Distinct Roles of Ephrin-B2 Forward and EphB4 Reverse Signaling in Endothelial Cells

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Objective—The transmembrane ligand ephrin-B2 and its receptor tyrosine kinase EphB4 are specifically expressed on arterial and venous endothelial cells, respectively, and bidirectional signals mediated by both proteins play an important role in vascular development. However, how such bidirectional signals are required for cell-cell adhesion or repulsion remains unclear.

Methods and Results—Using a cell line and sorted primary endothelial cells, we show that ephrin-B2 forward signaling through the EphB4 receptor inhibits cell adhesion, whereas EphB4 reverse signaling by the transmembrane ephrin-B2 ligand does not. Cell migration is also inhibited on immobilized ephrin-B2-Fc but not on EphB4-Fc protein.

Conclusions—Ephrin-B2 forward signaling and EphB4 reverse signaling differentially affect cell adhesion and migration between arterial and venous endothelial cells. (Arterioscler Thromb Vasc Biol. 2003;23:190-197.)

Key Words: ephrin-B2 ■ EphB4 ■ cell adhesion ■ spreading ■ vascular development

During vascularization and the maintenance of blood vessel integrity, cell adhesiveness and motility change at the endothelial cell level. Endothelial cells adhere to each other and to the extracellular matrix through interactions involving cell surface proteins. Changes in morphogenesis during vascularization can be considered a consequence of precise spatio-temporal expression of cell surface molecules controlling cell adhesion and motility. Several cell surface receptors that trigger intercellular signals regulating cell proliferation, differentiation, or migration in vascular development have been identified and characterized.1–3 The Eph receptor tyrosine kinases and their ephrin ligands mediate numerous developmental processes in both invertebrates and vertebrates.4 Multiple functions of Eph receptors throughout embryonic development are suggested by complex and highly dynamic expression patterns as well as by experimental evidence.5

The ephrins, unlike ligands for other receptor tyrosine kinases, must be membrane-anchored to activate Eph receptors.6 Based on how they are tethered to the membrane, ephrins are divided into 2 subclasses. The 5 members of the ephrin-A subclass (ephrin-A1 to -A5) are tethered via a glycosylphosphatidylinositol (GPI) anchor, whereas the 3 members of the ephrin-B subclass (ephrin-B1 to -B3) have a transmembrane domain and highly conserved cytoplasmic domains. Ephrin-B ligands interact primarily with the B subset of Eph receptors, which consist of at least 6 members.6,7 A characteristic of ephrin/Eph signaling is the potential for bidirectional signaling, that is, classical forward signaling by the Eph receptor via its intrinsic tyrosine kinase activity and reverse signaling by the transmembrane ephrin-B ligand via its cytoplasmic domain.

Recent work shows that ephrin-B2 ligand and its cognate EphB4 receptor are required for vascular development. Targeted disruption of the ephrin-B2 gene in mice leads to embryonic lethality at embryonal day (E) 10.5, attributable to a defect in both arterial and venous vessel remodeling.5,9 This defect is accompanied by a failure of intercalation between arteries and veins. EphB4 homozygous mutants have a similar phenotype with ephrin-B2 homozygous embryos in the vascular system.10 Interestingly, ephrin-B2 and EphB4 display a remarkable reciprocal expression pattern in endothelial tissue. Ephrin-B2 marks arterial endothelial cells, whereas EphB4 marks venous endothelial cells.11–13 Thus, signaling between arteries and veins mediated by ephrin-B2 and EphB4 is required for proper morphogenesis of the intervening capillary beds and network as well as for interdigitation and differential growth of arterial and venous vessels. These findings strongly indicate that ephrin-B2/EphB4 signaling plays an important role in vascular development, especially in the determination and boundary formation between arteries and veins. However, despite the remarkable reciprocal distribution of ephrin-B2 and EphB4 during early vascular development, it is not clear how interactions between ephrin-B2 and EphB4 occur in vascular development and whether ephrin-B2 forward signaling (ephrin-B2/EphB4 signaling) and EphB4 reverse signaling (EphB4/ephrin-B2 signaling) result in different functions.
Here we demonstrate that there are differences in ephrin-B2 forward signaling and EphB4 reverse signaling occurring in cell-cell interaction between endothelial cells.

Methods

Reagents and Cell Lines

Ephrin-B2-Fc and EphB4-Fc fusion proteins were prepared as described previously. A metalloproteinase inhibitor, o-phenanthroline, was purchased from Sigma-Aldrich. Murine brain-derived capillary endothelial cell line, b-End3, was grown in DMEM (GIBCO/BRL) supplemented with 10% FCS and 50 mmol/L 2-mercaptoethanol (ME). The murine pro-B cell line, BaF3, was cultured in RPMI 1640 (GIBCO/BRL) supplemented with 10% FCS. Brain-derived capillary endothelial cell line, b-End3, was grown in DMEM (GIBCO/BRL) supplemented with 10% FCS.

Cell Adhesion Assay

Petri dishes (35 mm, Falcon) were coated with a layer of nitrocel lulose (Schleicher & Schuell), as described previously. After washing, dishes were incubated with PBS(−) containing ephrin-B2-Fc, EphB4-Fc, and CD4-Fc or human immunoglobulin (Sigma-Aldrich) as control Fc protein for 2 hours before assay. Wells were washed with PBS(−) twice and blocked at 37°C with 1% BSA for 30 minutes. b-End3 cells were harvested with cell dissociation buffer (Gibco) or 1 mmol/L EDTA, washed twice in medium containing 1% BSA, and plated at a density of 1 × 10⁶ cells per well. After incubation at 37°C for 1 hour (or as specified), unattached cells were dislodged by washing with PBS(−) 3 times. The data from 3 independent experiments performed in triplicate are displayed as the fold of immobilized control-Fc protein.

Fc Protein Binding Assay

For the Fc protein assay, b-End3 cells were gently detached from dishes with EDTA. After cells were washed with PBS(−) 5% FCS twice, they were incubated with 2 μg/mL Fc-fusion protein for 30 minutes on ice. After washing with PBS(−) 5% FCS twice, cells were incubated with FITC-conjugated goat anti-human IgG(H + L) (Immunotech). Stained cells were analyzed by a FACS Calibur (Becton Dickinson).

Reverse Transcriptase–Polymerase Chain Reaction Analysis

RNA was extracted from sorted cells by FACs analysis using a RNaseasy Mini kit (Qiagen). RNA was reverse transcribed using an Advantage RT-for-PCR Kit (Clontech). cDNAs were amplified with an Advantage cDNA PCR Kit (Clontech) in a Gene Amp PCR System 9700 (Perkin-Elmer Inc) for 30 or 35 cycles. Sequences of specific primers for reverse transcriptase–polymerase chain reaction (RT-PCR) were as follows: ephrin-B2: sense: 5’TCTGGTGTTGGAA GTACCTGTGGGAGACTTT-3’; antisense: 5’TGTACCACTTCCT- AGCTTGAGGCTCTT-3’; EphB2: sense: 5’GGAGCCGCGGATG- GCTGTTG-3’; antisense: 5’TGGCGCTCTGTCAATGTTGG-3’; EphB3 and EphB4: DI4: sense: 5’ACCTTCAAGTCTGCGTGTC-3’; antisense: 5’GACTCTAATGTCGCGGAACTT-3’; Bmx sense: 5’GCAACATGCAATTTCCA-3’; antisense: 5’ AAGCATGCAATTTCCA-3’; β-actin: sense: 5’TGGGTGGTGACATCA- AAGAG-3’; antisense: 5’TGGAGATGAGGCCAAGATG-3’.

Each cycle consisted of 15 seconds of denaturation at 94°C and 3 minutes of annealing and extension at 68°C. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide.

Generation of an Anti-EphrinB2 Monoclonal Antibody

Rat monoclonal antibodies against ephrin-B2-Fc protein were produced using standard methods. In brief, an 8-week-old Wistar rat was immunized subcutaneously with 600 μg ephrin-B2-Fc protein in complete Freund’s adjuvant (Difco) and then administered 3 intraperitoneal shots of 100 μg ephrin-B2-Fc protein in Freund’s incomplete adjuvant in alternating weeks. Three days after an intravenous boost of 200 μg ephrin-B2-Fc protein, spleen cells were harvested and fused with murine myeloma cells, P3 × 63-Ag.8.1.1. Undiluted supernatants from hybridomas were screened by ELISA on plates coated with 50 ng/mL ephrin-B2-Fc. Positive hybridomas were cloned by the limiting dilution technique and were subcloned twice. Western blotting analysis was described previously.

Cell Preparation and Flow Cytometry

Cell preparation conditions for FACs analysis were as described. In brief, after dissecting E12.5 mouse embryos, tissues were homogenized to a single-cell suspension with an 18G needle and passed through a nylon mesh screen. These tissues were incubated with 0.05% collagenase S1 (Nitta gelatin) for 2 hours at 37°C and subsequently drawn through a 23G needle. Debris and cell aggregates were removed with nylon mesh again. Single cells from tissue were stained with Cy5-labeled anti-ephrin-B2 monoclonal antibody and anti-PECAM-1/CD31-PE (MEC13.3, rat anti-mouse monoclonal; Pharmingen). Stained cells were analyzed and sorted by a FACSAantage (Becton Dickinson).

Immunofluorescence Microscopy

After stimulation with Fc chimeric protein, b-End3 cells were fixed with 4% paraformaldehyde/PBS, pH 7.4, for 15 minutes at room temperature and, if necessary, permeabilized with 0.2% Triton X-100/PBS. After washing, cells were incubated with 1% BSA or 5% normal goat serum/1% BSA and exposed to FITC-conjugated goat anti-human IgG (H + L) (Immunotech) for detection of immobilized Fc-fusion protein. This treatment was followed by incubation with Rhodamine Red-X–conjugated anti-mouse IgG antibody (Jackson Immunoresearch). F-actin was detected by staining with rhodamine-conjugated phalloidin (Sigma-Aldrich). Cells were counterstained with DAPI (Dojin) to identify nuclei. Stained cells were mounted with 1,4-diazabicyclo-[2,2,2]-octane/glycerol. Images were obtained with fluorescent microscopy on an AX70 microscope (Olympus). Images were processed using Photoshop software (Adobe).

Cell Proliferation Assay

To determine the BrdU-labeling index, b-End3 cells were pulsed with 10 μmol/L BrdU for 1 hour. After washing with PBS(−) 3 times, BrdU incorporation was determined using a BrdU staining kit according to the manufacturer’s protocol (Oncogene research product). The colony assay was described previously. Two hundred b-End3 cells were plated onto 24 wells. After 7 days in culture, cells growing as small colonies were fixed and stained with 0.5% crystal violet.

Results

Cell Adhesion Induced by the Ephrin-B2/EphB4 Interaction

Previous observations that ephrin-B2 and EphB4 are expressed specifically in arteries and veins, respectively, suggested a potential role for ephrin-B2/EphB4 signaling in determination of the boundary between arteries and veins during vascular development. However, it is unknown whether ephrin-B2/EphB4 signaling is necessary for cell attraction or repulsion. To understand whether ephrin-B2/EphB4 bidirectional signaling affects cell-cell interactions, we used an endothelial cell line, b-End3 cells. As previously reported, EphB3 and EphB4 is expressed in venous endothelial cells, EphB2 is expressed in mesenchymal cells, and ephrin-B1 is expressed both in arteries and veins. Therefore, we examined the expression of ephrin-B2, EphB2, EphB3, and EphB4 in b-End3 cells using RT-PCR analysis. mRNA encoding ephrin-B2 and EphB4 but not EphB2 and EphB3
mRNAs was detected in b-End3 cells, whereas EphB2-B3 was detected in E12.5 embryo (Figure 1A). We confirmed expression of ephrin-B2 and EphB4 protein by Western blotting analysis by the method described previously14 (data not shown). Moreover, to estimate the potential capacity for transducing signaling of ephrin-B2 or EphB4, we examined the ability of recombinant ephrin-B2 or EphB4 protein to bind to b-End3 cells using a flow cytometric analysis (Figure 1B). These data showed that both ephrin-B2-Fc and EphB4-Fc fusion proteins bound to b-End3 cells (Figure 1B). CD4-Fc protein used as a control did not bind to b-End3 cells (data not shown). Therefore, we used b-End3 cells for additional analysis of both ephrin-B2 and EphB4 bidirectional signaling.

To determine whether the ephrin-B2-EphB4 signaling induced cell adhesion in endothelial cells, we plated b-End3 cells on immobilized EphB4-Fc, ephrin-B2-Fc, or control-Fc proteins. Adherence of b-End3 cells to immobilized EphB4-Fc and control-Fc fusion proteins was equivalent to their adherence to uncoated plates, and this adhesion was unchanged by varying the concentrations of immobilized EphB4-Fc or control-Fc proteins (Figure 1C). By contrast, immobilized ephrin-B2-Fc protein inhibited cell adhesion relative to controls in a dose-dependent manner (Figure 1C). This result indicates that ephrin-B2 forward signal is inhibitory for adhesion to cells expressing the cognate receptor.

**Differences in Signaling Between Ephrin-B2 and EphB4 in Primary Endothelial Cells**

To examine adhesion or repulsion in ephrin-B2/EphB4 signaling in primary endothelial cells, we made a monoclonal antibody against murine ephrin-B2 to use for FACS analysis. After screening, Keb2 (IgM) was isolated as a clone reacting specifically to the extracellular domain of ephrin-B2 protein by ELISA. Keb2 reacted specifically to ephrin-B2-transfected BaF3 cells but not to EphB4- or only vector-transfected BaF3 cells (data not shown). Moreover, the specific reactivity of Keb2 was confirmed by the finding that an excess molar ratio of ephrin-B2-Fc proteins completely blocked the reactivity of Keb2 with ephrin-B2 expressing BaF3 cells (data not shown). Using Keb2, we analyzed the expression of ephrin-B2 on CD31-positive endothelial cells in E12.5 whole embryos. As shown in Figure 2A, ephrin-B2 fraction expressed arterial-specific markers Dll4 and Bmx, whereas ephrin-B2 fraction expressed the venous endothelial specific marker, EphB4.

To test whether ephrin-B2/EphB4 signaling induces cell adhesion, we examined adhesion of sorted primary cells on immobilized ephrin-B2-Fc or EphB4-Fc. Immobilized EphB4-Fc proteins induced cell adhesion of ephrin-B2’ CD31’ cells (Figure 2C). Some of the adhered ephrin-B2’ CD31’ cells on EphB4-Fc proteins became flat (Figure 2Cb). By contrast, immobilized ephrin-B2-Fc proteins inhibited cell adhesion of ephrin-B2’ CD31’ cells (Figure 2Cc). Quantitative analysis showed that the number of adhering ephrin-B2’
cells to EphB4-Fc protein was 6 times greater than those adhering to ephrin-B2 or control-Fc protein (Figure 2Db). By contrast, adhesion rate of ephrin-B2+ cells on immobilized EphB4-Fc and ephrin-B2-Fc proteins (each 100 μg/mL). Micrographs show cells attached to immobilized EphB4-Fc (Ca and Cb) or ephrin-B2+ (Cb and Cd). Arrowheads indicate flat cells (Cb).

D, Numbers of adherent cells in ephrin-B2+ or ephrin-B2+ CD31+ cells on immobilized EphB4-Fc and EphB4-Fc protein. Ephrin-B2+ (Db) in CD31+ cells adhere to immobilized EphB4-Fc protein, whereas ephrin-B2+ (Da) in CD31+ cells were inhibited from adhering to immobilized ephrin-B2-Fc protein. Results are expressed as the fold of the number of cells adhering to a control-Fc area.

Ephrin-B2 Signaling Exhibits Repulsion Activities Toward Cell Migration
To additionally determine whether ephrin-B2/EphB4 bidirectional signaling can directly influence growth direction in endothelial cells, we examined how endothelial cells grew on immobilized ephrin-B2-Fc or EphB4-Fc proteins during short or long culture periods. For these experiments, small quantities of ephrin-B2-Fc or EphB4-Fc proteins were spotted on
nitrocellulose-coated dishes. After 12 hours of plating, elongation of filopodia in b-End3 cells had nothing to do in the presence of immobilized EphB4-Fc protein (Figure 4Aa). By contrast, immobilized ephrin-B2-Fc clearly induced the arrest of elongation of filopodia in b-End3 cells (Figure 4Ab). In long culture periods, numbers of cells were drastically different between immobilized EphB4-Fc proteins and immobilized ephrin-B2-Fc (Figure 4B). When b-End3 cells were plated on immobilized EphB4-Fc, b-End3 cells were evenly distributed (Figure 4Ba), whereas b-End3 cells did not move across immobilized ephrin-B2-Fc protein (Figure 4Bb). These data suggest the possibility that ephrin-B2 forward signaling induced cell repulsion by ephrin-B2/EphB4 interaction.

It was reported that ephrin-A2 forms a stable complex with the metalloprotease Kuzbanian in the absence of EphA receptors and that when ephrin-A2 ligand binds to the EphA receptor, Kuzbanian is activated and locally cleaves ephrin-A2 from the cell surface.24 To determine whether metalloproteinases are involved in the process activated by ephrin-B2 forward signaling, inhibition of cell adhesion by immobilized ephrin-B2-Fc was monitored in the presence of a metalloproteinase inhibitor, α-phenanthroline (Figure 4C). Cell adhesion and spreading were suppressed on immobilized ephrin-B2-Fc protein (Figure 4Ca through 4Cc), whereas treatment with α-phenanthroline significantly rescued inhibition of cell spreading in b-End3 cells (Figures 4Cd through 4Cf). These experiments suggest the possible involvement of

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**Figure 3.** Suppression of cytoskeletal changes by ephrin-B2 signaling. A and B, Cell morphology of ephrin-B2<sup>−</sup> or ephrin-B2<sup>+</sup> CD31<sup>−</sup> cells on indicated immobilized-Fc proteins. Ephrin-B2<sup>−</sup> (Aa through Ac and Ba through Bc) or ephrin-B2<sup>+</sup> (Ad through Af and Bd through Bf) CD31<sup>−</sup> cells were sorted and plated on 35-mm dishes coated with EphB4-Fc (Aa, Ad, Ba, and Bd), ephrin-B2-Fc (Ab, Ae, Bb, and Be), or control-Fc (Ac, Af, Bc, and Bf) proteins (100 μg/mL each). Cells were fixed in 4% paraformaldehyde after 2 hours (A) or 8 hours (B) of plating, permeabilized with 0.5% Triton X-100, stained with phalloidin-TRITC for detection of F-actin, and visualized using a fluorescence microscope. Arrowheads show some spreading (Ad). Bars in A and B represent 10 μm. C, Cell spreading of ephrin-B2<sup>−</sup> or ephrin-B2<sup>+</sup> CD31<sup>−</sup> cells on indicated Fc proteins. Cells were fixed in 4% paraformaldehyde after 8 hours of plating, stained with phalloidin-TRITC, and counted with spreading cells under fluorescence microscope. Results are expressed as the percentage of spreading cells in total cells. Representative results from 3 experiments are shown. The results are expressed as mean±SEM (n=4). *P<0.001.
These cells were then plated for the indicated times.

O-phenanthrolene (OPT) on immobilized ephrin-B2-Fc protein.

Treatment with immobilized ephrin-B2-Fc significantly reduced the size of colonies and the number of colonies formed compared with treatment with immobilized EphB4-Fc or control-Fc (Figure 5B). These results show that ephrin-B2 forward signaling inhibits cell proliferation. Moreover, we examined the possibility that apoptosis was induced by ephrin-B2 forward signaling by staining for annexin V and propidium iodide. Treatment with immobilized ephrin-B2-Fc did not induce apoptosis compared with immobilized EphB4-Fc or control-Fc (data not shown). Taken together, these results indicated that ephrin-B2 forward signaling inhibits cell proliferation and does not induce apoptosis.

**Discussion**

In this study, we examine functional differences between ephrin-B2 forward signaling (ephrin-B2/EphB4 signaling) and EphB4 reverse signaling (EphB4/ephrin-B2 signaling) in endothelial cells. To do so, we investigated cell adhesion using endothelial cells from murine embryos that are ephrin-B2<sup>+</sup> or EphB4<sup>+</sup>. Using these primary cells and an endothelial cell line, we made the following observations: (1) ephrin-B2 forward signaling prevented cell attachment, cell spreading, and cell migration; (2) EphB4 reverse signaling induced cell attachment, spreading, and migration; and (3) ephrin-B2 forward signaling suppressed cell proliferation.

Eph receptor tyrosine kinases and their membrane-bound ephrin ligands mediate cell-cell interactions and participate in several developmental processes. Bidirectional signaling mediated by ephrins and Eph has important functions in cell-cell recognition events, including those that occur during axon pathfinding and hindbrain segmentation. Moreover, ephrin/Eph signalings have recently emerged as essential regulators of cell adhesion in neuronal and endothelial cells. We have also shown that ephrin-B2/EphB4 signaling mediates reciprocal interactions between arterial and venous endothelial cells and surrounding cells to form vessels. However, there is little understanding of how such bidirectional signaling acts in vascular development and network formation. Previous studies have shown that signaling from ephrin through Eph activates cell attachment or detachment. To understand this question, we analyzed the cell-cell interaction using a pro-B cell line, BaF3 transfected with ephrin-B2 or EphB4. Heterophilic cell aggregation was observed after coincubation of BaF3/ephrin-B2 cells and BaF3/EphB4 cells (data not shown). Because these cells are nonendothelial cells, next we asked whether ephrin-B2/EphB4 signaling induced cell attachment or detachment in the vascular system.

To answer this question, we analyzed the signaling of ephrin-B2/EphB4 in both the b-End3 endothelial cell line, which expresses endogenous ephrin-B2 and EphB4, and primary endothelial cells sorted from murine embryos, which were either ephrin-B2<sup>+</sup> or EphB4<sup>+</sup>. CD31-positive cells in E12.5 embryos were clearly divided into 2 fractions by the ephrin-B2 MoAb. RT-PCR revealed that sorted ephrin-B2–

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**Figure 4.** Cell migration and proliferation on immobilized EphB4-Fc or ephrin-B2-Fc proteins. A, Immobilized ephrin-B2-Fc but not EphB4-Fc protein inhibits cell migration by b-End3 cells. Cells were detached with EDTA and plated for 12 hours on dishes coated with EphB4-Fc and ephrin-B2-Fc proteins (each 100 μg/mL). Cells were stained with FITC-conjugated anti-human immunoglobulin to visualize the Fc coating area (green), DAPI for nuclei (blue). Micrograph shows that b-End3 cells grow despite immobilized EphB4-Fc (Ba) but cell proliferation is inhibited on immobilized ephrin-B2-Fc coating (Bb). C, The effect of a metalloprotease inhibitor on ephrin-B2 forward signaling. b-End3 cells plated in the presence or absence of 25 μmol/L α-phenantrone (OPT) on immobilized ephrin-B2-Fc protein. These cells were then plated for the indicated times.

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Inhibition of Cell Proliferation by Ezhrin-B2 Signaling

To determine how ephrin-B2/EphB4 signaling affects cell proliferation, we assayed incorporation of 5-bromodeoxyuridine (BrdU) in b-End3 cells. After culturing on immobilized Fc protein for 24 hours, b-End3 cells were treated with BrdU 1 hour before fixation and immunostained to identify cells incorporating BrdU (Figure 5A). Treatment with immobilized ephrin-B2-Fc significantly reduced the labeling index compared with treatment with immobilized EphB4-Fc and control-Fc protein (10% versus 42%). Furthermore, to characterize the single cell proliferation against ephrin-B2/EphB4 signaling, we undertook a colony assay using endothelial cells. Treatment with immobilized ephrin-B2-Fc significantly reduced the size of colonies and the number of colonies formed compared with treatment with immobilized EphB4-Fc or control-Fc (Figure 5B). These results show that ephrin-B2 forward signaling inhibits cell proliferation.
positive cells express Dil4 and Bmx, whereas ephrin-B2-negative cells express EphB4. Thus, we regard the former as arterial endothelial cells and the latter as venous endothelial cells.

Ephrin-B2 forward signaling clearly induces repulsive effects in b-End3 cells and primary ephrin-B2-negative (EphB4-positive) endothelial cells. Cell spreading was inhibited on b-End3 cells and EphB4- cells from murine embryos on immobilized ephrin-B2-Fc but not on EphB4-Fc protein. This inhibition was accompanied by inhibition focal contact formation, indicating an important role of ephrin-B2 signaling in endothelial cell-cell interaction through cytoskeletal regulation. We have demonstrated that migration of b-End3 cells across immobilized ephrin-B2-Fc was inhibited and that cell proliferation was also inhibited on ephrin-B2-Fc but not EphB4-Fc protein. Inhibition of proliferation occurred not only through the repulsive effect of ephrin-B2 but by direct suppressive effects on endothelial cells, because delayed addition of ephrin-B2 suppressed BrdU uptake. Based on these findings, our results indicate that ephrin-B2 forward signaling has an inhibitory effect on attachment, migration, and proliferation of EphB4- cells. Recently, it was shown that EphB2 reverse signaling induced changes in cytoskeletal regulation and that the SH2/SH3 domain adapter protein Grb4 binds to the cytoplasmic domain of ephrin-B1 in a phosphorytrosine-dependent manner. The observation that the region of ephrin-B1 binding to Grb4 is conserved in ephrin-B2 cytoplasmic tail suggests that EphB4 reverse signaling through ephrin-B2 may be similar through EphB2 reverse signaling through ephrin-B1.

Hattori et al recently reported that ephrin-A2 forms a stable complex with the metalloprotease Kuzbanian in the absence of EphA. When EphA binds to ligand, Kuzbanian is activated and locally cleaves ephrin-A2 from the cell surface. Because a conserved motif, which exists at cleavage site by Kuzbanian is found in the extracellular region of ephrin-B2, it is possible that similar mechanisms operate in the case of repulsion by ephrin-B2 forward signaling. We show here that a metalloprotease inhibitor rescued inhibition of cell adhesion by ephrin-B2 forward signaling. However, we have not detected an ephrin-B2 cleavage fragment in this study. Therefore, it is necessary to investigate additionally the mechanisms of repulsion in ephrin-B2 forward signaling in EphB4- cells. Furthermore Lu et al described a new ephrin-B interacting PDZ-containing protein, PDZ-RGS3, that regulates Rho GTPases activity. These data suggest that downstream signaling in ephrin-B2 may interact with small G protein signalings.

The difference between ephrin-B2 forward and EphB4 reverse signaling is important for spatially and temporally appropriate vascular network formation. Capillaries consist of ephrin-B2-positive cells and ephrin-B2-negative cells. Our findings suggest that capillary formation is preceded by artery formation. EphB4- venous cells cannot connect to ephrin-B2+ cells, whereas ephrin-B2+ arterial cells can bind to EphB4- capillary network cells in capillary network formation. In Xenopus embryos, disruption of EphB4 signaling or misexpression of ephrin-B ligands results in intersomitic veins growing abnormally into the adjacent somitic tissue. Moreover, activin receptor-like kinase-1, Notch and Gridlock, which are up-stream molecules of Eph-B2, mutant as well as mice lacking ephrin-B2 and EphB4 reported the defect of vascular network formation. Therefore, our data and those of others indicate that such mutant phenotypes are caused by disruption of cell-cell interactions involving ephrin-B2 forward and EphB4 reverse signaling during vascular network formation. These results additionally suggest that downstream signaling in endothelial cells is important for appropriate vascular network formation.

In summary, we have demonstrated that ephrin-B2 forward signaling induced cell detachment and inhibited cell spreading, migration, and proliferation, whereas EphB4 reverse signaling induced cell attachment, spreading, and migration. Our data on cell-cell interactions in endothelial cells indicates that differential functions of bidirectional signaling play an important role in vascular development, network formation, and remodeling.

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