Forward EphB4 signaling in endothelial cells controls cellular repulsion and segregation from ephrinB2 positive cells

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Summary

Contact-dependent interactions between endothelial cells (ECs), as well as between ECs and mural cells, play a key role in the formation of a regular vascular system and the assembly of the vessel wall. Recent studies have identified ephrinB2 and EphB4 as markers and makers of arterio-venous differentiation during vascular development. On the basis of these findings, we hypothesized that Eph-ephrin interactions in the vascular system mediate distinct propulsive and repulsive effector functions that provide guidance signals for the proper spatial organization of the developing vasculature. Utilizing a set of specialized endothelial differentiation and angiogenesis assays, the present study was aimed at studying vascular morphogenic functions of endothelial EphB4 and ephrinB2 activation. EphrinB2-Fc acts anti-adhesively and induces detachment of ECs, as demonstrated by (1) inhibition of adhesion to ephrinB2-Fc-coated culture dishes, (2) detachment of ECs grown as differentiated 3D spheroids, and (3) endothelial denudation of explanted fragments of umbilical vein. Conversely, soluble ephrinB2-Fc inhibits lateral cell migration, vascular endothelial growth factor (VEGF) gradient-driven chemotaxis, capillary-like network formation and sprouting angiogenesis. In turn, soluble EphB4-Fc is pro-adhesive and stimulates EC migration and sprouting angiogenesis. EphrinB2-mediated repulsive signals are transduced by EphB4, as demonstrated by EphrinB2-Fc inhibition of sprouting angiogenesis of constitutively EphB4-overexpressing ECs. Confrontation experiments of EphB4-overexpressing ECs with ECs overexpressing full-length or truncated ephrinB2 that lacks the cytoplasmic catalytic domain demonstrated that forward EphB4 signaling with EphB4 tyrosine phosphorylation restricts intermingling of cells and supports cellular segregation. Taken together, these data identify distinct propulsive and repulsive effector functions of endothelial ephrinB2 and EphB4 that mediate spatial positional signals during angiogenesis and vessel assembly.

Key words: Endothelial cells, Angiogenesis, EphB4, EphrinB2, VEGF

Introduction

Eph receptors and their ephrin ligands comprise the largest family of receptor tyrosine kinases (Klein, 2001; Kullander and Klein, 2002). Originally identified as neuronal guidance and tissue-border formation mediating molecules, Eph and ephrin molecules have now been shown to be expressed by a number of non-neural cells, including ECs, hematopoietic cells and tumor cells (Adams, 2002; Dodelet and Pasquale, 2000). EphB receptors interact with their corresponding ephrinB ligands, which themselves act as transmembrane tyrosine kinase signaling molecules. Thus, EphB-ephrinB interactions are capable of mediating bidirectional signaling events upon cell-cell contact (Frisen et al., 1999; Kullander and Klein, 2002).

Genetic loss-of-function experiments in mice have revealed crucial roles of EphB receptors and ephrinB ligands in early developmental vascular morphogenesis (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Mice deficient in ephrinB2 or EphB4 have largely complementary phenotypes characterized by early embryonic lethality with disturbed arterio-venous differentiation. As such, ephrinB2 and EphB4, being asymmetrically expressed by arterial and venous ECs, respectively, do not just appear to be markers of arterio-venous differentiation, but are in fact makers of proper arterio-venous assembly. This conclusion is also supported by lineage-tracking experiments in the zebrafish embryo, which have suggested that asymmetric Eph-ephrin expression is genetically controlled and occurs prior to the formation of arteries and veins (Zhong et al., 2001). By contrast, embryonic artery-to-vein and vein-to-artery quail-chick grafting experiments have indicated that arterio-venous asymmetric Eph-ephrin expression has some plasticity until midgestation before the arterio-venous transdifferentiation potential becomes more restricted (Moyon et al., 2001). Likewise, developmental expression profiling of the vasculature in the human lung revealed that EphB4 and ephrinB2 do not distinguish between presumptive venous and arterial endothelium (Hall et al., 2002).

Mice that just lack the cytoplasmic catalytic domain of ephrinB2 have an early embryonic lethal phenotype similar to

total ephrinB2-knockout mice (Adams et al., 2001), indicating that bidirectional forward and reverse EphB4-ephrinB2 signaling is required for vascular morphogenesis and proper arterio-venous differentiation. EphrinB2 binds EphB2 and EphB3 in addition to EphB4. Correspondingly, some double EphB2- and EphB3-deficient mice die before E11.5 of embryonic development, indicating that EphB4-ephrinB2 signaling alone cannot fully compensate for the lack of EphB2 and EphB3 (Adams et al., 1999).

Functionally, Eph receptors have primarily been characterized as a repulsion-mediating signaling system. Neuronal Eph receptor activation inhibits axonal outgrowth and leads to growth cone collapse (Wilkinson, 2001). Repulsive Eph receptor signaling results as the consequence of crosstalk phenomena with integrin adhesion (Huynh-Do et al., 1999; Zou et al., 1999), as well as cytoskeletal organization involving Rho (Carter et al., 2002). Yet, recent work also suggests that Eph receptors might act in a bimodal manner, being capable of transmitting both pro-adhesive and anti-adhesive signals, which has been demonstrated by opposing functions of different splice forms of EphA7 (Holmberg et al., 2000). Similarly, reverse ephrinB signaling has been implicated in both attractive and repulsive functions (Kullander and Klein, 2002).

In contrast to the dramatic vascular phenotypes of EphB- and ephrinB-deficient mice, as well as the increasingly understood forward and reverse signaling mechanisms, little is known about the functional consequences of Eph and ephrin signaling in the vessel wall at the cellular level. Activation of endothelial EphB1 receptors was shown to promote endothelial capillary-like assembly, cell attachment and the recruitment of low-molecular-weight phosphotyrosine phosphatase (LMW-PTP) to receptor complexes (Stein et al., 1998). Likewise, activation of EphB4 supposedly stimulates EC migration and proliferation via activation of phosphoinositide 3-kinase (PI 3-kinase) (Steinle et al., 2002). Activation of endothelial ephrinB1 was shown to promote integrin-mediated migration, attachment and angiogenesis (Huynh-Do et al., 2002), which would imply that both endothelial EphB receptors and ephrinB ligands might be able to act in a pro-adhesive and pro-angiogenic manner. EphB4-Fc receptor bodies, activating ephrinB2, have been shown to act pro-angiogenicly (Adams et al., 2001). By contrast, EphB4 expressed by co-cultured stromal cells has been reported to inhibit vascular network formation of ephrinB2-expressing ECs (Zhang et al., 2001; Helbling et al., 2000).

In order to resolve these apparent discrepancies in some recent reports, the present study was aimed at specifying cellular functions mediated by forward and reverse EphB receptor and ephrinB ligand signaling during angiogenesis and vascular assembly. We applied a set of specialized 3D in vitro angiogenesis and EC differentiation assays in combination with more-conventional adhesion, lateral migration and chemotaxis experiments, and studied endogenous EphB receptor- and ephrinB ligand-expressing human umbilical vein ECs (HUVECs), as well as EphB4- and ephrinB2-overexpressing ECs. Collectively, the experiments demonstrate endothelial EphB-ephrinB signaling to act in a bidirectional antagonistic manner with EphB4 mediating anti-adhesive and repulsive signaling and ephrinB2 mediating attractive and pro-angiogenic activities.

Materials and Methods

Cell culture

EC growth medium (ECGM), EC basal medium (ECBM), smooth muscle cell growth medium 2 (SMCGM2), the corresponding media supplements and human umbilical artery smooth muscle cells (HUASMCs) were purchased from Promocell (Heidelberg, Germany). HUVECs were freshly isolated from human umbilical veins by collagenase digestion. Cells were cultured at 37°C, 5% CO_2, 100% humidity in the corresponding media containing 10% FCS (Biochrom, Berlin, Germany). Only HUVECs/HUASMCs cultured from passage 4 to 8 were used for experiments. Porcine aortic ECs (PAECs) were cultured in NUT.MIX.F-12 (Ham) with GLUTAMAX-1 (Invitrogen, Karlsruhe, Germany) including 1 μg/ml amphotericin-B (Invitrogen), 5 μg/ml gemtamycin sulfate (Biochrom) and 10% FCS (Biochrom).

Transfection of PAE cells

Full-length ephrinB2 was cloned by RT-PCR amplification from freshly isolated HUVECs using specific primers. EphrinB2 constructs lacking the cytoplasmic domain were generated using primers that only included the first 266 amino acid residues and lacked the C-terminal 67 amino acids (designated ΔephrinB2). Full-length murine EphB4 (mEphB4) cDNA was kindly provided by Ralf H. Adams (CRUK, London). Sequence-controlled cDNAs were subcloned into the pcDNA3.1$^+$ expression vector (Invitrogen) and transfected into PAECs 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 EC and EC-SMC co-culture spheroids

Spheroids of defined cell number were generated as described previously (Korff and Augustin, 1998; Korff et al., 2001). In order to generate co-culture spheroids, equal numbers of suspended HUASMCs and HUVECs (1500 of each per spheroid) were mixed. Spheroids were cultured for at least 24 hours and used for the corresponding experiments.

Attachment assay

Adhesive (Nunc, Wiesbaden, Germany) and non-adhesive (Greiner, Frickenhausen, Germany) 24-well plates were coated in the center of each well using 10 μl of either EphB4-Fc or ephrinB2-Fc (both R&D Systems, Wiesbaden, Germany) diluted in PBS (100 ng/μl each) and were incubated for 20 minutes. Suspended HUVECs were cultured for 24 hours on top of the coated plates, washed and fixed. Cellular attachment was quantified by measuring the area covered by HUVECs or computer-aided cell counting of DAPI- (1:5000; Hoechst, Frankfurt, Germany) stained nuclei analyzing at least five different microscopic fields of view inside the coated area per experimental group and experiment using the digital imaging software DP-Soft (Olympus, Germany)

Planar segregation assay

Equal numbers of different combinations of PAECs overexpressing ephrinB2, ΔephrinB2, EphB4 or control cells (mock) were plated and allowed to grow to confluence. Monolayers were paraformaldehyde-fixed after 3 days and analyzed following EphB4 and ephrinB2 receptor body staining.

Lateral cell migration

Planar migration was quantitated as described previously (Augustin-Voss et al., 1992). In brief, a silicon barrier (6 mm × 3 mm) was placed

in the middle of a tissue culture dish seeded with HUVECs. After removal of the barrier, cells were stimulated with either EphB4-Fc or ephrinB2-Fc (1 μg/ml dissolved in ECBM/10% FCS), or 50 ng/ml VEGF, or a combination of VEGF and ephrinB2-Fc, for 48 hours. After this, lateral cell migration was quantitated microscopically.

Boyden chamber assay

A modified Boyden chamber assay was performed using 48-well chambers (Neuroprobe, Gaithersburg, MD) using polyvinylpyrrolidone-free polycarbonate membranes with 8 μm pores (Costar, Cambridge, UK). Membranes were coated overnight in the appropriate cell culture media (RPMI1640, 0.5% FCS, 0.1% BSA), washed and coated for 1 hour with media containing 0.1% fibronectin (Sigma, Deisenhofen, Germany). VEGF (20 ng/ml; R&D Systems) was loaded in 36 wells of the lower compartment, whereas 12 wells were loaded only with media. Starved HUVECs were seeded into the upper compartment with either medium alone (control A: medium, upper and lower compartment; control B: medium, upper compartment, VEGF, lower compartment), ephrinB2-Fc (2 μg/ml) or EphB4-Fc (2 μg/ml). The cells were allowed to migrate for 4 hours. Following incubation, the cells on the upper side of the membrane were removed, the membrane was fixed and cells were stained with DAPI. Migration of cells across the membrane was quantified by computer-aided cell counting using the Olympus DP-Soft analysis software.

EC alignment assay

Alignment of ECs was studied by seeding 25,000 HUVECs on 350 μl polymerized Matrigel (B&D Biosciences, Heidelberg, Germany) in 24-well plates. Cells were stimulated with 2 μg/ml ephrinB2-Fc or EphB4-Fc for 24 hours, after which the cells were fluorescence labeled by treatment with 1 μg/ml calceinAM (Molecular Probes, Leiden, The Netherlands) for 30 minutes. Network formation was analyzed by automated computer-aided fluorescence microscopic image analysis quantitating the circumference of the network structures.

In vitro angiogenesis assay

In vitro angiogenesis in collagen gels was performed using EC spheroids as described previously (Korff and Augustin, 1999). In vitro angiogenesis was digitally quantitated by measuring the cumulative length of the sprouts that had grown out of each spheroid (ocular grid at 100× magnification) using the Olympus DP-Soft analysis software analyzing at least ten spheroids per experimental group and experiment.

Morphological and immunohistochemical analysis

Umbilical veins were cut into slices of approximately 2 mm, washed and incubated overnight in ECBM/10% FCS including either 2 μg/ml EphB4-Fc or ephrinB2-Fc. Explants were fixed and processed for paraffin embedding. Paraffin sections were stained for CD34 (1:25; Novocastra/Medac, Wedel, Germany), secondary antibody (biotinylated goat anti-mouse immunoglobulin antibody; Zymed, San Francisco, CA), exposed to streptavidin peroxidase, developed with diaminobenzidine as substrate, and weakly counterstained with hematoxylin. Positive staining was analyzed by measuring the $CD34^+$ area using the Olympus DP-Soft analysis software.

EphB4-Fc and ephrinB2-Fc receptor body staining

Cells were fixed, blocked with 3% BSA/PBS (albumin bovine fraction V; Serva, Heidelberg, Germany) and incubated with 1 μg/ml EphB4-Fc or ephrinB2-Fc. Binding was detected by a goat antibody specific for anti-human Fc conjugated to Cy3 (Sigma). Staining of the nuclei was performed using DAPI.

Immunoprecipitation and western blotting

EphB4-Fc (4 μg/ml) and ephrinB2-Fc (4 μg/ml) were coupled to 20 μl protein-G-agarose (Roche Diagnostics, Mannheim, Germany) in the presence of 250 μl lysis buffer. Cell lysate (1.0 ml) containing 2 mM Na_3VO_4 and protease inhibitor cocktail (Sigma) 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 and ephrinB2. Precipitates were washed, lysed and run on a 10% SDS-PAGE gel. Western-blotted gels were probed with a monoclonal phosphotyrosine (0.2 μg/ml; Santa Cruz, Heidelberg, Germany) and visualized by chemiluminescence. Equal loading of gels was confirmed by stripping and reprobing the blots with the corresponding EphB4 and ephrinB2 antibodies (0.2 μg/ml polyclonal antisera; both antibodies from R&D Systems).

Statistical analysis

All results are expressed as mean±s.d.; differences between experimental groups were analyzed by impaired Student's *t*-test and $P<0.05$ was considered as statistically significant.

Results

A subset of cultured HUVECs express ephrinB2

Expression of ephrinB2 in vivo is limited to arterial ECs, whereas EphB4 is expressed by venous ECs (Gale et al., 2001; Shin et al., 2001). By contrast, the expression pattern of cultured ECs and the functional consequences of ephrinB ligand and EphB receptor signaling in ECs are not well defined. To specify the Eph-ephrin expression pattern of cultured ECs, we analyzed HUVECs for their capacity to bind ephrinB2-Fc (binding to EphB2/3/4) or EphB4-Fc (binding to ephrinB2) receptor bodies. EphrinB2-Fc was found to bind to essentially all HUVECs, whereas EphB4-Fc only binds to a subset of approximately 40% HUVECs (Fig. 1A-C). Binding of EphB4-Fc is preferentially localized to intercellular contacts. By contrast, ephrinB2-Fc binding sites are uniformly distributed on the cell surface (Fig. 1C).

EphrinB2-Fc acts anti-adhesively on ECs

Eph-ephrin interactions play a key role in axonal guidance by transmitting attractive and repulsive signals (Kullander and Klein, 2002). Likewise, bidirectional signaling interactions of B-class ephrins with their corresponding B-class Eph receptors have been shown to orchestrate the invasive ingrowth of blood vessels and arterio-venous differentiation (Adams, 2002). In order to define propulsive and repulsive effector functions of ephrinB2 ligands and EphB4 receptors on ECs, HUVECs were seeded on ephrinB2-Fc- or EphB4-Fc-coated adhesive and non-adhesive tissue culture dishes. Coating with ephrinB2-Fc completely blocks adhesion of HUVECs to adhesive culture dishes, whereas EphB4-Fc does not interfere with EC adhesion to tissue culture dishes. In turn, when using EphB4-Fc-coated non-adhesive tissue culture dishes, a subset of HUVECs quantitatively corresponding to the $ephrinB2^+$ HUVEC subpopulation (Fig. 1B) was able to adhere (Fig. 2A). Similar results were obtained with microvascular EC populations, such as human dermal microvascular ECs (HDMVECs) (data not

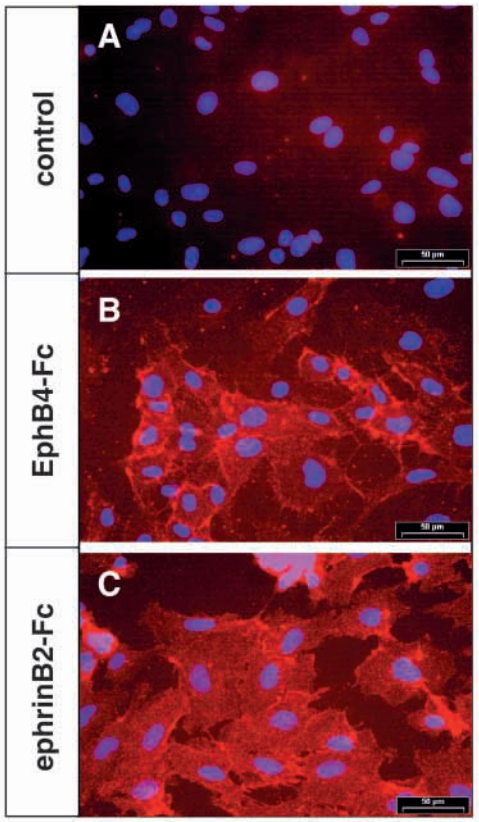

Fig. 1. Detection of EphB4 and ephrinB2 binding sites on cultured HUVECs by receptor body staining. Confluent HUVEC monolayers were fixed and incubated with EphB4-Fc (detecting ephrinB2) (B) or ephrinB2-Fc (detecting EphB2, EphB3 and EphB4) (C). Receptor body binding was visualized using a Cy3-coupled anti-Fc-antibody. Essentially, all HUVECs bind ephrinB2-Fc (C), whereas a subpopulation of cells binds EphB4-Fc (B), which is preferentially localized at intercellular contacts.

shown). Titration experiments of the anti-adhesive ephrinB2-Fc effect identified a steep dose-response curve with an EC_{50} of 12.2 ng/mm^2 ephrinB2-Fc (Fig. 2B). Clustering of ephrinB2-Fc receptor bodies with an Fc-specific secondary antibody prior to coating induced a left shift of the curve and an even steeper dose-response curve (EC_{50}: 8.5 ng/mm^2; hill slope: –2.4). Additional adhesion/detachment experiments showed that the anti-adhesive effect of ephrinB2-Fc on HUVECs is also exerted, albeit quantitatively attenuated, if ephrinB2-Fc is added to adherent cells or if suspended HUVECs are preincubated with ephrinB2-Fc prior to seeding on adhesive substrata (data not shown). Control experiments with smooth muscle cells and fibroblasts confirmed that the ephrinB2-Fc- and EphB4-Fc-mediated detachment-inducing and adhesion-mediating effects are specific for ephrinB2- and EphB4-expressing ECs (data not shown).

We have developed a spheroidal co-culture system of ECs and SMCs that mimics the 3D assembly of the normal vessel wall with a multicellular layer of SMCs and a surface monolayer of ECs (Korff et al., 2001). Exposure of differentiated EC/SMC co-culture spheroids to ephrinB2-Fc disrupts the integrity of the continuous layer of surface ECs and induces detachment of ECs (Fig. 3C). EphB4-Fc had no effect on EC monolayer integrity. Corresponding to these experiments, we performed experiments with in situ explanted fragments of umbilical cords. Treatment of fragments of explanted umbilical cord with ephrinB2-Fc led to detachment of ECs from their underlying extracellular matrix, which could be visualized and quantitated after CD34 staining (Fig. 3D,G). EphB4-Fc did not affect ECs in co-culture spheroids or in in situ explanted fragments of the umbilical cord (Fig. 3E,F). Together, the in vitro and in situ experiments indicate that ephrinB2-Fc, binding to EphB2, EphB3 and/or EphB4, is capable of acting anti-adhesively on ECs that are in their proper organotypic context with SMCs.

EphrinB2-Fc inhibits EC migration and sprouting angiogenesis, whereas EphB4-Fc stimulates migration and sprouting angiogenesis

EphrinB2 is strongly expressed by ECs during angiogenesis (Gale et al., 2001; Shin et al., 2001). On the basis of the observed anti-adhesive capacity of ephrinB2-Fc, we set out experiments aimed at functionally manipulating Eph-ephrin interactions during specific steps of the angiogenic cascade. A 2D lateral cell migration assay, as well as the vertical gradient-driven Boyden chamber, was used to study EC migration and chemotaxis. Baseline migration as well as VEGF-induced migration of ECs was inhibited by ephrinB2-Fc (Fig. 4A). By contrast, EphB4-Fc stimulates lateral EC migration (Fig. 4A). Correspondingly, the capacity of ephrinB2-Fc and EphB4-Fc to modulate VEGF-mediated chemoattraction of HUVECs was analyzed in a modified Boyden chamber assay. These experiments showed that ephrinB2-Fc, but not EphB4-Fc, inhibits VEGF-mediated chemoattraction (Fig. 4B). A 3D collagen gel assay was employed to quantitate the effect of soluble dimeric ephrin ligands and Eph receptors on sprouting angiogenesis. EphrinB2-Fc inhibited baseline as well as VEGF-induced sprouting angiogenesis, which was prominently stimulated by EphB4-Fc (Fig. 5A). Alignment of ECs cultured on top of Matrigel reflects some morphogenic properties of ECs during angiogenesis. EphrinB2-Fc strongly inhibits alignment of ECs grown on Matrigel, which is not affected by EphB4-Fc (Fig. 5B).

Repulsive forward EphB4 signaling is sufficient to induce EC segregation

The above experiments with exogenous soluble dimeric and clustered ephrinB2 and EphB4 receptor bodies had identified repulsive activities of the EphB4 receptor and propulsive activities of ephrinB2 expressed by ECs. In order to study the functional consequences of cell-cell contact-dependent Eph-ephrin signaling, we have generated constitutively EphB4- and ephrinB2-overexpressing EC lines (PAECs). Wild-type or mock-transfected PAECs do not express detectable levels of functional EphB4 or ephrinB2 as assessed by a negative receptor body binding assay, which was strongly and uniformly positive for EphB4- and ephrinB2-transfected cells, respectively (data not shown). Transfected PAECs show essentially the same responses to receptor body activation as the endogenous EphB4- and ephrinB2-expressing HUVECs. Yet, PAECs have a high baseline sprouting activity. As a consequence, sprouting of collagen gel-embedded spheroids of

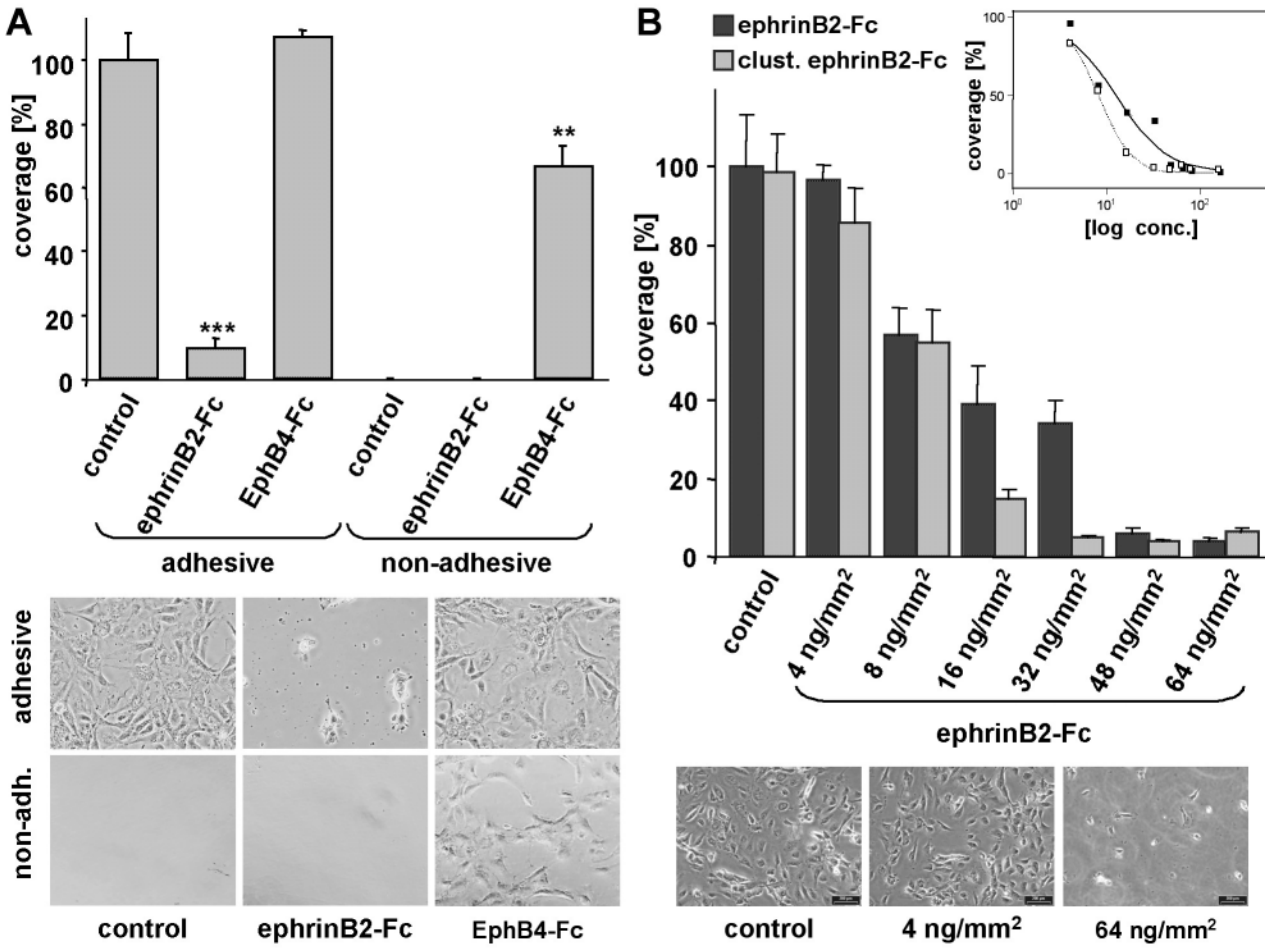

Fig. 2. Quantitative analysis of HUVEC adhesion to EphB4-Fc- and ephrinB2-Fc-coated adhesive and non-adhesive surfaces. (A) HUVECs were seeded on top of adhesive (left) or non-adhesive (right) plastic surfaces coated with ephrinB2-Fc (160 ng/mm^2) or EphB4-Fc (160 ng/mm^2). Adhesion of cells was assessed by digitally measuring the cell-covered area of the coated surface after 24 hours (representative pictures are shown below). HUVECs avoid adhesion to ephrinB2-Fc-coated adhesive plastic. In turn, EphB4-Fc promotes adhesion to non-adhesive plastic. (B) Dose dependency of ephrinB2-Fc-mediated anti-adhesive effects on HUVECs. EphrinB2-Fc (dark bars) inhibits HUVEC adhesion with an EC_{50} of 12.2 ng/mm^2 and a hill slope of –1.5 (representative pictures are shown below). Clustering of ephrinB2-Fc (light bars) induced a left shift with an even steeper dose-response curve (EC_{50}: 8.5 ng/mm^2; hill slope: –2.4) (insert). The figure shows the mean±s.d. of one out of three experiments with similar results. The coverage of control coated adhesive plastic was set to 100%. ***P<0.001 compared with corresponding control. Bars, 200 μm.

ephrinB2-transfected PAECs cannot be further enhanced by EphB4-Fc. In turn, ephrinB2-Fc strongly and selectively inhibits sprouting of EphB4-overexpressing PAECs (and not sprouting of mock-transfected cells) (Fig. 6A). EphrinB2-Fc stimulation of EphB4-expressing PAE cells is associated with strong tyrosine phosphorylation of EphB4 (Fig. 6B).

Cell-mixing experiments were performed with EphB4 and ephrinB2 transfectants as well as with PAECs transfected with a truncated ephrinB2 ligand that lacks the intracellular signaling domain (ΔephrinB2 PAECs). Planar co-cultures of either ephrinB2, ΔephrinB2, or EphB4 with mock-transfected PAECs led to uniform mixing of the cells, as demonstrated by the even distribution of fluorescent-labeled cells upon adhesion in tissue culture dishes (Fig. 7A-C). By contrast, mixing of ephrinB2- and EphB4-expressing PAECs led to the segregation of the cells upon adhesion and formation of clusters of ephrinB2$^+$ and EphB4$^+$ cells (Fig. 7D). Unidirectional forward EphB4 signaling was sufficient for the cellular segregation, as shown by the segregation of EphB4-expressing cells from ΔephrinB2 PAECs upon mixing (Fig. 7E). Biochemical analysis of the mixing experiments identified intense tyrosine phosphorylation of EphB4 in co-cultures of both EphB4 with ephrinB2-overexpressing cells and EphB4 with ΔephrinB2-overexpressing PAE cells (Fig. 7F,G).

Discussion

Genetic experiments have unambiguously demonstrated the key critical role of bidirectional EphB-ephrinB signaling for proper assembly of the vascular system. Yet, they have provided limited information about the cellular consequences of endothelial EphB receptor and ephrinB ligand activation. The present study was aimed at identifying effector functions of forward endothelial EphB and reverse ephrinB signaling. Towards this end, we have utilized a panel of established EC culture assays including specialized 3D spheroidal

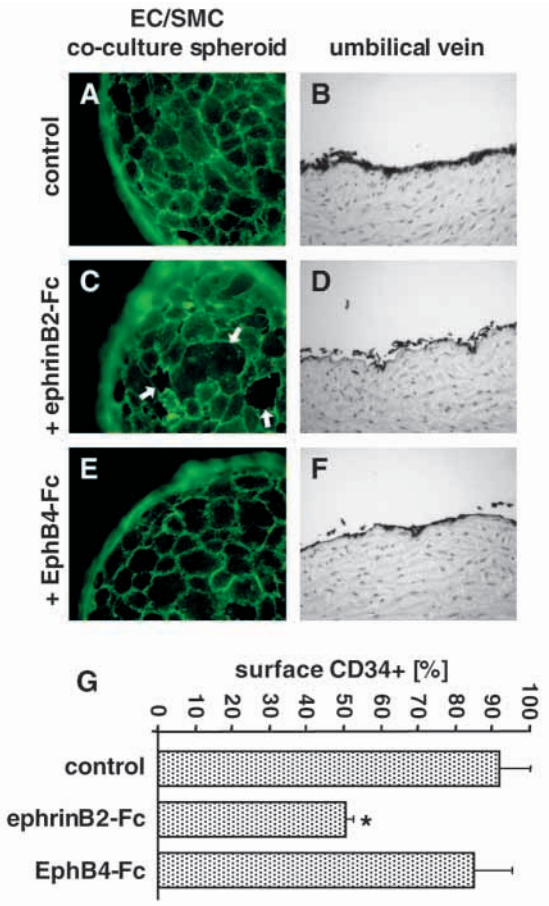

Fig. 3. EphrinB2-Fc-induced detachment of ECs from 3D co-culture spheroids of HUVECs and HUASMCs (EC/SMC; left) and from explanted fragments of umbilical vein (right). (A,C,E) EC/SMC co-culture spheroids were treated for 24 hours with ephrinB2-Fc (2 μg/ml) or EphB4-Fc (2 μg/ml), fixed and whole mount stained for the EC marker CD31. Control spheroids (A) and EphB4-Fc-treated spheroids (E) have an intact surface monolayer of $CD31^+$ ECs. EphrinB2-Fc (C) disintegrates the surface endothelial monolayer and induces EC detachment (arrows). (B,D,F) Explanted fragments of freshly isolated human umbilical cords were cultured for 24 hours in the presence of ephrinB2-Fc (2 μg/ml) or EphB4-Fc (2 μg/ml) after which they were fixed, paraffin embedded and stained for the EC marker CD34. Control (B) and EphB4-Fc-treated (F) umbilical veins have an intact monolayer of $CD34^+$ ECs. By contrast, ephrinB2-Fc (D) induces detachment and denudation of umbilical veins. (G) Quantitation of umbilical vein denudation induced by ephrinB2-Fc. Umbilical vein integrity was assessed by automated image analysis quantitating the relative SMC surface area that is covered by $CD34^+$ ECs. *P<0.05 compared with control.

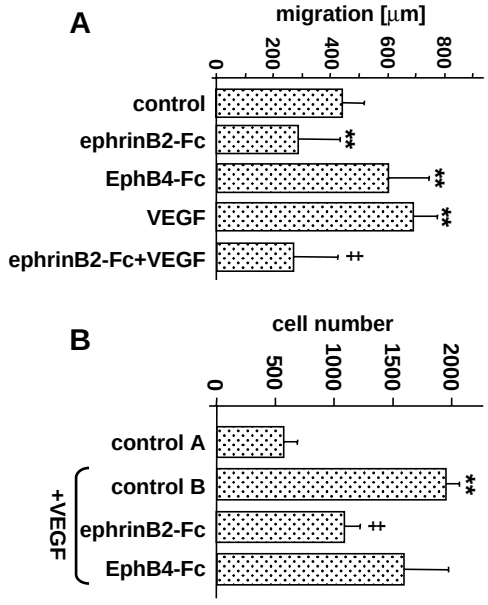

Fig. 4. Effect of EphB4-Fc and ephrinB2-Fc on lateral 2D migration of HUVECs (A) and VEGF-stimulated HUVEC chemotaxis (B). (A) EphB4-Fc (1 μg/ml) and VEGF (50 ng/ml) stimulate lateral EC migration (48 hours). In turn, ephrinB2-Fc (1 μg/ml) inhibits baseline as well as VEGF-induced lateral EC migration (mean±s.d.; analysis of four independent experiments performed in triplicates). (B) EphrinB2-Fc (2 μg/ml), but not EphB4-Fc (2 μg/ml), significantly inhibits VEGF- (20 ng/ml) mediated chemotaxis (4 hours) of HUVECs in a modified Boyden chamber assay (mean±s.d.; analysis of four independent experiments performed in triplicates). Control A: medium, upper and lower compartment; control B: medium, upper compartment, VEGF, lower compartment. **P<0.01 compared with baseline control; ‡P<0.05 compared with VEGF induction.

differentiation and angiogenesis assays (Korff and Augustin, 1999). Collectively, these experiments revealed that EC EphB4 activation transduces anti-adhesive, anti-migratory and anti-angiogenic signals. These findings are in line with signaling experiments that have shown that endothelial Eph receptor activation interferes negatively with VEGF and angiopoietin-1 signaling by suppressing mitogen-activated protein kinase (MAPK) activation (Kim et al., 2002). Contrasting with the forward EphB4 signaling findings, reverse ephrinB2 signaling acts pro-adhesively, stimulates EC migration and induces sprouting angiogenesis. These experiments were performed with HUVECs, which express a chimeric EphB4 and ephrinB2 phenotype (Fig. 1). Corresponding to these experiments, we studied aortic ECs that either constitutively overexpress EphB4 or ephrinB2, or a truncation mutant of ephrinB2 (ΔephrinB2) that lacks the intracellular signaling domain. Experiments with these transfectants revealed that forward EphB4 signaling transduces repulsive signals that restrict cellular intermingling in confrontation experiments. In fact, EphB4-expressing cells form clusters of cells that segregate from the ephrinB2- as well as the ΔephrinB2-expressing cells (Fig. 7). This segregation is associated with intense tyrosine phosphorylation of EphB4 and occurs independently from ephrinB2 reverse signaling as shown by essentially identical findings with ephrinB2- and ΔephrinB2-expressing cells.

The findings of our study support a model of endothelial propulsive and repulsive activities that mediate endothelial guidance signals during invasive angiogenesis as well as the positional control of EphB receptor- and ephrinB ligand-expressing cells towards each other (Fig. 8). This model is based on our functional data and takes into account published data on the repulsive guidance of ECs and neural crest cells by surrounding cells (Helbling et al., 2000; Krull et al., 1997; Oike

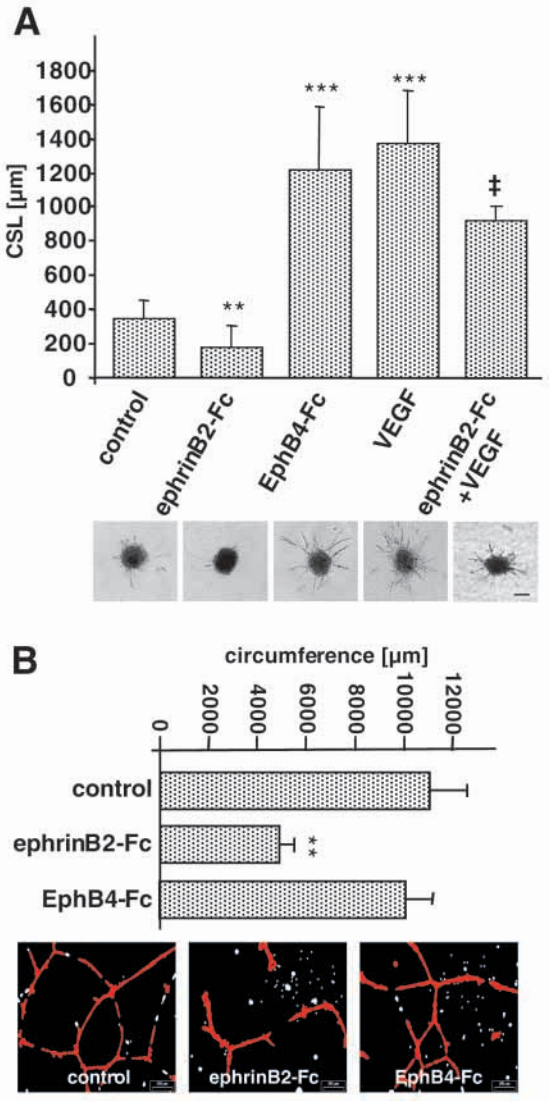

Fig. 5. Modulation of 3D sprouting angiogenesis (A) and endothelial alignment on Matrigel (B) by EphB4-Fc and ephrinB2-Fc. (A) Quantitative analysis of the cumulative sprout length (CSL, quantitated after 48 hours) originating from 10 collagen gel embedded HUVEC spheroids (one out of three independent experiments with similar results). Representatives of each experimental group are shown below the bar graph (bar, 100 µm). EphB4-Fc (1 µg/ml) as well as VEGF (50 ng/ml) induce capillary-like sprouting. By contrast, ephrinB2-Fc (1 µg/ml) inhibits baseline sprouting as well as VEGF-induced sprouting angiogenesis. (B) Quantitative cellular alignment analysis of ECs grown on Matrigel. HUVECs were grown on Matrigel for 24 hours after which the circumferential length of tube-like structures was quantitated by automated image analysis. EphrinB2-Fc significantly inhibits alignment of HUVECs. $^{**}P<0.01$; $^{***}P<0.01$ (compared with baseline control); $^{‡}P<0.01$ (compared with VEGF induction).

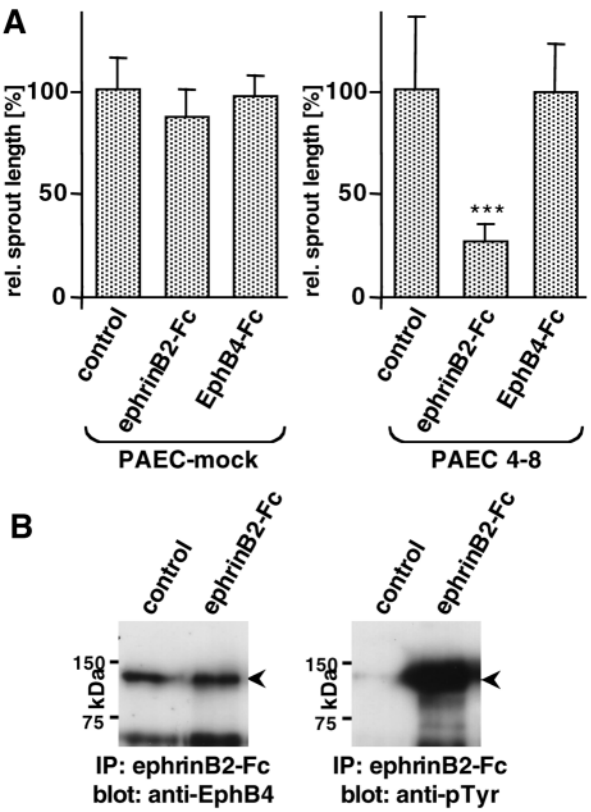

Fig. 6. Effect of EphB4-Fc and ephrinB2-Fc on sprouting angiogenesis of mock and EphB4 (clone 4-8)-transfected PAECs. (A) PAEC spheroids were embedded in collagen gels and stimulated with ephrinB2-Fc or EphB4-Fc for 48 hours. PAECs have a high baseline sprouting that was set to 100%. The sprouting activity of EphB4-transfected PAECs was significantly inhibited upon treatment with ephrinB2-Fc ($^{***}P<0.001$) but not upon EphB4-Fc treatment. By contrast, mock-transfected PAECs do not respond to either ephrinB2-Fc or EphB4-Fc treatment. (B) EphB4-overexpressing PAECs were stimulated with ephrinB2-Fc for 30 minutes and analyzed for EphB4 phosphorylation by immunoprecipitating EphB4 using ephrinB2-Fc. Blots were probed with an anti-EphB4 antibody (left) and reprobed with an anti-pTyr antibody (right). EphrinB2-Fc stimulation leads to prominent phosphorylation of EphB4 in PAECs overexpressing mEphB4.

et al., 2002; Wang and Anderson, 1997). EC guidance refers to Eph-ephrin signaling-mediated positional information between outgrowing ECs and surrounding cells as it occurs during intersomitic invasion of sprouting capillaries (Helbling et al., 2000). Correspondingly, EphB-ephrinB interactions have been shown to regulate EC and mural cell interactions (Oike et al., 2002). Likewise, mural cells (smooth muscle cells, pericytes) and other surrounding cells such as astrocytes in the retina have been proposed to act as guidance cells during sprouting angiogenesis (Nehls et al., 1998), which may similarly involve propulsive and repulsive Eph-ephrin signaling.

Eph-ephrin interactions also control the spatial organization of ECs towards each other as they are involved during vascular network formation (Fig. 8). EphB4 forward signaling restricts cellular intermingling and, thus, drives segregation of EphB4- and ephrinB2-expressing ECs, which controls proper asymmetric arterio-venous assembly of $EphB4^{+}$ and $ephrinB2^{+}$ ECs. The confrontation experiments indicate an arterio-venous push and pull orientation during angiogenesis, which supports an artery-to-vein model of sprouting angiogenesis. Likewise, the antagonistic propulsive and repulsive functions of ephrinB ligands and EphB receptors have led to speculations about a

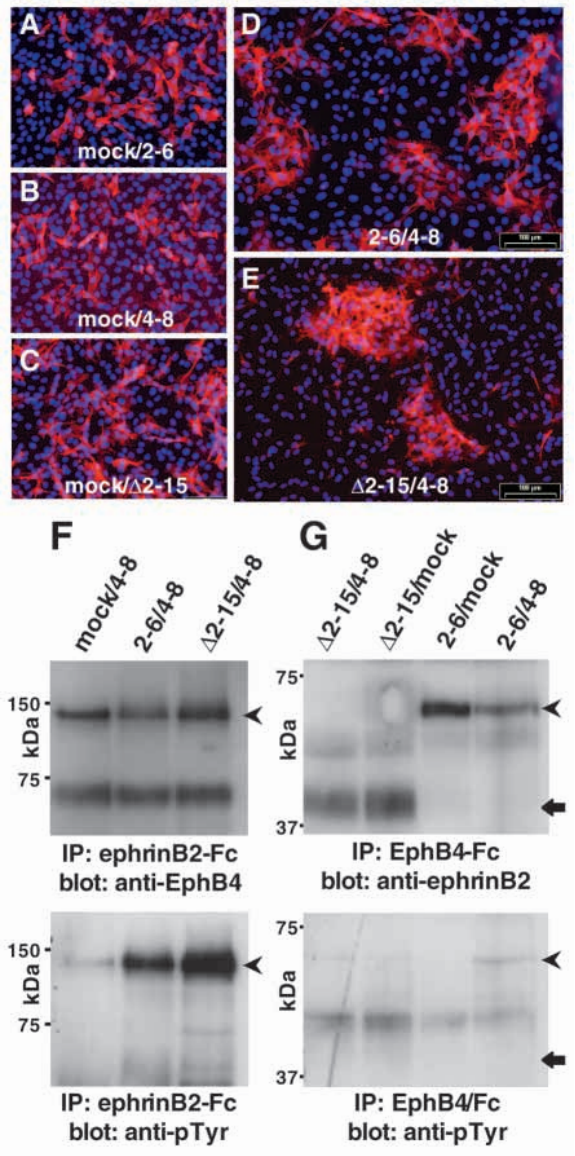

Fig. 7. Confrontation experiments of overexpressing cells EphB4- (clone 4-8), ephrinB2- (clone 2-6) and ΔephrinB2 (Δ2-15). Equal numbers of cells were mixed and seeded at confluent cell density. Combinations of mock-transfected PAECs with either ephrinB2 (A), EphB4 (B) or ΔephrinB2 (C) results in complete intermingling of the two cell populations. By contrast, combinations of ephrinB2 (D) or ΔephrinB2 (E) with EphB4-overexpressing cells leads to segregation of the two cell populations as demonstrated by island formation of EphB4-expressing cells (red staining in A and C: EphB4-Fc receptor body staining for ephrinB2 expression; red staining in B, D and E: ephrinB2-Fc receptor body staining for EphB4). EphrinB2- or ΔephrinB2-mediated segregation of EphB4$^+$ cells is associated with intense tyrosine phosphorylation of EphB4 (F). Biochemical analysis of confrontation experiments of mock-transfected cells with ephrinB4-transfected cells identified a weak phospho-EphB4 band. By contrast, co-culture of either ephrinB2 or ΔephrinB2 cells with EphB4 cells resulted in intense EphB4 tyrosine phosphorylation (F, arrowhead). In turn, analysis of ephrinB2 expression and phosphorylation identified abundant levels of full-length ephrinB2 and the truncated ΔephrinB2 (G, upper right arrowhead and arrow). Yet, a phospho-ephrinB2 band was only detectable in the confrontation experiments of ephrinB2/EphB4 co-cultures and in none of the other combinations (G, lower right arrowhead).

third ephrin$^-$ and Eph$^-$ cell population in between the ephrin$^+$ and Eph$^+$ cells (Oike et al., 2002). This conclusion is also supported by lacZ staining experiments suggesting that a significant proportion of capillary ECs may express neither EphB4 nor ephrinB2 (Visconti et al., 2002). In turn, despite the apparently antagonistic functions of EphB4 and ephrinB2, our experiments have shown that individual ECs can even co-express EphB receptors and ephrinB ligands as shown by the chimeric expression status of HUVECs. Furthermore, we have also performed triple-cell-type intermingling experiments (mock-transfected plus ephrinB2-transfected plus EphB4-transfected), which have shown that EphB4 cells fail to segregate under these conditions (data not shown). Thus, the positional orientation of EphB receptor$^+$, ephrinB ligand$^+$ and Eph-ephrin$^-$ ECs in different vascular beds awaits further analysis and might guide the identification of other EC Eph- and ephrin-mediated functions.

The proposed model (Fig. 8) summarizes the findings of the present study and is in line with published data obtained in genetic mouse models. Conceptually, it also corresponds to the propulsive and repulsive models established for Eph-ephrin-

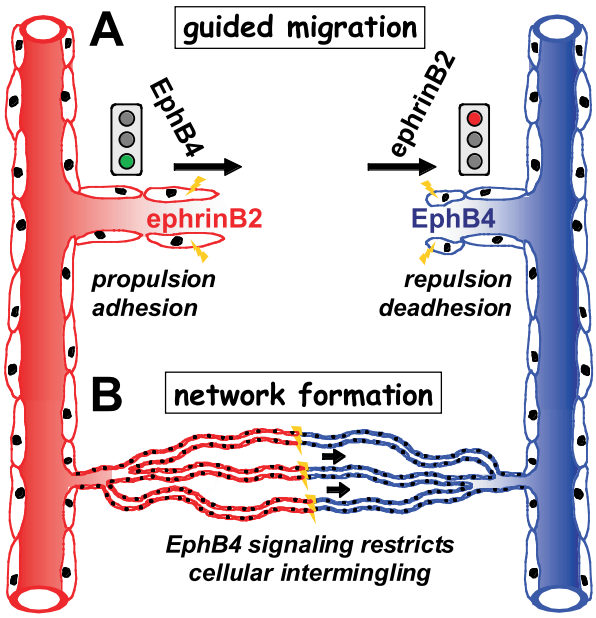

Fig. 8. Proposed model of the functional consequences of EC ephrinB2 and EphB4 signaling during guided migration (A) and capillary network formation (B). The model is based on the functional data summarized in this manuscript and takes into account published data on the repulsive guidance of ECs and neural crest cells by surrounding cells (Helbling et al., 2000; Krull et al., 1997; Oike et al., 2002; Wang and Anderson, 1997). Similar to guided nerve cell outgrowth, forward EphB4 signals may direct ECs in a repulsive manner upon activation by surrounding cells avoiding areas where ephrinB2 is expressed (guided migration, A, stop signal). The opposite, promotion of EC migration may occur if ephrinB2-expressing angiogenic ECs are activated by EphB4 (A, go signal). Additionally, these effects may segregate ECs from each other to limit cellular intermingling and control arterio-venous positioning of cells (network formation, B). Propulsive and repulsive EC forces during capillary morphogenesis indicate an arterio-venous push and pull situation that supports an artery-to-vein model of invasive angiogenesis.

mediated control of neuronal outgrowth (Cooke and Moens, 2002). It is also in line with the recent identification of EphB-receptor-mediated repulsive forces that separate proliferating and differentiating intestinal epithelial cells. Lack of repulsive forces in EphB2/EphB3-null mice leads to an intermingling of proliferating and differentiating intestinal epithelial cells (Batlle et al., 2002). Yet, the model also points to several key unanswered questions and unresolved discrepancies for Eph and ephrin functions in the vascular system. Capillary sprouting is believed to originate primarily from capillaries and venules (Burger et al., 1983). In turn, the expression of ephrinB2 by arterial ECs and ECs during angiogenesis has been considered as evidence that angiogenesis might have an arterial origin (Shin et al., 2001). Our data have shown that ephrinB2-expressing ECs have a propulsive and invasive phenotype corresponding to the properties of angiogenic ECs. Thus, it remains to be seen if ephrinB2 expression of angiogenic ECs indicates an arterial origin of angiogenesis or if angiogenic activation induces EC ephrinB2 expression in ephrin$^-$ cells or even a switch of the asymmetric arterio-venous ephrinB2 and EphB4 expression towards angiogenic ephrinB2 expression. Expression profiling experiments suggest the latter, as demonstrated by the intense upregulation of EC ephrinB2 expression following angiogenic activation by VEGF (G. Dandekar et al., unpublished). This observation also corresponds to the arteriolization associated with VEGF-induced angiogenesis (Stalmans et al., 2002).

Forward EphB4 signaling was sufficient for the segregation of EphB4-expressing cells as shown by the segregation-inducing effects of ΔephrinB2-expressing cells. Yet, reverse ephrinB2 signaling is capable of transducing propulsive and pro-angiogenic activities on ECs. The cytoplasmic domain of ephrinB ligands contains five conserved tyrosine residues and a PDZ-binding domain. Correspondingly, tyrosine phosphorylation-dependent and phosphorylation-independent ephrinB signaling has been reported: Src family kinases are involved in phosphorylation-dependent signaling (Palmer et al., 2002) and the SH2-SH3 domain adapter protein Grb4 acts as a downstream effector of ephrin B ligands (Cowan and Henkemeyer, 2001). Likewise, several PDZ-domain proteins have been reported to interact with ephrinB ligands including GRIP1, GRIP2 and syntenin, as well as Pick1, PDZ-RGS3 and the tyrosine phosphatase PTP-BL (Kullander and Klein, 2002). The latter proteins are of particular interest as these PDZ-domain molecules are themselves linked to a functional unit. Pick1 interacts with protein kinase C, PTP-BL can act as a negative regulator of ephrinB phosphorylation and Src activity (Palmer et al., 2002), and PDZ-RGS3 can negatively interfere with SDF-1/CXC-R4 chemokine signaling (Lu et al., 2001). Thus, given the different signaling pathways that may be entertained by reverse ephrinB signaling, it is intriguing to speculate that reverse ephrinB signaling can contribute to different angiogenic effector functions. Further ongoing experiments with ECs expressing different ephrinB2 truncation mutants might help to shed further light onto the complexity of endothelial EphB and ephrinB functions.

In summary, the present study has for the first time conclusively analyzed the functional consequences of EC ephrinB2 and EphB4 activation at the cellular level. The data support a model of propulsive and repulsive endothelial ephrinB2 and EphB4 signaling that contributes to providing positional cues that regulate guided EC migration and proper arterio-venous differentiation. The data support a model of artery-to-vein invasive angiogenesis and a forward push and reverse pull interaction between ephrinB2$^+$ and EphB4$^+$ ECs.

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Note added in proof

Since submission of this manuscript, Hamada et al. have published a paper on the adhesive and migratory effector functions of ephrinB2 and EphB4 on endothelial cells (Hamada et al., 2003). Corresponding to the findings presented in Fig. 2 and Fig. 4, Hamada et al. also report proadhesive and promigratory functions of EphB4-Fc and antiadhesive and antimigratory functions of ephrinB2-Fc.

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