Functional Ephrin-B2 Expression for Promotive Interaction Between Arterial and Venous Vessels in Postnatal Neovascularization

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Background—Ephrin-B2, one of the transmembrane ligands, is a genetic marker of arterial endothelial cells (ECs) at embryonic stages and is essential for cardiovascular development, but its roles in ischemic cardiovascular disease are not well understood. In this study, we focused on the function of ephrin-B2 in postnatal neovascularization.

Methods and Results—We found that ephrin-B2 is exclusively expressed and significantly upregulated in the arterial vasculature after the initial angiogenic responses in tissue ischemia. Upregulation of ephrin-B2 is also observed in EC cordlike formation in vitro. Interestingly, ephrin-B2 expression on ECs was enhanced by promotive angiogenic growth factors, such as vascular endothelial growth factor, basic fibroblast growth factor, and hepatocyte growth factor, whereas it was attenuated by angiopoietin-1, a factor for blood vessel maturation. Moreover, an ephrin-B2–rich environment was shown to induce neovascularization mainly through venous angiogenesis in an in vivo cornea micropocket assay.

Conclusions—Our study indicates that the ephrin-B2 ligand is likely to have functional expression on angiogenic arterial ECs and induce a subsequent promotive effect on venous vessels during postnatal neovascularization. (Circulation. 2005;111:2210-2218.)

Key Words: ischemia ■ ephrin-B2 ■ angiogenesis, postnatal ■ endothelial cells

Postnatal neovascularization is an important mechanism, especially in pathological processes in ischemic cardiovascular disease, such as myocardial and limb ischemia, tumorigenesis, and wound repair, as well as physiological processes in the female reproductive cycle. The identification of growth factors and receptors whose expression is largely restricted to endothelial cells (ECs), especially a family of receptor tyrosine kinase (RTK) ligands and receptors, has contributed significantly to unraveling the molecular mechanisms controlling the development of the vascular system. Vascular endothelial growth factor (VEGF) and its tyrosine kinase receptors appear to play a central role in angioblast specification and early vasculogenic development of the embryonic vasculature. On the contrary, angiopoietins and Tie-2 receptors appear to play a later role by controlling remodeling and maturation of the developing vasculature.

Following these steps, established blood vessels form a hierarchy consisting of arteries, capillaries, venules, and veins. Although the mechanisms that determine vascular heterogeneity have not been studied in detail, it has been shown recently that some of the members of the ephrin family of ligands and their Eph receptors are expressed in specific parts of the vasculature. These findings have implicated several Eph receptor family members as a third class of endothelial RTK implicated in the control of blood vessel hierarchy. Eph receptors, the largest family of RTK, and their ephrin ligands have a variety of functions, such as regulating axon guidance, cell migration, and cell attachment. Recently, one of the transmembrane ligands, ephrin-B2, was shown to be expressed specifically on arterial ECs but not on venous ECs in the embryo. The ephrin-B2 ligand and its receptor EphB4 were also shown to be crucial for cardiovascular development in the embryo. Our collaborators and others have also demonstrated postnatal expression of ephrin-B2 on arterial ECs and some smooth muscle cells in vascular organs of adult mammals.

In contrast to other RTK ligands, ephrin-B ligands show unique features: bidirectional signaling with their Eph receptors through cell-to-cell interaction and acting as receptor-like signaling molecules with tyrosine phosphorylation. Furthermore, a recent study indicated that the density of ephrin-B ligand modulates coupled Eph receptor signaling. Given these intriguing findings of ephrin ligands, we hypothesized that the ephrin-B2 ligand/Eph receptor system may play key roles in the process of postnatal blood vessel development.
development not only as landmark molecules but also as promotive factors for neovascularization. The present study focused on elucidating the role of ephrin-B2 by examining the expression profile of this ligand and its biological effect during postnatal angiogenesis.

Methods

Animals and Mouse Hindlimb Ischemia Model

All protocols described in this study were approved by the Division of Cardiovascular Research and Medicine, St Elizabeth’s Medical Center of Boston. We used ephrin-B2/LacZ heterozygous mice and wild-type mice for histochemical analysis of ephrin-B2 expression and also used C57BL/6J mice (Jackson Laboratories) for mRNA analysis. A hindlimb ischemia model was created in age-matched 8-week-old male mice as described previously and used C57BL/6J mice. Cells were cultured in modified MCD131 medium supplemented with 10% FBS and used for experiments between passages 3 and 6. For studies of stimulation by angiogenic growth factors, cells were maintained in medium with 10% FBS. After cells reached 80% confluence, the medium was changed to fresh medium containing 1% FBS for 48 hours. Then cells were stimulated with human (h)-VEGF, h-basic fibroblast growth factor (bFGF), h-scatter factor/hepatocyte growth factor (SF/HGF) (R&D Systems Inc), or h-angiopoietin-1 (Regeneron Pharmaceuticals Inc).

Matrigel Angiogenesis Assay

HMVECs that had reached 80% confluence were washed with PBS, trypsinized, and seeded onto 6-well culture plates coated with Matrigel Basement Membrane Matrix (Becton Dickinson Labware). Murine venous ECs were seeded on the layer of growth factor–reduced Matrigel (Becton Dickinson Labware) and treated with recombinant mouse ephrin-B2–FC (300 ng/mL) (R&D Systems Inc). At 1, 4, 7, and 13 hours later, cells on Matrigel were photographed and collected for mRNA analysis with the use of Matrispace Cell Release Solution (Becton Dickinson Labware).

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Quantitative Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA from cultured cells, mouse limb tissues, or corneal tissues was isolated with the use of a total RNA isolation kit (Ambion) according to the supplied instructions. Equal amounts of total RNA from each sample were converted to first-strand cDNA with a Superscript Preamplification System kit containing Superscript II reverse transcriptase (GIBCO-BRL). Then the cDNA template was amplified under nonsaturating polymerase chain reaction (PCR) conditions with the use of Advantage-GC cDNA polymerase (Clontech) and the following primers: human ephrin-B2 (sense, 5'-GACCAAGCAAGATG CAC-3'; antisense, 5'-ATTAAGG-CCACTTCGGGAACC-3'); mouse ephrin-B2, mouse EphB4 as described previously; mouse VEGF (sense, CCGAAGCTCTCGCAGAGTT-3; antisense, TTAACTCAAGCTCCTGCT-3'); mouse angiopoietin-1 (sense, 5'-TACAACACCGGGAGATGGAAG-3'; antisense, 5'-GTCGTTATCAGCATC TTCTG-3'). For relative quantitative reverse transcription (RT)–PCR, 18s ribosomal RNA primers and their competitors (competimer) (Ambion) were used as an internal control in PCR amplification with each specific primer. After amplification, PCR products were separated on 1.5% agarose gel containing ethidium bromide. Relative transcript levels of each gene were normalized by the relative amounts of 18s ribosomal RNA with the use of NIH Imaging software.

Western Blot Analysis

Whole-cell protein extracts from human ECs were separated on 12.5% SDS–polyacrylamide gel and transferred to nitrocellulose membrane. The membrane was probed with antibodies against human ephrin-B2 (Santa Cruz Biotech) and α-tubulin (Oncogene). A specific band for ephrin-B2 was initially confirmed by addition of blocking peptide (Santa Cruz Biotech) against ephrin-B2 antibody.

Statistical Analysis

Results from each experiment were obtained in at least 4 separate experiments and expressed as mean±SE. Statistical analysis was performed by unpaired Student t test.
Ephrin-B2 Is Expressed in Arterial Neovasculature in Hindlimb Ischemia

Our recent findings demonstrated that arterial-specific expression of ephrin-B2 was continuously detected in the vasculature in a variety of adult organs and also newly formed vessels in corneal angiogenesis. Therefore, we next examined whether arterial and venous neovasculature in the ischemic limb was distinguishable by ephrin-B2 expression. X-Gal histochemical staining (blue) with anti-PECAM antibody staining (brown) revealed that arterial (ephrin-B2⁺) and venous (ephrin-B2⁻) vessels existed in the ischemic limb (Figure 2A, 2B, 2C and 2D, 2E, 2F with higher magnification). A large number of blood vessels were observed with arterial (blue) and venous (brown) identity in severely ischemic areas near necrotic tissue (Figure 2B and 2E). X-Gal staining was strongly detected in these severely ischemic areas compared with the vasculature in normal healthy hindlimb muscle. Several arterial and venous communications were detected in such severely ischemic areas (Figure 2C and 2F). In contrast, fewer arterial (dark blue) and venous vessels (brown) were detected closely positioned to each other in relatively distant areas from necrotic tissue (Figure 2A and 2B). Arterial and venous communication was not observed in this area compared with severely ischemic areas. The density of blood vessels in such less ischemic areas (left half of Figure 2A) was similar to that in the contralateral healthy limb (data not shown).

To confirm these expression patterns of ephrin-B2 in the ischemic limb more clearly, double-fluorescence labeling with PECAM-1 (red) and β-galactosidase (green) was performed on mildly ischemic areas (Figure 2G through 2I) and...
severely ischemic areas (Figure 2J through 2L). In less ischemic areas, a small number of arterial vessels around muscle were detected as double positive for ephrin-B2 and PECAM-1 (Figure 2G through 2I). In contrast, greater numbers of vessels were observed in severely ischemic areas (Figure 2J), and arterial vessels (yellow) were closely positioned with venous vessels (red) (Figure 2L). To differentiate newly formed arterial blood vessels from preexisting arterial vessels, double fluorescence staining with an antibody against BrdU (red) and β-galactosidase (green) was performed on the ischemic limb in ephrin-B2/LacZ mice that were pretreated with BrdU injection, indicating that most of the arterial (ephrin-B2–expressing) vessels were double labeled with BrdU positivity in ischemic areas (Figure 2M through 2O). These histological findings of ephrin-B2, CD31 expression, and BrdU incorporation suggested that the arterial neovascularization increased along with total blood vessel number in hindlimb ischemia.

Expression of Ephrin-B2 Is Upregulated at Site of Postnatal Neovascularization

In ischemic muscles, strong expression of ephrin-B2 was detected in growing vessels compared with preexisting vessels in normal muscles of the contralateral limb (Figure 1E and 1F). These data, together with the recent finding that the density of ephrin-B ligand modulates its EphB receptor signaling,29 suggested the possibility that ephrin-B2 expression did not simply identify arterial and venous neovascularization but also was functionally regulated at sites of postnatal neovascularization. To determine this clearly, we created a hindlimb ischemia model in age-matched C57BL/6J male mice and collected mRNA from limb muscles in both the contralateral healthy limb and ischemic limb. At several time points after ischemia-inducing surgery, expression of ephrin-B2 mRNA was examined by quantitative RT-PCR analysis with specific primers for mouse ephrin-B2, VEGF, and angiopoietin-1. Strikingly, ephrin-B2 mRNA was significantly upregulated in the ischemic limb at 24 hours, after initial upregulation of VEGF at 6 hours (Figure 3A). The relative density of mRNA, the ratio of density in the ischemic limb to that in the contralateral limb, showed time-dependent upregulation of ephrin-B2 and VEGF mRNA in hindlimb ischemia (Figure 3B). On the other hand, expression of angiopoietin-1, which is known to have an important role at a later stage of angiogenesis,10 was downregulated in the ischemic limb from 6 hours (Figure 3A). However, this downregulation recovered at approximately day 7 (Figure 3B). These findings suggested that expression of ephrin-B2 in arterial vessels showed differential regulation with VEGF or angiopoietin-1 in the ischemic limb.

To confirm that upregulation of ephrin-B2 was actually detected on ECs in the developing vasculature, we analyzed ephrin-B2 mRNA in tube-forming HMVECs. At 4 hours, HMVECs started to show tubelike formation after stimulation with various growth factors and extracellular matrix proteins in Matrigel, such as bFGF, transforming growth factor-β, laminin, and collagen IV. Then these vascular tubes started to communicate with each other at 7 to 15 hours (Figure 3C). Quantitative RT-PCR analysis with specific primers for human ephrin-B2 demonstrated that ephrin-B2 transcription was significantly upregulated in tube-forming ECs from 4 to 15 hours during the process of vascular tube formation (Figure 3C), indicating that ephrin-B2 was upregulated in ECs under postnatal neovascularization.

Angiogenic Growth Factors Regulate Ephrin-B2 Expression on ECs

Next we addressed the question of whether ephrin-B2 expression on ECs was regulated by angiogenic RTK ligands such as VEGF, bFGF, SF/HGF, and angiopoietin-1 because several reports indicated that ephrin ligands showed cross-talk signaling with RTK ligand-receptor systems.34,35
transcriptional regulation of ephrin-B2, mRNA in HMVECs was analyzed by quantitative RT-PCR analysis. As shown in Figure 4D, ephrin-B2 mRNA was significantly upregulated at 6 to 12 hours after administration of VEGF, bFGF, or SF/HGF (100 ng/mL). We also examined the effect of angiopoietin-1 on ephrin-B2 expression on ECs. Ephrin-B2 expression on HMVECs was surprisingly downregulated by administration of angiopoietin-1 at both the protein and transcription levels (Figure 4E). These findings indicated that ephrin-B2 expression on arterial ECs was differentially regulated by angiogenic growth factors.

**Ephrin-B2 Ligand Directly Affects Arterial and Venous Neovascularization**

Recent reports indicated that the ephrin-B2 ligand had a stimulatory effect on migration of microvascular ECs in vitro. Therefore, we next addressed the question of how the ephrin-B2 ligand affects postnatal arterial and venous neovascularization in vivo. We performed a corneal micropocket assay on ephrin-B2/LacZ mice as an in vivo model of angiogenesis, showing that the ephrin-B2 ligand as well as VEGF induced postnatal neovascularization in corneal avascular areas (Figure 5A, 5B, 5D, and 5E). In contrast, vehicle pellets containing PBS did not induce neovessels from the corneal limbus (Figure 5C and 5F). Quantitative analysis of these neovessels showed that the ephrin-B2 ligand induced weaker circumferential neovascularization (angle of angiogenic area) than did VEGF (Figure 5M) but induced a similar length of neovascularization to VEGF (Figure 5N). This series of data was also consistent in another strain, C57BL/6J mice (data not shown), indicating less genetic heterogeneity of angiogenesis between these 2 strains. Next we questioned how the ephrin-B2 ligand affected arterial (ephrin-B2–positive) and venous (ephrin-B2–negative) neovascularization. Although the ephrin-B2 ligand as well as VEGF could induce arterial neovascularization (blue vessels) in X-Gal–stained cornea (Figure 5G through 5J), the arterial neovascularization induced by ephrin-B2 ligand was surprisingly limited, with a shorter length and fewer branches at the capillary level than that induced by VEGF (Figure 5H, 5I, and 5O). Interestingly, the capillary ratio between arterial neovessels and venous neovessels in corneal sections (Figure 5K and 5L) indicated that the ephrin-B2 ligand had a much stronger effect on venous neovascularization than did VEGF (Figure 5P). We further examined whether EphB4, a specific receptor for ephrin-B2, was regulated under the condition of ephrin-B2–induced venous angiogenesis. As shown in Figure 5Q, EphB4 mRNA was significantly upregulated in corneal tissues by ephrin-B2 stimulation for 3 days. In addition, upregulation of EphB4 mRNA was also observed in ephrin-B2–induced venous angiogenesis in Matrigel assay (Figure 5R). These observations support the concept that the ephrin-B2 ligand acts as an angiogenic growth factor, especially in venous neovascularization.

**Discussion**

The ephrin-B2 ligand was recently demonstrated to have an essential role in embryonic vascular development, with a unique expression pattern in arterial vessels. However,
the role of ephrin-B2 in postnatal neovascularization has not been investigated, whereas other RTK ligands such as VEGF and angiopoietins show an important role in both embryonic vascular development\textsuperscript{6,7,10,15} and postnatal neovascularization.\textsuperscript{1,2,3,33,41} We demonstrated here that ephrin-B2 is not simply a molecular landmark of arterial blood vessels in embryonic and postnatal life\textsuperscript{22,23,25,26} but is also a functional molecule that plays a role in the angiogenic cascade in postnatal neovascularization.

Upregulation of ephrin-B2 expression is observed at later time points than the initial upregulation of VEGF and is followed by recovery of angiopoietin-1 expression in vivo in response to tissue ischemia (Figure 3A and 3B). This suggests that the ephrin-B2 signaling system may be required for the subsequent response of triggered angiogenic ECs before blood vessel stabilization in the process of neovascularization. The in vitro ephrin-B2 expression on ECs supports this corollary. Ephrin-B2 expression is differentially regulated by angiogenic RTK ligands such as VEGF, bFGF, SF/HGF, or angiopoietin-1, which show therapeutic benefit in ischemic cardiovascular disease.\textsuperscript{3,6,9,14} Consistent with the recent findings that ephrin ligands showed cross-talk signaling with RTK ligand-receptor systems,\textsuperscript{34,35} there seems to be linked communications that ephrin ligands showed cross-talk signaling with angiogenic RTK ligands during postnatal neovascularization. Indeed, ephrin-B2 expression on microvascular ECs was induced by administration of VEGF as well as bFGF and SF/HGF (Figure 4A, 4B, and 4C), suggesting that these RTK ligands promote postnatal neovascularization via ephrin-B2 signaling on arterial ECs. In contrast, ephrin-B2 expression on ECs was downregulated by administration of a maturation factor, angiopoietin-1 (Figure 4E). In the present study, we focused on the regulation of ephrin-B2 on postnatal ECs. However, fetal ECs seem to have differential regulation by angiogenic growth factors such as bFGF and sonic hedgehog.\textsuperscript{42,43} Because discontinuous expression of ephrin-B ligands is necessary for proper development of the embryonic vasculature,\textsuperscript{44} this downregulation of ephrin-B2 by angiopoietin-1 seems to be important for proper blood vessel development, especially at the late stage of postnatal neovascularization. These findings raise the possibility that the expression and regulation of ephrin-B2 on arterial ECs is functionally important at sites of postnatal neovascularization, especially in governing the proper process of communication between arterial and venous neovascularization for structural maturation of blood vessels.

The increase in ephrin-B2 expression was not only the case after RTK ligand stimulation but was also observed throughout the process of tube formation of ECs cultured in Matrigel (Figure 3C). This indicates that endothelial sprouting or the following process requires ephrin-B2 ligand. Given the fact that the density of ephrin-B ligands modulates coupled EphB receptor signaling,\textsuperscript{70} a high density of ephrin-B2 ligand on arterial vessels may allow communication between arterial vessels and venous vessels under postnatal neovascularization via interaction with its coupled EphB receptors on venous vessels.

Among the angiogenic RTK ligands, ephrin-B2 is unique for its arterial-specific expression on vessels from the early embryonic stage to the adult. This unique expression pattern was also observed in postnatal neovascularization in ischemic tissues (Figure 2A through 2F). In this study we observed arterial neovascularization in tissue ischemia, with maintenance of its molecular identity from venous neovascularization even at the capillary level (Figure 2E and 2O), suggesting that vascular identity with respect to ephrin-B2 may restrict intermingling of arterial and venous vessels to avoid unexpected circulation between arterial and venous neovascularization in tissues. This notion is cited by others’ findings that expression patterns of ephrin ligands and Eph receptors in nerve systems restrict intermingling of different cell populations with bidirectional signaling.\textsuperscript{45} During the process of limb ischemia, peripheral perfusion of tissues recovers (Figure 1B), indicating the occurrence of communication between arterial and venous neovascularization for circulation. The pres-
ent histological findings clearly illustrated the communication between ephrin-B2–positive and –negative capillaries among the neovascularization (A, B, D, and E) and arterial-specific neovascularization (G through J) in murine cornea. Note that the control pellet did not induce neovascularization (C and F). Neovessels induced by ephrin-B2 (300 ng) or VEGF (300 ng) were visualized in live cornea (A and B) and fluorescent-lectin–stained cornea (D and E). Arterial neovessels (j) from preexisting corneal limbus artery (l) were detected as blue vessels (ephrin-B2 positive) in X-Gal–stained cornea (G through J). Note that limited arterial neovascularization was apparent in cornea with ephrin-B2 pellets (H and J). I and J are higher-magnification views of G and H. Double-immunofluorescence labeling with injected BS-1 lectin (red) and antibodies to β-galactosidase (green) in corneal sections reveal arterial vessels (double positive for BS-1 lectin and β-galactosidase) and venous vessels (single positive for BS-1 lectin) induced by VEGF (K) or ephrin-B2 (L). M through P, Corneal neovascularization induced by ephrin-B2 ligand or VEGF was quantified by its circumferential neovascularity (angle of angiogenic area) (K), length of neovessels (L), length of arterial neovessels (M), and capillary ratio between arterial neovessels and venous neovessels (N) (#P<0.01 between VEGF-stimulated group and ephrin-B2-stimulated group; n=12 in each group). Q and R, Expression and regulation of EphB4 receptor in ephrin-B2–induced corneal angiogenesis (Q) or tube-formed venous ECs in Matrigel (R) Comp indicates competitor; m, muscle.

**Figure 5.** Effect of ephrin-B2 ligand on postnatal neovascularization. A through L, Corneal micropocket assay was performed in ephrin-B2/LacZ mice. Administration of ephrin-B2 (B, E, H, J, and L) as well as VEGF (A, D, G, I, and K) stimulated postnatal neovascularization (A through J) in murine cornea. Note that the control pellet did not induce neovascularization (C and F). Neovessels induced by ephrin-B2 (300 ng) or VEGF (300 ng) were visualized in live cornea (A and B) and fluorescent-lectin–stained cornea (D and E). Arterial neovessels (j) from preexisting corneal limbus artery (l) were detected as blue vessels (ephrin-B2 positive) in X-Gal–stained cornea (G through J). Note that limited arterial neovascularization was apparent in cornea with ephrin-B2 pellets (H and J). I and J are higher-magnification views of G and H. Double-immunofluorescence labeling with injected BS-1 lectin (red) and antibodies to β-galactosidase (green) in corneal sections reveal arterial vessels (double positive for BS-1 lectin and β-galactosidase) and venous vessels (single positive for BS-1 lectin) induced by VEGF (K) or ephrin-B2 (L). M through P, Corneal neovascularization induced by ephrin-B2 ligand or VEGF was quantified by its circumferential neovascularity (angle of angiogenic area) (K), length of neovessels (L), length of arterial neovessels (M), and capillary ratio between arterial neovessels and venous neovessels (N) (#P<0.01 between VEGF-stimulated group and ephrin-B2-stimulated group; n=12 in each group). Q and R, Expression and regulation of EphB4 receptor in ephrin-B2–induced corneal angiogenesis (Q) or tube-formed venous ECs in Matrigel (R) Comp indicates competitor; m, muscle.
tion of venous ECs were driven by EphB4 receptor signaling with ephrin-B2 stimulation in vitro. Strikingly, our results showed that EphB4 expression was upregulated in ephrin-B2–induced venous angiogenesis (Figure 5Q and 5R). These results suggest that ephrin-B2 ligand mainly stimulates EphB4 forward signaling at early stages of postnatal neovascularization. This is further supported by a recent report from Henkemeyer et al., indicating that ephrin-B2 mediating forward EphB4 signaling, but not its reverse signaling, is required at the early stage of vascular development.

Recent findings also indicated that other EphB receptors were expressed on vascular ECs and their surrounding mesenchymal cells and can interact with ephrin-B2. Whereas EphB4 showed specific binding to ephrin-B2 with its essential role for vascular development, EphB3 and EphB2 show less specific binding to ephrin-B2 without their critical role for vascular development. From their expression patterns, ephrin-B2/EphB3 or ephrin-B2/EphB2 signaling is thought to mediate the interactions between arteries and their neighboring cells rather than between arteries and veins. In the present studies, it is still unclear how ephrin-B2 and EphB receptors modulate postnatal neovascularization, although our data indicated the possibility that ephrin-B2/EphB4 signaling has an important role in arterial and venous neovascularization in adults. Interestingly, the report by Zhang et al. showed that ephrin-B2–expressing stromal cells promote the growth of ephrin-B2–expressing ECs, indicating that more complex cell-to-cell interaction via EphB receptors was involved in the process of vascular maturation.

From our series of observations, the timing and activity of the ephrin-B2/EphB4 system in neovascularization seem to be crucial for balanced communication between the arterial and venous neovascularization, which will contribute to the formation of functional neoVessels. However, further studies still need to clarify when and how the ephrin-B2/EphB receptor system cooperates in postnatal neovascularization with other angiogenic RTK receptor ligands, such as VEGF and angiopoietin-1. Indeed, costimulation of ephrin-B2 with VEGF or angiopoietin-1 surprisingly suppresses the proliferation of in vitro venous ECs, indicating the possibility that ephrin-B2/EphB signaling cooperates in vascular maturation with other RTK receptor/ligand systems through promoting or suppressing endothelial proliferation.

In conclusion, the ephrin-B2 ligand is not simply a molecular marker of arterial neovascularization in tissue ischemia but also is functionally regulated by angiogenic growth factors, which is important for postnatal neovascularization. Enhanced ephrin-B2 ligand expression seems to induce the subsequent responses stimulating the interaction between arterial (ephrin-B2–expressing) and venous (EphB4-expressing) vessels for the further process of neovascularization and establishment of communication. These findings, together with its essential role in embryonic vascular development, suggest that this ligand seems to be functionally important for postnatal neovascularization, as well as other RTK ligands such as VEGF and angiopoietins. Therefore, the ephrin-B2 ligand and EphB receptor system is one of the key targets to clarify the molecular mechanisms of the postnatal neovascularization process and may be an important mediator of proper arterial and venous neovascularization in therapeutic angiogenesis.

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References


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