EPHB4 regulates chemokine-evoked trophoblast responses: a mechanism for incorporating the human placenta into the maternal circulation

Kristy Red-Horse[1,2], Mirhan Kapidzic[2], Yan Zhou[2], Kui-Tzu Feng[2], Harbindar Singh[3] and Susan J. Fisher[1,2,4,5,*]

[1]Biomedical Sciences Graduate Program, University of California San Francisco, San Francisco, CA 94143, USA
[2]Department of Cell and Tissue Biology, University of California San Francisco, San Francisco, CA 94143, USA
[3]Department of Physiology, Universiti Sains Malaysia, Kelantan, Malaysia
[4]Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA 94143, USA
[5]Department of Anatomy, University of California San Francisco, San Francisco, CA 94143, USA
*Author for correspondence (e-mail: sfisher@cgl.ucsf.edu)

Development 132, 4097-4106
Published by The Company of Biologists 2005
doi:10.1242/dev.01971

Accepted 7 July 2005

Summary

In humans, fetal cytotrophoblasts leave the placenta and enter the uterine wall, where they preferentially remodel arterioles. The fundamental mechanisms that govern these processes are largely unknown. Previously, we have shown that invasive cytotrophoblasts express several chemokines, as well as the receptors with which they interact. Here, we report that these ligand-receptor interactions stimulate cytotrophoblast migration to approximately the same level as a growth factor cocktail that includes serum. Additionally, cytotrophoblast commitment to uterine invasion was accompanied by rapid downregulation of EPHB4, a transmembrane receptor associated with venous identity, and upregulation of ephrin B1. Within the uterine wall, the cells also upregulated expression of ephrin B2, an EPH transmembrane ligand that is associated with arterial identity. In vitro cytotrophoblasts avoided EPHB4-coated substrates; upon co-culture with 3T3 cells expressing this molecule, their migration was significantly inhibited. As to the mechanisms involved, cytotrophoblast interactions with EPHB4 downregulated chemokine-induced but not growth factor-stimulated migration. We propose that EPHB4/ephrin B1 interactions generate repulsive signals that direct cytotrophoblast invasion toward the uterus, where chemokines stimulate cytotrophoblast migration through the decidua. When cytotrophoblasts encounter EPHB4 expressed by venous endothelium, ephrin B-generated repulsive signals and a reduction in chemokine-mediated responses limit their interaction with veins. When they encounter ephrin B2 ligands expressed in uterine arterioles, migration is permitted. The net effect is preferential cytotrophoblast remodeling of arterioles, a hallmark of human placentation.

Key words: Placenta, Cytotrophoblast, Vascular remodeling, Chemokines, EPHB4, Ephrin B2, Human, Pregnancy

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 extra-embryonic 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. 1). 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

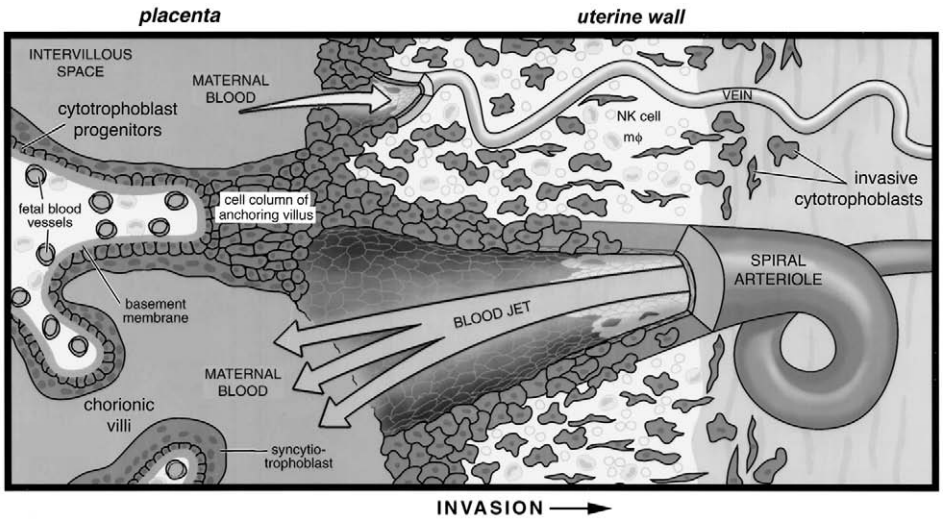


Fig. 1. Placental cytotrophoblasts invade the uterine wall where they breach veins and extensively remodel maternal spiral arterioles. The bulk of the placenta is composed of numerous tree-like projections termed chorionic villi where maternal-fetal exchange occurs. These structures mediate the passage 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. By contrast, cytotrophoblasts migrate up the lumina of spiral arterioles, eventually replacing the endothelial lining of the vessels and part of the muscular wall. This process encompasses the decidual and inner third of the myometrial segments of these vessels. NK, natural killer; mϕ, macrophage.

uterine blood flow to more than 30 liters per hour (Dickey and Hower, 1995; Metcalfe et al., 1955). By contrast, remodeling of veins, which establishes venous return, is limited to their termini.

Some of the molecules involved in endovascular invasion are known, and include adhesion molecules and cell surface receptors important for conventional vasculogenesis/angiogenesis (reviewed by 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 $\alpha6\beta4$ 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 $\alpha V\beta3$ and $\alpha1\beta1$) (Damsky et al., 1992; Zhou et al., 1997). 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, owing 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 ephrin B ligands and EPHA receptors bind ephrin A ligands. With regard to specific functions, EPHB4, a receptor tyrosine kinase, and its ligand, ephrin B2, 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 by 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 accordance with this theory, we found that, during pregnancy, maternal endothelial cells of uterine veins and arteries express EPHB4 and ephrin B2, respectively. Cytotrophoblast invasion was associated with acquisition of an arterial phenotype – downregulation of EPHB4 and sequential upregulation of ephrin B1 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 crucial roles in human placentation at two important junctures, first by generating repulsive signals that initiate cytotrophoblast invasion and later by patterning the interactions of cells with the uterine vasculature.

Materials and methods

Human tissue collection

Informed consent was obtained from all tissue donors. Placentas from elective terminations of pregnancy (6 to 22 weeks) or from normal term deliveries (34 to 40 weeks) were collected into 10% formalin (in situ hybridization) or 3% paraformaldehyde (immunofluorescence), or washed repeatedly in phosphate-buffered saline (PBS) containing antibiotics and placed on ice (cytotrophoblast isolation).

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 ephrin B1 (Beckmann et al., 1994) and ephrin B2 (Cerretti et al., 1995), or gene fragments for EPHB4 (nucleotides 412-1253) and EPHB2 (nucleotides 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) 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 μg/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]-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) GGATAC-CATCCCCGAAGAAT/AGGCACCAGCACACTCTCTT and (2) GGGAGGTGAGAGGTGATTT/GGGTGACGTAGGTGCCATAG. Similar results were obtained with each primer set.

Immunohistochemistry

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 week; n=3, 14-19 week; 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-ephrin B2 (Santa Cruz P-20).

Substratum choice assay

The preference of cytotrophoblasts for substrates containing either EPHB4 or ephrin B2 was assessed by using published methods (Birgbauer et al., 2001). Briefly, laminin was mixed with either ephrin B2-Fc, EPHB4-Fc or (control) human IgG (10 μg/ml) and fluorescein-conjugated goat-anti human Fcγ (10 μg/ml), and then spotted in 1 μl 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 μg/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 μM 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 μg/ml (murine) laminin (Invitrogen) or 100 μl 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). 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% 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 μg/ml human plasma fibronectin (Roche) and then washed with PBS. Cytotrophoblasts (2.5×10^5 cells in 250 μl serum-free medium) were added to the upper compartments, and the inserts were placed in 24-well plates that contained 500 μl medium with either vehicle alone (0.1% bovine serum albumin/PBS) or chemokines (10 and 1000 ng/ml). In some cases, the medium also contained EPHB4-Fc (10 μg/ml). Growth factor-induced migration was stimulated by adding to the lower chamber 125 μl endothelial cell growth medium (Clonetics, EGM-2) containing FCS, hEGF, VEGF, hFGF-B and IGF1 at the concentrations provided by the manufacturer to a final volume of 500 μl. 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 labeled 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% 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.

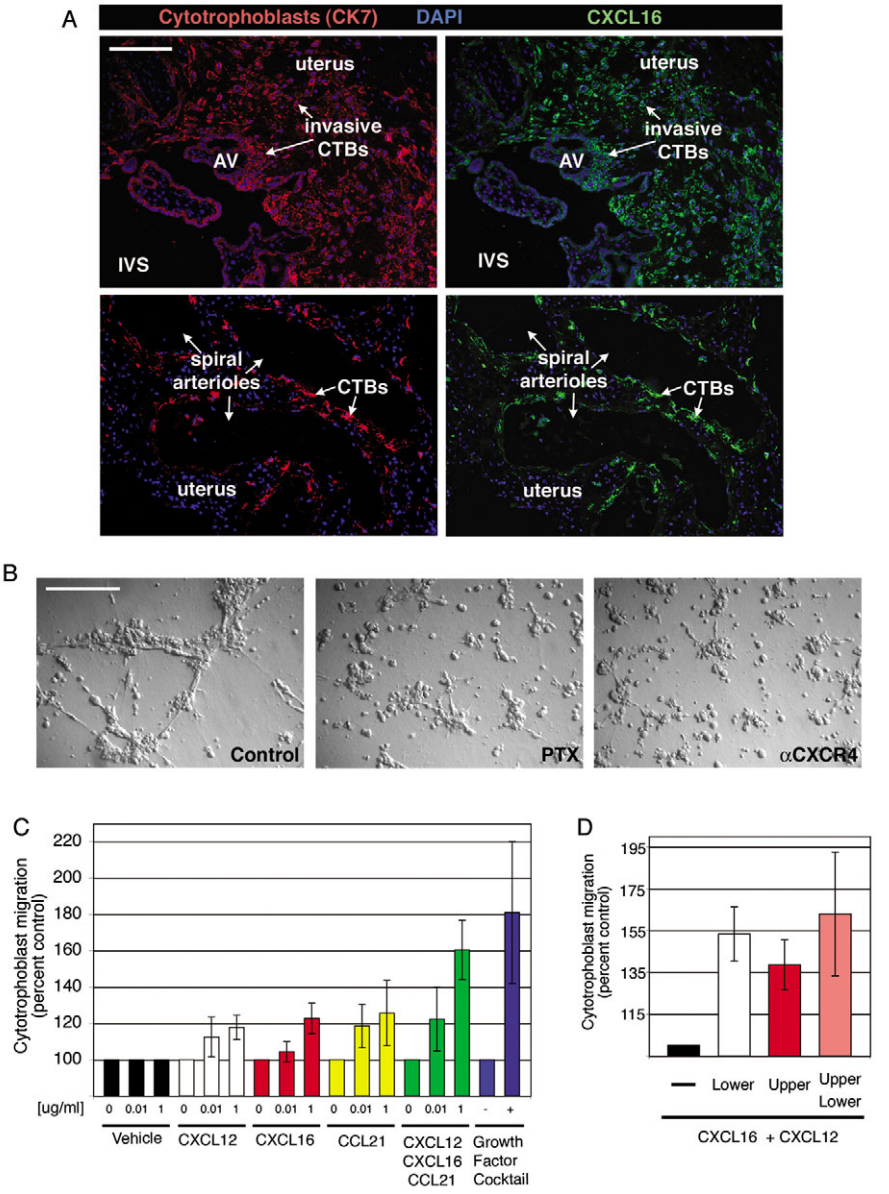


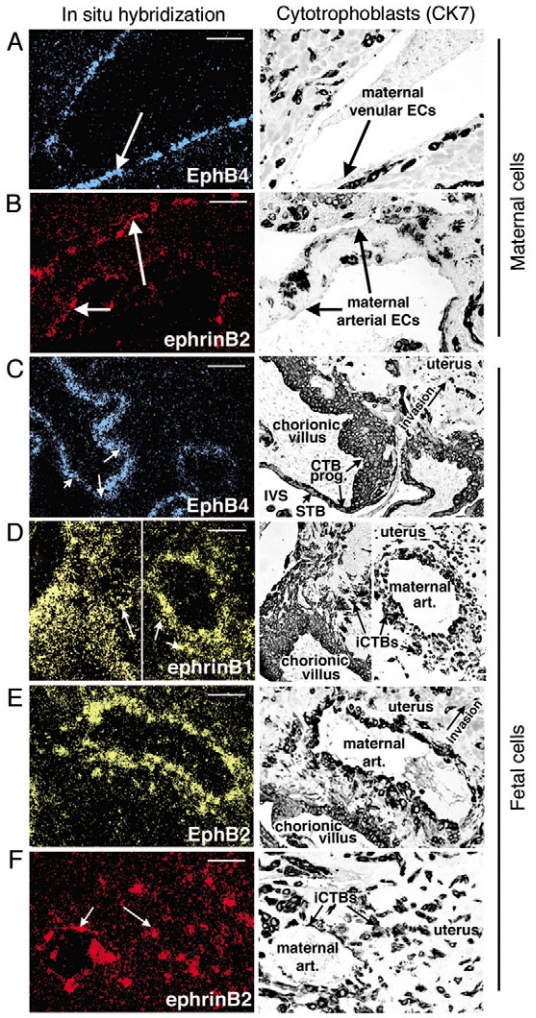
Fig. 2. Chemokines stimulate cytotrophoblast migration. (A) Cytokeratin 7 (CK7) (red) and CXCL16 (green) expression in the region of the maternal-fetal interface shown in Fig. 1. (A) CXCL16-specific antibody stained invasive and endovascular cytotrophoblasts (CTBs), identified by their cytokeratin 7 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 s.d. (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. Scale bars: 100 μm.

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. 2A), 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,



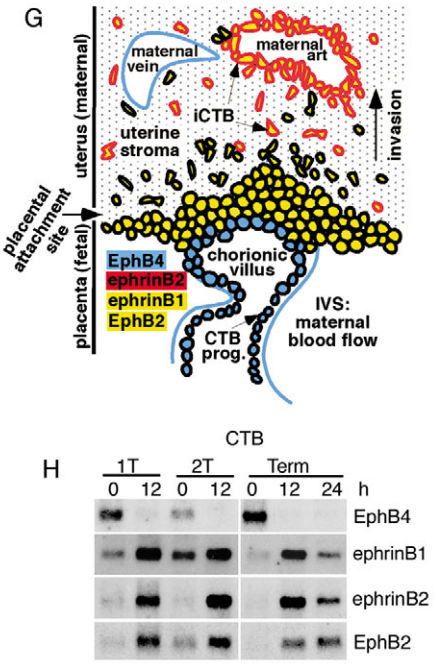


Fig. 3. Coincident with exit from the placental compartment, cytotrophoblasts switch from a venous to an arterial phenotype, as shown by their modulation of EPH and ephrin family members. (A-F) In situ hybridization; adjacent sections were stained with cytokeratin 7 (CK7), which identified trophoblasts. Venular and arterial endothelial cells (ECs) that lined uterine vessels expressed EPHB4 (A) and ephrin B2 (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 ephrin B1 (D) and EPHB2 (E) mRNAs. Subsequently, within the uterine stroma, ephrin B2 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 ephrin B1, B2 and EPHB2 after 12 hours in culture. IVS, intervillous space; T, trimester. Scale bars: 50 μm in A,B,F; 100 μm in C-E.

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. 2B).

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 with the addition of serum plus a growth factor cocktail (Fig. 2C). 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. 2D). 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. 3A) and ephrin B2 (Fig. 3B) 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. 3C). At the boundary where progenitors commit to differentiation, they abruptly downregulated EPHB4 expression and began to express ephrin B1 (Fig. 3D), which binds EPHB4 (Sakano et al., 1996), and EPHB2 (Fig. 3E), a receptor for the ephrin B ligands. These molecules continued to be expressed as the cells infiltrated the uterine stroma and occupied maternal arterioles (Fig. 3D,E). Finally, within the uterine wall, many of the interstitial and endovascular cytotrophoblasts upregulated ephrin B2 (Fig. 3F). Based on these expression patterns, we concluded that invasive cytotrophoblasts are equipped to interact with EPHB4, which is expressed by cytotrophoblast

progenitors and maternal veins, via the ligands ephrin B1 and B2. Additionally, invasive cytotrophoblasts express the EPHB2 receptor, a binding partner for ephrin B2 expressed on maternal arteries (Fig. 3G).

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. 3H). 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 ephrin B1, 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 ephrin B2

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 ephrin B2-Fc or EPHB4-Fc. The results showed that cytotrophoblasts differentially interpreted signals garnered from ligating ephrin B2 or EPHB4. On ephrin B2-spotted substrates (Fig. 4A,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. By contrast, regions that contained EPHB4 had significantly fewer cytotrophoblasts, with the outer edge of the spot appearing to

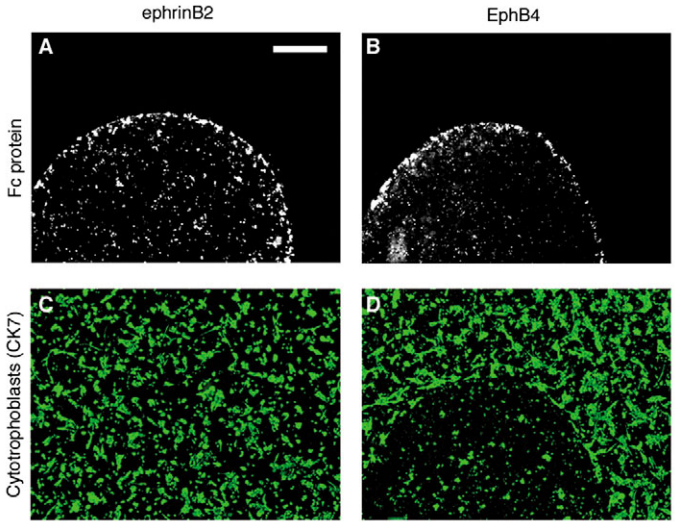


Fig. 4. Cytotrophoblasts avoid substrates containing EPHB4, but not ephrin B2. Laminin with either ephrin B2-Fc (A) or EPHB4-Fc (B) and fluorescent anti-human Fc was spotted on the plate before the entire surface was covered with laminin alone. (C,D) Cytokeratin 7 staining showed cytotrophoblast distribution on the tissue culture substrate. Initially, cytotrophoblasts were plated as a monolayer. (C) By 12 hours, the cells continued to associate with ephrin B2-Fc substrates, but (D) few cells were attached to EPHB4-Fc spots. Scale bar: 200 μm.

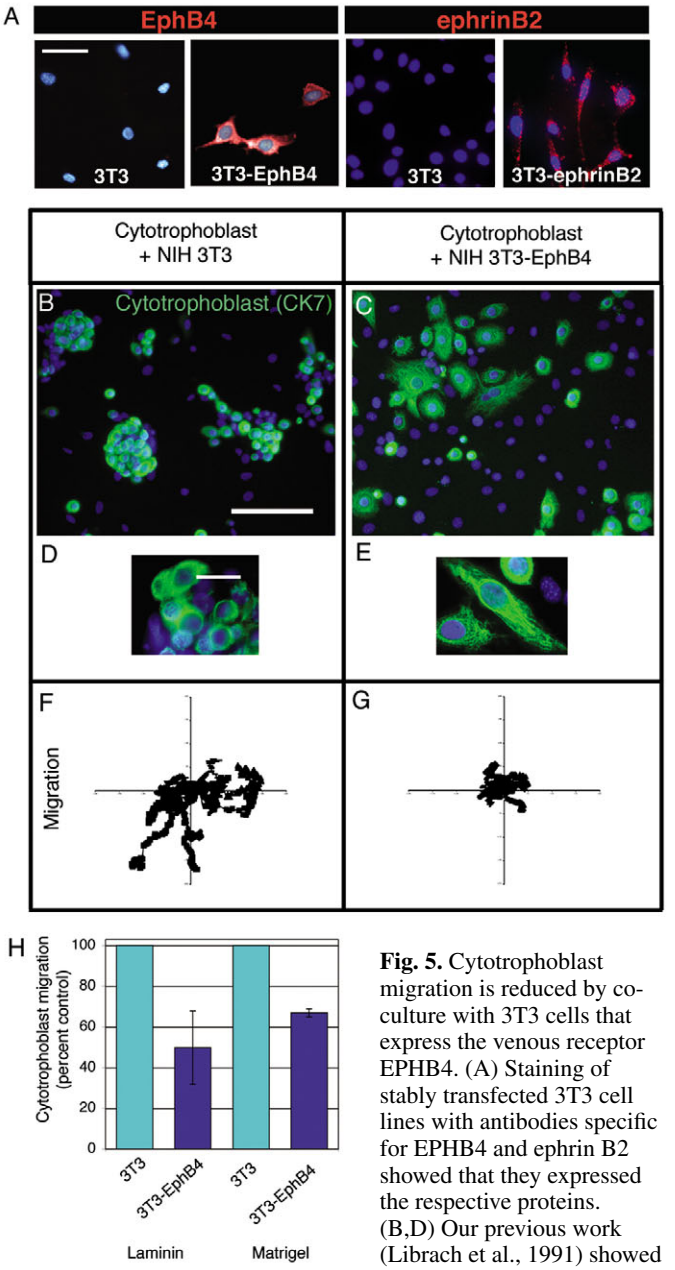


Fig. 5. Cytotrophoblast migration is reduced by co-culture with 3T3 cells that express the venous receptor EPHB4. (A) Staining of stably transfected 3T3 cell lines with antibodies specific for EPHB4 and ephrin B2 showed that they expressed the respective proteins. (B,D) Our previous work (Librach et 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) By 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 hours showed that, in comparison with 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 s.d. Scale bars: 20 μm in A; in B, 50 μm for B,C; in D, 10 μm for D,E.

form a boundary (Fig. 4B,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 ephrin B2 (Fig. 5A). In the presence of control (Fig. 5B) or ephrin B2-expressing (data not shown) 3T3 cells, the cytotrophoblasts rapidly migrated towards one another, forming aggregates that included both cell types. By 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. 5C). 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. 5B,D) and ephrin B2-expressing (data not shown) 3T3 cells had a rounded appearance, whereas those co-cultured with 3T3-EPHB4 cells spread on the matrix substrate (Fig. 5C,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. 5F) migrated on average twice as far as those that were cultured with the 3T3-EPHB4 line (Fig. 5G; quantification shown in Fig. 5H). Co-cultures on Matrigel gave very similar results (Fig. 5H). In summary, these data support the hypothesis that the interactions of cytotrophoblasts with EPHB4-expressing cells, such as those that line uterine veins, restrict their migration, whereas interactions with ephrin B2-expressing cells, such as those that line maternal arteries, do not.

EPHB4 specifically regulates chemokine-induced migration

As 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 ephrin B 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. 6A. 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. By 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. 6B). By contrast, addition of EPHB4-Fc had differential effects on chemokine and growth factor actions (Fig. 6B). Specifically, chemokine-induced migration was inhibited by ~60%, whereas growth factor effects were not significantly different from those of the controls (Fig. 6C).

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 ephrin B1 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

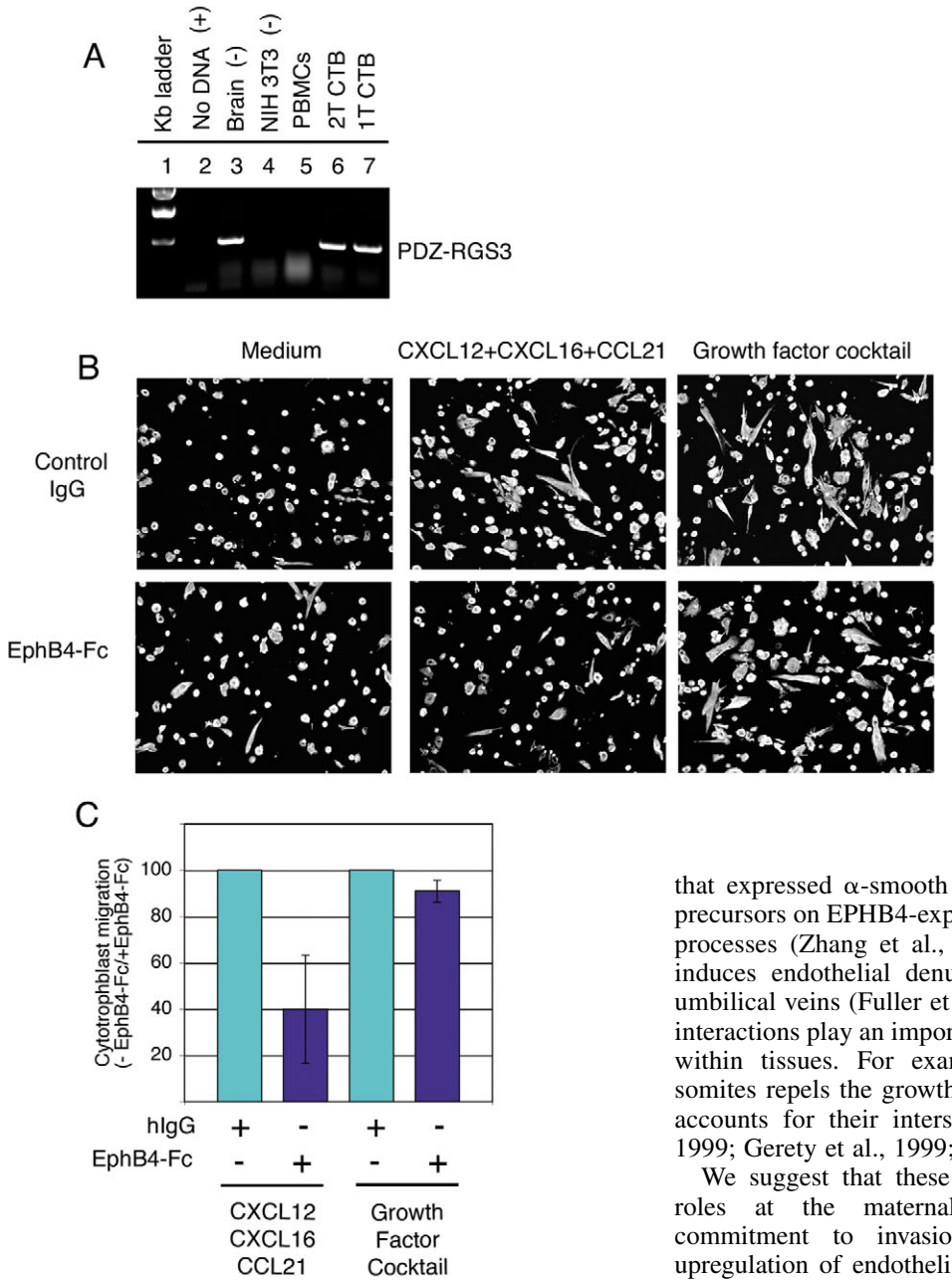


Fig. 6. EPHB4 decreases chemokine-stimulated cytotrophoblast migration. (A) RT-PCR detected expression of PDZ-RGS3 in RNA samples isolated from first trimester (1T) and second trimester (2T) cytotrophoblasts (CTB). PBMCs, peripheral blood mononuclear cells. +, positive control; –, negative control. (B) Cytokeratin 7 staining of cytotrophoblasts that had migrated to the underside of the transwell filter after overnight culture. EPHB4 decreased cytotrophoblast migration towards 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 s.d.

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-ephrin B2 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 ephrin B2-expressing OP9 stromal cells induced vascular network formation, as well as the proliferation of ephrin B2-positive arterial cells and the recruitment of a perivascular population that expressed α-smooth muscle actin. By contrast, plating precursors on EPHB4-expressing OP9 cells inhibited all these processes (Zhang et al., 2001). Additionally, ephrin B2-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, ephrin B 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., 1997) 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. 1) execute a dramatic downregulation of this transmembrane receptor and a concomitant upregulation of ephrin B1. Our data suggest that interactions between this receptor-ligand pair could form a boundary that orients cytotrophoblast invasion away from the placenta and towards the uterus (Fig. 7A). Our findings also suggest that acquisition of an arterial phenotype restricts cytotrophoblast migration into EPHB4-expressing uterine veins, resulting in asymmetrical vascular remodeling (Fig. 7B). 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

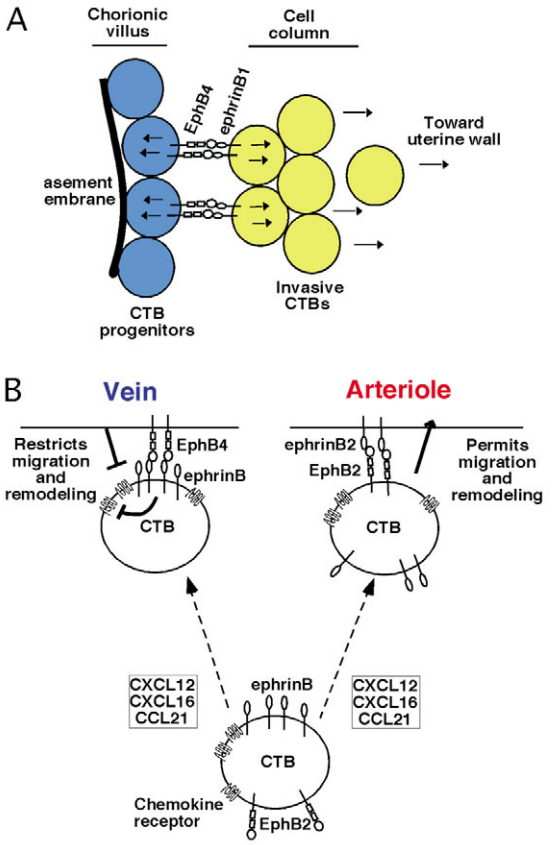


Fig. 7. Model describing the role of EPHs and ephrins in patterning chemokine-induced cytotrophoblast invasion. (A) A rapid switch in EPH and ephrin expression generates repulsive signals that orient cytotrophoblast (CTB) invasion away from the placenta and towards the uterine wall. (B) Within the uterine stroma, invasive cytotrophoblasts distinguish veins from arterioles based on their expression of EPHB4 and ephrin B2, 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. By contrast, interactions with ephrin B2-expressing cells permit migration. As a result, cytotrophoblast remodeling of the uterine vasculature is biased toward the arterial side of the circulation.

molecules. However, ephrin B2 and EPHB4-null mice die around the time period when the mature placenta begins to function, and ephrin B1 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 ephrin B2 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 ephrin B2 expression, which is restricted to invasive cytotrophoblasts.

Given the endothelial characteristics displayed by cytotrophoblasts, a similar interplay between ephrin Bs and chemokine receptors may occur during vasculogenesis and/or angiogenesis. Like EPHB4 and ephrin B2, 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-ephrin B2 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 ephrin B2 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, ephrin A1, is present on the uterine epithelium during the implantation window (Fujiwara et al., 2002) (K.R.-H. and S.J.F., unpublished). 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 onwards. 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. By contrast, partial defects in this process could lead to reduced arterial invasion, the hallmark of pre-eclampsia and of a subset of pregnancies complicated by intrauterine growth restriction. Thus, EPH and ephrin actions mediated by chemokines and their receptors could play crucial master regulatory roles in the initiation and continuation of human pregnancy.

We thank our collaborators for generously providing us with reagents: R. Zhou for ephrin B2 3T3 cells, D. Cerretti for ephrin B1 and B2 cDNA clones, and A. Welcher for an EPHB2 plasmid. We also thank D. Stretavan for technical advice on the substratum choice assay, T. Patel for assistance with time-lapse videomicroscopy, J. Cyster, C. Damsky and Z. Werb for their comments on the manuscript, and M. McKenney for excellent editorial advice. This work was supported by NIH grants R01 HD046744, R01 HL64597 and the National Institute of General Medical Sciences Minority Biomedical Research Support Research Initiative for Scientific Enhancement Grant Number R25 GM59298.

References

Adams, R. H., Wilkinson, G. A., Weiss, C., Diella, F., Gale, N. W., Deutsch, U., Risau, W. and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev.* **13**, 295-306.

Adams, R. H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U. and Klein, R. (2001). The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**, 57-69.

Adamson, S. L., Lu, Y., Whiteley, K. J., Holmyard, D., Hemberger, M., Pfarrer, C. and Cross, J. C. (2002). Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. *Dev. Biol.* **250**, 358-373.

Beckmann, M. P., Cerretti, D. P., Baum, P., Vanden Bos, T., James, L., Farrah, T., Kozlosky, C., Hollingsworth, T., Shilling, H., Maraskovsky,

E. et al. (1994). Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors. *EMBO J.* **13**, 3757-3762.
Birgbauer, E., Oster, S. F., Severin, C. G. and Sretavan, D. W. (2001). Retinal axon growth cones respond to EphB extracellular domains as inhibitory axon guidance cues. *Development* **128**, 3041-3048.
Caballero-Campo, P., Dominguez, F., Coloma, J., Meseguer, M., Remohi, J., Pellicer, A. and Simon, C. (2002). Hormonal and embryonic regulation of chemokines IL-8, MCP-1 and RANTES in the human endometrium during the window of implantation. *Mol. Hum. Reprod.* **8**, 375-384.
Cerretti, D. P., Vanden Bos, T., Nelson, N., Kozlosky, C. J., Reddy, P., Maraskovsky, E., Park, L. S., Lyman, S. D., Copeland, N. G., Gilbert, D. J. et al. (1995). Isolation of LERK-5: a ligand of the eph-related receptor tyrosine kinases. *Mol. Immunol.* **32**, 1197-1205.
Cowan, C. A., Yokoyama, N., Saxena, A., Chumley, M. J., Silvany, R. E., Baker, L. A., Srivastava, D. and Henkemeyer, M. (2004). Ephrin-B2 reverse signaling is required for axon pathfinding and cardiac valve formation but not early vascular development. *Dev. Biol.* **271**, 263-271.
Damsky, C. H., Fitzgerald, M. L. and Fisher, S. J. (1992). Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J. Clin. Invest.* **89**, 210-222.
Dickey, R. P. and Hower, J. F. (1995). Ultrasonographic features of uterine blood flow during the first 16 weeks of pregnancy. *Hum. Reprod.* **10**, 2448-2452.
Dominguez, F., Galan, A., Martin, J. J., Remohi, J., Pellicer, A. and Simon, C. (2003). Hormonal and embryonic regulation of chemokine receptors CXCR1, CXCR4, CCR5 and CCR2B in the human endometrium and the human blastocyst. *Mol. Hum. Reprod.* **9**, 189-198.
Drake, P. M., Gunn, M. D., Charo, I. F., Tsou, C. L., Zhou, Y., Huang, L. and Fisher, S. J. (2001). Human placental cytotrophoblasts attract monocytes and CD56bright natural killer cells via the actions of monocyte inflammatory protein 1 alpha. *J. Exp. Med.* **193**, 1199-1212.
Drake, P. M., Red-Horse, K. and Fisher, S. J. (2004). Reciprocal chemokine receptor and ligand expression in the human placenta: implications for cytotrophoblast differentiation. *Dev. Dyn.* **229**, 877-885.
Fischer, A., Schumacher, N., Maier, M., Sendtner, M. and Gessler, M. (2004). The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes Dev.* **18**, 901-911.
Fisher, S. J., Cui, T. Y., Zhang, L., Hartman, L., Grahl, K., Zhang, G. Y., Tarpey, J. and Damsky, C. H. (1989). Adhesive and degradative properties of human placental cytotrophoblast cells in vitro. *J. Cell Biol.* **109**, 891-902.
Fujiwara, H., Yoshioka, S., Tatsumi, K., Kosaka, K., Satoh, Y., Nishioka, Y., Egawa, M., Higuchi, T. and Fujii, S. (2002). Human endometrial epithelial cells express ephrin A1: possible interaction between human blastocysts and endometrium via Eph-ephrin system. *J. Clin. Endocrinol. Metab.* **87**, 5801-5807.
Fuller, T., Korff, T., Kilian, A., Dandekar, G. and Augustin, H. G. (2003). Forward EphB4 signaling in endothelial cells controls cellular repulsion and segregation from ephrinB2 positive cells. *J. Cell Sci.* **116**, 2461-2470.
Gao, P. P., Yue, Y., Cerretti, D. P., Dreyfus, C. and Zhou, R. (1999). Ephrin-dependent growth and pruning of hippocampal axons. *Proc. Natl. Acad. Sci. USA* **96**, 4073-4077.
Gerety, S. S. and Anderson, D. J. (2002). Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development* **129**, 1397-1410.
Gerety, S. S., Wang, H. U., Chen, Z. F. and Anderson, D. J. (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol. Cell* **4**, 403-414.
Goldman-Wohl, D., Greenfield, C., Haimov-Kochman, R., Ariel, I., Anteby, E. Y., Hochner-Celnikier, D., Farhat, M. and Yagel, S. (2004). Eph and ephrin expression in normal placental development and preeclampsia. *Placenta* **25**, 623-630.
Hanna, J., Wald, O., Goldman-Wohl, D., Prus, D., Markel, G., Gazit, R., Katz, G., Haimov-Kochman, R., Fujii, N., Yagel, S. et al. (2003). CXCL12 expression by invasive trophoblasts induces the specific migration of CD16- human natural killer cells. *Blood* **102**, 1569-1577.
Helbling, P. M., Saulnier, D. M. and Brandli, A. W. (2000). The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. *Development* **127**, 269-278.
Jaleel, M. A., Tsai, A. C., Sarkar, S., Freedman, P. V. and Rubin, L. P. (2004). Stromal cell-derived factor-1 (SDF-1) signalling regulates human placental trophoblast cell survival. *Mol. Hum. Reprod.* **10**, 901-909.
Kliman, H. J., Nestler, J. E., Sermasi, E., Sanger, J. M. and Strauss, J. F., 3rd (1986). Purification, characterization, and in vitro differentiation of cytotrophoblasts from human term placentae. *Endocrinology* **118**, 1567-1582.
Lawson, N. D., Scheer, N., Pham, V. N., Kim, C. H., Chitnis, A. B., Campos-Ortega, J. A. and Weinstein, B. M. (2001). Notch signaling is required for arterial-venous differentiation during embryonic vascular development. *Development* **128**, 3675-3683.
Librach, C. L., Werb, Z., Fitzgerald, M. L., Chiu, K., Corwin, N. M., Esteves, R. A., Grobelny, D., Galardy, R., Damsky, C. H. and Fisher, S. J. (1991). 92-kD type IV collagenase mediates invasion of human cytotrophoblasts. *J. Cell Biol.* **113**, 437-449.
Lu, Q., Sun, E. E., Klein, R. S. and Flanagan, J. G. (2001). Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. *Cell* **105**, 69-79.
Makinen, T., Adams, R. H., Bailey, J., Lu, Q., Ziemiecki, A., Alitalo, K., Klein, R. and Wilkinson, G. A. (2005). PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. *Genes Dev.* **19**, 397-410.
Metcalfe, J., Romney, S. L., Ramsey, L. H., Reid, D. E. and Burwell, C. S. (1955). Estimation of uterine blood flow in normal human pregnancy at term. *J. Clin. Invest.* **34**, 1632-1638.
Red-Horse, K., Drake, P. M., Gunn, M. D. and Fisher, S. J. (2001). Chemokine ligand and receptor expression in the pregnant uterus: reciprocal patterns in complementary cell subsets suggest functional roles. *Am. J. Pathol.* **159**, 2199-2213.
Red-Horse, K., Zhou, Y., Genbacev, O., Prakobphol, A., Foulk, R., McMaster, M. and Fisher, S. J. (2004). Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface. *J. Clin. Invest.* **114**, 744-754.
Sato, Y., Fujiwara, H., Zeng, B., Higuchi, T., Yoshioka, S. and Fujii, S. (2005). Platelet-derived soluble factors induce human extravillous trophoblast migration and differentiation: platelets are a possible regulator of trophoblast infiltration of spiral arteries. *Blood* **106**, 428-435.
Sakano, S., Serizawa, R., Inada, T., Iwama, A., Itoh, A., Kato, C., Shimizu, Y., Shinkai, F., Shimizu, R., Kondo, S. et al. (1996). Characterization of a ligand for receptor protein-tyrosine kinase HTK expressed in immature hematopoietic cells. *Oncogene* **13**, 813-822.
Salcedo, R. and Oppenheim, J. J. (2003). Role of chemokines in angiogenesis: CXCL12/SDF-1 and CXCR4 interaction, a key regulator of endothelial cell responses. *Microcirculation* **10**, 359-370.
Sapin, V., Bouillet, P., Oulad-Abdelghani, M., Dastugue, B., Chambon, P. and Dolle, P. (2000). Differential expression of retinoic acid-inducible (Stra) genes during mouse placentation. *Mech. Dev.* **92**, 295-299.
Sieg, D. J., Hauck, C. R., Ilic, D., Klingbeil, C. K., Schaefer, E., Damsky, C. H. and Schlaepfer, D. D. (2000). FAK integrates growth-factor and integrin signals to promote cell migration. *Nat. Cell Biol.* **2**, 249-256.
Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S. et al. (1998). The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* **393**, 591-594.
Wang, H. U., Chen, Z. F. and Anderson, D. J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* **93**, 741-753.
Wilkinson, D. G. (2001). Multiple roles of EPH receptors and ephrins in neural development. *Nat. Rev. Neurosci.* **2**, 155-164.
Zhang, X. Q., Takakura, N., Oike, Y., Inada, T., Gale, N. W., Yancopoulos, G. D. and Suda, T. (2001). Stromal cells expressing ephrin-B2 promote the growth and sprouting of ephrin-B2(+) endothelial cells. *Blood* **98**, 1028-1037.
Zhou, Y., Fisher, S. J., Janatpour, M., Genbacev, O., Dejana, E., Wheelock, M. and Damsky, C. H. (1997). Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J. Clin. Invest.* **99**, 2139-2151.
Zhou, Y., McMaster, M., Woo, K., Janatpour, M., Perry, J., Karpanen, T., Alitalo, K., Damsky, C. and Fisher, S. J. (2002). Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am. J. Pathol.* **160**, 1405-1423.