Endothelial EphrinB2 Is Controlled by Microenvironmental Determinants and Associates Context-Dependently With CD31

Thomas Korff, Gudrun Dandekar, Dennis Pfaff, Tim Füller, Winfried Goettsch, Henning Morawietz, Florence Schaffner, Hellmut G. Augustin

Objective—The EphB ligand ephrinB2 has been identified as a critical determinant of arterial endothelial differentiation and as a positive regulator of invading endothelial cells during angiogenesis. This study was aimed at identifying determinants of endothelial cell ephrinB2 expression.

Methods and Results—Arteriovenous asymmetrical endothelial cell ephrinB2 expression in vivo is lost on transfer into culture with aortic endothelial cells becoming partially ephrinB2-negative and saphenous vein endothelial cells becoming partially ephrinB2-positive. Contact with smooth muscle cells and angiogenic stimulation by vascular endothelial growth factor lead to an increased endothelial cell ephrinB2 expression. Quiescent, smooth muscle-contacting endothelial cells express ephrinB2 uniformly on their luminal surface. In contrast, monolayer endothelial cells translocate ephrinB2 to interendothelial cell junctions, which is strongly enhanced by EphB4-Fc-mediated receptor body activation. Functional ephrinB2 colocalizes and coimmunoprecipitates with CD31.

Conclusions—This study identifies distinct regulatory mechanisms of endothelial ephrinB2 expression and cellular distribution in quiescent and activated endothelial cells. The data demonstrate that endothelial cell ephrinB2 expression is controlled by microenvironmental determinants rather than being an intrinsic endothelial cell differentiation marker.

Key Words: endothelial cells | angiogenesis | EphB4 | ephrinB2 | VEGF | smooth muscle cells

The Eph receptors comprise the largest family of receptor tyrosine kinases. Their ligands, the ephrins, are membrane-associated molecules that are expressed as GPI-anchored peripheral membrane molecules (ephrinA) or as transmembrane molecules (ephrinB). B class ephrin ligands are capable of acting as signal-transducing molecules themselves, a process referred to as “reverse signaling.” Originally, Eph receptors and ephrin ligands have been identified as neuronal guidance and tissue border-forming molecules. More recently, Eph and ephrin molecules have been shown to be expressed by a number of non-neural cells, including endothelial cells, intestinal epithelial cells, hematopoietic cells, and tumor cells.

Genetic loss of function experiments in mice have revealed critical roles of EphB receptors and ephrinB ligands in early developmental vascular morphogenesis\(^4\)–\(^7\) and in lymphangiogenesis. Mice deficient in ephrinB2 or EphB4 have largely complementary phenotypes characterized by early embryonic lethality with disturbed arteriovenous differentiation. As such, ephrinB2 and EphB4, being asymmetrically expressed by arterial and venous endothelial cells, respectively, are not just markers of arteriovenous differentiation, but appear to be makers of proper arteriovenous assembly. Endothelial cells (ECs) in the adult maintain their asymmetrical arteriovenous expression of ephrinB2 and EphB4,\(^9,10\) suggesting that the EphB/ephrinB system may play a role in controlling vascular homeostasis. Yet little is known about the regulation and function of endothelial EphB receptor and ephrinB ligand expression in the adult vasculature. Genetic lineage tracking experiments in zebrafish suggest that vascular ephrinB2 expression may be an intrinsic property of arterial ECs. In contrast, embryonic artery to vein and vein to artery quail-chick grafting experiments have indicated that arteriovenous asymmetrical Eph-ephrin expression has some plasticity until midgestation when the arteriovenous transdifferentiation potential becomes more restricted.\(^12\) Analysis of ephrinB2 expression in the adult vasculature indicates that angiogenic EC express ephrinB2, which has been considered as evidence that angiogenic EC may be of arterial origin or that angiogenesis is an arteriolizing process.\(^9,10\) Likewise, recent microarray experiments have shown that at least some traits of organ-specific and caliber-specific EC differentiation...
are maintained even on prolonged culture in vitro. In turn, it is well-established that isolated EC rapidly lose their organ-specific and vessel caliber-specific properties on transfer into tissue culture, which suggests that microenvironmental cues may be required to maintain endothelial phenotypic differentiation. For example, shear stress has been identified as a major determinant of arteriovenous differentiation that affects endothelial ephrinB2 expression. Likewise, recent experiments in mice have shown that hypoxia controls ephrinB2 expression in endothelial cells.

Taken together, the recent literature provides evidence that arterial ephrinB2 expression is partially controlled by intrinsic genetic forces and in part by microenvironmental cues. To shed further light into the intrinsic versus microenvironmental regulation of arterial and angiogenic ephrinB2 expression, we have traced ephrinB2 expression in vivo and in vitro. We have also pursued manipulatory experiments in 2-dimensional and 3-dimensional EC culture and differentiation systems. The experiments demonstrate that arterial and angiogenic EC ephrinB2 expression is not an intrinsic property of distinct EC populations, but rather controlled by microenvironmental forces. Furthermore, the identification of luminal and intercellular junctional expression of ephrinB2 and the context-specific association of ephrinB2 with CD31 provide a strong rationale for specific functions of EC ephrinB2 in controlling vascular homeostasis and the trafficking of circulating cells.

Materials and Methods

For detailed methodology, please see http://atvb.ahajournals.org. Briefly, EC ephrinB2 expression in vivo and into culture was traced using reverse-transcription polymerase chain reaction (RT-PCR), in situ hybridization, Western blotting, immunocytochemistry, and immunohistochemistry. RT-PCR was performed as described previously. In situ hybridizations were performed using sense and antisense probes corresponding to bp 7 to bp 1028 from NCBI sequence NM-004093. Immunoprecipitations were performed using EphB4-Fc receptor bodies coupled to Protein-G-agarose. EphrinB2 polyclonal antibodies (R&D Systems) were used for Western blotting experiments. Cytochemical and immunohistochemical detection of ephrinB2 protein in cells and tissue sections was performed using EphB4-Fc receptor bodies and polyclonal ephrinB2 antibodies as detecting probes.

EphrinB2 mRNA and protein expression was studied in tissues and cultured ECs. Paraffin-embedded tissue sections were processed according to standard immunohistochemical techniques and used for in situ hybridizations, as well as EphB4-Fc receptor body and ephrinB2 antibody staining. Cellular expression of ephrinB2 mRNA and protein expression were traced in standard monolayer culture, as well as in differentiated 3-dimensional spheroidal cell culture techniques as described previously. Spheroidal EC culture included 3-dimensional coculture spheroids of EC and smooth muscle cells (SMCs), which mimic the spatial assembly of the physiological vessel wall in an inside-out orientation. This technique was particularly used because it represents the hitherto most advanced cellular model of the quiescent EC phenotype.

Results

Arteriovenous Asymmetrical EphrinB2 Expression In Vivo Is Lost on Transfer of ECs Into Culture

RT-PCR screening of different EC populations cultured in vitro showed that all analyzed EC populations, irrespective of their arterial or venous origin, express ephrinB2 mRNA (Figure I, available online at http://atvb.ahajournals.org). To more closely compare the in vivo and in vitro ephrinB2 expression pattern of individual ECs, in situ hybridization experiments were performed on sections of human aortas and saphenous veins, as well as on human umbilical arteries and veins (Figure 1). Corresponding to the reported mouse developmental arteriovenous asymmetrical expression pattern of ephrinB2, ECs in the adult human aorta are uniformly ephrinB2-negative (A, green arrows). Cultured ECs are all partially ephrinB2-positive, irrespective of their arterial or venous origin (A and B, green arrowheads). Scale bar, 100 μm.

Figure 1. Expression of ephrinB2 in adult human aorta (A), saphenous vein (A), fetal umbilical artery (B), and fetal umbilical vein (B) in vivo (left) and in vitro (right). Sections from human aorta (HAoEC) (A), human saphenous vein (HSaVEC) (A), umbilical artery (HUaEC) (B), and umbilical vein (HUVEC) (B) were analyzed histochemically for the expression of CD34 (IHC-CD34) and in situ hybridization for ephrinB2 (ISH-EB2). Correspondingly, monolayer of cultured human aortic EC (A), human saphenous vein EC (A), umbilical artery EC (B), and umbilical vein EC (B) were pelleted, embedded, and sectioned for the analysis of immunohistochemical CD31 detection (IHC-CD31) and in situ hybridization for ephrinB2 (ISH-EB2). Human aortic EC (HAoEC) in vivo are uniformly ephrinB2-positive (A, green arrows). In contrast, human saphenous EC (HSaVEC) in vivo are uniformly ephrinB2-negative (A, red arrows). ECs in the umbilical artery and in the umbilical vein are both ephrinB2-positive (B, green arrows). Cultured ECs are all partially ephrinB2-positive, irrespective of their arterial or venous origin (A and B, green arrowheads). Scale bar, 100 μm.
are the most frequently used human ECs studied in culture. We therefore assessed the expression of ephrinB2 by in situ hybridization in the human umbilical cord. Surprisingly, ephrinB2 shows no arteriovenous asymmetrical expression pattern in the vessels of the umbilical cord. ECs in both the umbilical artery and the umbilical vein are uniformly positive for ephrinB2 (Figure 1B).

Arterial and venous ECs lose their arteriovenous asymmetrical ephrinB2 expression pattern when they are removed from their microenvironment and cultured in vitro. EphrinB2 is expressed in subpopulations of all cultured EC populations independent of their arterial or venous origin (Figure 1A, 1B), indicating that arteriovenous asymmetrical EC ephrinB2 expression is not an intrinsic property of arterial cells, but rather controlled by microenvironmental cues.

Contact With Smooth Muscle Cells as Well as Stimulation by VEGF Control Endothelial EphrinB2 Expression

Based on the above findings, we next analyzed environmental conditions that may control EC ephrinB2 expression. It has been shown that contact with SMCs controls differentiation of ECs and induces EC quiescence. To analyze the capacity of SMC to modulate ephrinB2 expression in ECs, human umbilical vein endothelial cells (HUVECs) were cocultured with SMCs (human umbilical artery SMCs) in 3-dimensional EC/SMC coculture spheroids. Within these spheroids, ECs organize as surface monolayer enclosing a core of SMCs. EphrinB2 expression was analyzed by in situ hybridization and immunofluorescence staining using EphB4-Fc receptor bodies (Figure 2). ECs cultured in spheroids without SMCs do not or only minimally express ephrinB2 mRNA (Figure 2A, upper left) and protein (Figure 2A, lower left), respectively. In contrast, interaction of ECs with SMCs for 24 hours in EC/SMC coculture spheroids induces an increased expression of ephrinB2 in EC (Figure 2A, upper right [mRNA]; lower right [protein]). SMCs did not express detectable levels of ephrinB2 protein under any of the conditions used in this study (Figure II, available online at http://atvb.ahajournals.org).

In vivo experiments have shown that the upregulation of ephrinB2 in EC correlates with their angiogenic activation. Because VEGF is one of the most potent and the most specific inducers of angiogenesis, we analyzed if VEGF stimulation induces ephrinB2 expression in HUVECs cultured as standard monolayer. Approximately 60% of unstimulated early-passage HUVECs express detectable amounts of ephrinB2 (Figure 2B). Stimulation of ECs with VEGF increases the number of ephrinB2-positive cells close to 100% (Figure 2B).

Polarized Quiescent ECs Express EphrinB2 on Their Luminal Surface

These experiments indicate that contact with SMCs controls the expression of EC ephrinB2. Based on these findings, we performed high-resolution double-labeling experiments for ephrinB2 and CD31 to gain insights into the subcellular localization of ephrinB2 in quiescent endothelial cells in vivo and in vitro (Figure 3). Analysis of cross-sections of the umbilical vein identified the primarily junctional localization of CD31 (Figure 3A) and the luminal expression of ephrinB2 (Figure 3C and 3E). Corresponding double-labeling experiments of differentiated EC/SMC coculture spheroids similarly demonstrated ephrinB2 staining on top of the junctional and abluminal CD31 staining in the quiescent surface EC layer (Figure 3B, 3D, and 3F).

Eph-B4 Receptor Body Stimulation Induces the Junctional Translocation of EphrinB2

On confluence, ephrinB2-expressing HUVECs accumulate ephrinB2 at interendothelial cell junctions (Figure 2B, lower right; Figure 4A). Stimulation of confluent HUVEC monolayer with EphB4-Fc receptor bodies for 30 minutes leads to endocytosis of the resulting EphB4/ephrinB2 complex (Figure 4B versus 4A), confirming the reported resolution of cell
contact-dependent EphB4–ephrinB2 interactions by endocytosis. To probe the requirement of reverse ephrinB2 signaling for receptor body-mediated ligand endocytosis, we stimulated HUVECs expressing cytoplasmically truncated ephrinB2 with EphB4-Fc receptor bodies. Deletion of the cytoplasmic domain of ephrinB2 prevents endocytosis of the EphB4/ephrinB2 complex. Instead, the resulting EphB4/ephrinB2 complex translocates to cellular junctions (Figure 4D versus 4C). Based on these findings, we tested EphB4-Fc–mediated cellular ephrinB2 redistribution in porcine aortic endothelial cells (PAECs), which do not express endogenous ephrinB2. Intriguingly, noncontacting subconfluent PAECs express ephrinB2 in a clustered manner at lamellipodial cell protrusions, likely reflecting the invasive and migratory phenotype of tip cells in angiogenic sprouts, which is not affected by EphB4-Fc stimulation (Figure 4H versus 4G). Instead, ephrinB2 translocates primarily to interendothelial junctions (F), which is even more pronounced in ΔC-ephrinB2–expressing PAECs (H). Scale bar, 50 μm.

**Figure 3.** Luminal expression of ephrinB2 in quiescent EC in vivo and in vitro. Sections of the umbilical vein (left) and differentiated EC/SMC coculture spheroids (right) were double stained for CD31 (CD31) and ephrinB2 (ephrinB2). CD31 accumulates at interendothelial cell junctions in quiescent EC. EphrinB2 is located on top of the CD31 staining in vivo and in vitro indicating a luminal expression of ephrinB2 in quiescent, smooth muscle contacting EC (merge). Scale bars, 50 μm.

**Figure 4.** Interendothelial junctional accumulation of ephrinB2 following EphB4-Fc–mediated receptor body stimulation. HUVECs and PAECs expressing full-length ephrinB2 (ephrinB2) or cytoplasmically truncated ephrinB2 (ΔC-ephrinB2) were stimulated for 30 minutes with EphB4-Fc after which monolayers were fixed and the cells stained for ephrinB2. Unstimulated HUVECs express ephrinB2 on their cell surface and at cell–cell contacts (A, C). Stimulation of full-length ephrinB2-expressing HUVECs with EphB4-Fc leads to endocytosis of the receptor-ligand complex (B, arrow). In contrast, ΔC-ephrinB2–expressing cells are not capable to endocytose the receptor-ligand complex, but translocate ephrinB2 to interendothelial junctions (D). Similar effects were observed in PAECs. Yet, PAECs show only minimal receptor-ligand complex endocytosis (F, arrow). Instead, ephrinB2 translocates primarily to interendothelial junctions after EphB4-Fc stimulation (F), which is even more pronounced in ΔC-ephrinB2–expressing PAECs (H). Scale bar, 50 μm.

**CD31 Associates Context-Dependently With EphinB2 in ECs**

Based on the observed junctional translocation of ephrinB2 in confluent and particularly EphB4 receptor body-stimulated ECs, we performed colocalization experiments of ephrinB2 with the junctional molecule CD31. As shown in Figure 5, CD31 (Figure 5A) and ephrinB2 (Figure 5B) colocalize at interendothelial junctions in contacting HUVECs (Figure 5C). Based on these findings, we performed coimmunoprecipitation experiments to examine if ephrinB2 and CD31 interact directly in endothelial cells. Immunoprecipitation of...
ephrinB2 from HUVECs followed by ephrinB2 Western blot identifies only a faint ephrinB2 band corresponding to the weak expression of ephrinB2 in unstimulated HUVECs (Figure 4D, lane 1). Yet this weak ephrinB2 signal coprecipitated a prominent CD31 band (Figure 4D, lane 1). Stimulation of monolayer ECs with VEGF or coculture of HUVECs with SMCs in spheroids leads to a more intense ephrinB2 precipitation band (Figure 5D, lanes 2 and 3), confirming the observed positive regulation of EC ephrinB2 expression by contact with SMC (Figure 2A) and VEGF (Figure 2B). Precipitation of ephrinB2 from VEGF-stimulated HUVECs leads to prominent coprecipitation of CD31 (Figure 5D, lane 2). Yet coculture of ECs with SMCs in 3-dimensional coculture spheroids leads to barely detectable CD31 coprecipitation with ephrinB2 (Figure 5D, lane 3). Coculture of ECs with SMCs in 3-dimensional spheroids mimics the quiescent EC phenotype. We therefore pursued ephrinB2/CD31 coinmunoprecipitation experiments from ECs freshly scraped from intact umbilical cords. These in vivo-derived HUVECs express abundant ephrinB2 (Figure 5D, lane 5). Yet these quiescent EC do not coprecipitate CD31.

To further validate and study the observed association of ephrinB2 and CD31, we pursued coinmunoprecipitation experiments with PAECs expressing full-length ephrinB2 and cytoplasmically truncated ephrinB2. As in HUVECs, ephrinB2-expressing PAECs coinmunoprecipitate CD31 with ephrinB2 (Figure 5E, lane 1). Stimulation with EphB4-Fc leads to a transient increase of ephrinB2/CD31 complex formation (Figure 5E, lanes 2 and 3) corresponding to the receptor body stimulated translocation of ephrinB2 to interendothelial cell junctions (Figure 4). Surprisingly, ΔC-ephrinB2–expressing PAECs show only barely detectable CD31 coinmunoprecipitation (Figure 5E, lane 4). Yet intense association of ephrinB2 and CD31 is detectable in ΔC-ephrinB2 PAEC on EphB4-Fc receptor body stimulation.

Discussion

EC expression of the EphB ligand ephrinB2 is required for embryonic vessel formation. EphrinB2-deficient mice are not capable to support proper arteriovenous differentiation and subsequently vessel assembly.4–7 These genetic studies have identified ephrinB2 as a molecular marker of arterial ECs that is expressed at the earliest onset of arterial vascular differentiation. Yet little is known about the role of ephrinB2 in the adult vasculature, even though it is well-established that the arteriovenous asymmetrical expression of ephrinB2 is maintained in the adult vasculature.9,10 Sustained arteriovenous asymmetrical expression of ephrinB2 in the adult vasculature could point to important homeostatic maintenance functions, for example by supporting the cross-talk between ECs and SMCs. Alternatively, endothelial ephrinB2 in adults could be involved in controlling interactions of circulating cells with the vessel wall. For example, several EphB receptors and ephrinB ligands have been shown to be involved in leukocyte and lymphocyte function.27–29 Similarly, EphB/ephrinB interactions have been shown to regulate platelet aggregation.30

To shed further light into the mechanisms controlling arteriovenous differentiation and to guide experiments on the functional role of vascular EphB/ephrinB interactions in adults, this study was aimed at exploring the expression of the arterial and angiogenic marker ephrinB2 in ECs. Using a combination of in vivo and ex vivo analytical and in vitro manipulatory techniques, the experiments have shown that:
(1) the asymmetrical arteriovenous expression of ephrinB2 in vivo is lost on transfer into culture; (2) contact with SMCs (quiescent phenotype) and stimulation with VEGF (angiogenic activation) upregulate ephrinB2 expression in ECs; (3) quiescent resting ECs express ephrinB2 on their luminal cell surface; (4) ephrinB2 accumulates at interendothelial cell junctions on EphB4 receptor body activation; and (5) junctional ephrinB2 associates with CD31.

The findings of this study are likely to have important functional and conceptual implications. First, adding to the complexity of intrinsic versus microenvironmental endothelial cell phenotypic regulation, the present study has shown that ECs ephrinB2 expression is not an intrinsic determinant of the arterial EC phenotype, but rather needs to be maintained by local microenvironmental cues. Arteriovenous differentiation has been characterized as a primarily genetically driven process during development. Correspondingly, gene array experiments have shown that cultured ECs maintain some traits of their original vascular bed even when maintained in culture for prolonged periods of time. In turn, it is well-established that organ-specific and caliber-specific EC differentiation and phenotypic heterogeneity is also under microenvironmental control. In line with these findings, the present study has demonstrated that the expression of the developmental arterial marker ephrinB2 in the adult must be maintained by local microenvironmental cues.

Second, the search for microenvironmental factors that control EC ephrinB2 expression identified contact with SMCs and stimulation by VEGF as strong positive regulators of ephrinB2 expression. These findings suggest that constitutive arterial and activation-associated angiogenic EC expression of ephrinB2 may be 2 mechanistically related, albeit functionally and temporally different, ephrinB2 expression states of the vascular endothelium. VEGF-mediated angiogenic activation is associated with ephrinB2 expression and an arteriolizing EC phenotype. These findings correspond to cellular experiments demonstrating that EC ephrinB2 expression mediates invasive and propulsive signals which are compatible with a proangiogenic phenotype. Conversely, the observed prominent regulation of ephrinB2 expression by contact with SMCs appears to reflect the quiescent arterial EC phenotype. This corresponds also to the recently observed prominent ephrinB2 expression in ECs of collecting lymphatic vessels that are in contact with SMCs.

Third, the present study has identified distinct spatial distribution pattern of EC ephrinB2. Quiescent cells in vivo and in a 3-dimensional spheroid culture model of ECs and SMCs that mimics many of the properties of the quiescent monolayer in vivo express ephrinB2 in a strictly luminal expression pattern. In turn, confluent monolayer EC expression ephrinB2 at intercellular junctions, which is enhanced by stimulation with EphB4-Fc receptor bodies. Prolonged stimulation with EphB4-Fc leads to endocytosis of the receptor–ligand complex, as has been reported during the EphB/ephrinB-mediated termination of adhesion after contact-mediated cell–cell repulsion. The luminal localization experiments of this study were limited to the umbilical cord and ECs cultured in EC/SMC coculture spheroids. Light microscopic analysis does not allow the precise dissociation of the luminal and the abluminal EC surface in most vascular bed with flattened EC. In turn, ultrastructural techniques and perfusion labeling techniques are not yet established to examine ephrinB2 expression in other vascular beds. It therefore remains to be demonstrated if ephrinB2 is luminally expressed in all ephrinB2-positive quiescent vascular beds. These will be critical experiments because the luminal expression of ephrinB2 in quiescent ECs would strongly imply EphB/ephrinB2 interactions in the control of circulating cell interactions with the vessel wall, as has recently been shown for the interaction of lymphocyte-expressed EphA receptors with high endothelial venule-expressed ephrinA. Correspondingly, EphB-expressing monocytes adhere preferentially to ephrinB2-expressing EC (D.P., unpublished results). EphB receptor body stimulation leads to junctional translocation of surface expressed EC ephrinB2. This may hint at a role of the EphB/ephrinB2 system in gating adherent cells to interendothelial cell junctions to prime them for transendothelial cell migration.

Fourth, a role of EC ephrinB2 in regulating the trafficking of adherent cells is also supported by the observation that junctional ephrinB2 associates with CD31. CD31 is an important adhesion molecule of ECs that has been shown to be involved in several signaling pathways that include the participation in the maintenance of adherens junction integrity and permeability, organization of the intermediate filament cytoskeleton, and regulation of catenin localization and transcriptional activities. The experiments have firmly established the association of CD31 with ephrinB2 in endothelial cells. Yet it remains to be seen if this is a direct interaction or if CD31 and ephrinB2 coimmunoprecipitate within a junctional complex that may involve other molecules such as VE-cadherin and VEGFR-2. Future work will be aimed at mechanistically dissecting the role of EC expressed ephrinB2 in controlling EC junctional organization, leukocyte recruitment, and leukocyte transmigration.

In summary, despite its inherently analytical nature, this study has shed important novel light into the regulation and possible roles of ECs expressed ephrinB2. EC ephrinB2 expression is under microenvironmental control (VEGF, contact with SMCs). Quiescent ECs express ephrinB2 on their luminal cell surface, from where it can be translocated to junctional complexes to associate with CD31. Collectively, these findings suggest important functions of the EphB/ephrinB system in controlling vascular homeostasis.

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Supplementary Fig. I

Supplementary Figure I. EphrinB2 expression in cultured vascular cells. The expression of ephrinB2 was analyzed by PCR in cultured adult human arterial and venous endothelial cells (HAoEC, HSaVEC) and embryonal endothelial cells isolated from human umbilical artery (HUAEC) and vein (HUVEC). EphrinB2 is expressed in the endothelial cell populations tested as well as in cultured smooth muscle cells isolated from the umbilical artery (HUASMC). The housekeeping gene TBP (TATA box binding protein) was used as loading control.
Supplementary Figure II. Comparison of ephrinB2 expression in cultured HUVEC and HUASMC. HUVEC, but not HUASMC express detectable levels of ephrinB2 (left: Western blot analysis, right: immunocytochemistry).
Supplementary Figure III. Expression of ephrinB2 in subcellular, non-contacting and in semi-confluent, barely contacting PAEC. Unstimulated cells (A,C) and cells stimulated for 60 min with EphB4-Fc (B,D) were fixed and stained for ephrinB2. Non-contacting PAEC express ephrinB2 at lamellipodial protrusions (A,B, arrows). Semi-confluent PAEC express ephrinB2 uniformly on their cell surface (C). Stimulation with EphB4-Fc induces the translocation of ephrinB2 which accumulates at intercellular contacts (D, arrow). Scale bars: 20 µm.
Detailed Materials and Methods:

Antibodies, growth factors and reagents

Recombinant mouse Eph-B4/Fc chimera, anti-EphrinB2 antibody and human recombinant VEGF were obtained from R&D Systems GmbH (Wiesbaden, Germany). Methylcellulose (4,000 centipoises, cat# M-0512) was from Sigma (Deisenhofen, Germany). The monoclonal mouse anti-hCD31 antibody was obtained from Dako (Glostrup, Denmark), the mouse anti-hCD34 was from Novocastra Ltd (England). Soluble monomeric EphB4 was produced by extracting and reverse transcribing RNA from renca tumors using primers specific for the extracellular domain of EphB4. The sequence was amplified by PCR and verified by sequencing. Then the fragment was subcloned in frame with 6×His-Myc tags into KS vector (NcoI digest). The extracellular domain of EphB4 plus the 6×His-Myc tags was subcloned into pVL1392 (Bioscience) vector. Sf9 insect cells were then infected with Baculogold DNA (Pharmingen pACGP67A) and pVL1392mEphB4-Myc. The protein produced in the Sf9'supernantant were purified by coupling with agarose beads (Quiagen) overnight at +4°C. The samples were passed through a column washed and eluted by adding 40 mM imidazole. Different fractions were analysed by silver staining, dialysed against PBS and stored at -20°C.

Cell culture

Human umbilical vein endothelial cells (HUVEC), human umbilical artery endothelial cells (HUAEC), human aortic endothelial cells (HAoEC), human saphenous vein endothelial cells (HSaVEC) and human umbilical artery smooth muscle cells (HUASMC) were purchased from Promocell (Heidelberg, Germany) and cultured at 37°C using endothelial cell growth media (ECGM) and endothelial cell population specific growth supplements according to manufacturers instructions. Only primary human cells up to passage 5 were employed in the experiments. Porcine aortic endothelial cells were cultured in NUT.MIX.F-12 (Ham) with GLUTAMAX-1 (Invitrogen, Karlsruhe, Germany) including 1 µg/ml Amphotericin-B
(Invitrogen, Karlsruhe, Germany), Gemtamycin Sulfate 5 µg/ml (Biochrom, Berlin, Germany) and 10% fetal calf serum (Biochrom, Berlin, Germany).

**Transfection of PAE cells**

Full length ephrinB2 was cloned by RT-PCR amplification from freshly isolated HUVE cells using specific primers. Sequence controlled cDNAs were subcloned into pcDNA3.1+ expression vector (Invitrogen, Karlsruhe, Germany) and transfected into PAE cells by electroporation. Individual 500 µg/ml G418 (PAA, Cölbe, Germany) resistant clones were isolated and expanded. Expression of the transfected cDNA was confirmed by RT-PCR and corresponding receptor body staining.

**Generation of endothelial cell and EC-SMC co-culture spheroids**

Spheroids of defined cell number were generated as described previously. In order to generate co-culture spheroids, equal numbers of suspended SM and HUVE cells (1,500 HUASMC and 1,500 HUVEC per spheroid) were mixed. Spheroids were cultured for at least 24 hours and used for the corresponding experiments.

**RT-PCR Analysis of Eph-B and ephrinB2**

cDNA was reverse transcribed from 0.5 µg RNA of EC or leukocytes and amplified with specific primers for EphB or ephrinB2 as described before by Kim et al. For semiquantitative analysis of gene expression TBP (5’-ATG GAT CAG AAC AAC AGC CTG-3’ and 3’-CCCTGT GTT GCC TGC TGG GA-3’) was used as standard in an separate reaction. Quantification of band intensitiy was performed by scion image analysis and ratio to TBP was calculated. As negative control cDNA reaction without reverse transcriptase was used.

**In Situ Hybridization**

Human ephrinB2 riboprobe was produced from pcDNA3 vector containing bp 7 to 1028 from NCBI sequence NM_004093. Antisense strand and sense control were transcribed by Sp-6
polymerase after linearization by BamHI digestion or by T-7 polymerase after NotI digestion, respectively and labeled with digoxigenin (digoxigenin RNA labeling kit, Roche). RNA was subsequently precipitated with ethanol and ammonium acetate. In situ hybridization was performed generally as described previously by Othman-Hassan and colleagues. In brief 4 µm paraffin-sections on SuperFrost Plus slides (Menzel) were deparaffinized, hydrated and postfixed with 4 % paraformaldehyde, treated with proteinase K (Sigma) and refixed with 4 % PFA. Hybridization mixture contained 40% formamide, 25 % 20 x SSC 1 % Denhardt’s solution 1 % tRNA and 1 % hering sperm DNA and DEPC-water. 100 ng of labeled antisense or sense probe were added to 60 µl of this mix and incubated on each slide over 72 hours at 65°C. Digoxigenin was detected by an anti-digoxigenin antibody conjugated to alkaline phosphatase (1: 4000, at 4°C overnight, Roche). BCIP/NBT (Roche) revealed a blue reaction product with alkaline phosphatase after 3 to 5 days. Sense controls did not develop any signal.

**Eph-B4-Fc staining**

Cells were fixed with cold methanol for 15 minutes at 4°C. After 30 minutes blocking with 10% goat serum in PBS (Sigma) 1 µg/ml Eph-B4-Fc (R & D) was applied for 1 hour at room temperature. As second antibody an anti-human Fc specific antibody from goat conjugated to Cy3 was used (Sigma, 1:100, 30 minutes room temperature). Nuclei stainings were performed with DAPI 1:5000 (Höchst) for 10 minutes. Stained cells were embedded in Kaiser’s glycerin gelatin from Merck.

**Immunohistochemical analysis**

Spheroids, pelleted monolayer cells and umbilical cords were fixed in phosphate buffered 4% formaldehyde and embedded in paraffin. For histochemical analyses, paraffin sections (5 µm) were cut, deparaffinized, and rehydrated. After washings in PBS, the sections were incubated for 30 min with blocking solution (10% normal rabbit serum) followed by incubation with the primary antibodies (anti-EphrinB2, anti-CD31, anti-CD34) diluted according to
manufacturer instructions in a humid chamber at 4°C overnight. They were then incubated with secondary antibody (biotinylated rabbit anti-goat or rabbit anti-mouse antibody (DAKO, Glostrup, Denmark), exposed to RPE labeled streptavidin (fluorescence staining) or streptavidin peroxidase. Peroxidase treated samples were developed with diaminobenzidine as substrate, and weakly counterstained with hematoxylin.

**Immunoprecipitation and Western blotting**

To precipitate ephrinB2, EphB4-Fc (4 µg/ml) was coupled to 20 µl Protein-G-agarose (Roche Diagnostics, Mannheim, Germany) in the presence of 250 µl lysis buffer. Cell lysates (1.0 ml) containing 2 mM Na₃VO₄ and protease inhibitor cocktail (Sigma, Deisenhofen, Germany) were precleared with 20 µl Protein-G-agarose for 2 hours at 4°C. The cleared cell lysates were incubated with the Protein-G-agarose coupled EphB4. Precipitates were washed, lysed, and run on a 10% SDS-PAGE. Western blotted gels were probed with anti-CD31-antibody (Santa Cruz, Heidelberg, Germany), visualized by chemiluminescence, stripped and reprobed with the anti-ephrinB2 antibody (0.2 µg/ml).

**References**


4. Kim I, Ryu YS, Kwak HJ, Ahn SY, Oh JL, Yancopoulos GD, Gale NW, Koh GY. EphB ligand, ephrinB2, suppresses the VEGF- and angiopoietin 1-induced Ras/mitogen-