now, there has been no *in vivo* model of the vascular component of cytotrophoblast invasion. For example, the mechanism whereby cytotrophoblasts replace the maternal endothelial lining of uterine arterioles and intercalate within the surrounding smooth muscle layer is unknown (Hamilton and Boyd, 1960). In addition, the possibility that cytotrophoblasts interact with resident lymphatic vessels has yet to be addressed.

In humans, trophoblast remodeling of arterioles is more extensive than in many other species, including mice. With regard to the mechanisms involved, these placental cells undergo an ectodermal to vascular transformation that involves a dramatic switch in their repertoire of cell adhesion molecules (Damsky and Fisher, 1998). In previous work, we established that the distinct patterning of vascular invasion is attributable to a switch

from a venous to an arterial phenotype in terms of the cells' expression of Eph and ephrin family members that control vessel identity (Red-Horse et al., 2005). We also showed that human trophoblasts express a broad range of factors that regulate conventional vasculogenesis and angiogenesis, including vascular endothelial growth factor (VEGF)-C and its receptors (VEGFR1 and -3) and angiopoietin-2 (Ang-2) (Zhou et al., 2003; Zhou et al., 2002). The latter findings were unexpected since these molecules are largely involved in lymphatic development within embryonic and adult tissues (Gale et al., 2002; Karkkainen et al., 2004; Makinen et al., 2001). Gene deletion studies in mice showed that VEGF-C/VEGFR3 and Ang-2 are required for these processes and ectopic expression of the two ligands elicits lymphohyperplasia (Jeltsch et al., 1997; Morisada et al., 2005).

Relatively little is known about uterine lymphatics in either the non-pregnant or the pregnant state. Given its importance in other organs and tissues, this circulation could play a crucial role in establishing and maintaining pregnancy. For example, the lymphatic system returns excess interstitial fluid to the bloodstream and organizes adaptive immune responses by providing a vascular-type network for trafficking of lymphoid cells for surveillance purposes (Oliver, 2004). As a result, individuals with lymphatic defects are highly susceptible to debilitating lymphedema and chronic unresolved infections (Karkkainen et al., 2000; Rockson, 2001). Given these critical functions, lymphatic vessels, which are present in most tissues, are particularly abundant at sites that come in contact with the external environment where microbial pathogens reside.

Thus, it is surprising that the endometrium, the mucosal surface of the uterus, is thought to lack lymphatic vessels, which are instead restricted to the deeper myometrial and serosal segments of this organ. This arrangement, which has been observed in mice (Saban et al., 2004), rats (Head and Lande, 1983), rabbits (Otsuki et al., 1990), and humans (Koukourakis et al., 2005), is thought to isolate the endometrium from the lymphatic system. In support of this supposition are data showing that dyes and cells are readily taken up from the myometrial region and transported to local lymph nodes, whereas introduction of the same reagents into the lumen or endometrium results in uterine localization (Head and Lande, 1983; Maroni and de Sousa, 1973). Thus far, the reasons for excluding lymphatics from the endometrium remain obscure but could include the complexities of organizing, on a monthly basis, both angiogenesis and lymphangiogenesis. In contrast to the non-pregnant state, it is unclear whether lymphatic vessels are present in the gravid endometrium, termed decidua. However, anatomists have noted hyperplasia of the lymphatic vessels of the pelvic region during pregnancy (Gray, 2000), suggesting that lymphangiogenesis could be part of the decidualization process.

To address mechanisms of trophoblast-mediated arterial remodeling and, possibly, lymphangiogenesis, we devised a novel *in vivo* model to study both processes—implantation of chorionic villi into the mammary fat pads or under the kidney capsules of SCID mice. The results showed histological evidence of robust cytotrophoblast invasion of vessels in both regions. Further analysis showed that cytotrophoblasts induced apoptosis only in the endothelial and smooth muscle layers of arteries, revealing additional insight into the mechanisms that allow the cells to colonize

these vessels rather than veins. With regard to lymphangiogenesis, we show that the human decidua contains numerous lymphatic vessels. A similar response was not observed in the decidua of pregnant mice. *In vivo*, cytotrophoblast invasion of the kidney capsule was accompanied by a robust infiltration of murine lymphatic endothelial cells and subsequent vessel formation. Thus, placental cells in the uterus during pregnancy induce both arteriole-specific apoptosis of the resident maternal cells and a dramatic lymphangiogenic response. Each of these processes is likely to play a crucial role in the formation of the maternal-fetal interface.

Results

An in vivo *model of cytotrophoblast invasion shows that placental cells induce arterial apoptosis*

To assess the relationship between invasive cytotrophoblasts and vascular cells *in vivo*, we implanted human placental villi under the kidney capsules or into the 5th mammary fat pads of SCID mice. Both locations supported cytotrophoblast invasion. However, we primarily used the mammary fat pads as the implantation site to analyze vascular invasion because of their larger vessel size, which allowed us to identify arteries and veins using morphological criteria. In this location, cytokeratin-positive cytotrophoblasts extensively invaded the stroma and blood vessels (Figure 18A, D). There, they interacted with both arteries and veins, as demonstrated by an antibody that specifically recognizes murine PECAM (CD31) expressed by endothelial cells. Arteries

were identified by their more extensive smooth muscle actin distribution (Figure 18B). By 3 weeks, the cells had traveled up to 250 μm from the site where the villi were implanted.

Further analysis showed that the integrity of several arteries was disrupted, as demonstrated by extravascular platelet deposition visualized by staining with an antibody that recognizes CD41 (Figure 18E). TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling) staining showed that the cytotrophoblasts induced apoptosis of arterial smooth muscle and endothelial cells (Figure 18C, F). In contrast, stromal cells and veins lacked TUNEL reactivity (Figure 18C, F). Finally, no apoptosis among invasive cytotrophoblasts was detected (Figure 18C, F), an impression that was confirmed by co-staining additional serial sections with cytokeratin and TUNEL reagents (data not shown). In summary, these experiments established a novel *in vivo* model of cytotrophoblast invasion and vascular remodeling during which interactions between placental cells and murine arteries are easily differentiated from those with veins.

Human pregnancy induces endometrial lymphangiogenesis

Immunostaining tissue sections of the non-pregnant human endometrium (Figure 19A) and decidua (Figure 19B-E) with the lymphatic endothelial specific marker, LYVE-1, revealed that lymphatics were normally only present in the pregnant uterus (Table 1). Not all decidual biopsy samples included LYVE-1-positive vessels; nevertheless, they were abundant in all the regions of this tissue (diagrammed in Figure 19G) (Table 1).

Lymphatic vessels were prominent in the regions of the uterus that were invaded by fetal cytotrophoblasts, including the decidua basalis (Figure 19C,D), where the placenta attaches, and the decidua capsularis, adjacent to the extraembryonic membranes (Figure 19E). Although they are routinely in close proximity, we failed to observe cytotrophoblast invasion of decidual lymphatic vessels (Figure 19D). In regard to distribution, the decidual capsularis contained the highest vessel density, but there was no correlation between this parameter and gestational age (Table 1). LYVE-1 also labeled cells of the monocyte/macrophage lineage, including Hofbauer cells, within the placenta (Figure 19C). In contrast to human decidual samples, LYVE-1-positive vessels were restricted to the myometrial layer of the pregnant murine uterus (Figure 19F).

Because LYVE-1 is expressed on venous sinusoids in some tissues (Banerji et al., 1999; Prevo et al., 2001), we examined the expression of additional markers specific for lymphatic endothelium (CCL21 and integrin $\alpha 9\beta 1$) to confirm the identity of these vessels. *In situ* hybridization revealed CCL21 mRNA expression (Figure 20A). Immunostaining adjacent sections with LYVE-1 established that this molecule and CCL21 co-localized (Figure 20A); the same distribution was observed for integrin $\alpha 9\beta 1$ (Figure 20B). In addition, we investigated the localization of ephrinB2, which is expressed in murine dermal lymphatic vessels (Makinen et al., 2005). A pan-ephrin antibody failed to label decidual lymphatic vessels. Instead, staining was localized to cytotrophoblasts within the uterine wall (Figure 20C), the expected result based on our previous work describing the cells' extensive repertoire of these molecules (Red-Horse et al., 2005). Taken together, these data established that pregnancy is associated with a

robust growth of lymphatic vessels into the uterine lining and that invading cytotrophoblasts do not breach these structures.

Cytotrophoblasts stimulate lymphatic endothelial cell migration in vitro

Given our previous work showing that invasive cytotrophoblasts produce lymphangiogenic molecules (Zhou et al., 2002), we tested the hypothesis that placental cells trigger the new appearance of uterine lymphatic vessels during pregnancy. We tested the ability of cytotrophoblast-conditioned medium to stimulate lymphatic endothelial cell migration *in vitro*. Cytotrophoblast-conditioned medium from first- or second-trimester cells dramatically increased the migration of purified LYVE-1-positive human lymphatic endothelial cells (Figure 21). In this regard, the highest activity, similar to that of a growth factor cocktail, was associated with the first-trimester samples.

An in vivo *model of cytotrophoblast invasion shows that placental cells induce lymphangiogenesis*

Initially, we explored the utility of the mammary fat pad implant model for studying cytotrophoblast-induced lymphangiogenesis. However, the large number of macrophages that expressed LYVE-1 made interpretation of the results difficult. Therefore, we explored the possibility of transplanting chorionic villi under the kidney capsules of SCID mice as an alternative.

Cytotrophoblast invasion in the kidney was even more robust than that in the mammary fat pads, extending up to 500 μm at 1 week post-implantation (Figure 22A). After 3 weeks, the invasion front reached 2 mm, with a few cell clusters migrating to even deeper regions (Figure 22B); this phenomenon is also observed during the early stages of human placentation. Reminiscent of cytotrophoblast interactions with decidual cells, zones that were occupied by trophoblasts also contained intact kidney tubules that were not undergoing apoptosis, as demonstrated by the lack of TUNEL staining (data not shown). As early as the 1-week time point, staining for CD31 expression revealed the new appearance of murine vascular networks that were detected only in areas of cytotrophoblast invasion (Figure 22C). As to the route, cytotrophoblast migration was restricted to the peritubular spaces (Figure 22D, E). Double staining with CD31 and cytokeratin revealed that the cells were closely associated with blood vessels that travel in these areas (Figure 22F). Staining with CD41 showed that the cells also breached these vessels, as demonstrated by platelet deposition that occurred only in areas of cytotrophoblast invasion (Figure 22G). In control kidney tissue, CD41 localization was restricted to cells within blood vessels, presumably platelets (data not shown). This pattern is similar to cytotrophoblast invasion of the uterine wall *in situ* in which the cells breach decidual blood vessels and activate platelet adhesion (Sato et al., 2005). The cytotrophoblasts displayed a molecular phenotype, in terms of stage-specific antigens, that was the same to the equivalent population within the uterine wall *in situ*. Specifically, they stained with antibodies specific for HLA-G (Figure 22H), the cells' distinctive MHC molecule, and human placental lactogen (data not shown). We also noted differences in nuclear size of the cytotrophoblast progenitors that were associated

with the original villous implants and the cells that had invaded the kidney tissue. Specifically, the nuclear diameter in the invading cells increased substantially (Figure 22I and insets), a phenotype associated with the hyperdiploid state of invasive cytotrophoblasts (Weier et al., 2005).

To test the hypothesis that trophoblast-derived lymphangiogenic molecules induce the development of a decidual lymphatic circulation, we used the kidney capsule implant model described above. In initial experiments, we stained sections of the implant sites with LYVE-1. At 1 week post-implantation, a few LYVE-1-positive structures were detected in association with vessels located beyond the front of cytotrophoblast invasion (Figure 23A and insets), a pattern that was similar to that observed in control tissues (data not shown). LYVE-1 also stained CD45-positive macrophages within the kidney capsule (Figure 23A-F). In contrast, after 2 weeks, cells that stained for LYVE-1 began to infiltrate the implants (Figure 23B), a phenomenon that further increased at the 3-week interval (Figure 23C). The possibility that these cells were macrophages was excluded by staining for CD45, which was negative (Figure 23E, F). In addition, the cells were murine in origin, since the antibody we used did not react with human LYVE-1 in other tissues (data not shown). Infiltrating lymphatic endothelial cells were closely associated with cytotrophoblasts and contained many extended processes (Figure 24A). By 3 weeks, some LYVE-1-positive structures had the morphological appearance of lymphatic vessels, including the presence of lumens (Figure 24B).

Discussion

Here, we report the development of a novel *in vivo* model of human cytotrophoblast-mediated vascular remodeling and offer evidence of its utility in studying this process, which is difficult, if not impossible, to model *in vitro*. First, using this technique, we showed that placental villi that were implanted into the mammary fat pad gave rise to invasive cytotrophoblasts that specifically induced apoptosis in arterial smooth muscle and endothelial cells. Within the uterine wall, cytotrophoblasts remodel maternal spiral arterioles by replacing their endothelial layers and intercalating within the surrounding tunica media. The data we present give additional insights into the molecular mechanisms underlying this enigmatic process. Specifically, our findings suggest that apoptosis of the resident arterial cells is a key element of vascular remodeling of uterine spiral arterioles. Implicit in this finding is the concept that placental cells play a key role in triggering this process, which, in ectopic pregnancy, can involve vessels outside the uterus. Previously, *in vitro* assays suggested that the Fas/Fas ligand system functions during vascular remodeling (Ashton et al., 2005). Interrupting receptor-ligand interactions decreased endothelial apoptosis in explanted vessels infused with isolated cytotrophoblasts. It would be interesting to investigate whether the expression patterns of molecules in this family are consistent with this hypothesis and whether these molecules function during *in vivo* remodeling of the murine vasculature, experiments made possible by our new model.

In additional work we showed that the human non-pregnant endometrium lacks lymphatic vessels and that pregnancy induces lymphangiogenesis in the decidual portions of the uterus. In parallel analyses we failed to detect lymphatic vessels in pregnant murine endometrium. The reasons for this difference are obscure, but could lie in the

structural and functional variations between the human and mouse placenta and decidua. In addition, the fact that murine pregnancy occurs over a 20-day period rather than the 9 months of gestation that is typical in humans may also be responsible for species-specific differences in pregnancy-associated lymphangiogenesis.

What are the functional implications of the lack of a uterine lymphatic circulation in the non-pregnant state and the rapid assembly of these vessels in the decidua? With regard to the non-pregnant state, exclusion of lymphatic vessels from the endometrium could protect a woman from monthly exposure of her immune system to the menses, which includes apoptotic cells and their contents, a possible source of autoantigens. At the same time, this arrangement could prevent production of antibodies against sperm, an important risk factor associated with infertility (Naz and Menge, 1994).

The development of a decidual lymphatic system during pregnancy also implies possible functional relevance to pregnancy outcome. For example, maintaining fluid balance in this region is of critical importance. During pregnancy, placenta-induced vascular changes vastly increase uterine blood flow, which perfuses the placenta. This process is likely accompanied by increased vascular leakage, made even more probable given the relining of these vessels by cytotrophoblasts, which based on scanning electron microscopic analyses of remodeled arterioles is incomplete in some regions (K. Red-Horse and S. J. Fisher, manuscript in preparation). In the fetal compartment, amniotic fluid levels must be tightly regulated, because changes in normal volumes increase perinatal morbidity and mortality (Barkin et al., 1987; Barss et al., 1984). Excess amniotic fluid is removed by fetal swallowing and absorption through extraembryonic membranes where it is taken up by fetal blood and maternal tissues (Gilbert et al., 1997).

In this context, it is interesting to note that, within the decidua, the region adjacent to the chorionic and allantoic membranes had the greatest density of lymphatic vessels.

Lymphangiogenesis in the pregnant uterus may also have implications for maternal-fetal immunity. During this time, the mother must balance protection of the fetus with tolerance of its hemiallogeneic tissues. Enhanced surveillance of the maternal-fetal interface could help prevent infections, which are associated with preterm labor (Lamont, 2003). Additionally, these results suggest possible mechanisms for establishing maternal tolerance. We failed to find evidence that placental cells enter the lymphatic circulation; however, dendritic cells, also numerous at the maternal-fetal interface, can traffic to regional lymph nodes for the purpose of presenting fetal antigens. These pathways could include the production of factors that dampen impending anti-fetal responses and/or the genesis of a regulatory population of immune cells.

In addition to the mammary fat pad, we also established an *in vivo* model that involves implantation of placental tissue under the kidney capsule. These implants gave rise to invasive cytotrophoblasts that breached murine blood vessels. Using this assay, we were able to provide evidence that the placenta plays a governing role in establishing the uterine lymphatic circulation during pregnancy. Specifically, these implants induced the infiltration of LYVE-1-positive lymphatic endothelial cells that formed vessels. Experiments in progress suggest that decidual explants lack this activity, additional evidence that this is a cytotrophoblast-driven phemonenon. Furthermore, cytotrophoblast-conditioned medium stimulated lymphatic endothelial cell migration (Podgrabinska et al., 2002) *in vitro*. The mechanism of vessel formation in the kidney may share some components with a similar process that occurs in the cornea, where there

is an initial infiltration of cells that form vascular structures *de novo* rather than sprouting from preformed vessels (Maruyama et al., 2005). However, there are also differences, as the founder cells in the corneal model were macrophages. An as yet unexplored possibility is that the placenta may enhance its own ability to induce uterine lymphangiogenesis by attracting decidual leukocytes (Drake et al., 2001), which have been reported to express VEGF-C (Li et al., 2001). Immune cell production of VEGF-C and -D is critical for lymphangiogenesis in the lung during *Mycoplasma pulmonis* infection (Baluk et al., 2005). Following corneal injury, macrophages are required to induce associated lymphangiogenesis (Cursiefen et al., 2004) and may differentiate into lymphatic endothelial cells participating in vessel formation (Maruyama et al., 2005). The fact that the vessel-associated LYVE-1-positive cells that were recruited to sites of cytotrophoblast invasion in our *in vivo* model were CD45 negative suggests that the latter phenomenon is not a major component of the mechanism whereby lymphatic vessels are formed in our *in vivo* murine model.

In summary, we established two *in vivo* assays that recapitulate human cytotrophoblast invasion and vascular remodeling by implanting placental villi into either the mammary fat pads or beneath the kidney capsules of SCID mice. In the mammary fat pad, arterial and venular vessels are easily identified by morphological criteria, making this location amenable to studies aimed at evaluating cytotrophoblast interactions with both sides of the circulation. In comparison, cytotrophoblasts implanted under the kidney capsule invaded much further into murine tissues, suggesting that this site may provide the extracellular matrix cues that better support trophoblast migration. These new *in vivo*

models will be a valuable adjunct to our *in vitro* systems for analyzing the crucial process by which the maternal-fetal interface forms during human pregnancy.

Table 1. **Lymphatic vessels in the uterus**

	Donors (no.)	Tissue sections examined (no.)	LYVE-1-positive vessels: Average vessel number/section	Number of sections with no vessels	Number of sections with <10 vessels	Number of sections with ≥10 vessels
Non-pregnant						
Endometrium						
proliferative phase	6	6	0	6	0	0
secretory phase	11	11	0	10	0	1[a]
Pregnant						
1st trimester decidua	10	22	4	8	8	6
2nd trimester						
decidua basalis	12	16	5.4	4	8	4
decidua capsularis	5	5	>25	1	1	3
3rd trimester						
decidua basalis	13	13	3.25	2	9	2
decidua capsularis	7	7	>30	1	0	6
Murine decidua	5	5	0	5	0	0

[a] sample contained a large number of CD45-positive cells indicating possible inflammation

Figure 18

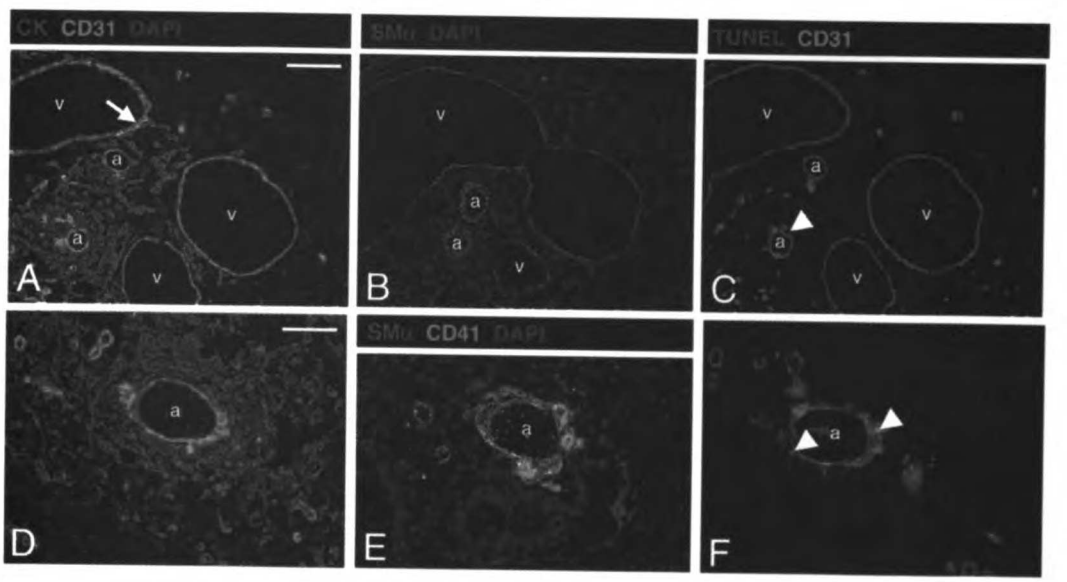


Figure 19

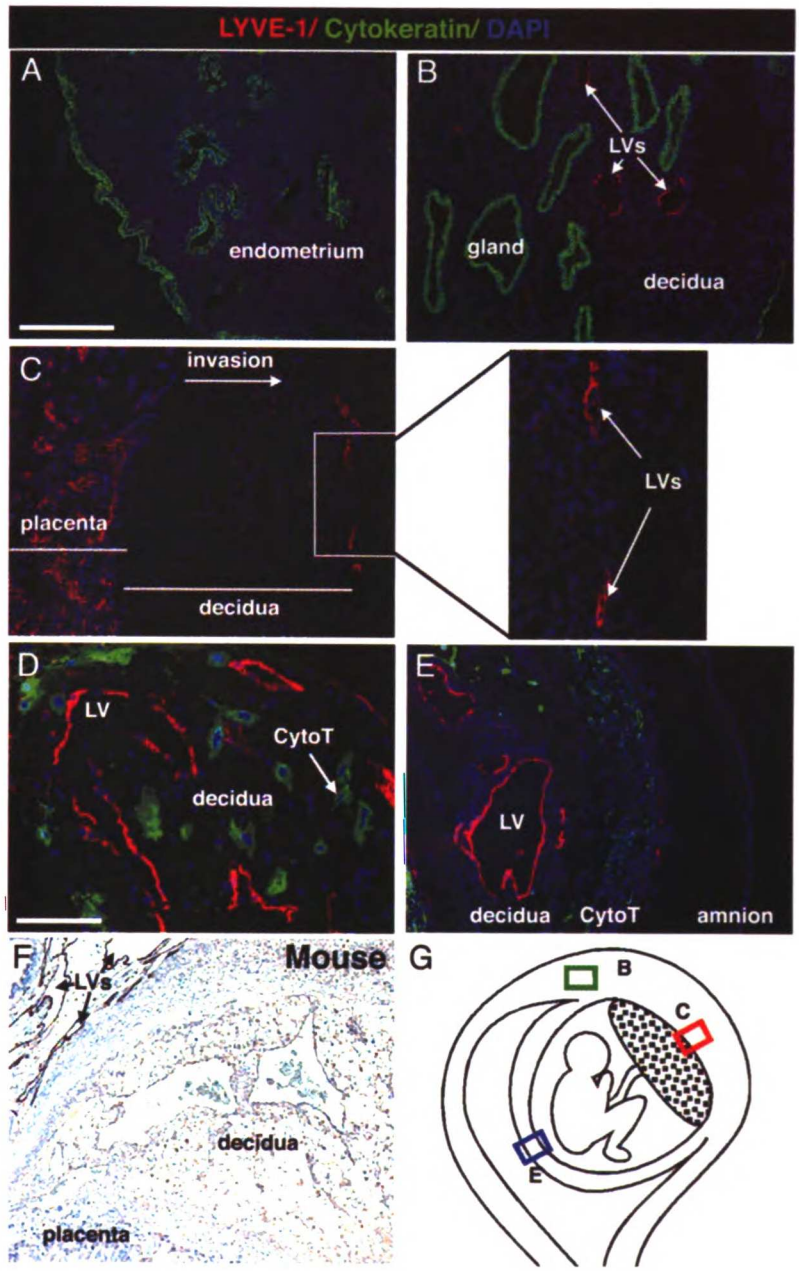

LYVE-1/ Cytokeratin/ DAPI
A
endometrium
B
LVs
gland
decidua
C
invasion
placenta
decidua
LVs
D
LV
CytoT
decidua
E
LV
decidua
CytoT
amnion
F
Mouse
LVs
decidua
placenta
G
B
C
E

Figure 20

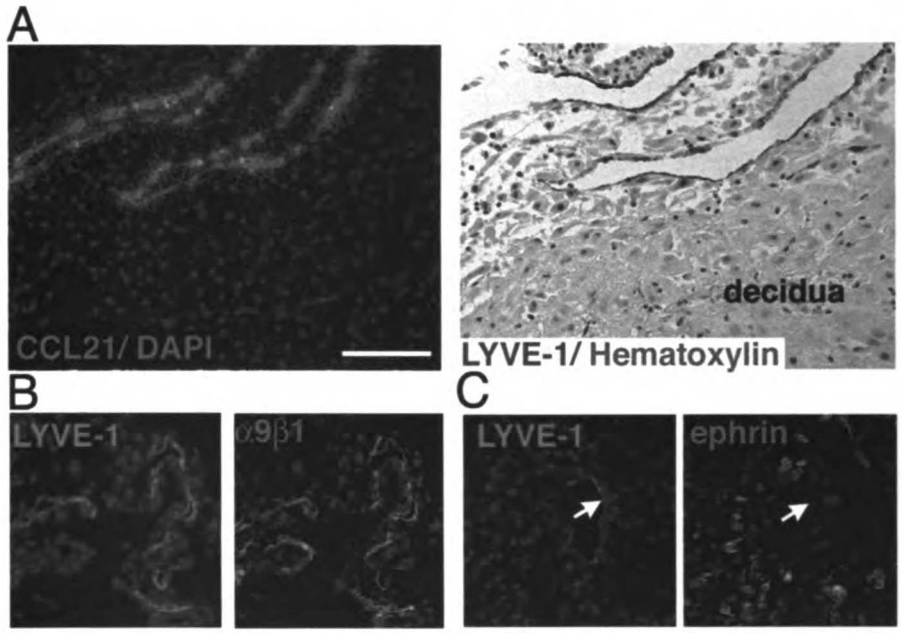

A
CCL21/ DAPI
decidua
LYVE-1/ Hematoxylin
B
LYVE-1
α9β1
C
LYVE-1
ephrin

Figure 21

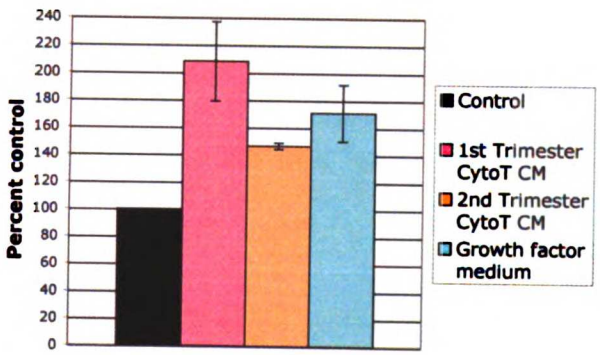

Percent control
240
220
200
180
160
140
120
100
80
60
40
20
0
Control
1st Trimester CytoT CM
2nd Trimester CytoT CM
Growth factor medium

Figure 22

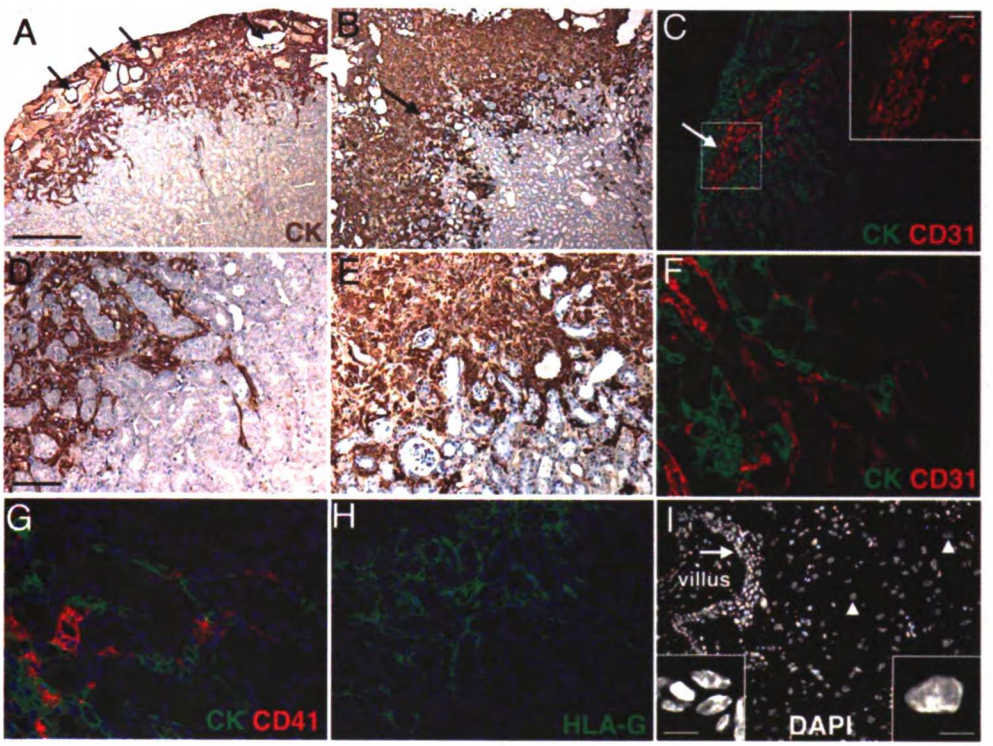


Figure 23

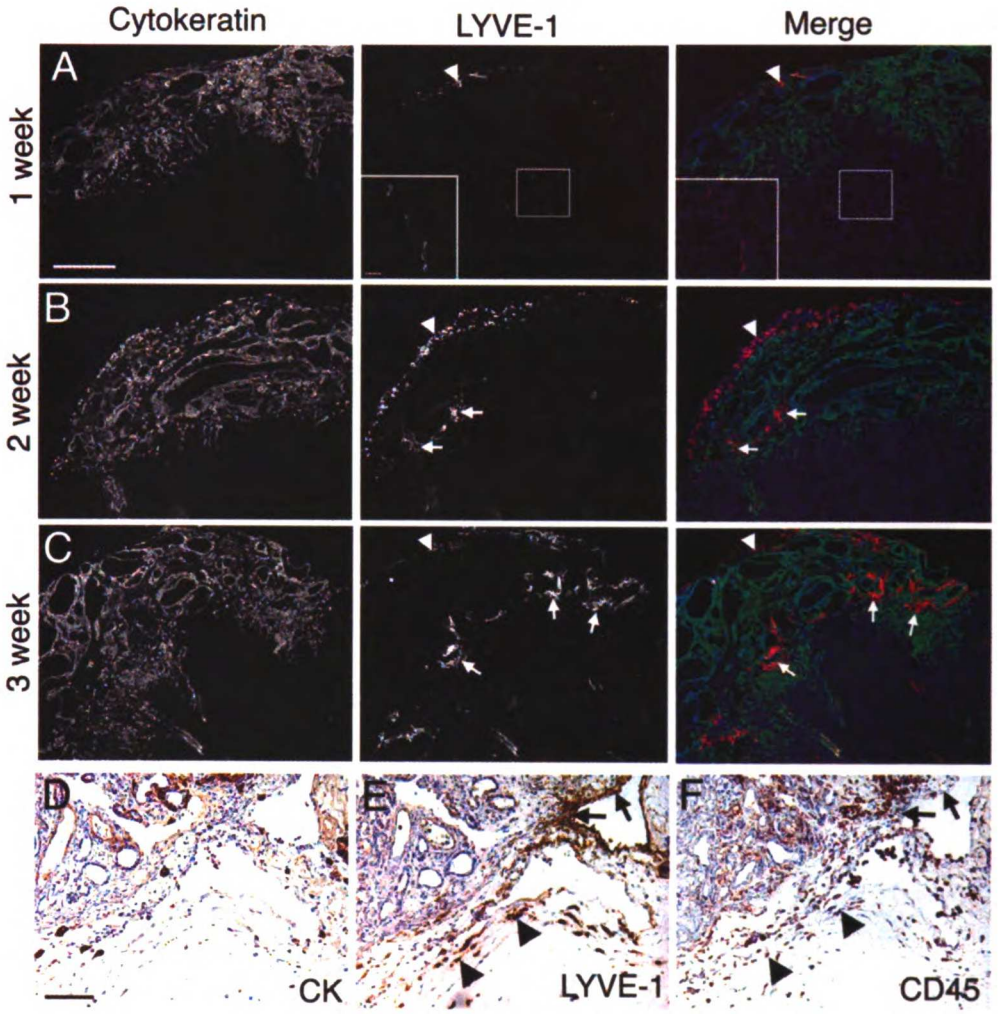

Cytokeratin
LYVE-1
Merge
A
1 week
B
2 week
C
3 week
D
CK
E
LYVE-1
F
CD45

Figure 24

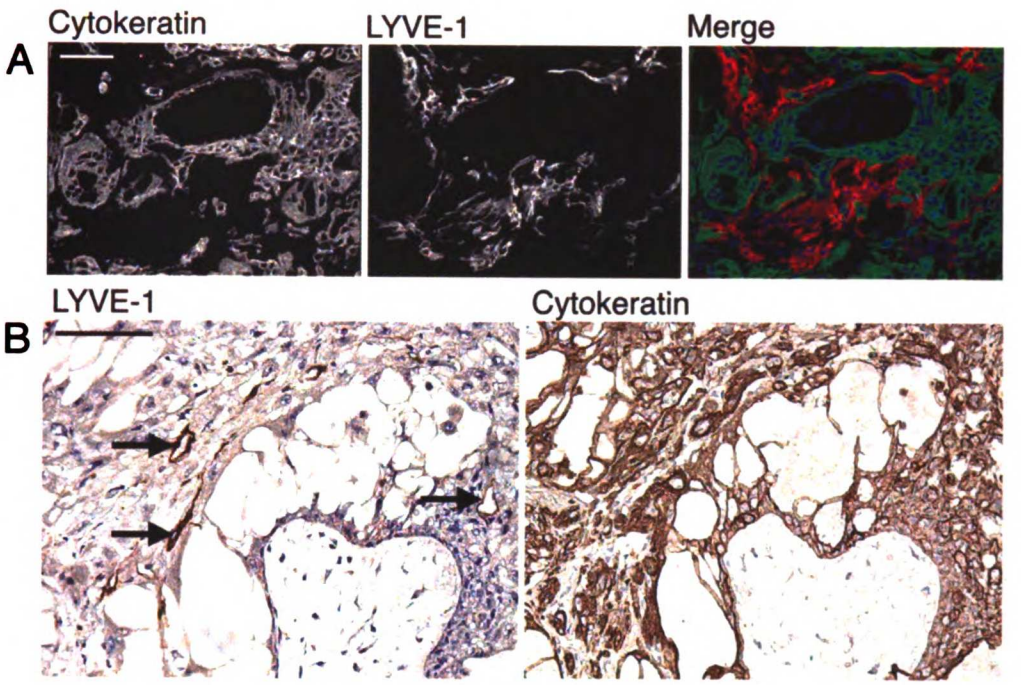


Figure Legends

Figure 18. Cytotrophoblasts induce arterial apoptosis during vascular remodeling *in vivo*. Human placental villi were implanted into the mammary fat pads of SCID mice, which were maintained for 3 weeks before implants were excised by dissection and prepared for histological examination. (A, D) Cytokeratin 7-positive cytotrophoblasts (green) invade murine stromal tissue, where they interact with arteries (a) and veins (v) (CD31, endothelial cells, red). The arrow denotes a point (yellow) where the cells appear to intercalate within the wall of the vein. The nuclei of cells, which were stained with DAPI, appear blue. (B) Smooth muscle actin distribution (green) in adjacent sections identifies arteries, which have a more well developed tunica media than veins. (E) Cytotrophoblast invasion disrupts arterial integrity and results in platelet deposition, which was visualized by staining with an antibody that recognizes CD41 (red). The distribution of smooth muscle actin is shown in green. (C, F) TUNEL labeling (green, arrowheads) reveals that cytotrophoblasts induced apoptosis of arterial smooth muscle and endothelial cells, but veins were not affected. Scale bars: A-C, 50 mm; D-F, 40 mm.

Figure 19. Endometrial lymphangiogenesis occurs during human pregnancy. (A-E) Immunostaining tissue sections of the non-pregnant endometrium (A) and the pregnant endometrium/decidua (B-E, diagrammed in G) with the lymphatic endothelial-specific marker LYVE-1 (red) revealed that these vessels (LV) were present only in the pregnant uterus. Nuclei were stained with DAPI and appear blue. Glands (A, B) and

cytotrophoblasts (CytoT) (D, E) were labeled with an antibody that reacts with cytokeratin (green). (C) LYVE-1 also labeled cells of the monocyte/macrophage lineage, including Hofbauer cells, within the placenta. (F) LYVE-1-positive vessels (brown, arrows) were excluded from the decidua during murine pregnancy. (G) Diagram showing the decidual regions analyzed in the coordinately labeled panels. B, decidua parietalis; C, decidua basalis; E, decidua capsularis. Scale bars: A, B, C, E, F, 200 mm; panel D and inset C, 50 mm.

Figure 20. LYVE-1-positive vessels in the decidua express other markers of lymphatic endothelium. (A) Adjacent sections were analyzed. (left panel) In situ hybridization with a CCL21-specific probe (green) showed that (right panel) LYVE-1-positive vessels (brown) in the decidua express this chemokine. (B) Immunostaining of adjacent sections reveals the co-localization of LYVE-1 (red) and integrin a9b1 (green). (C) In contrast, a pan-ephrin antibody (green) failed to label decidual lymphatic vessels (red, arrows), but stained invasive cytotrophoblasts (Red-Horse et al., 2005). Scale bar: A-C, 50 mm.

Figure 21. Cytotrophoblasts stimulate lymphatic endothelial cell migration *in vitro*. The addition of cytotrophoblast (CytoT)-conditioned medium from either first- or second-trimester cells to the lower compartment of a Transwell chamber significantly increased lymphatic endothelial cell migration in comparison to controls. Note that the highest activity, similar to that stimulated by a growth factor cocktail, was associated with the first-trimester samples. The graph depicts means ± SEM of multiple experiments.

Figure 22. Implantation of placental villi under the kidney capsule as an *in vivo* model of cytotrophoblast invasion. Placental explants were surgically placed under the kidney capsules of SCID mice, which were maintained for 1 week (A, C, D, F, G, and H) or 3 weeks (B, E, I) before histological analyses. (A) Examination of the tissue sections revealed the villous cores (arrows), which mark the original sites of implantation. By 1 week post-implantation, cytotrophoblasts, identified by cytokeratin 7 expression (brown), invaded murine renal tissue. (B) After 3 weeks, the amount of renal parenchyma occupied by cytotrophoblasts increased dramatically, with the invasion front extending well into the cortex and a few cell clusters migrating to even deeper regions. Many of the kidney tubules within the area occupied by the cytotrophoblasts remained intact (arrow). (C) Staining for CD31 expression (red) revealed the new appearance in areas of cytotrophoblast invasion (green) of vascular networks (arrow) that had a very different morphology from the resident renal vessels (compare inset with panel F). (D, E) Higher magnifications of panels A and B showed the migration route of invasive cytotrophoblasts, which was restricted to the peritubular spaces. (F) Double staining with CD31 (red) and cytokeratin (green) revealed that the cells were closely associated with blood vessels that course through these areas. (G) The cells also breached these vessels, as demonstrated by platelet deposition (CD41 staining, red), which occurred only in areas of cytotrophoblast invasion (green). (H) Cytotrophoblast expression of stage-specific antigens mimicked the pattern observed during human uterine invasion. For example, the cells stained with an antibody specific for HLA-G. (I) In addition, nuclear volume increased, indicative of the amplification in chromosome number that occurs as the cells

invade. This phenomenon is illustrated by the relatively small nuclear diameter of the progenitor cells in the location marked with an arrow (shown at higher magnification in the left inset), as compared to the invasive cells, two of which are marked with arrowheads (one shown at higher magnification in the right inset). Scale bars: A-C, 500 mm; C inset, 20 mm; D-I, 50 mm; I insets, 5 mm.

Figure 23. Cytotrophoblasts induce lymphatic endothelial cell infiltration. (A-C) Double staining of histological sections of placental implants. Cytotrophoblasts were visualized by cytokeratin 7 staining (left panels), and lymphatic endothelial cells were labeled with LYVE-1 (middle panels). Panels on the right side show the merged images. (A) At 1 week post-implantation, preexisting LYVE-1-positive vessels were detected beyond the front of cytotrophoblast invasion (white box and inset). Anti-LYVE-1 also stained CD45-positive macrophages within the kidney capsule (arrowheads). (B) After 2 weeks, cells that stained for LYVE-1 began to infiltrate the implants (arrows). (C) By 3 weeks, infiltration had increased (arrows). (D-F) Immunostaining of adjacent sections showed that within the renal parenchyma, which the cytotrophoblasts invaded (D), LYVE-1 expression (E, arrows) did not co-localize with CD45 staining (F, arrows). Tissue macrophages within the capsule that stained for CD45 (F, arrowheads) also reacted with LYVE-1 (E, arrowheads). Scale bars: A-C 500 μm; A insets, 20 μm; D-F, 50 μm.

Figure 24. Cytotrophoblasts induce lymphatic vessel formation. (A) Double staining labeled cytotrophoblasts (cytokeratin 7, left panel) and lymphatic endothelial cells

(LYVE-1, middle panel) revealed the close association of the two cell types (merged image). (B) After 3 weeks, the implant sites contained LYVE-1-positive structures that had the morphological appearance of lymphatic vessels, including the presence of lumens (arrows).

CHAPTER FIVE: Ultrastructure of endovascular cytotrophoblasts

Introduction

During pregnancy, the placenta is the first organ to differentiate, a reflection of its critical role in transporting a myriad of substances between the embryo/fetus and uterine blood. Thus, to function normally the placenta must gain access to the maternal circulation. Different species have solved this problem in different ways as shown by the various classifications of placental structure. For example, the human placenta is categorized as hemochorial: the chorion (placenta) is in direct contact with maternal blood. In this arrangement the placenta becomes part of the maternal circulation.

How does this happen? Cytotrophoblasts, the specialized cells of the human placenta, invade the uterine wall and remodel the maternal blood vessels they encounter (Figure 25). At a molecular level the invasive subpopulation of cytotrophoblasts, placental cells of ectodermal origin, undergo an epithelial-to-endothelial transition in which they upregulate the expression of vascular-type adhesion receptors (Zhou et al., 1997a) and growth factors that regulate conventional vasculogenesis and/or angiogenesis (Zhou et al., 2002). At a morphological level, one of the most striking features of vascular remodeling is cytotrophoblast tropism for arteries rather than veins. The cells breach both vessels, thereby diverting maternal blood flow to the placenta. Once this connection is made, remodeling of the venous side of the circulation is halted. In contrast, cytotrophoblasts migrate in a retrograde fashion up the lumina and walls of spiral arterioles thru the inner third of the myometrium, eventually replacing the vessels'

endothelial lining and intercalating within the surrounding smooth muscle layer (tunica media). A subset of the invasive cytotrophoblasts within maternal vessels undergo a morphological transformation as well. These cells, which initially have the extended appearance associated with migration, spread on the underlying basal lamina with a more flattened morphology. Together, these changes have important functional consequences as the uterine vessels are transformed into highly specialized large-bore arteries that by term deliver several liters of blood per minute to the placenta.

During vascular remodeling, cytotrophoblast migration is unique in that it occurs in a retrograde fashion, e.g., against the direction of blood flow. This process has been observed *in vitro*; macaque trophoblast cells migrate against laminar flow at shear stresses up to 30 dynes/cm^2 (Soghomonians et al., 2002). The mechanisms underlying this unusual type of migration are not well understood, but likely involve cytotoskeletal components and adhesion molecules that are regulated by shear stress (McCue et al., 2004). In fact, macaque trophoblasts upregulate b1 integrin expression when cultured under flow (Soghomonians et al., 2002).

To investigate the morphological features at the ultrastructural level associated with counterflow migration, we examined the morphology of cytotrophoblasts within uterine arteries using scanning electron microscopy. In comparison to unmodified vessels, remodeled uterine arterioles that were occupied by cytotrophoblasts exhibiting heterogeneous phenotypes were larger and less uniform in size. Rounded cells clustered along the vessel wall, most likely the migratory population, were interconnected at points of cell-cell contacts by numerous filipodia. These structures may play a functional role in

stabilizing cell-cell interactions as the endovascular popultation of cytotrophoblasts migrates en masse against arterial blood flow.

Results

Ultrastructure of spiral arterioles before and after cytotrophoblast modulation

To investigate the morphological changes that occur during vascular remodeling, we collected tissue specimens of the maternal-fetal interface from first and second trimester placentas that contained uterine blood vessels and analyzed them by using scanning electron microscopy. Consistent with typical histology (Figure 26A), an unmodified arteriole, 10-20 mms in diameter, was surrounded by a uniform smooth muscle layer (Figure 26B,C). The endothelial cells were orientated longitudinally with respect to blood flow (Figure 26C, D), the typical arrangement (Figure 26E).

In contrast, arterioles that were in contact with the intervillous space, which were much larger in diameter, were much less uniform in size. The ultrastructural features of these vessels were consistent with the histology of uterine arterioles that were completely remodeled by endovascular cytotrophoblasts (Figure 27A). Scanning electron micrographs showed that the lumina of these modified vessels contained cells of variable sizes and morphologies (Figure 27B, C). In addition, irregular clumps of placental cells protruded into the lumens, a phenomenon that we observed previously (Zhou et al., 1997a). The surfaces of endovascular cytotrophoblasts also exhibited a larger number of microvillious protrusions (Figure 27D).

Next, we examined the ultrastructural features of the endovascular cytotrophoblasts, which displayed diverse morphologies. As observed in tissue sections stained with cytotrophoblast-specific antibodies, the cells lining the vessel wall had either a rounded or flattened appearance. Both types were commonly seen in our scanning electron micrographs and several distinct structural features were noted. For example, cells with a rounded phenotype lining the vessel wall were interconnected by numerous columns of filopodia (Figure 28B-D). Flattened, endothelial-like cells were covered by microvilli-type structures (Figure 28E). Intraluminal protrusions were encased in a thick layer of acellular material, mostly akin to the fibrinoid material that is typically deposited at the maternal-fetal interface (Figure 28F).

Discussion

The uterine circulation undergoes a dramatic transformation during pregnancy when placental cytotrophoblasts invade resident blood vessels and extensively remodel spiral arterioles. A stepwise process, vascular remodeling, begins when invasive cytotrophoblasts make contact with uterine blood vessels. Our previous work shows that these placental cells use an Eph/ephrin system to distinguish between arterioles and veins. Interactions with veins are actively inhibited while those with arteries are allowed (Red-Horse et al., 2005). Thus, cytotrophoblasts migrate up the arterial side of the circulation and eventually replace the maternal endothelial lining as they intercalate within the surrounding smooth muscle, which they also partially replace. This process involves nearly the entire intrauterine course of arterioles adjacent to the placenta. Here,

we studied the ultrastructural features of these unusual cells as a way of gaining insights into this unique process, which is impossible to study *in vivo* and difficult to model *in vivo*.

This study uncovered unusual structural elements that are unique to the endovascular population of cytotrophoblasts that reside within maternal vessels. These cells were connected with each other by columns of numerous filipodia. In other cells, filopodia are actin-based membrane protrusions that are induced during periods of active cell migration by activation of cdc42. Filopodia are commonly formed at the leading edges of migrating cells including fibroblasts and neurons, in which case they localize to the highly motile growth cone.

The fact that these structures were frequently observed suggested that filopdia may be involved in cytotrophoblast migration in a direction counter to maternal blood flow, but finding their definitive role awaits functional studies. Primary macaque trophoblasts migrate in a counterflow direction when cultured on uterine microvascular endothelial cells (Soghomonians et al., 2005). It would be interesting to investigate whether under these conditions migrating trophoblasts interact via filopodial columns and whether cdc42 activity is required. During static culture, our work shows that human cytotrophoblasts communicate using very long filipodia-like structures, which can extend greater than 10 times the length of the cell. These specialized processes appear to function in cell-cell communication during aggregate formation by attaching to smaller clusters of cytotrophoblasts, which they draw into larger groups of cells.

During placental morphogenesis, cytotrophoblast aggregates are also frequently observed jutting out into the lumina of uterine blood vessels. In first trimester specimens,

these clusters appear to form plugs that occlude blood flow to the placenta, which are thought to maintain a hypoxic environment that promotes placental growth (Genbacev et al., 1997). Our data suggest that eventual canalization of these vessels may be incomplete since a portion of the cells remain as much smaller clusters that become surrounded by a fibronoid-like material. This is in agreement with previous observations that intravascular invasion triggers platelet deposition (Sato et al., 2005). However, we cannot exclude the possibility that these structures appear as a result of cytotrophoblast delamination from the blood vessel wall. Finally, we note that acellular material was not associated with cytotrophoblasts located in closer proximity to the vessel wall, perhaps because the cells are not subjected to turbulent blood flow.

In summary, we have used scanning electron microscopic to study the human uterine arterioles that are colonized by placental cytotrophoblasts. In keeping with the unusual nature of this process, these vessels and the embryonic/fetal cells that line them also have very unusual morphologic features as compared to non-pregnant arterioles. Whether the ultrastructural anatomy of this region changes in certain pregnancy complications is an interesting question we hope to answer in the future.

Figure Legends

Figure 25. Placental cytotrophoblasts invade the uterine wall where they extensively remodel maternal spiral arterioles. The bulk of the placenta is composed of numerous tree-like projections termed chorionic villi where maternal-fetal exchange occurs. These structures mediate the passage of nutrients, gases, and wastes between fetal blood, which

circulates through the villous core, and maternal blood, which circulates through the intervillous space. The uteroplacental circulation is established by cytotrophoblasts that acquire an invasive phenotype as they leave the placenta and enter the uterine wall. Here, invasive cytotrophoblasts migrate up the lumina of spiral arterioles, eventually replacing the vessels' endothelial lining and a portion of the muscular wall. This process incorporates the placenta into the uterine circulation and vastly increases maternal blood flow, actions that are critical for successful maternal-fetal exchange. NK, natural killer; mϕ, macrophage.

Figures 26. Ultrastructure of uterine spiral arterioles in the absence of cytotrophoblast invasion. Uterine tissue specimens containing blood vessels were dissected and analyzed by using scanning electron microscopy. A histological section stained with hematoxylin and eosin (A) and SEM images (B-D) of the uterine wall show that unmodified spiral arterioles (a), which are 10-20 μm in size, are surrounded by a uniform smooth muscle layer (brackets). (C and D) Flattened endothelial cells (ec) lining these vessels were oriented longitudinally with respect to blood flow (arrow in D). (E) This morphology is typical of arterial vessels as shown in the SEM image of a fetal arteriole (a) located within the placenta. Bars represent: A, 100 μm; B and C, 10 μm; D, 2 μm; E, 10 μm.

Figures 27. Ultrastructure of uterine spiral arterioles remodeled by placental cytotrophoblasts. Uterine tissue specimens containing invasive cytotrophoblasts were dissected and analyzed using SEM. (A) A histological section stained with cytokeratin 7 (black), which labels cytotrophoblasts (CytoT), showed that remodeled arterioles (a) were

much larger in diameter and less uniform in size as compared to unmodified vessels. (B) Similar findings were observed in SEM images, which also showed that the vessels contained irregular clumps of placental cells that protruded into the lumina (arrow). (C an D) Cytotrophoblasts lining the lumina of these vessels were of variable sizes and morphologies (C) and their surfaces exhibited a larger number of microvillious protrusions (D). Bars represent: A and B, 100 μm; C, 10 μm; D, 5 μm.

Figures 28. Ultrastructural features of endovascular cytotrophoblasts. (A) A histological section stained with an antibody that recognizes cytokeratin 7 (black) labeled cytotrophoblasts and showed that placental cells lining uterine blood vessels have either a rounded (arrow) or flattened (arrowhead) appearance. (B-E) SEM images of cells displaying both phenotypes. Cytotrophoblasts with a rounded morphology were interconnected by numerous columns of filopodia (B-D, arrows). Flattened, endothelial-like cells were covered by microvilli-type structures (E). (F) Cytotrophoblasts (arrow) within intraluminal protrusions were encased in a thick layer of acellular material. Bars represent: A, 100 μm; B, 10 μm; C, D, E, 5 μm; F, 50 μm.

Figure 25

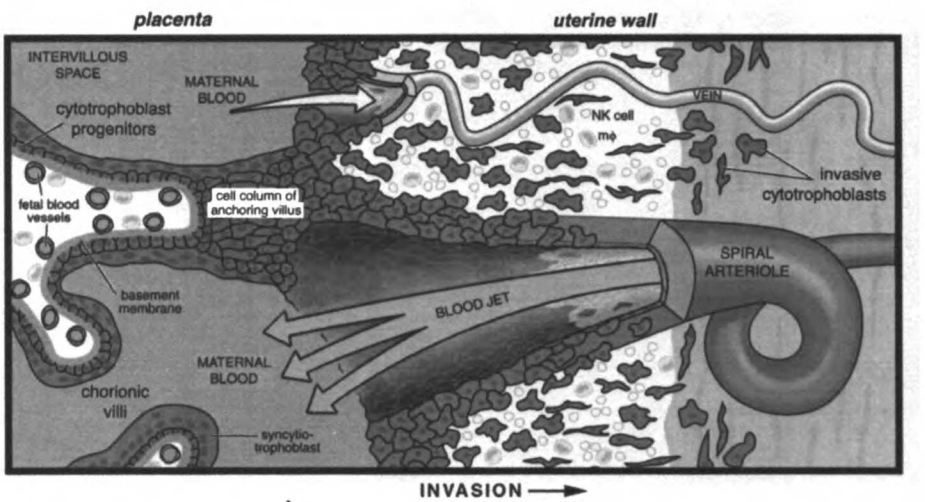

placenta
uterine wall
INTERVILLOUS SPACE
MATERNAL BLOOD
cytotrophoblast progenitors
VEIN
NK cell
mϕ
invasive cytotrophoblasts
fetal blood vessels
cell column of anchoring villus
SPIRAL ARTERIOLE
basement membrane
BLOOD JET
MATERNAL BLOOD
chorionic villi
syncytio-trophoblast
INVASION

Figure 26

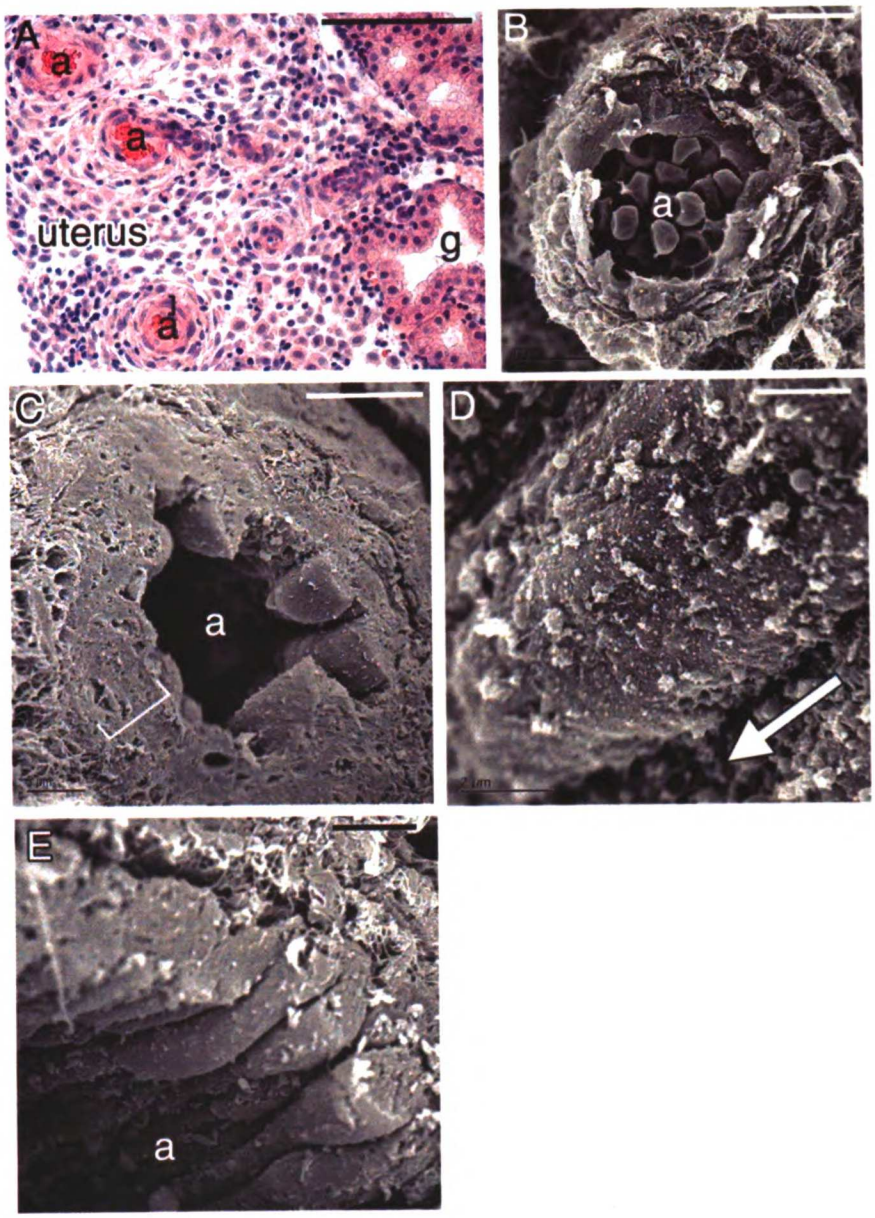


Figure 27

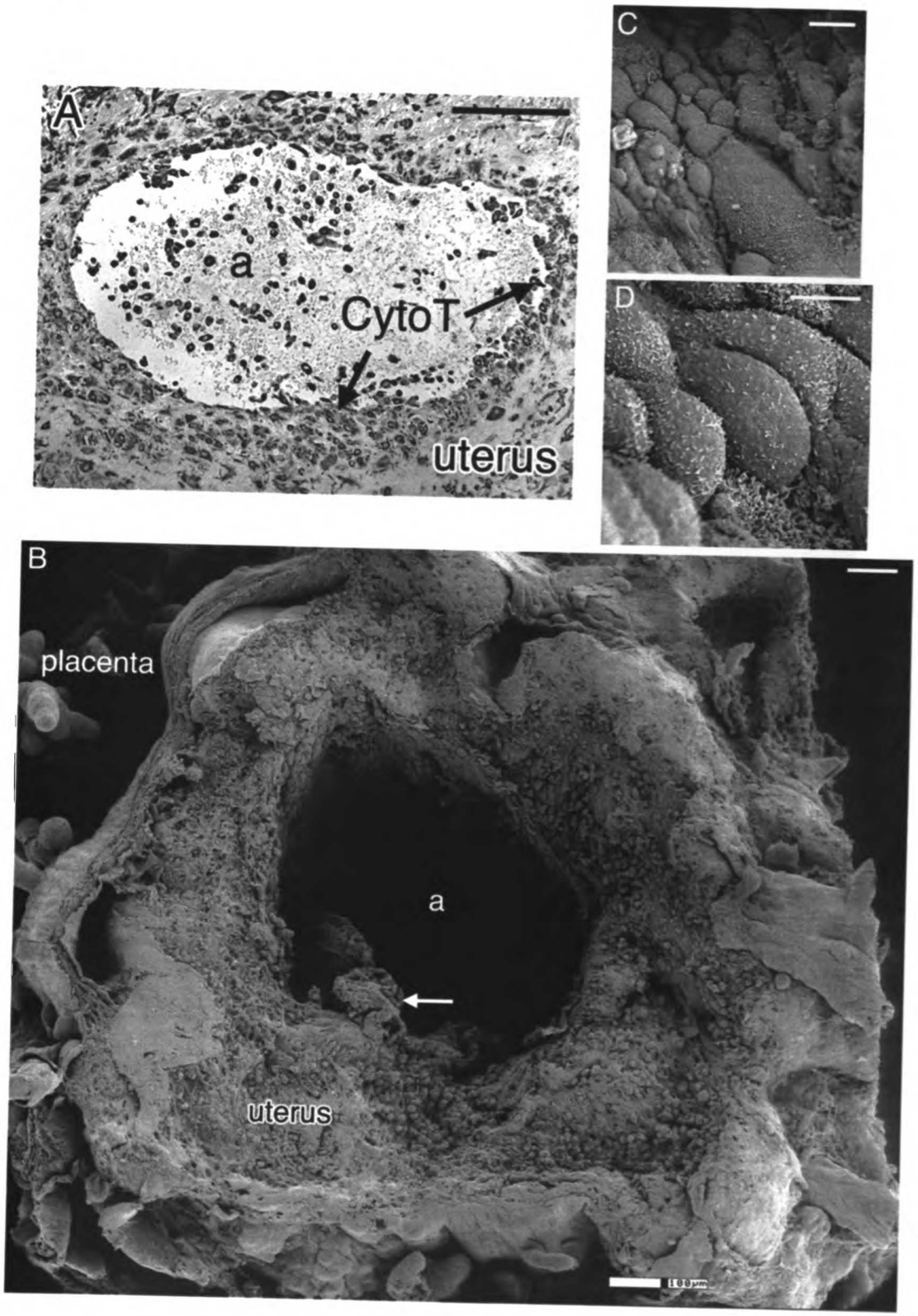
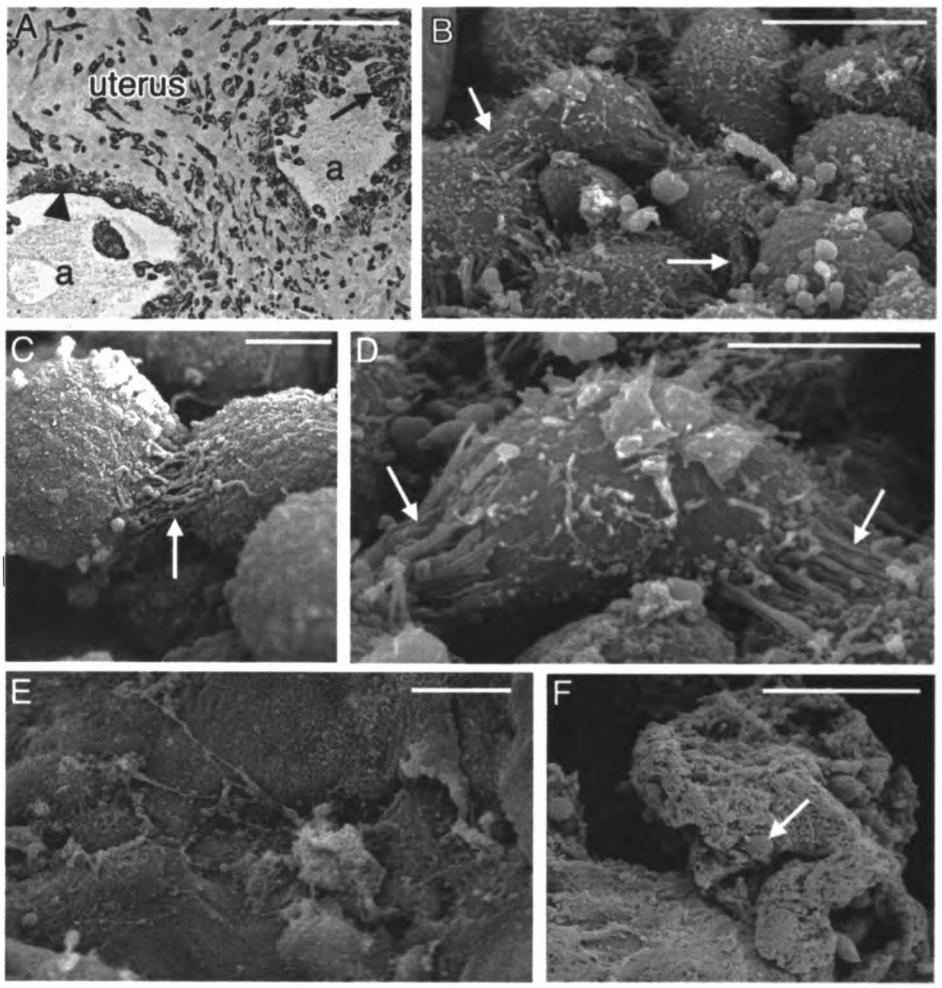


Figure 28



CHAPTER SIX: Conclusions and Future Directions

Data in this thesis significantly advances our knowledge of human placental biology. It provides a better understanding of the mechanisms by which this organ connects the fetus to maternal blood vessels, a function that is critical to successful pregnancy. Additionally, this work describes the first *in vivo* model of placental invasion and cytotrophoblast-mediated vascular remodeling. This model has the potential to vastly expand our understanding of placental development, particularly in regards to pregnancy complications that arise from deficient interstitial and arterial invasion.

Cytotrophoblast-blood vessel interactions

To date, most of the information concerning the mechanisms that direct placental morphogenesis has been derived from a combination of *in situ* and *in vitro* methods. Using similar strategies, we demonstrate that cytotrophoblasts utilize genetically predetermined differences in arterial and venular endothelial cells to pattern vascular invasion. Specifically, EphB4, a cell surface receptor specifically expressed on veins, restricts cytotrophoblast adhesion and migration by regulating chemokine responses, which are active in the uterine wall. This suggests that EphB4 is an important factor that limits venular remodeling in the uterus during pregnancy. Thus, the arterial bias of vascular remodeling is not only influenced by presumptive attractive mechanisms associated with spiral arterioles, but also by negative influences exerted by veins.

The existence of a vein-specific molecule that blocks cytotrophoblast invasion has several implications. For example, the restrictive mechanism(s), must act downstream from the phenotypic switch that takes place within cell columns in which cytotrophoblasts change their phenotype from an epithelial to a pseudo-vascular state. The data presented here are consistent with this paradigm. Control of venular remodeling also suggests that defects in this process would be deleterious to pregnancy. In this regard we hypothesized that defects in Eph and/or ephrin receptor expression are associated with pregnancy complications associated with faulty vascular remodeling, of which preeclampsia and intrauterine growth restriction are the prime examples. Initial experiments suggest no major alterations in preeclampsia (data not shown), but this issue deserves much closer examination. At a functional level, it is not known why invasion is skewed towards arterioles; however, extensive venular remodeling could impact the mother's systemic blood pressure, which is already dramatically altered during pregnancy. At a teleological level, veins, which lack a well-developed muscular layer, may not be able to support cytotrophoblast invasion, which could potentially destroy this side of the circulation, with obvious deleterious affects on pregnancy.

Given the important impact of EphB4 on cytotrophoblast function, future experiments are aimed toward further characterizing its role *in vivo*. Specifically, because a new animal model of human placentation was developed during the course of work described in this thesis, it is now possible to assess cytotrophoblast invasion of murine arteries and veins in the presence of EphB4-blocking reagents using this model. Since *in vitro* experiments suggest that EphB4 limits venular remodeling, we expect that

blocking function of this molecule will result in more extensive interactions between cytotrophoblasts and veins.

With regard to the feasibility of these future experiments, evidence to date supports the utility of our new *in vivo* assay for studying the mechanisms that pattern arterial and venular invasion. Specifically, we observed a conservation of mechanisms between species in that human cytotrophoblasts displayed different relationships with the two sides of the murine circulation. The placental cells induced apoptosis specifically in arterial-associated components including the endothelium and surrounding smooth muscle layer. It will be interesting to define the molecular pathways conferring this exquisite specificity with regard to programmed cell death and whether Ephs and ephrins are required in this process.

The observation that cytotrophoblasts initiate arterial apoptosis strongly suggests that this activity is part of the mechanism that enables the cells to colonize uterine spiral arterioles. Since several pregnancy complications result from deficient arterial remodeling, future experiments will test whether placental implants isolated from these cases properly induce arterial apoptosis *in vivo*. One of the most relevant complications is preeclampsia, which is the leading cause of maternal mortality and fetal morbidity worldwide. Preeclampsia, which is characterized by maternal hypertension, edema, and proteinuria, can progress to a severe form of the syndrome, eclampsia, which is associated with seizures and even death. To date, the cause of this syndrome is not known and the only cure is delivery of the placenta. Our *in vivo* model will be a valuable asset in the search for a better understanding of this condition. Initial experiments will involve implanting villi from preeclamptic placentas and aged-matched controls in order

to compare cytotrophoblast invasion and vascular remodeling in the two groups of animals. We are also interested in the possibility that trophoblast overburden, the state in twin pregnancies that are susceptible to preeclampsia, is also a risk factor for developing this conditions. Our *in vivo* model is also amenable to testing this hypothesis as both the kidney capsule and mammary fat pad can physically accommodate many more anchoring than we have implanted thus far. Finally, we realize the enormous utility of an animal model of preeclampsia. The potential applications are exciting. For example, it may be possible to study the steps in real time by which this syndrome develops and the molecular determinants of the important transitions. An explication of this process will then allow screens of various agents that interfere with the pathways thus identified for their utility in prevention of the full-blown syndrome.

We are also interested in using our newly developed model to discover whether arteries have positive signals that play an important role in patterning vascular remodeling by attracting cytotrophoblasts to this side of the vasculature. The most obvious candidate is ephrinB2, a ligand for EphB4 that is specifically expressed on arterial smooth muscle and endothelial cells. We showed that cytotrophoblasts upregulate ephrinB2 and its receptor EphB2 upon differentiation down the invasive pathway. Thus, we also hypothesize that receptor-ligand interactions provide signals that stimulate arterial invasion and that blocking these signaling pathways will decrease cytotrophoblast-arterial interactions *in vivo*.

Experiments aimed at discovering general attractive signals that are associated with the blood vasculature are also important. It is thought that cytotrophoblast invasion is directed toward these structures, which suggests a diffusible molecule that acts over an

appreciable distance. Our data show that chemokines, including CXCL12, stimulate cytotrophoblast migration. This chemokine is present in high amounts in the circulation suggesting the possibility that blood-derived CXCL12 could be one of the signals that attract cytotrophoblasts toward blood vessels. We can now test this possibility by blocking murine chemokines during blood vessel invasion in our *in vivo* model.

Finally, work from our group and other laboratories suggest that VEGF family members play a very large role in many aspects of pregnancy. For example, an imbalance of VEGF family members can induce cytotrophoblast apoptosis *in vitro* (Zhou et al., 2002), a phenomenon that is also associated with cytotrophoblasts in basal plate biopsies obtained from patients with preeclampsia (DiFederico et al., 1999). In further support of the importance of VEGF family members, levels of sFLT (soluble VEGF receptor 1) increase and a VEGF ligand (placental growth factor) decreases in the blood of women who go on to develop this syndrome (Levine et al., 2004). It will be very interesting to determine if inducing an imbalance in VEGF family members produces a preeclampsia-like syndrome in our animal model, including cytotrophoblast apoptosis, and whether VEGF therapy ameliorates this condition.

Cytotrophoblasts and lymphangiogenesis

In addition to being helpful for studying cytotrophoblast-blood vessel interactions, placental implants also revealed the impact of these cells on uterine lymphatic vessels. Initially, we repeated the longstanding observation that the nonpregnant endometrium lacks a significant lymphatic circulation. Then we went on to show that the decidua

contains numerous lymphatic vessels. *In vitro*, cytotrophoblast conditioned-medium induced the migration of lymphatic endothelial cells. *In vivo,* assayed in the novel murine model described above, cytotrophoblast invasion was accompanied by a significant influx of these same cells, which formed vessels. Repeating these experiments in the presence of blocking reagents will be helpful for delineating the specific molecules involved. Likely candidates include the lymphangiogenic factors produced by the placenta including VEGF-C, Ang-2, and FGF-2.

Interestingly, murine and human placentation differed in that only the latter was associated with lymphangiogenesis. Experiments in progress are aimed at delineating whether this is due to differences in the ability of the two trophoblast populations to recruit lymphatic vessels. Implantation of murine primary trophoblasts or trophoblast cell lines under the kidney capsule will allow us to determine whether the cells differ from human placental cells with respect to their lymphangiogenic capacity. Another possibility is that exclusion of lymphatic vessels from the murine decidua is regulated at the level of the uterine stroma. The reverse experiment, implanting human placental villi into the murine uterus before assessing lymphangiogenesis, could address this possibility.

In addition to mechanisms of decidual lymphangiogenesis, the function of decidual lymphatic vessels is another potentially important and unexplored area of investigation. We are particularly interested in the implications with regard to maternal-fetal immunity. Normally, the uterine lining is sequestered from systemic immunity by restricting movement from this site into local lymphatic tissue (Head and Lande, 1983). Our observations suggest that in humans this state changes dramatically during pregnancy. To begin to understand the impact of decidual lymphangiogenesis on

maternal immune functions during pregnancy, we want to define the leukocyte subsets that are present in decidual lymphatic vessels with the presumption that they may be en route to local lymph nodes. Initial observations show that these cells are leukocytes as they express the common leukocyte antigen, CD45. By analogy with other systems, they likely include a dendritic cell population. $CD56^{bright}$ NK cells may also traffic from the decidua since they are the most abundant subset of maternal leukocytes in this location during pregnancy. It is interesting to note that, beyond dendritic cells, leukocyte trafficking in tissue lymphatic vessels is not well understood. The pregnant uterus may provide a new model that will allow us to learn more about this process in a physiological context. For example, the $CD56^{bright}$ NK cell population is found in secondary lymphoid tissue. Their function in this location, which is thought to be regulatory, is not well understood (Cooper et al., 2001; Fehniger et al., 2003).

Identifying the cells that traffic within decidual lymphatic vessels will lead to hypotheses about their functions and the pathways that are involved. At other sites, activated dendritic cells that take up antigens in the surrounding environment move into lymphatic vessels, a part of the circuitry that ultimately enables an adaptive immune response. Occurrence of a similar phenomenon in the pregnant human uterus would suggest that the placental environment activates dendritic cells, an interesting possibility that could be assessed by evaluating the expression of activation-specific markers by decidual leukocytes. Since preliminary evidence suggests that these cells are carrying trophoblast antigens (K. Redhorse and E. Mack, unpublished observations), one of our goals is to identify the type of response these cells stimulate. Our hypothesis is that decidual dendritic cells acquire trophoblast antigens and transport them to local lymph

nodes where they stimulate the production of regulatory T cells, which may regulate tolerance to hemi-allogeneic fetal tissues. In this regard, it is interesting to note that regulatory T cells are required for normal allogeneic, but not syngeneic, pregnancies in some mouse stains (Aluvihare *et al.*, 2004). Future experiments that test elements of this theory include isolating human decidual dendritic cells and assessing the types of T cells responses they stimulate *in vitro*.

Placental genetics

Placental cells have unique genetic properties. For example, in mice, the invasive trophoblast population undergoes endoreduplication replicating their chromosomes multiple times without undergoing mitosis. Invasive cytotrophoblasts in humans acquire random aneuploidies as they differentiate and invade the uterine wall. Analyses completed to date suggest that this phenomenon most commonly involves the X-chromosome (Weier et al., 2005). The functional consequences of invasive trophoblast hyperdiploidy are not well understood in either mice or humans. We hypothesize that this very unusual genetic state might limit their ability to undergo mitosis within the uterine wall. In addition, it is possible that achieving an aneuploid state plays an important role in limiting invasion and circumscribing lifespan. Thus, in the special case of pregnancy, the acquisition of aneuploidies in the invasive cytotrophoblast subpopulation plays an *anti*-tumorogenic role, *i.e.*, has the opposite effect of cancer-associated aneusomies.

In histological sections of kidney implantation sites, we noted differences between the nuclear size of the cytotrophoblast progenitors that were associated with the original villous implants and the cells that invaded the murine tissue. In addition, preliminary FISH data show that many of the invasive cytotrophoblasts contained ≥ 2 copies of the X chromosome. Thus, this *in vivo* model, in which implants can be maintained for up to 3 months, is a tractable system for studying the molecular mechanisms that lead to cytotrophoblast acquisition of aneuploidies as they complete their last mitotic cycle and become invasive. For example, placental implants could be treated with agents that increase chromosomal instability before assessing their ability to invade *in vivo*. Additionally, effects on lifespan could also be evaluated. Alterations in either parameter would suggest that the normal process by which cytotrophoblasts acquire aneusomies is integral to both processes.

Summary

In conclusion, the results presented in this thesis provide new mechanistic insights into the patterning of cytotrophoblast endovascular invasion and describe a novel *in vivo* murine model of human placentation for studying, in real time, formation of the maternal-fetal interface including the enigmatic process whereby cytotrophoblasts remodel the vasculature.

The data that were generated significantly advance our understanding of basic elements of human placentation that often go awry in common pregnancy complications. Specifically, this work uncovered mechanisms whereby cytotrophoblasts remodel blood

vessels and induce formation of a decidual lymphatic vasculature. These observations lay the foundation for numerous other studies. For example, it will be interesting to determine if particular elements of the apoptotic-driven mechanism that allows cytotrophoblasts to replace the endothelial lining of arterioles fails in preeclampsia. It will be equally as interesting to delineate the function of uterine lymphangiogenesis and determine whether elements of this unusual process fail or contribute to pathological conditions that affect both the pregnant and non-pregnant uterus.

The new chimeric model that was developed in which human anchoring villi invade either the murine kidney or the mammary fat pad was instrumental to proving several hypotheses that were tested. We envision that this model will be used by many investigators to address numerous aspects of human placental biology and immunology that cannot be studied by employing the currently available *in situ* and/or *in vitro* cell and organ culture methods.

Thus, this work opens many new doors for other investigators in the Fisher group, in particular, and for the field, in general.

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